Sildenafil Inhibits Cardiac Hypertrophy In Vivo: Role of the RhoA/rho Kinase Pathway

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Increased cyclic GMP and thus activation of PKG has been implicated in modulating the RhoA/Rho kinase (ROCK) pathway in endothelial cells and vascular smooth muscle cells. In the heart, enhanced RhoA/ROCK is thought to contribute to hypertrophy. Here we tested whether prevention of calcineurin (CaM kinase II) or adenylate cyclase (PKA) inhibition by expression of the alpha-myosin heavy chain promoter of the ROCK inhibitor, Rock2 (ROCK2-SRF), in mouse neonatal cardiomyocytes would inhibit cardiomyocyte hypertrophy. ROCK2-SRF inhibited the hypertrophic phenotype induced by both. Interestingly, sildenafil blunted the hypertrophic phenotype induced by both. Interestingly, sildenafil blunted the treatment of neonatal rat cardiac myocytes results in changes in the fetal gene program consistent with pathologic hypertrophic response. Here we show that TRPC1 mRNA levels and TRPC5 mRNA levels are up-regulated 6 fold and 8 fold respectively in human heart failure. Moreover, we show that TRPC blockade in neonatal rat cardiac myocytes prevents the activation of the fetal gene program in response to beta-adrenergic stimulation. These results suggest that TRPCs are an important component of the pathways involved in the induction of fetal gene program during pathologic hypertrophy.

The Transcriptional Repressor NAB1 is a Specific Regulator of Pathological Cardiac Hypertrophy

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A wide variety of neurohumoral and other stimuli have been described as potent inducers of cardiac hypertrophy. However, little is known about mechanisms which serve to limit cardiac hypertrophy. Here, we have recently shown that expression of the C. elegans transcriptional repressor NAB1 (NAB-1, a NAB family member) in neonatal rat cardiomyocytes blunts hypertrophy in response to beta-adrenergic stimulation as it blocks the expression of the co-called fetal gene program. It has recently been shown that NAB1 is a repressor of fetal gene program activation in human cardiac myocytes. Here, we report that NAB1 in transgenic mice with increased expression of NAB1 (NAB1-TG) results in a significant increase in hypertrophy in the mouse heart, enhanced RhoA/ROCK is thought a contributor to hypertrophy. Here we tested whether prevention of calcineurin (CaM kinase II) or adenylate cyclase (PKA) inhibition by expression of the alpha-myosin heavy chain promoter of the ROCK inhibitor, Rock2 (ROCK2-SRF), in mouse neonatal cardiomyocytes would inhibit cardiomyocyte hypertrophy. ROCK2-SRF inhibited the hypertrophic phenotype induced by both. Interestingly, sildenafil blunted the hypertrophic phenotype induced by both. Interestingly, sildenafil blunted the treatment of neonatal rat cardiac myocytes results in changes in the fetal gene program consistent with pathologic hypertrophic response. Here we show that TRPC1 mRNA levels and TRPC5 mRNA levels are up-regulated 6 fold and 8 fold respectively in human heart failure. Moreover, we show that TRPC blockade in neonatal rat cardiac myocytes prevents the activation of the fetal gene program in response to beta-adrenergic stimulation. These results suggest that TRPCs are an important component of the pathways involved in the induction of fetal gene program during pathologic hypertrophy.

Cardiac Hypertrophy and Dilation Caused by a Dominant-Negative Mutation of Delta-Sarcoglycan

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Loss of function, recessive sarcoglycan gene mutations produce cardiomyocyte and skeletal myofiber degeneration. A unique case of familial dilated cardiomyopathy has also been associated with a heterozygous S151A delta-sarcoglycan gene mutation (Tsuchita et al. JCI 2000). Four independent transgenic mouse lines were generated that express the delta-sarcoglycan S151A mutant gene. A single line was generated that expressed wildtype protein (WT). In addition, NAB1 suppressed pressure overload induced cardiac hypertrophy (transverse aortic constriction) by 70% (NAB1-TG vs. WT). We then assessed the role of NAB1 in physiological hypertrophy caused by chronic running exercise (running wheel exercise with 6.9 km/day for 21 days). In sharp contrast to the repression of pathological hypertrophy by NAB1, physiological growth in response to exercise was not affected. By gene array analysis, we have identified a subset of target genes specifically regulated by this growth-controlling pathway. Our results imply the NAB1-Egr-1-axis as an important mechanism regulating pathological growth of the myocardium.

TRPC Channels and beta-Adrenergic-Mediated Activation of the Fetal Gene Program

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Transient receptor potential channels (TRPC) constitute a family of channels that have been proposed to function as store-operated or second messenger-operated channels in a variety of cell types. Recently, TRPC3 has been implicated in the calcineurin-mediated response in skeletal muscle, making it a likely candidate for mediating hypertrophy in the heart. Activation of the fetal gene program during pathologic hypertrophy is characterized by changes in gene expression that includes repression of genes that are expressed during adult development (α-Myc, Myh9, Nppa), and sarcoplasmatic reticulum ATPase 2a (Sarc2a2a), and re-expression of genes that are present during the fetal development (β-Myc, Myh6, Atf3, and brain natriuretic peptide (Bnp), and Bnp, and skeletal β-actin). We have recently shown that dilated cardiomyopathy patients (DCM) patients changes in gene expression that recapitulate the fetal gene program, and that these changes are partially reversed in patients that favorably respond to β-blocker therapy. We have also shown that β1-adrenergic stimulation in neonatal rat myocytes results in changes in the fetal gene program consistent with pathologic hypertrophic response. Here we show that TRPC1 mRNA levels and TRPC5 mRNA levels are up-regulated 6 fold and 8 fold respectively in human heart failure. Moreover, we show that TRPC blockade in neonatal rat cardiac myocytes prevents the activation of the fetal gene program in response to β-adrenergic stimulation. These results suggest that TRPCs are an important component of the pathways involved in the induction of fetal gene program during pathologic hypertrophy.
Mice with Heart-Restricted β-Catenin Deletion Develop a Modest Cardiac Hypertrophic Phenotype but a Normal Hypertrophic Response to Angiotensin II

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Constitutive activation of cardiac glycoce synthase kinase-3β (GSK-3β) is known to suppress cardiac hypertrophy in vivo. GSK-3 phosphorylates a variety of substrates including β-catenin leading to its degradation. Here, we studied the effect of specific β-catenin deletion in adult cardiac hypertrophy after angiotensin II (Ang II) stimulation. Mice lacking β-catenin ubiquitously die at embryonic day 5.5. To avoid this lethality, we employed a cardiac-specific mifepristone-inducible Cre transgene to induce β-catenin deletion in cardiomyocytes. Recombination of loxP sites flanking exons 3 to 6 as confirmed by PCR of genomic heart extracts, resulted in a decrease of about 70% of cytosolic β-catenin protein. Mice with cardiac specific β-catenin deletion were viable. Surprisingly, these mice exhibited a phenotype of modest cardiac hypertrophy at baseline conditions as assessed by echocardiography and histology. Additionally, expression of hypertrophic markers was increased e.g. atrial natriuretic peptide (ANP, fold expression vs sham, 2.8±0.7 (mean±SEM), brain natriuretic peptide (BNP, 4.3±1.5), β-MHC (2.0±1.0) and α-skeletal actin (2.9±0.9) as measured by real-time RT-PCR. Infusion of Ang II by osmotic minipumps induced cardiac hypertrophy in wild type mice as expected. Interestingly, a similar response was observed in transgenic mice as judged by histologic examination using Masson trichrome staining and by mRNA levels of ANP (fold expression vs sham, 10.0±3.6 and 9.8±3.4, control and transgenic mice, respectively), BNP (4.0±1.4 and 10.0±3.1, respectively), β-MHC (4.1±2.3 and 3.5±0.5, respectively) and α-skeletal actin (5.9±0.5 and 6.5±0.7, respectively. In conclusion, β-catenin deletion in adult heart leads to a modest re-activation of fetal gene expression accompanied by a phenotype of mild cardiac hypertrophy while it does not affect Ang II-induced cardiac hypertrophy. The effect of constitutive active GSK3β on cardiac hypertrophy is not mediated through β-catenin.

SH2-Containing Protein Tyrosine Phosphatase Functioning in LIF-Induced Signaling Pathway Inhibits Skeletal α-Actin Gene Expression in Cardiomyocytes

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Background and Aim: Leukemia inhibition factor (LIF), an interleukin-6 related cytokine, induces cardiomyocyte hypertrophy distinctly from that observed after stimulation with G protein-coupled receptor (GPCR) agonists. GPCR agonist such as endothelin-1 increases cell shape of cardiomyocyte. However, the mechanism of distinct a-SKA gene expression between GPCR agonists- and LIF-mediated signaling remains unknown. Here, we report the specific role of SHP2 in LIF-dependent a-SKA gene regulation. Methods and Results: In neonatal rat cardiac myocytes, activation of small G protein RhoA was observed after stimulation with ET-1, but not with LIF. Adenosine-mediated overexpression of dominant-negative RhoA (DN-RhoA) abrogated ET-1-induced upregulation of a-SKA mRNA, indicating that ET-1 promotes a-SKA gene expression via RhoA. We hypothesized SHP2, which is a key downstream molecule in LIF-induced signaling, inhibits a-SKA gene expression. To test this hypothesis, we examined the involvement of SHP2 in downstream signaling activated by GPCR agonists and LIF. SHP2 was tyrosine-phosphorylated by LIF, but not by GPCR agonists including norpeinephrine, angiotensin II and ET-1. To decipher the role of SHP2, we tested the effect of adenovirus vector expressing wild-type SHP2 (AdShp2)26, phosphatase inactive SHP2 (AdShp2S)27, and beta-galactosidase (AdGal) on a-SKA gene expression after stimulation. LIF overexpression a-SKA mRNA in myocytes infected with AdShp2S26, but not in those infected with AdGal or AdShp226. In contrast, neither AdShp226 nor AdShp2S26 did not affect ET-1-induced a-SKA gene expression. Overexpression of DN-RhoA and pretreatment of Rho kinase inhibitor Y27632 abrogated LIF-induced upregulation of a-SKA mRNA in myocytes infected with AdShp2S26. Conclusion: these findings indicate that SHP2 inhibits RhoA-dependent a-SKA expression in LIF-mediated signaling, probably resulting in elongative shape of myocytes distinct from a-SKA upregulated myocytes.

Protemic Analysis of Ischemic Heart Failure Patients Reveals Increases in a Myosin Assembly Protein (UNC-45)

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This study examined alterations to the human heart proteome as a result of ischemic heart failure. Two dimensional gel electrophoresis was used to compare the protein expression of end-stage ischemic heart patients (n=5) with age-matched controls (n=5). By optimizing tissue extraction and IE conditions using different zwitterionic detergents, we were able to obtain higher protein representation of membrane / cytoplasmic proteins than previous 2-DE analysis of human heart. Over 300 highly abundant protein spots with a molecular weight greater than 20 kDa were resolved upon silver staining. Seventy-two of these protein spots have been identified by MALDI-TOF MS and are from various biological categories including receptor tyrosine kinases, cytoskeleton (actin, vinculin, desmin); mitochondria (acyl-CoA dehydrogenase, ATP synthase α/β); and the endoplasmic reticulum (GRP78, GRP94). Fifteen of these proteins exhibited altered protein abundance between failing and normal hearts. These changes reveal new markers in ischemic heart failure which is consistent with previous proteomic results highlighting a switch to glycolysis in the failing heart. Also, changes in the abundance of myofilament associated proteins were observed. Specifically, striated muscle UNC-45 (a putative myosin assembly chaperone) was found to exhibit a 2.6 fold increase in O.D. in the failing hearts (0.09±0.02 to 0.21±0.02). This is the first in vivo observation of the translated protein which was previously only defined as a putative gene product in humans. We hypothesize that UNC-45 is a critical component of the failing heart’s hypertrophic response as it is important for myosin assembly. The importance of this protein in the heart was confirmed in siRNA experiments on cultured neonatal cardiomyocytes that demonstrated reduced myosin expression and a lack of myosin filaments as observed by confocal microscopy.

First Proteomic Association between a Single Nucleotide Polymorphism in a A142D*14-Diencoy-α Isozyme and Ischemic Heart Failure

Brian A Stanley, Johns Hopkins Univ, Baltimore, MD; Rani P Cruz, Univ of British Columbia, Vancouver, Canada; Dawn Chen, Johns Hopkins Univ, Baltimore, MD; Peter Liu, Univ of Toronto, Toronto, Canada; Bruce M McMullan, Univ of British Columbia, Vancouver, Canada; Jennifer E Van Eyk, Johns Hopkins Univ, Baltimore, MD

Proteomic analysis of myocardium from normal versus end stage ischemic cardiomyopathic (ICM) and dilated cardiomyopathic (DCM) patients demonstrated a heterogeneity in the presence / absence of two proteins (A and B) which differed by a 0.3 isolectric point (pI) shift. These proteins were both identified as A142D*14-dienoyl-α isomerase (ECH), an auxiliary enzyme involved in fatty acid oxidation. In-depth mass spectrometric analysis (ES-MS/MS and MALDI-TOF MS) revealed that these proteins differed by an E41A substitution caused by a single nucleotide polymorphism (SNP : GAG→GGA). The correlation between spot pattern and genetic polymorphism was confirmed through a TaqI restriction digest using genomic DNA. Consequently, the homozygous C/C allele of ech1 is strongly associated with patients presenting with ischemic heart failure (Table). Molecular modeling demonstrated that an E41A substitution results in a significant reduction in the electronegative field surrounding the N-terminus. We believe that this SNP may predispose a patient to a negative outcome following an ischemic episode as well as contribute to lipidopaxy in the failing heart. Further, we demonstrate that a proteomic methodology can be used as an unbiased method for SNP detection.

Expression of a Dominant Inhibitory Mutant of a Tcf/Lef-1 Family Member in Mouse Heart Blocks Physiological Myocyte Growth and Leads to Ventricular Dilatation and Failure

Sergei P Shevtsov, Tufts Univ Sch of Med, Boston, MA; Ronglih Liao, Lei Cui, Boston Univ, Boston, MA; Robert N Salomoni, Xin Chen, Richard Patten, Thomas Force; Tufts Univ Sch of Med, Boston, MA

Elements of the Wnt/β-catenin signaling network have been shown to play a critical role in many biological processes, such as cell proliferation, differentiation, development, and cancer. This lab and others have shown that GSK-3β is a negative regulator of the β-catenin pathway, reduces both physiologic and pathologic cardiomyocyte hypertrophy. To determine whether inhibition of β-catenin signaling may be one mechanism by which GSK-3β negatively regulates heart growth, we generated a cardiac-specific transgenic mouse expressing a dominant inhibitory mutant of the transcription factor Lef-1, an obligate downstream of β-catenin expression of which promoted induction of cardiac dependent genes. Hearts of transgenic mice were slightly smaller than wild type on day 5 after birth. However, by 5 wks of age, echocardiography revealed significantly thinner LV posterior wall (PW 0.62 ± 0.25 mm vs. 0.79 ± 0.3 mm; p < 0.05). Isolation of cardiac ventricle confirmed markedly reduced myocyte width in cells expressing DriL compared to wild type, consistent with the inability to undergo concentric hypertrophy. In addition, the left ventricles of the transgenics were significantly dilated (VEDV 4.1±1.0 vs. 3.1±0.1 mm; p<0.05) and systolic function was markedly depressed (FS 20.7±4.4 vs 41.8±1.7%; p<0.05). In conclusion, β-catenin/Tcf-Lef-dependent gene expression...
Blocking Cardiac Growth in Hypertrophic Cardiomyopathy Induces Cardiac Dysfunction and Decreased Survival in Only Males

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Mutations in myosin heavy chain (MyHC) can cause familial hypertrophic cardiomyopathy (HCM) characterized by cardiac hypertrophy, histopathology, and contractile dysfunction. The signaling pathways involved in the pathogenesis of this disease are not known and while cardiac hypertrophy alone is an independent risk factor for heart failure, an unresolved question is whether blocking cardiac growth may be maladaptive or beneficial in this disease. Because constitutively active glycogen synthase kinase-3β (caGSK-3β) had been shown to suppress cardiac hypertrophy and heart failure in vivo, we investigated whether short-term overexpression of a constitutively active form of GSK-3β (caGSK-3β) would block the development of cardiac hypertrophy in response to a genetic HCM mutation as well as suggest that blocking the ability of the heart to undergo hypertrophy in response to an HCM mutation may only be beneficial in females.

GFP-FRNK Expression in the In Vivo Adult Rat Heart Induces Cardiac Hypertrophy

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Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase involved in integrin-mediated signal transduction and is an important mediator of cardiomyocyte hypertrophy. We previously showed that inhibition of FAK phosphorylation by adenovirally-mediated expression of GFP-FRNK, the noncataltatic, C-terminal domain of FAK, displaced FAK from focal adhesions and induced apoptosis in neonatal rat ventricular myocyte cultures. Although the role of FAK in vivo is becoming increasingly clear, the importance of FAK in the intact adult heart remains largely unknown. In order to investigate the role of FAK in vivo, replication-defective adenoviruses (Adv) encoding GFP-FRNK, Y397FAK (an autophosphorylation site mutant of FAK), or GFP were injected into the aortic root of adult Sprague Dawley rats via catheterization of the right carotid artery. Opticon echocardiographic controls were used as a delivery agent for the adenoviruses. Real-time pulse-wave Doppler ultrasound disrupted Optison echocontrast microbubbles and released the adenoviruses into the ventricular myocardium. Transthoracic echocardiography was performed prior to and 1 week following adenoviral gene transfer. Hearts were harvested for Western blotting or histological sectioning. Both GFP-FRNK and Y397FAK were highly expressed in the heart 1 week after gene transfer. Epifluorescence microscopy revealed robust GFP-fluorescence in myocytes isolated from Adv-GFP and Adv-GFP-FRNK infected hearts. Data analyzed from M-mode echocardiographic measurements revealed that expression of GFP-FRNK markedly increased ventricular function, as assessed by an increase in LV fractional shortening. In addition, GFP-FRNK expression induced LV hypertrophy (increased LV mass/BW). Expression of Y397FAK had no effect on either LV fractional shortening or LV mass/BW as compared to Adv-GFP infected hearts. Real-time RT-PCR revealed that SERCA2 mRNA levels were markedly reduced following GFP-FRNK expression as compared to GFP- or Y397FAK-expressing hearts. This is consistent with prior reports describing SERCA2 downregulation during cardiac hypertrophy. In summary, these data provide evidence for an important structural and functional role of FAK in the in vivo adult heart.

Serine Phosphorylation and Nuclear Redistribution of FAK and FRNK in Cardiac Myocytes

Jibin Zhou, Xian Ping Yi, Lu Huber, Jiaxiang Qu, Kelly Graber, Xuejun (XJ) Wang; Cardiovascular Rsch Inst, Sioux Falls, SD

Recent studies in skeletal muscle demonstrate that catabolic triggers induce atrophy by activating FOXO transcription factors. We hypothesized that FOXO proteins through a mechanism requiring the PI3K/Akt pathway. Consistent with these findings, over-expression of FOXO1 proteins in cardiomyocytes blocked both angiotensin II-induced hypertrophic growth and calcineurin activation. We conclude that FOXO transcription factors are negative modulators of cardiac hypertrophy and that inactivation of FOXO may be a key element in the development of cardiac hypertrophy and failure.

Remodeling of the Intercalated Disk in Cardiac Specific β-Catenin Knockout Mice

Faqian Li, Jibin Zhou, Xian Ping Yi, Kelly Graber, Jiaxiang Qu, Lu Huber, Xuejun (XJ) Wang; Cardiovascular Rsch Inst, Sioux Falls, SD

Recent progresses in signal transduction have revealed how β-catenin signaling controls embryonic development, tumorigenesis, cell shape, and polarity. The role of this pathway in myocyte shape regulation during cardiac hypertrophy and failure is, however, not clearly defined. Since homozgyous knockout of β-catenin is embryonic lethal, we have deleted β-catenin genes specifically in the heart of adult mice by crossing homozygous loxp-flanked β-catenin mouse with α-MHC-MerCreMer mice. Administration of tamoxifen induces the deletion of β-catenin only in the cardiac myocytes. Immunolabeling with β-catenin antibody demonstrates that 90% of cardiac myocytes completely lose their β-catenin expression, but maintain normal rod-shape morphology. The intercalated disk of cardiac myocytes without β-catenin is morphologically unremarkable with normal distribution of vinculin, N-cadherin, desmoplakin, ZO-1, connexin43, α-, γ- and β120 catenins. The expression level of these proteins except γ-catenin is also similar in tamoxifen treated and control mice. Western blots reveal that γ-catenin increases in the heart of loxp-flanked β-catenin mice treated with tamoxifen compared to untreated controls. Confocal microscopy also demonstrates that γ-catenin has significantly increased in the intercalated disks of cardiac myocytes lacking β-catenin. Echocardiography shows the knockout mice maintain normal ventricular geometry and cardiac function. This model provides an important tool to investigate the roles β-catenin in cardiac hypertrophy and heart failure during hemodynamic challenge.

FOXO Transcription Factors are Negative Modulators of Cardiac Hypertrophy

Yan G Ni, Kambez Benjeni, Asim Day, Jun Cheng, Guangrong Lu, Robert D Gerard, Beverly A Rothermel, Joseph A Hill; Univ of Texas Southwestern Med Ctr, Dallas, TX

Cellular hypertrophy requires coordinated regulation of pro-growth and anti-growth mechanisms. Hypertrophic stimuli induce cell growth by promoting protein synthesis. Recent studies in skeletal muscle demonstrate that catabolic triggers induce atrophy by activating FOXO transcription factors. Here, we report that FOXO-1 and -3a transcription factors are inactivated by hemodynamic stress in hearts undergoing hypertrophic growth. We also show that multiple hypertrophic agonists trigger the inactivation of these FOXO proteins through a mechanism requiring the PI3K/Akt pathway. Consistent with these findings, over-expression of FOXO1 proteins in cardiomyocytes blocked both angiotensin II-induced hypertrophic growth and calcineurin activation. We conclude that FOXO transcription factors are negative modulators of cardiac hypertrophy and that inactivation of FOXO may be a key element in the development of cardiac hypertrophy and failure.

Withdrawn
The hearts of male C57Bl/6J mice display features of dilated eccentric hypertrophy when compared to male A/J mice. Likewise, we have verified that the length of isolated adult cardiomyocytes (CMs), a variable known to correlate closely with the size of left ventricular cavity, was greater in C57Bl/6J male mice than in their A/J counterparts (see Table). Whereas the length of CMs from the B6AF1 cross (resulting from the cross between a female C57Bl/6J and a male A/J mouse) was similar to that of CMs from male A/J mice (129 ± 0.8), the length of CMs of male mice resulting from the reciprocal AB6F1 cross was 144 ± 1.0, and thus significantly higher than that of CMs from the B6AF1 cross. To test whether the chromosome Y (MMUY) from C57Bl/6J may contribute to these various differences, we compared the length of CM as well as left ventricular chamber characteristics (as measured by echocardiography) of consomic C57Bl/6J-Chr Y/NaJ mice (where MMUY from A/J has been introgressed into the C57Bl/6J genetic background) to that of the parental strains. In the consomic strains, the values of CM length, end-diastolic dimension of the left ventricle as well as mitral flow were intermediate between that of C57Bl/6J and A/J mice (see Table), thus confirming the contribution of MMUY from C57Bl/6J to the morphology of the left ventricle. Additional experiments are underway to elucidate the molecular mechanisms responsible for these differences. These findings may also explain why certain cardiac transgenes display sex-specific phenotypes when expressed into the C57Bl/6J background.

### CARDIAC VARIABLES OF MALE MICE IN PARENTAL AND CONSOMIC STRAINS

<table>
<thead>
<tr>
<th>Variable</th>
<th>C57Bl/6J</th>
<th>C57Bl/6J-Chr Y/NaJ</th>
<th>A/J</th>
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<tr>
<td>Cardiomyocyte length (μm)</td>
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<td>End-diastolic LV diameter (mm)</td>
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<td>Mitral velocity (m/s)</td>
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### Adenose A1 Receptor Modulates Cardiomyocyte Hypertrophy and Signaling to ERK Map Kinase

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Left ventricular hypertrophy results from increased mechanical load and/or soluble factors that promote cardiomyocyte growth. The ras/raf/MEK/ERK cascade plays a prominent role in hypertrophic growth signaling in response to both mechanical and chemical stimuli. Adenose A1 receptor (A1R) agonists can attenuate pressure overload-induced hypertrophy in vivo and also block hypertrophy of cultured cardiomyocytes in response to adrenergic stimuli. To determine the effect of adenose receptor activation on the ras/raf/MEK/ERK cascade, activation of ERK was observed in the presence or absence of adenose A1R agonists in a neonatal rat ventricular cardiomyocyte (NRVM) model of hypertrophy. Treatment of NRVMs with the α-adrenergic agonist phenylephrine (PE) induced hypertrophy and increased ERK phosphorylation. Treatment with a stable analogue of adenose (2-chloro adenosine-3,5-di-O-phosphate) blocked PE induced hypertrophy and adrenergic stimulation of phospholamban phosphorylation but, surprisingly, increased ERK phosphorylation. The CADO induced increase in ERK activation was blocked by the specific adenose non-receptor antagonist DPCPX or pertussis toxin suggesting an A1 receptor independent mechanism. To further substantiate a role for the A1R in modulating adrenergic signaling to ERK, A1 receptor levels were depleted using RNAi. Depletion of A1R blocked the CADO induced increase in PE signaling to ERK, and prevented the reduction of PE induced phosphorylation of phospholamban. Interestingly, cells with depletion of the A1R also had decreased basal levels of raf activation, diminished cell area and misshapen myofilaments, suggesting that the A1R's effect is occurring in a signaling and cytoskeletal organization even in the absence of a receptor stimulus. Together these results reveal an unexpected role for the Adenosine A1 receptor in cytoskeletal structure and the activity of the ras/raf/MEK/ERK cascade during both basal conditions and in response to hypertrophic stimuli.

### Identification of BRCA1-Associated Protein 2 as a Potential Modulator of Myocardial Hypertrophy and Heart Failure

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BRCA1 (breast cancer related antigen) part of a conserved signal transduction pathway, known as a tumor suppressor gene with antiproliferative effects. The antiproliferative features of BRCA1 are related to its nuclear translocation. BRAP2 was reported to translocate newly synthesized BRCA1 via binding to its NSL-domain to the cytosol thereby inhibiting BRCA1 signaling. The aim of this study was to analyze the role of BRAP2 in myocardial hypertrophy and heart failure. By using Real-Time PCR, Western Blots, 2D-Gei analysis and massspectrometry, expression and posttranslational modifications of BRAP2 were analyzed in two animal models of right ventricular (RV) hypertrophy (Monocrotaline (MCT)-induced RV hypertrophy and pulmonary bands induced RV hypertrophy) in rats. Further, the expression of BRAP2 was analyzed in samples of non-failing and failing human myocardium. BRAP2 was found to be upregulated in the hypertrophied RV myocardium of both rat models (BRAP2/GAPDH mRNA ratio MCT 0.0051 ± 0.0009 vs. control 0.0031 ± 0.0003, p < 0.002 (n = 8); pulmonary banding 0.0047 ± 0.0008 vs. control 0.0037 ± 0.0002, p = 0.02 (n = 7)). Western blot results confirmed the mRNA data 20-Gelectrophoresis and massspectrometry revealed dephosphorylation of BRAP2 in the hypertrophy groups compared to the controls. Further, mRNA analysis of human myocardium showed a significant higher expression of BRAP2 in failing human myocardium (12 months compared to non failing and non-inflamed and inflamed adult hypertrophy). Further, expression analysis of murine myocardium showed a higher expression of BRAP2 in failing murine myocardium (both 12 weeks and 24 weeks) compared to non failing and non-inflamed and inflamed adult hypertrophy. Immunohistochemistry confirmed the higher expression of BRAP2 in failing murine myocardium. These findings suggest that BRAP2 is a potential modulator of myocardial hypertrophy and failure. Regression of Sarcoplasmic Reticulum Ca2+ ATPase Gene Transcription by Protein Kinase C Isoenzymes in Neonatal Rat Ventricular Myocytes

Kalpana Vijayan, Loyola Univ Med Ctr, Maywood, IL; Sara Danzi, Irwin Klein, North Shore Univ Hosp, Manhasset, NY; Allen M Samarel; Loyola Univ Med Ctr, Maywood, IL

Sarcoplasmic reticulum Ca2+ ATPase (SERCA2) is a calcium regulatory protein that is critical for the maintenance of normal cardiac function. Downregulation of SERCA2 expression is an essential feature of pathological hypertrophy that contributes to impaired cardiac contractility. Previous studies have shown that specific PKC isoforms regulate SERCA2 expression in neonatal rat ventricular myocytes (NRVM). Specifically, overexpression of wtPKCε and wtPKCγ down-regulated SERCA2 mRNA levels while wtPKCζ did not. The present investigation explored the effects of PKC isoforms on SERCA2 expression. Both wtPKCε and wtPKCγ were transcriptionally mediated. NRVM were infected with adenosine receptor agonist expressing cells, and cell volume measured using a LUC reporter gene. In summary, the NE-induced remodeling process of the heart is regulated by PKC isoforms. Our data indicate that PKCζ transcriptionally regulates SERCA2 expression while PKCε likely modulates SERCA2 expression via a post-transcriptional mechanism.

### Tissue Inhibitor of Matrix Metalloproteinases-1 Expression Correlated with Collagen Expression in Norepinephrine-Induced Remodeling of the Mouse Heart

Wilfried Briesl, Henning Meier, Grit Marx, Alexander Dettten, Heinz-Gerd Zimmer; Univ of Leipzig, Leipzig, Germany

Norepinephrine (NE)-induced hypertrophy of the left ventricle (LV) in the rat is associated with remodeling of the extracellular matrix (ECM). In the present study it was analyzed, whether the NE-induced changes of functional and molecular parameters of the heart are comparable in rats and mice. The NE-content in the plasma was elevated from 1.08 ± 0.25 to 19.4 ± 5.26 pmol/ml in Sprague Dawley rats after 3 days of i.v NE-infusion (0.1 mg/kg/h). NE was administered in ca balic mice with increasing concentration (0.13, 0.25, 0.38 mg/kg/h). The NE-content was elevated to 0.6–0.7 to 12.9–2.9 pmol/mg in mice after NE-treatment (0.25 mg/kg/h). While the right ventricular (RV) systolic pressure (RVS) was elevated in rats and mice, the left ventricular (LV) SP was only elevated in mice. Also in mice, there was a concentration-dependent elevation of the LV weight/bodyweight ratio after 3 days of NE-stimulation (19% at 0.38 mg/kg/h). This was accompanied by an elevation of the atrial (ANP) and brain natriuretic peptide (BNP) mRNA expression and a remodeling of the ECM. The mRNA expression of collagen type I and type III was elevated after 3 days of NE-application predominantly in the LV. The matrix metalloproteinase (MMP) expression was elevated from 5.1 ± 1.6 to 65.2 ± 4.2 pmol/ml by NE treatment (0.25 mg/kg/h) although the mRNA expression of MMP-2 was not changed. However, the mRNA of the tissue inhibitor of MMP (TIMP)-1, and not TIMP-2, TIMP-3 or TIMP-4 was elevated concentration-dependently in the LV at this time point. There was a strong correlation of the mRNA expression with the protein expression of TIMP-1, which was detected in the cardiac tissue extract with ELISA. It was elevated from 29.7–6.6 to 346.158.0 pg/mg after NE-treatment (0.25 mg/kg/h). The best correlation for this elevated TIMP-1 mRNA expression was found with collagen I mRNA expression (r = 0.88, p < 0.001) (n = 24). In summary the NE-induced remodeling process of the heart is comparable in mice and rats. The observation that TIMP-1 expression correlated with elevated collagen-1 expression suggests that TIMP-1 may play an important role in the induction of collagen production in the heart that extends beyond their traditional role as MMP inhibitors.
26 Downregulation of Cardiac Apelin System in Hypertrophied and Failing Hearts: Possible Role of Angiotensin II-Angiostatin Type 1 Receptor System

Yoshitaka Iwana, National Cardiovascular Ctr, Suita, Osaka, Japan; Hiroi Ayu Takenaka, Toru Kita, Yasuaki Kihara; Kyoto Univ Graduate Sch of Med, Kyoto, Japan

Background: Cardiac apelin has been recently suggested to contribute to the pathophysiology of heart failure (HF) in humans. However, its regulation and relationship with other neurohumoral factors contributing to deterioration of HF remain unknown. Accordingly, we investigated the role and regulation of cardiac apelin system in the diseased heart using in vitro experiments with rat isolated ventricular myocytes (IVM) and in vivo experiments using apelin receptor (APJ) blocking antibodies.

Results: In IVM, apelin mRNA expression was down-regulated in hypertrophic vs. control hearts. In vivo, APJ mRNA showed no change compared with the control animals, while these treatments significantly increased cardiac apelin expression. The effect was further enhanced in APJ receptor-knockout mice. Ex vivo, cardiac apelin mRNA expression was decreased after 24 hr administration and its restoration was obtained by APJ treatment. Conclusion: These results indicate that cardiac apelin system is markedly down regulated in experimental HF and may be regulated by angiotensin II-angiotensin type 1 receptor system directly. Renin-angiotensin system inhibition may have beneficial effects, at least in part, through the restoration of cardiac apelin system in the treatment of HF.

27 Compartment of Protein Kinases and Phosphoprotein Phosphorilation in Pressure-Overload-Induced Hypertrophic Rat Hearts

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Objective: In order to estimate whether protein phosphorylation and dephosphorylation in nuclei play roles in the development of myocardial hypertrophy, distribution of protein kinases and phosphatases in cell fractions were determined. Methods: The model of rat cardiac hypertrophy was established by abdominal aortic constriction. Velocity and isovolumetric gradient centrifugation was employed to fractionate myocardium to membrane, cytosol and nucleus. Enzymatic methods were employed to determine the activity of kinases and phosphatases. Results: Compared with control group, the activity of mitogen activated protein kinase (MAPK) increase by 16.83% (p<0.05) in nuclear, decreased by 51.23% (p<0.01) in cytosolic fraction. The activity of protein kinase A (PKA) in different fractions were close to those of control. The activity of P53 in membranes and cytosol fractions, changed without significance in nuclear and cytosolic fractions, changed without significance in nuclear and nuclear fractions, whereas declined by 50% in membranes fraction. Conclusion: The increase of activity of nuclear PKA, P53 and P53 in different fractions were close to those of control. Further, the increase of activity of nuclear MAPK, P53 and P53 might be involved in developing overload-induced cardiac hypertrophy.

28 Role of the Transient Outward Current in Regulating Ventricular Action Potential and Contractility: A Dynamic Clamp Study

Min Dong, Xiaowei Sun, Hong-Sheng Wang; Univ of Cincinnati, Cincinnati, OH

The transient outward current (Ito) is a major repolarizing current in the heart, and is responsible for phase 1 repolarization of the action potential. Reduction of Ito density is consistently observed in human heart failure and animal models of failure, and is accompanied by significant action potential duration (APD) prolongation. It has also been proposed that Ito density regulation may contribute to the impaired intracellular Ca2+ handling and contractility in heart failure. Due to limitations in available techniques such as computer modeling or the pharmacologic approach, the role of Ito in regulating APD is not well understood. We investigated this question using a novel approach, the dynamic clamp. This technique allows “insertion” of simulated conductances in real, living cells, providing a powerful method of experimental electrophysiology. “Insertion” of a canine epicardial-level of Ito in canine endocardial cells or guinea pig ventricular cells, where the native Ito is small or lacking, produced a prominent phase-1 notch, but did not significantly affect the APD. Increasingly larger Ito densities moderately prolonged, and then dramatically shortened the APD. Simulations of three unrelated Ito models produced near identical effects on the APD, suggesting that our findings are not linked to a specific Ito formulation. We also used the dynamic clamp to subtract the native Ito in canine epicardial cells. Such “blockade” eliminated the epicardial action potential notch, but had no significant effect on the APD. We conclude that Ito, while being a key regulator of phase 1 repolarization, does not significantly affect the APD of canine ventricular myocytes. We further conclude that, contrary to the findings of earlier studies, Ito does not produce a density-dependent shortening of the APD in guinea pig ventricular cells. We also investigated the role of Ito in regulating the contractile properties of ventricular myocytes.

29 Presenilin 2 Regulates the Systolic Function of the Heart by Modulating Calcium Signaling

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Genetic studies of families with familial Alzheimer’s disease have implicated presenilin 2 (PS2) in the pathogenesis of this disease. PS2 is ubiquitously expressed in various tissues including hearts, but the physiological functions of PS2 in hearts have not yet been characterized. In this study, we examined cardiac phenotypes of PS2 knockout (PS2KO) mice in order to elucidate a role of PS2 in hearts. PS2KO mice are viable and fertile and have a normal appearance. Histological examination of the PS2KO hearts showed no evidence of cardiac hypertrophy or fibrosis. There were no differences in the ratios of heart weight to body weight and left ventricular weight to body weight between PS2KO and wild type (WT) mice. Although functional improvements were similar among the three treated groups 6 months after treatment, the restoration of cardiac apelin and APJ expression was observed only in ARB group. Furthermore, in angiotensin II - infused rats, cardiac apelin mRNA was decreased after 24 hr administration and its restoration was obtained by ARB treatment. Conclusion: These results indicate that cardiac apelin system is markedly down regulated in experimental HF and may be regulated by angiotensin II-angiotensin type 1 receptor system directly. Renin-angiotensin system inhibition may have beneficial effects, at least in part, through the restoration of cardiac apelin system in the treatment of HF.

Our preliminary studies strongly suggest that, contrary to the current view, Ito acts as a negative rather than a positive regulator of myocyte contractility. Taken together, our findings have important implications for understanding the role of Ito in both cardiac physiology and heart failure.
Calcium Signaling in Cardiotrophin-1–Induced Cardiac Myofibroblast Cell Migration

Darren H Freed, Larry V Hyslop, Univ of Manitoba, Winnipeg, Canada; Wayne R Giles, Univ of California, San Diego, La Jolla, CA; Ian M Dixon; Univ of Manitoba, Winnipeg, Canada

Background: Cardiac fibroblast migration is an integral part of post-myocardial infarction wound healing. Cell movement is a complex process involving actin-mycin cross bridge cycling which is at least partly regulated by myosin light chain phosphorylation, which in turn is regulated by calmodulin/myosin light chain kinase and intracellular calcium. Cardiotrophin-1 (CT-1), a member of the IL-6 family of cytokines is expressed very early in the infract zone following coronary artery occlusion and induces cell proliferation and reduced collagen expression. The relationships between CT-1, membrane potential, intracellular calcium and cell migration have not been explored. Materials and Methods: Cardiac fibroblasts were isolated from the ventricles of Sprague-Dawley rats and used after the first passage (myofibroblasts). Cell migration was assessed with a Boyden chamber. The status of myosin light chain phosphorylation was assessed using Western analysis. Membrane potential was assessed using potentiometric Dibac (4)3 dye and intracellular calcium was estimated with Fluo-3. CT-1 induced migration of cardiac fibroblasts. The migratory response could be inhibited with ML-7, a myosin light chain inhibitor. CT-1 treatment caused increased myosin light chain phosphorylation which could be attenuated by treatment with ML-7, removal of extracellular calcium or inhibition of extracellular calcium (BAPTA). CT-1 induced complex membrane potential changes consisting of initial depolarization followed by profound hyperpolarization. CT-1 caused a modest increase in intracellular calcium, although it was sufficient to produce near maximal myosin light chain phosphorylation. Conclusions: Although CT-1 is a modest chemokine, it induces unusual changes in cell membrane potential and causes a rise in intracellular calcium. These changes culminate in cell movement. The mechanism of intracellular calcium flux and whether the rise in intracellular calcium was from extra or intra-cellular sources is not clear.

Situs inversus with G-Protein Receptor Kinase-2 Gene Ablation Suggests a Critical Regulatory Role for α-adrenergic Receptor Signaling in Embryonic Asymmetry

Eric W Brunskll, Justin Klalance, Daniel Hammer, Gerald W Dorn, II; The Univ of Cincinnati College of Med, Cincinnati, OH

Situs inversus with G-protein receptor kinase-2 gene ablation suggests a critical regulatory role for α-adrenergic receptor signaling in embryonic asymmetry. Background: The G protein-coupled receptor kinase (GRK) family mediates agonist-dependent phosphorylation of membrane G protein-coupled receptors. Gene targeting experiments revealed important functions for the seven known GRKs in diverse biological processes. Unlike other GRK knockouts, deletion of GRK2 resulted in embryonic lethality (~ E13.5), suggesting a unique and essential function for this GRK in fetal development. The specific mechanism for this essential GRK function is unknown. Results/Methods: A conditional knockout of GRK2 was created, placing loxP sites flanking exons 3 and 6. Cre-mediated deletion of these exons results in a frame shift, altering protein coding. To verify GRK2 deletion, we tested whether ubiquitous deletion of GRK2 recapitulated the previously demonstrated phenotype. GRK2−/− mice were crossed with β-actin-Cre transgenic mice that ubiquitously express Cre recombinase to generate heterozygous GRK2+/− mice. Analysis of multiple litters derived from heterozygous GRK2+/− crosses failed to generate the expected Mendelian ratios of 1:2:1, resulting instead in 70% (28/40) GRK2−/−, 30% (12/40) GRK2+/−, and 0% (0/40) GRK2+/+ animals. Ten-day embryos from timed pregnancies of heterozygous GRK2+/− litters were compared to wildtype embryos. Strikingly, there was a ~50% incidence of situs inversus in GRK2−/− embryos, indicating randomization of asymmetry determination in the absence of GRK2. It has previously been reported that treatment of cultured early rat embryos with phorbol ester or norepinephrine results in a randomization of asymmetry. Summary: Our results, combined with the previous observation that adrenergic agonists can induce situs inversus, suggest a novel regulatory pathway in embryonic left/right axis determination. We are testing the hypothesis that loss of GRK2 function results in loss of G protein-α-adrenergic activity, and hence loss of the regional differences in calcium that are critical for the expression of left-right dependent signaling molecules.

Redox Regulation of Protein Kinase C in Myocardial Ischemia/Reperfusion

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Protein kinase C (PKC), a key signaling kinase, is one of those subjected to redox control representing a new paradigm of the alternate signaling principle. We were among the first to establish that ROS directly act on PKC, releasing chelated zinc ions from the zinc finger of the regulatory domain. Our studies led to the unexpected and intriguing hypothesis suggesting that in addition to serving a structural function, zinc ions are likely to play a dynamic regulatory role. The evidence obtained by us clearly defines cysteine-rich domain as a redox sensor and a reversible redox switch. We extend our findings to the whole heart model and now demonstrate physiological implication of zinc movements during myocardial ischemia/reperfusion. Methods: Langendorff perfused adult rat and mouse hearts were subjected to 15 min global ischemia followed by 20 min reperfusion (Rf). Free Zn2+ in isolated cardiomyocytes and cryo-sections was assessed by confocal microscopy using TSM as a probe. Results: TSM fluorescence in cryo-sections originated from sarcomeric units, cell periphery, and intercalated disks similar to the pattern observed in isolated cells. Oxidatively triggered Zn2+ release was reduced by reversion with N-acetylcysteine. Areas with irregular morphology were observed in I/R tissue sections. TSM decorated zones of cell contacts, as well as patchy areas containing granulated vesicle-like structures. Fascinatingly, overall fluorescence intensity was much lower in I/R stressed heart compared to control. PMA treatment significantly increased TSM fluorescence of tissue sections obtained from control hearts (90% increase) but not from the ones after I/R (14% increase). The transgenic mouse model with altered function of PKCε revealed preservation of zinc response by activated kinase. Conclusions: Being an integrated composite of redox signaling systems, free zinc reflects the protein redox status and serves a valid biomarker of stressed tissue and its capacity to respond to stimulus. Our approach to investigate functional zinc and its movements in situ would apply to human myocardial tissue samples. We believe that this set up the prototype model for the potential future studies from those samples would provide clues to individual approach to cardioprotection.

Ubiquitination and Degradation of Protein Kinase C ε is Modulated by Phosphorylation of This Protective Protein Kinase C (PKCe). The ubiquitination and degradation of PKCe was assessed by immunoprecipitation (IP) and immunoblotting (IB) with ubiquitin and PKCe antibodies. The rate of PKCe degradation was found to be tightly coupled with its ubiquitination and was significantly inhibited by two structurally independent inhibitors of the proteasome system, clasto-lactacystin β-lactone and MG132. Selective activation of PKCe in cardiac cells enhanced PKCe phosphorylation and reduced PKCe protein degradation, demonstrating that phosphorylation of PKCe renders this kinase less susceptible to degradation. The loss of effect of phosphorylation on proteasome dependent degradation was also confirmed by phosphorylation site mutagenesis studies on PKCe. The half-life of PKCe and PKCe T566A (a mutation which prevents phosphorylation of this residue) in COS7 cells was determined by pulse-chase experiments to be ~22 and ~8 hours respectively. Furthermore, cardiac tissues from PKCe transgenic mice exhibiting a cardioprotective phenotype showed increased PKCe activity, elevated PKCe phosphorylation, decreased PKCe ubiquitination, and reduced PKCe degradation compared with those in wild type control mice. In contrast, cardiac tissues from I-1 expressing dominant negative I-1 transgenic mice displayed reduced PKCe activity, attenuated PKCe phosphorylation, and enhanced PKCe ubiquitination. Taken together, these data demonstrate that the ubiquitin-proteasome system modulates PKCe degradation in cardiac cells and that activation of PKCe in cardioprotection attenuates the degradation of this kinase, suggesting a possible critical role of the UPS in sustained activation of protective proteins and thereby cardioprotection.

Functional Significance of a New PKC-ε Phosphorylation Site on Inhibitor-1 of Cardiac Protein Phosphatase-1

Patricia Rodriguez, Bryan Mitton, Evangelia Kranias; Univ of Cincinnati, Cincinnati, OH

Protein phosphatase 1 (PP1) plays a pivotal role in the development of heart failure. PP1 is regulated in vivo by inhibitor-1 (I-1). When I-1 is phosphorylated at Thr-35 by PKA it inhibits PP1. However, recent studies reported that I-1 can also be phosphorylated by PKC-ε on Ser-67 and suggested that this may decrease the ability of I-1 to inhibit PP1. Given that both PKC-ε and PP1 activities are implicated in cardiac failure, we further examined PKC-ε mediated phosphorylation of I-1, using purified proteins. We cloned and expressed cDNAs, encoding human I-1 or an I-1 mutant with alanine substitution at Ser-67. The recombinant proteins were purified and the GST-tag was removed. PKC-ε phosphorylation of the pure proteins indicated that +P incorporation into the mutant was decreased but not completely abolished in comparison to the I-1 wild type, suggesting that there may be another PKC-ε phosphorylation site. For identification of this putative PKC-ε site, phosphorylated human I-1 was subjected to matrix-assisted laser desorption ionization mass spectrometry in combination with Edman degradation. These analyses revealed threonine-75 as a new PKC-ε site on human I-1. To confirm these data, I-1 mutants with alanine substitutions at Thr-75 (T75A), and Ser-67 plus Thr-75 (S67A/T75A) were generated. PKC-ε treatment of I-1 and its mutants showed reduced +P incorporation into both S67A/T75A and none in the S67A/T75A +P. Further analyses by two-dimensional electrophoresis corroborated that: 1) Thr-75 is a PKC-ε site; and 2) Ser-67 and Thr-75 are the only residues phosphorylated by PKC-ε on human I-1. To determine the functional significance of Thr-75 phosphorylation, the PKC-ε assay were performed. Phosphorylation of I-1 or I-1 mutants by PKA was
associated with inhibition of PP1. However, PKCα phosphorylation of I-1 had no effect on its activity. Furthermore, PKC-δ phosphorylation had no effect on the PKA-mediated inhibitory function of I-1. These in vitro findings suggest that there exists an additional PKC-κ site in human I-1 but its phosphorylation has no significant effect on its inhibitory activity. Future in vivo experiments may further elucidate the functional role of this new I-1 phosphorylation site.

Omega-3 Fatty Acids Inhibit Protein Kinase A and Calcium Calmodulin Kinase II Activities and Improve Survival Following Myocardial Infarction

Rafat A Siddiqui, Nargiz Ruzmetov, Kevin A Harvey, Mustapha Zerouga, Colin Terry, Neal Patel, Methodist Rsch Inst, Indianapolis, IN; Gary P Zalogo, Methodist Rsch Inst, Indianapolis, IN

Epidemiological and clinical data suggest that omega-3 polyunsaturated long chain fatty acids (n-3 PUFAs) decrease sudden death in patients with coronary artery disease following myocardial infarction. However, the mechanisms for the beneficial effects of n-3 PUFAs are unknown. The objectives of the present study were to confirm the findings from clinical trials using an animal model of myocardial infarction in which dietary intake could be closely controlled and to utilize this model to investigate molecular mechanisms for the beneficial effects of n-3 PUFAs. Using an animal model of myocardial infarction (i.e. coronary ligation), we report that a diet high in n-3 PUFAs was associated with a significant improvement in six-month mortality compared with animals consuming a diet high in n-6 PUFAs [86.5% (32 out of 37) vs 64.9% (24 out of 37), p < 0.01]. Plasma samples and cardiac tissues of animals maintained on n-6 PUFAs or n-3 PUFAs diets exhibited significantly elevated I-6 PUFAs or n-3 PUFAs levels, respectively. The improved survival was associated with decreased activity of proteins kinase A and calcium-calmodulin dependent kinase II. Total kinase activity was decreased by 40% (n = 10/group, P < 0.05) whereas PKC activities did not change significantly. We also found that heart-specific ryanodine receptor 2 phosphorylation was decreased in the omega-3 diet group by ~20% (n = 10/group, P < 0.05) and by 46% (n = 10/group, P < 0.05), respectively, whereas PKC activity did not change significantly.

AMPK-Kinase Phosphorylation Correlates with Arrest Duration and Return of Spontaneous Circulation in a Murine Model of Cardiac Arrest

Kimberly R Wojcik, Jason P Alvarado, Danhong Zhao, Huashan Wang, Benjamin S Univ, Indianapolis, IN; Gary P Zalogo, Methodist Rsch Inst, Indianapolis, IN

Cardiac arrest accounts for approximately 300,000 deaths each year in the United States. Efforts to improve survival require an understanding of the cellular mechanisms of intra- and post-arrest injury, which may impact return of spontaneous circulation (ROSC) and post-resuscitation injury. We established a murine model of KCl-induced circulatory arrest to investigate the role of the stress kinase AMPK. AMPK is a highly conserved heterotrimeric kinase consisting of one catalytic subunit (AMPK-α) and two regulatory subunits (AMPK-β and AMPK-γ). This kinase is activated by stimuli that increase cellular AMP-ATP ratio and is thus a sensor of cellular energy status. Phosphorylation of AMPK-α at Thr172 and AMPK-β1 at Ser108 are required for the complete activation of the enzyme in this role as a critical regulator of energy levels within eukaryotic cells. As ischemia is known to decrease AMP levels, we examined the phosphorylation state of both AMPK-α and AMPK-β1 in the hearts of mice in response to the effects of induced cardiac arrest.

Phosphorylation at the major regulatory site (Thr172) of AMPK-α was seen following either 5 or 8 min of arrest. Upon ROSC, this phosphorylation level decreased and reached baseline levels by 2 hr post-ROSC. Phosphorylation of AMPK-β1 at Ser108 followed similar patterns. Furthermore, phosphorylation levels of both AMPK-α and AMPK-β1 remained elevated in cardiac tissues of mice that failed to achieve ROSC. We have previously reported data showing that Akt phosphorylation declined immediately upon arrest and returned to high levels shortly after ROSC. Our work supports previous reports suggesting an inverse relationship in Akt and AMPK activity in conditions of cardiac ischemia/reperfusion injury. Interactions between this stress and survival kinase may be major determinants of ROSC and post-resuscitation injury and survival from cardiac arrest.

Gene Dysregulation on Bicuspid Aortic Valve

Salah A Mohamed, Hans H Sievers; Univ zu SH-Luebeck, Luebeck, Germany

Background: The bicuspid aortic valve (BAV) is the most common congenital cardiac malformation, caused by fusion of valve cushions at the onset of valvulogenesis. The pathogenesis is still unclear and the genetic basis still unknown. Search for a potential candidate gene was suggested the UFD1L gene was the fusion of zone in Embryogenesis. Knock-down of the UFD1L gene as orthologous to those found in zebrafish is discussed. Method:Aortic valves were collected during surgery from 38 patients with BAV (mean age 36.8 ± 18.1 years) and from 38 patients with tricuspid aortic valves (TAV, mean age 61.7 ± 16.1 years). Fluorescence in situ hybridisation (FISH) was performed for microdeletion, reverse transcriptase polymerase chain reaction (RT-PCR) for gene expression, Western blot for protein analysis, and antisense morpholino oligonucleotides for knock-down studies. Results: There was a microdeletion on the critical region of chromosome 22 containing the UFD1L gene. UFD1L gene expression was significantly reduced in BAV (median 786.88 fold change) compared with TAV (10886.79 fold change, p < 0.001). UFD1L protein was also significantly reduced in BAV (3.9 ± 2.6 vs 6.4 ± 4.8 optical density, p < 0.05). Zebrafish embryos injected with Ufd1l specific antisense morpholino oligonucleotides showed fatal heart malformations detected by 72 hours postfertilization resulting in loss of cells and an increase of apoptosis. Conclusions: UFD1L gene is not deleted but its expression is downregulated in BAV, supporting the hypothesis that BAV may be a genetic disorder with the UFD1L gene as one potential candidate gene involved in BAV pathogenesis. UFD1L gene knock-down in zebrafish embryos suggests its central role in the migration and coordination of cardiac neural crest cells.

Accumulation of 4977 BP Deletion in the Aging Heart

Salah A Mohamed, Thorsten Hanke, Michael Scharfschwerdt, Hans H Sievers; Univ zu SH-Luebeck, Luebeck, Germany

Objective: Mitochondrial DNA (mtDNA) mutations proposed as a potential contributor of aging. However, in an age-dependent increase of the common deletion (4977 bp deletion) of the human mtDNA still many unanswered questions remain. Purpose of this study was the analysis of mtDNA copy number and deletion in three regions of the heart which were compared with other neuronal tissues. We also analyzed the effects of oxidative stress (OS) on human transformed fibroblasts (WI38VA). Materials and Methods: Tissues from 69 patients (age 48 ± 25.22) were studied (16 familial cases were subjected to DNA extraction and 60% of mtDNA was detected by real-time PCR). On the other hand, OS induced morphological damage of mitochondria, programmed suicide cell death (apoptosis). Conclusion-
Bone Marrow Mesenchymal Stem Cells (MSCs) have been used in myocardial regeneration. The fate of these cells after in vivo injection needs further studies. Our goal is to track and examine the gene expression of autologous pig MSCs injected into pig myocardium. Pig MSCs were cultured. They were labeled by overnight incubation in the culture medium with 2.5 μM of ferumoxides injectable solution (Feridex, Berlex Labs.) to allow for subsequent in vivo visualization using Magnetic Resonance Imaging (MRI). The 52 x 10^6 cells were injected into the anterior wall of one pig heart via the coronary sinus using a novel continuous infusion system induced murine hypertension in adults through a novel transfection agent.

### Tracking and Gene Expression Profile of Bone Marrow Mesenchymal Stem Cells Injected into Pig Myocardium

**Elie R Chemaly, Davide Gianni, Djamel Lebeche, Irina Pomerantseva, Massachusetts General Hosp, Charlestown, MA; Ryuichi Yoneyama, Yoshiaki Kawaue, Kozo Hoshino, Motoya Hayase, Massachusetts General Hosp, Boston, MA; Federica del Monte, Roger J Hajjar; Massachusetts General Hosp, Charlestown, MA**

Bone Marrow Mesenchymal Stem Cells (MSCs) have been used in myocardial regeneration. The fate of these cells after in vivo injection needs further studies. Our goal is to track and examine the gene expression of autologous pig MSCs injected into pig myocardium. Pig MSCs were cultured. They were labeled by overnight incubation in the culture medium with 2.5 μM of ferumoxides injectable solution (Feridex, Berlex Labs.) to allow for subsequent in vivo visualization using Magnetic Resonance Imaging (MRI). The 52 x 10^6 cells were injected into the anterior wall of one pig heart via the coronary sinus using a novel continuous infusion system induced murine hypertension in adults through a novel transfection agent.

### A Direct and Easy Method in Drug Screening

**Bingsheng Chang; UT Southwestern Med Ctr, Dallas, TX**

A novel drug screening model is developed in this study. Imaging ellipsometry is a new optical surface-sensitive method, which is used to study the macromolecules adsorption on solid surfaces and the cross-talk of biomolecules. Compared with traditional surface based methods like immunohistochemistry, immunofluorescence and enzyme-linked immunoabsorbent assays, reagents' labeling is not needed in this new method. And most importantly, the state of interaction among the biomolecules is directly observed via the computer monitor. Human interleukin 6 (hIL-6), which displays a broad range biological activities on many kinds of cells, is a kind of important cytokine. Moreover, hIL-6 is involved in many pathology processes of disease such as inflammation, rheumatoid arthritis, tumor, cardiovascular diseases and autoimmune diseases. hIL-6 exhibits its role on target cells through a double-stranded receptor complex consisting of IL-6R and a signal transduction subunit. In this study, human interleukin 6 was first adsorbed in the surface of silicon slide, and then blocked with BSA contained 0.2% Tween 20. The soluble human interleukin 6 receptor isolated from S. lividans, natural products and anti-human interleukin6 receptor antibody were subsequently added. Then the slide was monitored by a biosensor based on imaging ellipsometry. To identify the antagonist of sIL-6R from natural products, we have already developed a screening model with recombinant hIL-6 and hIL-6R from insect cells as target based on the competitive receptor-ligand interactions before (Chang BS, Li Y, Acta Academiae Medicinae Sinciae, 2001 Feb;23(1):36-9). Using that sandwich-like screening model, we have screened more than 15000 microorganisms metabolic products and found 10 positive candidates. These findings can be easily confirmed in this new model. These two methods double-checked the possible candidates. This new method can be used in biological molecules high throughput screening. And it will provide an easy, efficiency and reliable approach in drug study in the near future.

### Selective Inhibition of cGMP-Dependent Protein Kinase 1x In Vivo in Adult Mice Induces Endothelial Dysfunction and Hypertension

**Ping Lu, Kian-Keong Poh, Marcy Silver, Gangian Qin, Michael E Mendelsohn, Yan Zhu, St. Elizabeth’s Med Ctr and Tufts Univ Sch of Med, Boston, MA**

**Introduction:** Nitric Oxide and its derivatives regulate blood pressure through eventual activation of cyclic guanosine monophosphate dependent protein kinase (PKG). However, the role of PKG and its downstream target(s) remained unclear. Though conventional PKG knowledge supports its involvement in the regulation of PKG in adult has not been demonstrated previously. We aim to derive selective inhibitors of PKG by fusing membrane-permeable TAT-domain and an eight amino-acid PKG-inhibitor domain, LRK0000H, previously identified by Dostmann and coworkers, and delivering the peptide in vivo to elucidate its role in vasorelaxation and blood pressure regulation. Methods: An expression vector was constructed using polymerase chain reaction-based mutagenesis to produce the inhibitor peptide in bacteria. Affinity purified peptide was tested in ex vivo vascular ring assays for its ability to prevent endothelium-dependent relaxation and delivered in vivo by a micro-osmotic pump to evaluate its role on blood pressure regulation in adult mice. Results: In vitro study demonstrated that the TAT-PKG inhibitor peptide effectively blocked phosphorylation of the kinase. Ex vivo, using vascular ring assays, nitric oxide dependent relaxation in murine aorta was inhibited by 25% with TAT-PKG inhibitor (sham 76±11%, n=8, TAT-PKG inhibitor 51±13%, n=8). The in vivo effect of TAT-PKG inhibitor on murine blood pressure was investigated by continuous intravenous infusion of 500 μg of the inhibitor over 72 hours. This was delivered using micro-osmotic pump implanted subcutaneously and connected via tailored polyethylene tubing inserted into the right internal jugular vein. In 20-week-old mice (8) infused with the inhibitor, the average blood pressure increased by 36±24/31±30 mmHg from 108±14/92±19 to 145±13/123±19 mmHg; whereas in mice infused with either saline (4) or TAT-green fluorescent protein (4), the average blood pressure remained the same, from 117±21/101±26 to 119±22/196±30 mmHg, ANOVA P<0.001. Conclusions: High specificity peptide inhibitor of PKG in vitro when delivered by a novel continuous infusion system induced murine hypertension in adults through inhibition of nitric oxide mediated relaxation.

### Nitric Oxide Synthase-3 is Essential for Antihypertrophic Effect of Chronic Phosphodiesterase 5A Inhibition

**Eki Takimoto, David A Kass; Johns Hopkins Med Inst, Baltimore, MD**

Phosphodiesterase (PDE) 5A is a cyclic GMP catalytic enzyme and its inhibitors are widely used to treat erectile dysfunction. PDE5A also exists in cardiac myocytes and modulates

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**CT FOR MYOSIN HEAVY CHAIN (β-MHC) SAMPLES**

<table>
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<tr>
<th>Cultured MSCs</th>
<th>Cardiac Tissue</th>
<th>LCM interstitial</th>
<th>LCM myocardium</th>
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<td>u-Actin</td>
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**CT for Connexin 43 samples**

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<th>LCM interstitial</th>
<th>LCM myocardium</th>
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<td>26.74</td>
<td>15.51</td>
<td>38.10</td>
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cardiac physiologic response to various stimuli. We recently reported that PDE5 inhibition (PDE5A-I) potently blunts acute beta-adrenergic stimulated cardiac contractility by nitric oxide synthase-3 (NOS3) dependent mechanism. We also reported that chronic PDE5A-I attenuates pressure load-induced cardiac hypertrophy. To test the hypothesis that NOS3 is essential for cGMP modulation by PDE5A-I in attenuation of cardiac hypertrophy, we examined the effect of PDE5 inhibitor (EMD 3652) on isoproterenol induced- or load induced- cardiac hypertrophy in wild type or NOS3 null mice (NOS3-/-). Concomitant administration of EMD 3652 (3.5g/kg/day) largely blunted this response in WT, but not in NOS3-/- mice. In vivo heart analysis revealed that increases in heart rate, systolic blood pressure (SBP) and contractility induced by ISO were all attenuated by PDE5A-I in WT, while no effect of PDE5A-I was observed in NOS3-/-.

Concomitant administration of hyaluridine (50mg/L in drinking water) to WT partially reduced SBP increase induced by chronic ISO, but did not change hypertrophic response, suggesting that PDE5A-I directly inhibits ISO-induced myocardial hypertrophy. PDE5A activity is upregulated by increased intracellular cGMP levels in many different cell types. To test the hypothesis that PDE5A-I inhibits ISO-induced cardiac hypertrophy we measured in the vasculature and kidney of a separate group of normal and failing dogs.

BNP leads to a similar, yet more consistent increase in UNaV (H11022 approached BL values (28.7±14 vs. 15.1±14 vs 138±83 pg/mol). Following Sild (H11001) 25mg/kg p.o., UNaV measured in the vasculature and kidney of a separate group of normal and failing dogs. BNP).

Heart failure.

PDE5A inhibition may represent a novel therapeutic target both acutely and chronically in kidney. This may in part explain natriuretic peptide resistance in heart failure, and why reduced following sild (H11001) dramatically from BL to CHF (6.6±0.6 pg/mol; *).

Increased (H11006) BNP reduced mPAP (-8.3±3.6 mmHg; *), and PCWP (17±4.1 mmHg), while ISO (0.05 VS TAC) 0.05 VS ISO, † P 0.02). In controls no significant phosphorylation in any enzyme was observed. FLT-1 positive capillaries were significantly decreased at 1, 1.5, and 3 months post transplantation. In contrast, ERK1/2-activity phosphorylation remained unchanged throughout the experiment (p>0.2). Akt/PKB activity significantly increased after 60 min CA by 8.5±2.2 (p=0.006) and decreased thereafter to reach baseline levels at 120 min post CPB. In contrast, ERK1/2-activity remained unchanged during ischemia but increased significantly during 30 min repurifusation by 4.8±6.1U (p<0.02). In controls no significant phosphorylation in any enzyme tested was observed. Conclusions: CA activates myocardial eNOS and thus contributes to cardiac NO generation. Although ERK1/2 has been suggested to inactivate NOS by eNOS phosphorylation, our results do not support this hypothesis. Therefore, improving myocardial protection during CA/CPB by manipulating the eNOS-NO-pathway will have to focus on Akt/PKB.

The hemodynamic and renal derangements of CHF progress despite marked increases in BNP levels, implying a state of natriuretic peptide resistance. This may be due

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The hemodynamic and renal derangements of CHF progress despite marked increases in BNP levels, implying a state of natriuretic peptide resistance. This may be due
Early Coronary Endothelial Apoptosis in Cardiac Transplants

Uwe M Fischer, Albert Antonyan, Hans J Geiseler, Univ of Cologne, Cologne, Germany; Wilhem Blobch, German Sports Univ, Cologne, Germany; Uwe Mellhorns; Univ of Cologne, Cologne, Germany

Objectives: Coronary endothelial dysfunction is a marker for cardiac allograft vasculopathy (CAV) in orthotopic heart transplant recipients. Apoptosis activation in endothelial cells of the graft has been suggested as a symptom of acute rejection. We screened endomyocardial biopsies taken during the first year post transplantation for myocardial apoptosis induction in heart transplant recipients. Methods: We analyzed endomyocardial biopsies from 20 heart transplant recipients, taken for routine rejection monitoring during the first twelve months post-transplantation (7 and 14 days, 1, 6 and 12 months, respectively). LV specimen were immunocytochemically stained against activated caspase-3 (apoptosis key-enzyme). Cardiomyocytes were then quantitatively investigated using TV densitometry and caspase-3 positive capillaries were counted per viewfield (cpv). In addition, ISHL grade was determined. Results: After one week the amount of caspase-3 positive capillaries was increased by 2.36 ± 0.98 cpv and decreased significantly during the second week (p = 0.001). During the first six months caspase-3 positive capillaries increased at 1st month 0.89 ± 1.34 cpv and at 6th month 2.89 ± 1.39 cpv, respectively) to finally decrease at 1 year post-transplantation (0.08 ± 0.51 cpv, p < 0.001 vs 1st week). There was no correlation between the number of caspase-3 positive capillaries and the corresponding ISHL grade. TV densitometry did not show caspase-3-activation in cardiomyocytes. Conclusions: Our data show apoptosis induction in heart transplant coronary endothelium but not in cardiomyocytes. Caspase activity did not reflect rejection status. However, as coronary endothelial dysfunction is an important symptom of cardiac allograft vasculopathy (CAV), assessment of vascular apoptosis induction might serve as an early marker for CAV independent of the ISHL grading system.

Inducible NOS Does Not Influence the Chronic Response to Systolic Overload in Mice

Yingjie Chen, Ping Zhang, Xin Xu, Robert J Bache; Univ of Minnesota, St. Paul, MN

Previous studies have demonstrated that inducible nitric oxide synthase (iNOS) can be expressed in cardiac myocytes and endocardial endothelium of patients and animals with hypertrophy and heart failure. However, the effect of overexpression of iNOS on cardiac function is controversial. Thus, one study reported that cardiac-specific overexpression of iNOS had no effect on heart size or ventricular function, while another study reported that cardiac-specific overexpression of iNOS resulted in myocardial hypertrophy and fibrosis, ventricular dilatation, block and sudden death. The effect of iNOS on the response to cardiac pressure overload has not been previously reported. Consequently, this study was performed to determine whether iNOS influences the development of ventricular hypertrophy in the overloaded heart. Studies were performed in male iNOS deficient and wild type mice of similar genetic background. There was no difference in heart weight, lung weight, aortic pressure or left ventricular (LV) function between iNOS deficient mice and wild type mice under unstressed conditions. Moderate LV pressure overload was produced by transverse aortic constriction (TAC) by ligating the aorta over a 25G needle. Four weeks after TAC heart size and the ratio of heart weight to body weight were increased over 50% in both strains. Although iNOS deficient mice had a higher left ventricular hypertrophy than wild type mice, this difference was not significant. No differences were observed in cardiac function measured echocardiographically or in myocardial fibrosis. However, early mortality was observed in iNOS/- mice (4 of 11), as compared with no mortality in wild type mice. Western blot demonstrated that both eNOS and dimethylenine nine dimethylaminohydrolase 1 were increased after TAC, while caveolin-1, PRMT1 and nNOS were unchanged with no differences between the groups. iNOS was undetectable in both iNOS deficient and wild type mice 4 weeks after TAC when chronic compensated hypertrophy was present. Our data imply that iNOS does not exert a negative impact on the chronic response to moderate LV pressure overload that resulted in stable compensated hypertrophy, but did appear to influence the acute response to systolic overload.

Cardiac Myosin Binding Protein-C Phosphorylation and Cardiac Function

Sadayappan Satkivel, James Gulick, Hanna Osinska, Raisa Klevitsky, Lisa A Martin, Wilhelm Blobch, German Sports Univ, Cologne, Germany; Uwe Mehlhorn; Univ of Cologne, Cologne, Germany; Uwe M Fischer, Albert Antonyan, Hans J Geisseler, Univ of Cologne, Cologne, Germany; Uwe Mellhorns; Univ of Cologne, Cologne, Germany

Background. The cardiac isoform of myosin binding protein-C (MyBP-C) differs from the skeletal isoform in that it has a small insertion near the carboxyl terminus that contains 3 phosphorylatable serines (Ser-273, -282, -302). However, the functional correlates of skeletal isoform in that it has a small insertion near the carboxyl terminus that contains 3 phosphorylatable serines (Ser-273, -282, -302). However, the functional correlates of MyBP-C phosphorylation remain obscure. Hypothesis. The loss of MyBP-C phosphorylation will lead to altered cardiac function. Methods. We generated transgenic mice with cardiac-specific expression of a mutated MyBP-C in which the 3 serines were changed to alanines (MyBP-C-AllPminus). These mice were then bred with MyBP-C nulls (MyBP-C/WT) to ensure the absence of endogenous phosphorylatable MyBP-C. An animal that expressed the normal cardiac isoform, MyBP-C-WT, was also bred to the nulls to serve as a control.

Results. MyBP-C-AllPminus was incorporated normally into the cardiac sarcomere. Phosphorylation is essential for normal MyBP-C function as MyBP-C-AllPminus was unable to rescue the null phenotype. The mice developed signs of heart failure with myofibrillar disarray and fibrosis, similar to what was observed in the null animals. In contrast, MyBP-C/WT in the MyBP-C(WT) background resulted in complete rescue of the null phenotype. Echocardiography showed significantly depressed shortening fractions in both the MyBP-C-AllPminus and the MyBP-C(WT) background, with no differences between the groups. MyBP-C phosphorylation was decreased by 40–70% in various heart failure models, which included transverse aortic constriction, ischemic reperfusion, the calcium transgenic mouse, and the MLP knock-out mouse models. Electron microscopy showed severe alterations in the M-line as well as focal areas of sarcocemeter disorganization in the MyBP-C-AllPminus hearts compared to MyBP-C/WT. These data suggest that MyBP-C-phosphorylation is essential for normal cardiac function.

Novel Cardioprotective Effects by the Small Heat Shock Protein Hsp20

Guo-Chang Fan, Qunying Yuan, Jian Qian, Persoula Nicolau, Univ of Cincinnati, Cincinnati, OH; Guojie Song, Univ of Dublin, Dublin, Ireland; Guoli Chen, Guoxiang Chu, Evangelia K Kranias, Univ of Cincinnati, Cincinnati, OH

Small heat-shock proteins have been shown to render cardioprotection against stress-induced injury. We recently reported that a small heat-shock protein Hsp20 was associated with β-adrenergic stimulation of the heart, and adenosine-mediated gene delivery of Hsp20 protected isolated cardiomyocytes against β-adrenergic-induced apoptosis. To investigate whether Hsp20 exerts protective effects in vivo, we generated a transgenic mouse model with cardiac-specific overexpression of Hsp20 (TG). Transgenic (TG) and wild-type (WT) hearts were then subjected to global no-flow ischemia/reperfusion (IF: 45min/120min). Compared with controls, TG hearts exhibited improved recovery of contractile performance over the entire reperfusion period. This improvement was accompanied by a 2-fold decrease in lactate dehydrogenase released from TG hearts. The extent of infarction and apoptotic cell death was also significantly decreased, which was associated with increased ratio of Bcl-2/Bax and reduced Caspase-3 activity in TG hearts. To further test the effects of Hsp20 on the hypertrophic response induced by β-adrenergic signaling, we infused isoproterenol (5mg/g/day) to TG and WT mice for 14 days. TG hearts displayed an attenuated cardiac hypertrophic response, as evidenced by decreases in 1) heart weight/body weight; 2) myocyte cross-sectional areas; and 3) mRNA levels of ANF and BNP. Both Mason’s trichrome staining and immunostaining with anti-collagen type I/II revealed marked reduction of interstitial fibrosis in TG hearts. In addition, the number of TUNEL positive cells in TG hearts was reduced by 2 fold, compared to WT hearts. Furthermore, chronic β-adrenergistimulation resulted in impaired heart function in WT mice, whereas Hsp20-TG mice maintained their myocardial function. Taken together, our findings indicate that overexpression of Hsp20 in the heart 1) protects the heart against ischemia/reperfusion injury, possibly due to its potent antiapoptotic properties; 2) attenuates the induction of myocardial hypertrophy in response to sustained β-adrenergic stimulation. Thus, Hsp20 may represent a promising therapeutic target for cardiac diseases.

Relaxin Targets Cardiac Myofilaments through a Protein Kinase C-Dependent Pathway and Decreases Myofilament-Associated Protein Kinase C-α and -β (II)

Eryn E Shaw, Fenghua Yang, W Glen Pyle; Univ of Guelph, Guelph, Canada

Circulating relaxin (RLX) increases in the first trimester of human pregnancies, correlating with increased cardiac output. A similar increase in RLX occurs in the failing heart, at levels that parallel the degree of failure. Changes in cardiac myofilament function often underlie myocardial dysfunction associated with heart failure. It is unknown if RLX produces its effects through alterations in cardiac myofilaments, or that intracellular molecular signals transduce its message in myocardium. The purpose of this study was to determine what effect RLX has on myofilament function, and to elucidate the intracellular signaling cascade activated by RLX in the ventricles. Murine ventricles were treated with 30 nM recombinant H-2 RLX and the myofilament fraction isolated by centrifugation. Ca2+-dependent actomyosin ATPase activity was not altered by RLX, nor was myofibrillar Ca2+ sensitivity. However, inhibition by chelex of Ca2+ mobilization by RLX treatment revealed an increase in myofilament Ca2+-sensitivity. Immunoblot analysis of myofilament fractions found that PKC-α and PKC-β levels were decreased by 31% and 26% respectively after RLX treatment. Troponin I, troponin T, and myosin binding C-protein all had increased levels of phosphorylation following RLX treatment. CHEL abolished all changes in myofilament protein phosphorylation, except for troponin T. These results demonstrate for the first time that RLX targets cardiac myofilaments through a PKC-dependent pathway, as well as an undetermined cascade. Moreover, we found that RLX inhibits myofilament-association of two putative signals of heart failure, namely...
PKC-α and PKC-β2. Previous studies showing a positive inotropic effect of RLX, coupled with our results showing no change in actomyosin ATPase activity, suggests that RLX may increase myocardial efficiency. In short, these results are consistent with the hypothesis that RLX is a compensatory player in the failing heart.

Curcumin Attenuates Neointimal Development and Collagen Content Following Arterial Injury in Rats

Nair Sreerajay, Xiaoping Yang, D Paul Thomas, Susan X Zhang, Bruce W Culver, Jun Ren, Univ of Wyoming, Laramie, WY; David Tulie, North Carolina Central Univ, Durham, NC

Curcumin (diferuloyl methane), the major component of the spice Turmeric (Curcuma longa), is known for its potent anti-inflammatory and antioxidant activities. We have previously shown that curcumin is a potent inhibitor of platelet-derived growth factor (PDGF)-induced migration and proliferation of vascular smooth muscle cells. Since migration and proliferation of smooth muscle cells are critical events in the pathogenesis of atherosclerosis and restenosis following angioplasty, we investigated the ability of curcumin to alleviate the response to vascular injury in vivo. Rats were subjected to left common carotid artery balloon-injury and the exposed artery was topically treated with polyethylene glycol (PEG) hydrogel or PEG hydrogel containing curcumin (72 μg) in random fashion. Animals were sacrificed and tissues were obtained fourteen days post-injury and used for morphometric analysis. Neointimal formation was significantly attenuated by perivascular curcumin compared to vehicle controls (neointima to medial area ratios 1.00 ± 0.09 versus 1.43 ± 0.03 for curcumin treated and controls, respectively, p = 0.012; n = 10). Collagen content in injured curcumin-treated vessels was significantly lower compared to injured vehicle treated vessels as assessed by Western blotting 2 days post-injury (densitometry data: 0.240 ± 0.03 versus 0.345 ± 0.08, respectively; p < 0.05). In vitro data support these results by showing curcumin inhibits PDGF-stimulated migration, proliferation, collagen synthesis and cytoskeletal reorganization of vascular smooth muscle cells. Taken together, these data strongly suggest that curcumin may be an effective multi-functional therapeutic agent used for minimizing or preventing intimal growth following balloon injury.

Development of AAV-Mediated Gene Therapy for Murine Models of Inherited Diseases Affecting the Heart

Christina A Pacak, Cathryn Mah, Gabriel Gaitsch, Melissa Lewis, Raquel Torres, Rahul Kanadia, Maurice Swanson, Univ of Florida, Gainesville, FL; Kevin Campbell, Univ of Iowa, Iowa City, IA; Glenn Walter, Barry Byrne; Univ of Florida, Gainesville, FL

Introduction: The long term goal of this project is to develop a clinically relevant gene therapy approach for the treatment of genetic diseases affecting the heart. The phenotypes of 3 murine models for cardiac dysfunction; the alpha-sarcoglycan (ASG-/-) knockout model for limb girdle muscular dystrophy Type 2D (LGMD-2D), the muscular dystrophy (MBNL-/-) knockout model for Myotonic Dystrophy and the acid-alpha glucosidase (GAA-/-) knockout model for Pompe Disease are currently being characterized. Due to its small size, safety and proven ability to persist for long periods of time in muscle, adeno-associated virus (AAV) has emerged as a promising cardiac gene delivery vehicle. We have sought to determine the most advantageous combination AAV serotype vector and delivery route for the specific transduction of cardiomyocytes in vivo and are in the process of treating our disease models. Methods: Our 3 murine models of inherited myopathies have been characterized functionally and morphologically using MRI, ECG and histological techniques. To assess optimal AAV-mediated cardiac transduction, both intra-venous (iv) and intra-cardiac (ic) injection routes were compared by injecting 1x10^11 and 5x10^10 transgene particles of AAV-CMV-LacZ per mouse neonate of 3 different serotypes AAV1, AAV8 and AAV9. Tissue analysis included both x-gal staining on tissue sections to visualize expression and the quantitative β-galactosidase enzyme detection assay. Results: Our results show that of the serotypes and delivery route combinations analyzed, iv administration of AAV9 results in 30-fold more efficient transduction of cardiac tissue. Moreover, hearts injected with AAV9 displayed a global distribution of transgene expression suggesting this serotype has no transduction site preference within cardiac tissue. In contrast, regional abundance of AAV1 and AAV8 transgene expression was suggestive of cardiac uptake. There was a weak correlation between ME and NE cardiac transduction gradient in HF (479±346 pg/ml) was ~four-fold higher versus values for non-HF. After tilt stress, in contrast to further increase in NE trans-cardiac gradient, ME trans-cardiac gradient was reduced to ~20±9 pg/ml (n=9, p=0.04), suggestive of cardiac uptake. There was a weak correlation between ME and NE trans-cardiac gradients (R2=0.04, p=0.05, n=8). Conclusions: 1. Myocardial ME but not ARF-Phe is reduced in end stage HF. 2. Serum levels of ME are reduced in HF. 3. Reduction of ME in HF coincides with sympathetic neural over-activity.

Decreased Cardiac Met-Enkephalin Levels Coincide with Sympathetic Overactivity in Human Heart Failure

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Met-enkephalin (ME) is the main peptide derived from proenkephalin and is produced by heart cells and by neurons terminating in the heart. ME is co-released with noradrenaline (NE) from sympathetic neurons and counteracts the chronotropic, vasoconstrictive, and positive inotropic effects of NE. In heart failure (HF), sympathetic nervous system over-activity augments NE release, increasing “spill-over” into coronary sinus (CS) plasma, and depletes cardiac tissue of NE despite increased NE production. CS and cardiac levels of ME in HF have not been previously measured. Aims: 1. To measure cardiac and serum ME levels in HF and non-HF patients. 2. To compare trans-cardiac gradients of ME and NE in HF following tilt table-induced stress. Methods: Cardiac biopsies were obtained from explant (end-stage) HF hearts (n=12) post-transplantation and from non-HF hearts (n=10). In a separate cohort of patients with moderate to severe HF, CS and radial artery blood samples (n=9) were obtained. ME and ME-Arg-Phe levels were measured via 125I radioimmunoassay. ME was determined using HPLC. Results: Cardiac ME content (pg/g tissue, measured by Western blotting) was lower in wet weights of HF hearts compared to non-HF hearts (LA: 777±910 vs 1564±953, p=0.09; RA: 1149±1700 vs 1789±1307 p=0.17; LV: 606.5±438 vs 12637±6274, p=0.001; RV: 518.3±310 vs 1821.8±1227, p=0.002). In contrast to ME, regional abundance of ME-Arg-Phe (also derived from proenkephalin) did not differ significantly between HF and non-HF hearts. In vitro, before tilt table-induced stress (10min, 20°tlt), CS and arterial (n=9) ME levels were 19.9±3 pg/ml and 10.9±1 pg/ml, respectively, compared to 52.25±36 pg/ml in venous samples from healthy subjects (n=6, p=0.01). NE trans-cardiac gradient in HF (479±346 pg/ml) was ~four-fold higher versus values for non-HF. After tilt stress, in contrast to further increase in NE trans-cardiac gradient, ME trans-cardiac gradient was reduced to ~20±9 pg/ml (n=9, p=0.04), suggestive of cardiac uptake. There was a weak correlation between ME and NE trans-cardiac gradients (R2=0.04, p=0.05, n=8). Conclusions: 1. Myocardial ME but not ARF-Phe is reduced in end stage HF. 2. Serum levels of ME are reduced in HF. 3. Reduction of ME in HF coincides with sympathetic neural over-activity.

Differentially Expressed Sarcoplasmic Reticulum Proteins in Human Heart Failure

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Abnormalities of sarcoplasmic reticulum (SR) proteins modulating Ca2+ handling are known to produce profound effects on cardiac function and heart failure (HF). In the complex environment of the SR, protein-protein interaction and post translational modifications may have an important impact in the process of HF. We used a combination of subcellular fractionation and proteomics to analyze differentially expressed SR proteins and their post-translational modifications. We identified 13 differentially expressed SR proteins obtained from hearts explanted from 5 patients with dilated cardiomyopathy (DCM) and 5 non-failing “donor” hearts. We modified a previously described SR preparation method of sequential ultra-centrifugation to adapt it for the purification of SR vesicles from human myocardium. SR proteins were extracted using a combination of detergents such as C12E8, DPHC and CHAPS. SR ATPase activity was measured to validate the integrity of proteins using different detergents. The supernatant and pellet fractions were then separated on 2-0 and 1-0 gels respectively. Gels were stained with Sypro Ruby and image analysis was performed using Progenesis® software to identify differentially expressed proteins. Annotated proteins were excised and analyzed by LC-MS/MS for protein identification and post-translational modifications. We obtained purified SR vesicles from 10 g of frozen myocardial tissue (protein range: 0.7–1.7 mg/ml, ATPase activity range: 32–130 mmol/mg/min). Image analysis of the 10 gels revealed different band distribution in samples prepared using DPHC compared to samples prepared with C12E8 suggesting different extraction capabilities. Over 40 protein spots were significantly differentially expressed in failing vs non-failing hearts. In summary, the use of the combination of detergents appears to have the potential to improve the analysis of cardiac SR proteins. The initial analysis is focusing on the changes in protein expression levels and identification of post-translational modifications associated with the development of HF. Unraveling the protein complexity of SR in HF may help unravel the mechanisms for the development and progression of HF as well as devise appropriate treatment strategies. 1. Stokes et al JBC 1996

Lipid-Oxidizing Chlamydia Antibodies as a New Drug Target in the Treatment of Atherosclerosis

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A sub set of Chlamydia antibodies which cross-react with human low density lipoproteins, LDL, and cause oxidation of both of these targets were isolated from human atherosclerotic lesions and from the serum of patients with clinical complications of atherosclerosis. These antibodies catalyzed an oxidation of LDL in apparent enzymatic fashion, called further lipoxidizing antibodies. Their Kd was 0.69 ± 0.012 mg LDL cholesterol. Their Vmax was 0.83 ± 0.105 μM of malondialdehyde per hour, i.e. in a range of physiological concentration of the substrate and in a “real-time” range of bio-catalysis. The role of these Antibodies in pathogenesis of Atherosclerosis was validated in a number of clinical trials where it was shown that their catalytic oxidative activity coincided with the stenosis of both arteries in patients with Coronary Heart Disease, CHD, and Carotid Ischaemic Disease. Moreover, this activity correlated with the severity of these conditions and was most prominent in patients with unstable anghia, myocardial infarction and stroke. The final validation data came from a randomised open trial, Phase Ila, when the Abzyme
subendothelial layer; internal elastic lamina disruption, the smooth muscle cells invaded mitochondrion swelling, chromatin concentrating; matrix and collagen fibers hyperplasia in cells dropping, endothelial cell membrane breaking, endoplasmic reticulum dilatation, significantly. Under transmission electron microscope, external iliac arteries showed endothelial of the ultrastructural lesion in smooth muscle cells support the concept that endothelium-dependent dilation but also endothelium-independent dilation. The evidence

5.93% vs 23.70
7.02%, p

After 80 days, the ultrastructural changes of external iliac arteries were observed through high resolution ultrasound. endothelium dependent/independent dilation were examined by high resolution ultrasound. Nitric oxides

Conclusions

Characterization of a Cyclical Dosing Model of Cardiotoxicity

Stefanie Bonigut, Xiaochen Wang, Theresa Deisher; Amgen, Seattle, WA

In order to rapidly screen for compounds, which might prevent doxorubicin-induced cardiac toxicity, we utilized a single high dose retro orbital injection of DOX, which induces cardiac dysfunction within two to five days. The compounds were dosed every two days using this schedule. We discovered that DOX treatment was cardiotoxic, as evidenced by increased caspase 3 activity and decreased fractional shortening. However, when DOX was administered every five days, cardiac function was restored. These results suggest that cyclical dosing of DOX may be a valuable strategy for reducing cardiotoxicity.

Active ROCK-1 is Generated by Caspase 3 Cleavage in Human and Mouse Heart Failure and Disruption of ROCK-1 Attenuates Myocyte Apoptosis

Jiang Chang, Inst of Biosciences and Technology, Texas A&M Univ System Health Science Ctr, Houston, TX; Lei Wei, Min Xie, Baylor College of Med, Houston, TX; Viraj R Shah, Inst of Biosciences and Technology, Texas A&M Univ System Health Science Ctr, Houston, TX; Michael D Schneider, Mark L Entman, Baylor College of Med, Houston, TX; Robert J Schwartz, Inst of Biosciences and Technology, Texas A&M Univ System Health Science Ctr, Houston, TX

Recent findings suggest that rho-associated kinase, ROCK-1, is a direct substrate of caspase 3 and is cleaved into a 130 kDa fragment in conferring cytotoxicity in apoptotic non-cardiac cells. We evaluated the contribution of ROCK-1 cleavage to cardiomyocyte apoptosis and heart failure. ROCK-1 protein levels were assessed from patients with end-stage heart failure and normal hearts. ROCK-1 was cleaved into a 130 kDa fragment in failing hearts but remained uncleaved in normal hearts and was restored in the failing hearts with left ventricular assist device. ROCK-1 cleavage was detected in neonatal rat cardiomyocytes subjected to doxorubicin, a potent apoptotic inducer, or doxorubicin plus caspase 3, ROCK-1 cleavage was blocked in the presence of a caspase 3 inhibitor, indicating that caspase 3 activation is responsible for ROCK-1 cleavage in cultured cardiomyocytes. We further analyzed hearts from three transgenic mouse lines, cMHC-Gq, cMHC-HK and bi-transgenic cMHC-Gq-HK. The bi-transgenic mice displayed a significant increase in caspase 3-mediated apoptosis rates compared to other two lines. A significant increase in caspase 3 activity was observed in bi-transgenic mice which paralleled ROCK-1 cleavage. This was not observed in HK, Gq and wild type mice. These results suggest that ROCK-1 cleavage is associated with increased caspase 3 activity in human and murine failing hearts. To explore the functional significance of ROCK-1 in apoptosis, ROCK-1 knockout mice were generated. The deficiency of ROCK-1 significantly decreased cardiac apoptotic levels induced by pressure overload. Consistent with these results, in cultured cardiomyocytes, ROCK-1 active mutant mimicking caspase 3-mediated cleavage is sufficient to induce caspase 3 activation and myocyte apoptosis; the specific blockade of ROCK-1 by siRNA dramatically attenuated ceramide-induced apoptosis. Furthermore, phosphorylation of serum response factor (SRF) by active ROCK-1 facilitated SRF cleavage by caspase 3, turning SRF into a dominant negative transcription factor. Our studies indicate that caspase 3-dependent cleavage and activation of ROCK-1 may play a central role in the development of heart failure through facilitating cardiomyocyte apoptosis.

Cardio-Specific Overexpression of Sir2 (Silent Information Regulator 2) Induces Antiapoptotic and Antiaging Effects in Transgenic Mice

Ralph Alcendor, Chull Hong, Jing Lui, Jurichi Sadoshima; UMDNJ, Newark, NJ

Sir2, a class III histone deacetylase, functions in a variety of cellular processes, including gene silencing, cell differentiation, and life span extension in many organisms, including yeast, nematodes, and fruit flies. Sir2 deacetylates p53 and the Forkhead box class O family transcription factors, thereby regulating transcription of anti-oxidants and apoptotic cell death in mammalian cells. We have recently shown that overexpressing Sir2 in transgenic mouse hearts protects cultured neonatal cardiac myocytes from serum starvation-induced apoptosis in vitro. To investigate whether or not Sir2 overexpression exerts anti-apoptotic and anti-aging effects in the heart in vivo, we generated transgenic mice with cardiac specific overexpression of Sir2 α (Tg-Sir2 α), using the α-myosin heavy chain promoter. We made two lines, in which Sir2 α was overexpressed specifically in the heart. In both lines, the heart exhibited normal morphology and left ventricular (LV) function compared to non-transgenic mice (NTg). To investigate whether Sir2 α exerts anti-apoptotic effects, we performed TUNEL staining. Hearts from Tg-Sir2 α exhibited less TUNEL positive myocytes than those from NTg after thoracic aortic banding for 10 days (0.10% vs 0.18%). Tg-Sir2 α showed a significantly higher expression of anti-apoptotic proteins, including Bcl2 (2.6 fold), and Bcl-xL (1.9 fold) suggesting that Tg-Sir2 α mice are protected from apoptosis. We also investigated the effect of Sir2 α overexpression on the cellular aging markers, p15 and p19. Hearts from Tg-Sir2 α exhibited significant increase in caspase 3 activity and reduced fractional shortening. These results suggest that Sir2 α not only protects the heart from apoptosis but also has anti-aging effects in cardiac myocytes in vivo.

Role of Nuclear Calcium/Calmodulin-Dependent Protein Kinase II Delta B in Doxorubicin-Induced Cardiomyopathy

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Calcium/calmodulin-dependent protein kinases (CaMKs) are calcium-activated enzymes that play a central role in cardiac contractility. Among them, the delta B subunit of CaMKII (CaMKII δB) is the predominant isoform in heart muscle. However, the role of CaMKII δB in pathophysiological processes remains to be elucidated. We report that nuclear CaMKII δB regulates gene transcription in heart muscle and is a novel target of the antineoplastic drug doxorubicin (Adriamycin). Microarray and quantitative RT-PCR analyses revealed that CaMKII δB is down-regulated in the hearts of rats that develop cardiomyopathy following chronic treatment with doxorubicin. The loss of kinase parallels the decrease in fractional shortening measured by transthoracic echocardiography as well as tissue damage estimated by electron microscopy. In primary neonatal rat cardiomyocytes, constitutive active CaMKII δB increases MeF2-dependent transcription and activity of multiple clusters of genes, including the tumor suppressor protein p53 and apoptosis mediators. This study identifies CaMKII δB as a novel target of doxorubicin and suggest that cardiac toxicity and apoptosis induced by...
oxurubicin are mediated by silencing of the CaMK pathway and possibly by class II HDAC recruitment.

Bcl2 is a Preferred Gene Target for the Transcription Factor GATA4

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Doxorubicin (DOX) is a potent anti-tumor drug known to cause heart failure after chronic chemotherapy. The cardiac enriched transcription factor GATA-4 regulates the expression of many cardiac-specific genes. Recent studies have shown that GATA4 protects cardiomyocytes from DOX toxicity. The anti-apoptotic protein Bcl-xl is suggested to mediate the protective effect of GATA4. This study aims to examine if the prototype anti-apoptotic protein Bcl2 is also a downstream drager of GATA4. We show that Bcl2 and Bcl-xl are depleted by DOX in cultured neonatal rat ventricular cardiomyocytes (NRVC), and this depletion is reversed by adenovirus-mediated GATA4 over-expression. In contrast, the basal protein levels of Bcl2 and Bcl-xl are moderately increased by GATA4, suggesting that both Bcl2 and Bcl-xl are potential downstream effectors of GATA4 in cultured NRVC. However, we show that the preferred gene target of GATA4 compared to Bcl-xl. An adenovirus encoding a short hairpin RNA is used to specifically knock down GATA4 in NRVC. The results show that the RNAi-mediated GATA4 knockdown reduces Bcl2 protein levels, but does not have any effect on Bcl-xl levels, suggesting that GATA4 is required only for Bcl2 but not for Bcl-xl constitutive gene expression despite the fact that GATA4 over-expression is able to up-regulate both genes in cultured NRVC. At the whole animal level, both Bcl2 mRNA and protein levels are drastically up-regulated in GATA4 expressing transgenic (TG) hearts compared with wild type (WT) hearts. Surprisingly, the Bcl-xl mRNA and protein levels are not affected by GATA4 over-expression. Together, these findings suggest that Bcl2 is a preferred target gene that may mediate the protective effect of GATA4 in vitro and in vivo. Consistent with this notion, injection of DOX (20 mg/kg) into mice markedly reduces the mRNA and protein levels of Bcl2 in WT hearts but not in GATA4 TG hearts. In contrast, DOX does not affect Bcl-xl mRNA and protein levels in either WT or GATA4 TG hearts, suggesting that Bcl-xl may not be involved in DOX-induced cardiotoxicity in vivo. Our ongoing research will attempt to establish a novel molecular paradigm in which GATA4 protects the heart from DOX toxicity through up-regulation of the anti-apoptotic gene Bcl2.

LATS2 Induces Cardiac Myocyte Apoptosis: The Role of the Mst1-hWW45-LATS2 Signaling Complex in Mediating Cardiac Myocyte Apoptosis

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Mammalian sterile 20-kd kinase 1 (Mst1) plays an important role in mediating apoptosis in cardiac myocytes. Persistent activation of Mst1 in the heart leads to dilated cardiomyopathy in vivo. Thus far, the downstream signaling mechanism mediating the proapoptotic effect of Mst1 is poorly understood. Hippo, a Drosophila homologue of Mst1, forms a signaling complex with Salvador and Warts, which in turn stimulates cell death and inhibits cell proliferation in fruit flies. Thus, the goal of this study was to clarify whether the proapoptotic effect of Mst1 is mediated by hWW45 and LATS2, suggesting that LATS2 plays an important role in mediating the proapoptotic effects of Mst1 in cardiac myocytes. LATS2 might be a promising therapeutic target to prevent Mst1-mediated cardiac myocyte apoptosis.

Endonuclease G Serves as an Ischemic Necrosis Cell Death Factor During Human MI

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The diagnosis of an acute MI in patients who die suddenly can be difficult because the infarct heart tissue will appear normal post-mortem. Usually, gross and histological evidence of ischemic (e.g. coagulative) necrosis is not seen at autopsy unless the MI victim survives many hours before dying. During this time interval, endogenous nuclease(s) extensively degrade RNA and DNA in the dying myocyte that alter the histological staining of the cell and make the cell appear necrotic. The nuclease(s) that destroy nuclear acids during ischemic necrosis and the mechanism by which they become active remain poorly understood. In order to identify these activities, we collected small samples of left ventricular heart tissue from a series of 30 randomly selected human autopsies. Five of these cases showed histologic evidence of an acute MI. Three of these five cases had elevated serum troponin levels prior to death. The other 25 hearts appeared unremarkable. For all 30 cases, we prepared nuclear and cytoplasmic fractions of protein from each heart sample, and carried out a variety of biochemical assays to identify nuclease activity in vitro. We identified potent DNA endonuclease activity in the nuclear and cytoplasmic fractions of the five MI cases in the other 25 cases. Western blot analysis and the enzymatic and biochemical traits of the nuclease recovered from the infarct indicate that it corresponds to Endonuclease G, a 31-kDa Mg2+−dependent DNA/RNA endonuclease that largely resides in the intermede space of mitochondria. Endonuclease G has been shown by others to be released from mitochondria and to translocate to nuclei during apoptosis in non-myocytes. Our study strongly suggests that Endonuclease G likely serves as a death factor in ischemic necrosis in the heart. In addition to Endonuclease G, we have also identified two distinct mitochondrial exonuclease activities in the nuclear fraction during infarction. These results indicate that during human MI, mitochondrial nuclease activities are apparently largely responsible for the extensive breakdown of nuclear DNA that occurs during an early stage of ischemic necrosis. Remarkably, these activities remain active many hours after death and detectable in heart samples taken at autopsy.

Regulation of Nuclear Factor κB Gene Transcription by Class I HDACs in Ventricular Myocytes

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Programmed cell death or apoptosis has been recognized to play a central role in a number of cardiovascular pathologies including heart failure. Transcription factor NF-κB is known to activate a survival pathway in mammalian cells. In this report we show that the transcription potential of the p65 subunit of NF-κB is reduced in the class I histone deacetylases (HDACs). We demonstrate that expression of the p65 subunit of NF-κB resulted in a 10 fold increase in NF-κB dependent transcription in post-natal ventricular myocytes that was repressed to basal level by HDAC 1 and HDAC 2 activity. Inhibition of HDAC activity with trichostatin A (100nM TSA) abrogated the inhibitory effects of HDAC on NF-κB dependent gene activation. Mutations in the catalytic domains of HDAC 1 (H141A), crucial for de-acetylase, de-repressed the inhibitory effects of HDAC on NF-κB gene activation. Immunoprecipitation assays revealed that HDAC1 but not HDAC 2 formed protein-protein interactions with p65 subunit of NF-κB. Inhibition of HDAC activity was observed in post-natal ventricular myocytes subjected to hypoxia. To our knowledge the data provide the first evidence that survival pathways mediated by NF-κB are regulated by class I HDACs in post-natal ventricular myocytes.

Reversal of Amyloid-Induced Heart Failure in a Conditional Model of CryAB R120G Desmin-Related Cardiomyopathy

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Background. An R120G missense mutation in the small heat shock protein alpha-B-crystallin (CryAB) causes a desmin-related cardiomyopathy (DMR) that is characterized by the formation of aggregates containing CryAB and desmin. The mutant CryAB protein leads to the formation of inclusion bodies, which contain amyloid oligomer intermediates (amyloid oligomer), as found in Alzheimer’s patients as well as other amyloid-related degenerative diseases. Hypothesis. Mechanisms of amyloid corexist in the cardiomyocyte. Removal of the primary pathogen, CryAB R120G, after symptoms of heart failure have developed will result in recovery that is concomitant with a decrease of the toxic amyloid oligomer. Methods and Results. To address the relationship between amyloid oligomer formation, cardiac function and survival in CryAB R120G DMR, an inducible transgenic mouse was created using the Tet activator and modified alpha myosin heavy chain promoter responder constructs. Mice expressing the mutant CryAB protein in the cardiomyocytes showed CryAB positive aggregates, which also contained the oligomer amyloid. These mice also showed decreased fractional shortening, as well as other symptoms of developing heart failure. Blockade of mutant CryAB expression in the symptomatic mice improved cardiac function and prevented premature death in all of the mice. This rescue was accompanied by significant decreases in amyloid levels in the cardiomyocytes. In contrast, there was no obvious decrease in inclusion body levels.

Abstract Presentations
Conclusion. These data, taken together with data showing cellular toxicity of the CryAB R120G oligomer amyloid in vitro demonstrate that it is the amyloid oligomer that is the primary toxic entity in CryAB R120G DRM and not the inclusion bodies. Blocking oligomer formation may be a new therapeutic strategy for rescuing the DRM as well as other types of amyloid-like degenerative diseases.

Mechanism for In Vivo and In Vitro Transcriptional Regulation of the Nix Mitochondrial Death Gene in Cardiac Myocytes

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Background: Nix, a pro-apoptotic member of the Bcl-2 family, was first identified as being upregulated in Gq-mediated cardiac hypertrophy. Nix is necessary for peripartum apoptosis. Cardiac myocytes have a high mitochondrial content and are prone to ischaemic injury. In this study, we examined the transcriptional regulation of Nix by agents that influence mitochondrial function.

Methods/Results: We cloned the complete Nix gene promoter. Computational analysis revealed numerous Sp1 consensus binding sites and GC boxes, typical of other TATA-less promoters. No HFF-1 binding sites were found. Five serial 5' deletion Nix promoter (Nix-P) constructs were generated and cloned into the pGL3Basic luciferase vector for transfection into neonatal rat cardiac myocytes (NRMCs). A Nix promoter activity decreased with length of the promoter constructs. To test PKC reactivity, Nix-P-transfected NRMC were treated with 10 nM PMA or vehicle. PMA (24h) significantly increased Nix luciferase activity for -5386, -3877, -1012, and -377 Nix-P, but not -210 Nix-P. PMA-induced transcriptional activation was inhibited by Milrinonyl, a compound that prevents Sp1 binding to DNA. In vivo determinants of Nix expression were evaluated in two separate transgenic mouse lines expressing -5386 Nix-luciferase. Basal Nix reporter gene activity was modest in all organs sampled, consistent with ubiquitous low-level gene expression seen in many tissue Northern blot. To evaluate stimulated Nix gene expression, Nix-P-luc mice were subjected to infarction-reperfusion (I/R; 1h/24h), transverse aortic coarctation (TAC; 4 days), 108 9 mm Hg), and cross-breeding with Gq overexpressers. Gq-Nix-P hearts showed a 3.2±0.4 fold increase in luciferase activity. Likewise, TAC hearts showed a 2.8±0.4 and a 2.3±0.5 fold increase in luciferase activity in two different lines. In contrast, VR did not increase Nix luciferase activity in the heart. Summary: Cardiac Nix expression is induced by pressure overload and Gq overexpression, both Gq-mediated events. PKC, which is downstream of Nix overexpression, likely working through Sp1.

Cardioprotective Role of the Mitochondrial ATP-Binding Cassette Protein-1

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The mechanism by which mitochondria exert protection against ischemia and oxidant stress is not clear. We recently showed that a partially pure mitochondrial fraction containing five co-immunoprecipitating proteins (succinate dehydrogenase, adenine nucleotide translocator, ATP synthase, inorganic phosphate carrier and mitochondrial ATP-binding cassette protein 1 or mABC1) displayed mitoKATP channel activity. mABC1, a member of the ABC family of proteins, is the only protein in this complex whose function is not known. A yeast homologue of mABC1 protein, Mdt1p, was recently identified to have a novel role for induction of cellular resistance to oxidant stress. Based on these observations, we hypothesized that mABC1 plays a key role in protection of myocardial cells against oxidant stress. We studied the function of mABC1 by modulating the levels of this protein in neonatal rat cardiomyocytes (NRMCs) using various molecular techniques. To functionally assess the effects, mitochondrial membrane potential was measured by flow cytometric analysis of tetramethylrhodamine ethyl ester (TMRE)-loaded cells. DNA interference results in reduced protein levels, and was associated with significantly attenuated loss of TMRE fluorescence under basal conditions. In contrast, adenoviral-mediated expression of mABC1 resulted in protection against oxidant stress induced loss of TMRE fluorescence. Mdt1p, inhibitors (5-hydroxydecanote and glybenclamide) failed to reverse the effects of mABC1 overexpression, suggesting that mABC1 probably exerts its protective effects independently of mdt1p. These observations support the notion that mABC1 protein plays a major role in cellular protection against oxidant stress, identifying mABC1 as a novel target for cardioprotective therapeutics.

Intracellular Angiotensin II-Induced Cell Proliferation Independent of Angiotensin Type-1 Receptor

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We recently reported that angiotensin II (Ang II) could act as an intracellular agonist and produce cardiac hypertrophy when generated intracellularly. AT1 receptor antagonist (losartan) did not inhibit intracellular Ang II-induced myocyte growth and cardiac hypertrophy. To further determine the requirement of AT1, for intracellular effects, we studied Ang II-induced cell proliferation in CHO-K1 and CHO-AT1 (CHO-K1 stably expressing AT1) cells. Radioligand binding assay showed that native CHO-K1 cells expressed insignificant levels of AT1, and did not respond to extracellular Ang II. CHO-AT1 cells, expressing AT1 (S395 = 1229 fmol/mg protein), showed cell proliferation (54 ± 14 %) when exposed to extracellular Ang II. However, when transiently transfected with an expression vector that produced Ang II intracellularly, both cell types showed enhanced proliferation (31 ± 11 % and 35 ± 10 % for CHO-K1 and CHO-AT1, respectively), as measured by Wst-1 assay. Losartan (10 M), added to the media, failed to inhibit extracellular Ang II, but did not have any effect on cell proliferation. To further confirm these findings, CHO-K1 and CHO-AT1 cells were stably transfected for intracellular Ang II expression. Stable clones producing similar levels of intracellular Ang II, CHO-K1 and CHO-AT1-A (from CHO-K1 cells), and CHO-AT1-B (from CHO-AT1 cells), were chosen for further studies. Cells were seeded in 24-well plates, placed in serum-free medium and numbers counted by Coulter counter. The cells were counted every 24 h up to 72 h. CHO-K1 and AT1-A, cells showed a 1.85 ± 0.23 and 1.73 ± 0.24 fold increase in cell number after 72 h, compared to CHO-K1 and CHO-A1, respectively. These observations were confirmed by flow cytometry maintained by a novel autoregulatory feedback loop. As mitoKATP transfection, losartan did not attenuate the faster growth rate of CHO-K1 and CHO-A1, cells. As we had reported with cardiac myocytes, extracellular Ang II showed an additional growth effect in CHO-K1-A1 cells; which could be selectively blocked by losartan. These data suggest AT1-independence of growth promoting effects of Ang II, which may have significant implications for understanding the renin-angiotensin system.

ERK Activation Prevents the Positive Feedback Loop of Phosphodiesterase 3 and Induces CAMP Early Repressor and Attenuates Cardiomyocyte Apoptosis

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Myocyte apoptosis plays an important role in pathologic cardiac remodeling and the progression of heart failure. CAMP is crucial in the regulation of myocyte apoptosis. PDEs play important roles in regulating the amplitude, duration, and the compartmentalization of CAMP. We have previously found that expression of cAMP hydrolizing PDE3A was significantly reduced in human failing hearts, accompanied by upregulation of inducible cAMP early repressor (ICER) expression. Neurohormonal stimulators, such as angiotensin II (Ang II) and β-adrenergic agonists isoproterenol (ISO), also induced persistent PDE3A downregulation and concomitant ICER upregulation in vitro, which is important in Ang II- and ISO-induced cardiomyocyte apoptosis. We report here that the persistent PDE3A reduction and ICER induction in vivo were validated by a novel autoregulatory feedback loop between PDE3A and ICER (named PDE3A-ICER feedback loop). ICER induction repressed PDE3A gene transcription. PDE3A downregulation activated cAMP/ PKA signaling, leading to ICER upregulation via PKA-dependent stabilization of ICER. We further show that PDE3A-ICER feedback loop is essential for Ang II-induced cardiomyocyte apoptosis. ISO and PDE3 inhibitors also induced PDE3A-ICER feedback loop and subsequent apoptosis. In contrast, insulin-like growth factor-1 (IGF-1) activates ERK5 kinase and prevents PDE3A-ICER feedback loop and apoptosis via upregulation of PDE3A. To determine the physiological relevance of ERK5 activation on PDE3A-ICER feedback loop, we investigated the role of ERK5 activation in PDE3A-ICER feedback loop, cardiomyocyte apoptosis, and cardiac function in transgenic mice with cardiac-selective overexpression of CA-MEK5a. We found that PDE3A downregulation and ICER upregulation induced by chronic pressure overload was prevented by MEK inhibition. Cardiomyocyte apoptosis and cardiac dysfunction were also significantly inhibited in CA-MEK5a transgenic mice. Our findings demonstrate a key role of ERK5-mediated PDE3A induction in the regulation of PDE3A-ICER feedback loop and cardiomyocyte survival, and suggest that strategies to block the PDE3A-ICER feedback loop will reduce apoptosis and benefit heart failure.

Apoptosis Signal-Regulating Kinase 1 is Involved Not Only in Apoptosis But Also in Nonapoptotic Cardiomyocyte Death

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Necrosis and apoptosis are the two major and distinct types of cell death in cardiomyocytes. Both necrosis and apoptosis have been reported to be involved in myocardial injury after ischemia-reperfusion. We recently demonstrated that the necrotic cell death, but not apoptotic cell death is a critical event in ischemia-reperfusion injury by examining a role of Cyclophilin D-dependent mitochondrial permeability transition. Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase that plays an important role in stress-induced apoptosis. In this study, we studied ASK1(−/−) mice to examine the role of ASK1 in ischemia-reperfusion injury. In the wild-type heart, ischemia-reperfusion resulted in necrotic injury, whereas infarct size was drastically reduced in the ASK1(−/−) heart. The necrotic injury was not accompanied with any evidence of apoptosis such as an increase in TUNEL, positive cells, DNA fragmentation, or the activation of caspase-3. ASK1(−/−) cardiomyocytes were more resistant to H2O2 or Ca2+ -induced apoptotic and non-apoptotic cell death compared with wild-type cells. These data suggest that ASK1 is involved in necrosis as well as apoptosis and that ASK1-dependent necrosis is likely to contribute to myocardial cell death in the ischemia-reperfusion heart.
Neurohormonal Regulation of Gq-Stimulated Cardiac Apoptosis through Multiple Kinase Signaling Pathways

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Background: Cardiac specific overexpression of Gq promotes cardiac apoptosis, leading to LV dilation, fulminant decompensation and lethal heart failure. Apoptotic cardiomyopathy in the peripartum period of Gq mice suggests a pregnancy-related neurohormonal stimulus. Therefore, the dopamine receptor 2 (D2) agonist bromocriptine, which is used clinically to suppress peripartal prolactin secretion, was administered to pregnant Gq/H9251 mice to assess the role of dopamine in cardiac remodeling of the peripartum state. Methods: Bromocriptine treatment recapitulates many of the physiological features of apoptotic peripartum cardiomyopathy in Gq mice. The distinct signaling profiles of peripartum cardiomyopathy and bromocriptine-treated Gq hearts suggest that both are caused by the dysregulation of Gq signaling. Results: Bromocriptine treatment resulted in abnormal diastolic performance, increased heart rate, and increased LV remodeling, MI expansion (Fig.) and cardiomyocytes hypertrophy to a greater degree than Gq mice in the absence of Gq. The addition of chronic pharmacological Gq stimulation might enhance the therapeutic effects of bromocriptine treatment in the heart. Conclusions: Pharmacological Gq stimulation recapitulates many of the physiological features of apoptotic peripartum cardiomyopathy in Gq mice. The distinct signaling profiles of peripartum cardiomyopathy and bromocriptine-treated Gq hearts suggest that both are caused by the dysregulation of Gq signaling.

Withdrawn
Acute ischemia/reperfusion experimental porcine model.

Reperfusion (min)

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importance of Sarcoplasmic Reticulum Calcium Handling in Vascular Smooth Muscle Proliferation

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Neointimal VSMC proliferation constitutes a primary cause of vascular disorders but, despite increasing knowledge about the cell-cycle regulation of VSMC, the molecular mechanism governing VSMC proliferation remains elusive. Acquisition of the proliferating phenotype by VSMC is associated with alterations in Ca2+-handling supported by modification of Ca2+-transporters expression, particularly, loss of the sarcoplasmic/endoplasmic reticulum calcium pump SERCA2a. To determine the importance of Sr Ca2+-handling in neointima formation, we have restored SERCA2a expression by gene transfer in the rat carotid artery after balloon injury. Morphometric and immunohistochemical analysis performed 2 weeks after gene delivery demonstrated that the lumen/media ratio was significantly lower in SERCA2a infected than in injured non-infected or injured giga-infected carotids (0.29±0.04 vs 0.89±0.19 and 0.72±0.14 respectively p<0.05), and was comparable to that observed in control carotids (0.21±0.03). The SERCA2a-infected carotids displayed a quiescent differentiated phenotype defined by its smooth muscle myosin heavy chain phenotype. Forced expression of SERCA2a suppressed serum-induced VSMC proliferation in culture and blocked cell cycle at the G1 phase. SERCA2a expression inactivates the Ca2+/calmodulin-dependent phosphatase PP2B and the transcriptional activity of its downstream target-NFAT (Nuclear Factor of activated T-cells), VIT, a NFAT-competing peptide, decreased PP2B function and inhibited proliferation. These data strongly suggest that NFAT transcriptional activity is necessary for VSMC proliferation. Furthermore, we found that SERCA2a also increased transcriptional activity of CREB (cAMP Responsive Element Binding Protein), favoring production of its downstream target, the cell death. Two protein families, the inner membrane adenine nucleotide translocase (ANT) and the outer membrane voltage dependent anion channel (VDAC), have garnered significant attention in the field of myocardial ischemia-reperfusion. The latter is known to contribute to the pathophysiological changes during various pathological situations such as myocardial ischemia-reperfusion. In this study, we investigated the role of VDAC in this process.

The Antioxidant EUK-8 Protects the Harlequin Mouse Mutant from Pressure Overload-Induced Heart Failure

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Background: Oxidative stress has been postulated to cause cell death and pathological remodeling in heart failure. We recently demonstrated that the Apoptosis-Inducing Factor-deficient harlequin mouse mutant has normal baseline myocardial function and morphology, but displays excessive myocardial oxidative stress in response to pithotic stimuli, which correlated with accelerated progression to heart failure. Results: We were able to demonstrate that AIF expression is downregulated in various pathophysiological situations such as pressure overload and in the MDP-deficient heart failure model. To examine whether antioxidant treatment can prevent cell death and heart failure due to AIF downregulation, harlequin mice and their wildtype counterparts were subjected to pressure overload (transverse aortic constriction) for 4 weeks, myocardial geometry and function was assessed by serial echocardiography, and mice were given low-dose EUK-8 (25 mg/kg,1.5-1) or vehicle for the duration of the study. EUK-8 prevented myocardial oxidative stress, attenuated necrotic and apoptotic cell death, improved left ventricle end-systolic dimensions, fractional shortening, and attenuated cardiac hypertrophy and fibrosis in mutant and wildtype mice. EUK-8 also improved survival in both harlequin AIF-deficient mutants and pressure overload injured mice. Conclusions: EUK-8 may be a therapeutic tool to treat pressure overload-induced pathological remodeling and cardiac decompensation.

No Catch Up of Physiological Cardiomyocyte Proliferation in Neonatal Rat Hearts after Suppression by Glucocorticosteroids

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Background: Glucocorticosteroids (mostly dexamethasone) are widely used to prevent pathologic cellular stresses, such as cardiac ischemia/reperfusion, can induce a defect known as mitochondrial permeability transition (MPT). MPT involves permeabilization of the inner membrane, mitochondrial swelling, and cytochrome c release and can lead to cell death. Two protein families, the inner membrane adenine nucleotide translocase (ANT) and outer membrane voltage dependent anion channel (VDAC), have garnered significant attention in the setting of MPT because of their abilities to function as non-selective channels. Despite recent studies that implicate prevention of MPT as a potential cardioprotective intervention, the molecular basis of this phenomenon in the heart remains unclear. In the present study we tested the hypothesis that a lower number of cardiomyocytes later in life is caused by a reduced cardiomyocyte proliferation and/or by early cell death. Pathological cellular stresses, such as cardiac ischemia/reperfusion, can induce a defect known as mitochondrial permeability transition (MPT). MPT involves permeabilization of the inner membrane, mitochondrial swelling, and cytochrome c release and can lead to cell death. Two protein families, the inner membrane adenine nucleotide translocase (ANT) and outer membrane voltage dependent anion channel (VDAC), have garnered significant attention in the setting of MPT because of their abilities to function as non-selective channels. Despite recent studies that implicate prevention of MPT as a potential cardioprotective intervention, the molecular basis of this phenomenon in the heart remains unclear.
unclear. To analyze intact MPT pore complexes, Percoll gradient-purified mitochondria were treated with the cognate cross-linker disuccinimidyl suberate (1:50, DDS-Protein) to fix proteins, membranes disrupted with 1% dodecylmaltoside to release the fusion complexes, and the complexes purified by sequential immunoprecipitation for ANP and VDAC. Following SDS-PAGE separation, proteins were identified by LC/MS/MS analysis of tryptic peptides and database mining. We observed multiple intact complexes containing putative MPT pore proteins, including pore-forming unit complex VDAC as well as other unidentified unrecognized members, such as K + channel beta 1 subunit and ATP synthase beta -transporter. Immunoblotting (IB) detected enhanced levels of creatine kinase and ATP synthase beta subunit in specific supramolecular complexes following treatment with the cross-linker, indicating the use of this approach to immobilize native complexes. To examine the relevance of these intact complexes to the phenomenon of MPT, we quantitatively analyzed (via IB) specific complexes containing ANP and VDAC in the normal heart and in hearts from genetically-cardioprotected mice (PKcε transgenic mice). The data indicate decreased formation of ANP-VDAC complexes in the protected heart, supporting the notion that decreased assembly of MPT complexes may promote cell survival. These studies represent, to our knowledge, the first proteomic characterization of the MPT pore subproteome in the heart and provide a framework of distinct native MPT pore complexes.

**Analysis of MCIP1 and MCIP2 Null Mice Suggests That MCIP Functions as an Enhancer of Calcineurin Signaling In Vivo**

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Modulatory Calcineurin-Interacting Proteins (MCIP) are a family of related proteins that are expressed preferentially in brain and striated muscles where they interact with the calcium/calmodulin-dependent phosphatase calcineurin. Overexpression of a truncated form of MCIP1 in the heart by transgenesis demonstrated a negative regulatory role toward cardiac hypertrophy through a mechanism involving suppression of endogenous calcineurin, while Mcip1 null mice were shown to both reduce assembly and calcineurin-regulated hypertrophic growth. To more carefully examine the functional role of MCIPs in vivo we generated Mcip1 and Mcip2 null mice, as well as double null mice. Loss of either Mcip1 or Mcip2, or both Mcip1/2 together each significantly blunted the cardiac hypertrophic response induced by pressure overload, suggesting that MCIPs might function as calcineurin activators at physiologic levels in vivo. Mcip1/2 null mice also showed reduced cardiac hypertrophy following thyroid hormone treatment, exercise induced by a forced swimming protocol, and AngII infusion. These reductions in heart weight were associated with a reduction in myocyte size as assessed by cross-sectional area measurements from histological sections. To further investigate the regulatory role of MCIPs in vivo, ischemia-reperfusion induced cell death in the heart was examined since calcineurin is thought to be protective. Loss of Mcip1, but not Mcip2, predisposed the heart to greater ischemia-reperfusion injury suggesting a positive regulatory role for MCIP1 toward calcineurin signaling in regulating cell death. Calcineurin also regulates fiber-type specificity of skeletal muscle. Loss of Mcip1/2 inhibited slow/oxidative fiber-type switching induced by swimming, similar to that observed in calcineurin Ab and Aa null mice. Taken together these data suggest that MCIPs are positive effectors of calcineurin signaling in vivo.

**Angiotensin II Destabilization of Cardiac Kv4.3 K + Channel mRNA Is Mediated by NADPH Oxidase**

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Hypertrophied cardiac myocytes exhibit prolonged action potentials and decreased transient outward potassium current mediated by Kv4 K + channels. We reported that Angiotensin II (Ang II), a known inducer of cardiac myocyte hypertrophy, down-regulates Kv4.3 K + channel mRNA and protein in cultured cardiac myocytes. Subsequent studies led us to hypothesize that Ang II acts indirectly through Ca2+ and PKC to destabilize Kv4.3 mRNA. Here, we implicate the 3' UTR (3' untranslated region) of Kv4.3 in the Ang II response and show that NADPH oxidase mediates this effect. RT-PCR was used to clone the 5 kb 3' UTR of the Kv4.3 gene in order to generate a 3' UTR-luciferase reporter construct. After transient transfection into primary cultured rat cardiac myocytes, Ang II was shown to destabilize Kv4.3 mRNA. Following-up experiments suggest that the Ang II effect is present in renal vascular smooth muscle, but not in nonmyocyte cell lines. Our studies of Ang II regulation of Kv4.3 suggest that NADPH oxidase acts via an ROS sensitive protein that is expressed specifically in cardiovascular myocytes to induce mRNA destabilization.

**Overexpression of an SHP2 Mutation in the Developing Mouse Heart Causes Ventricular Noncompaction and Ventricular Septal Defect**

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Mutations within SHP2, a ubiquitously expressed protein tyrosine phosphatase, are causative for a large proportion of Noonan syndrome (NS) cases. The gross morphological presentation often includes typical facies, developmental delay and short stature. Cardiac heart disease presents in approximately 80–90% of these patients. The heart defects cover a spectrum of morphological and functional deficits, with the most common being dysplastic pulmonary valve, AV septal defects or hypertrophic cardiomyopathy. The Q79R mutation acts as a gain of function similar to what would be expected from excessive SHP2. To elucidate the pathogenic role of the SHP2 mutation on the embryonic and postnatal heart, we did in vivo analysis using cardiomyocyte-specific transgenic expression both before and after birth. We expressed the wild type (WT) or mutant (Q79R) mouse SHP2 cDNAs using either the alpha- or beta-musomyosin heavy chain (MHC) promoters. Late-stage (E15.5) hearts from mice with cardiac specific expression of wild-type ventricular noncompaction and ventricular septal defects in an almost protein expression dose-dependent manner. Ventricular noncompaction persisted until the adult stage at 13% of the mice dying of congestive heart failure by 3 months. In contrast, neither the alpha-MHC Q79R nor beta-MHC WT SHP2 embryonic hearts showed any abnormality. In parallel with the histological findings, echocardiography of beta-MHC Q79R SHP2 mice at 3 months demonstrated ventricular dilatation, wall thinning and decreased contractility, whereas cardiac Q79R in the alpha-MHC and beta-MHC WT SHP2 mice was normal. Erk1/2 phosphorylation in the beta-MHC Q79R SHP2 mice was abolished exclusively in the embryonic stage. C-Myc, downstream of Erk 1/2, was upregulated following the activation of Erk1/2. Immunostaining also confirmed the c-Myc activation in the ventricle and trabeculae of the embryonic heart. Our data suggest development-specific effects of SHP2 cardiomyocyte expression and suggest that sustained overexpression of Erk and/or c-Myc in the later embryonic stages contributes to ventricular noncompaction.

**Role of p90 Ribosomal S6 Kinase in Cardiac Troponin I Phosphorylation and Possible Involvement in Cardiac Dysfunction after Ischemia-Reperfusion**

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Protein kinase C (PKC)-induced phosphorylation of cardiac troponin I (cTnI) can depress the acto-myosin interaction and may be important during heart failure. A downstream effector of PKC is p90 ribosomal S6 kinase (p90RSK). Since Ser23 and 24 of cTnI are contained in putative consensus sequences of p90RSK phosphorylation sites, we hypothesized that p90RSK could be downstream of PKC. We used mTg mice with cardiac specific p90RSK overexpression, but not with cardiac specific PKCε overexpression, to test the hypothesis that p90RSK is critical in ischemia-reperfusion, through a mechanism not related to cTnI Ser 23/24 phosphorylation, but NTG mice had increased phosphorylated cTnI. Since post-ischemic phosphorylation of cTnI were similar between NTG and control mice, we took together these data suggest that p90RSK phosphorylation of cTnI is sufficient to activate p90RSK and lead to phosphorylation of cTnI. In our study, ischemia-reperfusion, through a mechanism not related to cTnI Ser23/24 phosphorylation, but NTG mice had increased phosphorylated cTnI. Since post-ischemic phosphorylation of cTnI were similar between NTG and control mice, we took together these data suggest that p90RSK phosphorylation of cTnI is sufficient to activate p90RSK and lead to phosphorylation of cTnI. Results from our study in p90RSK-Tg mice detected with MALDI-TOF mass spectrometry analysis, myocytes infected with lentiviral shRNA to knock down p90RSK or dominant negative p90RSK were used to examine cTnI phosphorylation via p90RSK. Inhibition of p90RSK prevented H2O2-mediated cTnI phosphorylation. Mice with cardiac specific p90RSK overexpression, but not with cardiac specific PKCε overexpression, p90RSK phosphorylated cTnI in vitro with high substrate affinity, but not cTnI. Dual phosphorylation of Ser23/24 on cTnI vip was detected with MALDI-TOF mass spectrometry analysis, myocytes infected with dominant negative p90RSK were used to examine cTnI phosphorylation via p90RSK. Inhibition of p90RSK prevented H2O2-mediated cTnI phosphorylation, but not Erk1/2 or PKCε phosphorylation, supporting the hypothesis that p90RSK is critical in H2O2-mediated cTnI phosphorylation. Despite cardiac specific p90RSK-Tg higher cTnl Ser23/24 phosphorylation. To examine the functional consequence of p90RSK overexpression in vivo isolated hearts were challenged with 20 min of global ischaemia followed by 30 min reperfusion with normoxic mouse (WT) recovered 92 ± 11% of basal developed pressure within 10min of reperfusion, while p90RSK-Tg retained only 30% of developed pressure and dP/dt max. Compared to baseline. cTnl Ser23/24 phosphorylation was unchanged in p90RSK-Tg mice after ischemia-reperfusion, but NTG mice had increased phosphorylated cTnl. Since post-ischemic phosphorylation of cTnl were similar between NTG and p90RSK-Tg mice, we hypothesized that phosphorylation of Ser43/45 in p90RSK-Tg mouse may lead to contractile dysfunction after ischemia-reperfusion. Current studies are testing this hypothesis. In summary, PKCε, but not p90RSK, can activate p90RSK and phosphorylate troponin I at Ser23/24. Functionally, overactivation of p90RSK results in contractile dysfunction after ischemia-reperfusion, through a mechanism not related to cTnl Ser23/24 phosphorylation, but possibly phosphorylation of other sites.

**Cyclic AMP-Dependent Effect of the Small GTPase Rac in Cardiac Myocyte**

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Among various small G proteins, the Rho family has become a focus of interest since they have been shown to play key roles in the generation of cytoskeletal structures in cardiac
cells. As cAMP participates to the main regulations of cardiac function, we investigated the role of this second messenger on the regulation of Rho GTPase in cultured cardiomyocytes. Here, we show that stimulation of β-adrenergic receptors with isoproterenol leads to Rac activation as determined by affinity precipitation. To examine the involvement of cAMP in Rac activity, neonatal rat cardiac myocytes and HL-1 atrial cells were treated with forskolin (10 μM), a potent activator of adenylyl cyclase, or the phosphodiesterase inhibitor, IBMX (100 μM). Both compounds mimicked the effects of isoproterenol since they caused a strong increase in the amount of Rac-GTP. The stimulatory effect of isoproterenol on Rac was mimicked by a cAMP analog, 8-Bromo-cAMP (10 μM). We also show the cAMP-guanine-nucleotide exchange factor (GEF), Epac activates Rac activation. Indeed, activation of endogenous Epac with a selective activator of this GEF, 8-CPT-2′′-O-Me-cAMP (8-CPT) increased Rac GT-p-loading in rat cardiac myocytes. Similarly, infection of cardiomyocytes with an adenovirus encoding Epac95 (Ad.Epac95) significantly enhanced Rac GT-p-loading compared to control cells infected with GFP. As Rac has been found to be involved in cardiac myocyte hypertrophy, we next tested the potential involvement of Epac in this process. Transient transfection of Epac95 as well as a constitutive active form of Rac (racQ79L) increased the transcriptional activity of hypertrophic gene markers such as the atrial natriuretic factor (ANF) and the skeletal muscle α-actin. Accordingly, endogenous expression of ANF mRNA was significantly increased in ventricular cardiomyocytes infected with Ad.Epac95 as compared to cells infected with control Ad.GFP. The involvement of Rac in Epac signaling pathway controlling cardiomyocyte hypertrophy was further supported by the observation that Ad.RacQ79L, a negative dominant form of Rac inhibited Epac-induced ANF expression. Our results thus open new insights into the signaling pathways by which cAMP may mediate its biological effect.

The Hyperproliferative Effect of the Noonan Syndrome Mutation Q79R-Shp2 on Valve Primordia is Mediated by ERK Signaling

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Noonan syndrome is an autosomal dominant disease characterized by dysmorphic features and cardiac abnormalities such as pulmonary valve stenosis and hypertrophic cardiomyopathy. In severe cases, the congenital defects can result in heart failure in children. Genetic studies revealed that in approximatively 60% of the families gain-of-function mutations (e.g. Q79R) in the protein tyrosine phosphatase Shp2 are responsible. However, a direct mechanistic link between enhanced Shp2 activation and aberrant valve formation has not been identified. Therefore, our aim was to study the effects of wild-type Shp2 and Q79R-Shp2 on endocardial cushion development in an in vitro model. We focused on downstream MAPK signaling since Shp2 is a known modulator of ERK and p38 MAPK pathways. Endocardial cushions of the aorticoventricular canal and outflow tract of E4.5–E5 chick embryos were excised, infected with wild-type Shp2 or Q79R-Shp2 adenovirus and embedded in Matrigel®. Three-dimensional cultures, 97Shp2, but not WT-Shp2 expression resulted in increased outgrowth of cells from the expanded cushions into the gel matrix. This effect was completely abolished by treatment with the ERK-inhibitor U0126. In contrast, the p38–MAPK pathway inhibitor SB203580 did not inhibit Q79R–Shp2’s effect on cushion explant growth. Co-infection with Q79R–Shp2 and a dominant negative mutant of MEK-1 prevented enhanced endocardial cushion cell outgrowth, whereas expression of constitutively active MEK-1 could mimic the effect of Q79R–Shp2. Furthermore, BrdU incorporation was increased in dissociated endocardial cushion cells if infected with Q79R–Shp2, but not with WT–Shp2. This pro-mitotic effect was eliminated in the presence of U0126. These results demonstrate that the Shp2–ERK pathway is both necessary and sufficient to mediate the hyperproliferative effect of a gain-of-function mutation of Shp2 on mesenchymal cells in valve primordia.

Identification of an Ischemia-Inducible Receptor-Independent Activator of G-Protein That Interfaces with Gjγ

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Recent data indicate the existence of signal regulators that directly influence the activation state of heterotrimetric G-proteins (G) independent of a receptor. As part of a broader effort to define the role of such entities in signal integration, we asked if they were involved in the signals responsible for myocardial adaptation to ischemia. We used a yeast-based functional platform expressing human Gαq in place of the yeast Gαq that allowed us to rapidly screen mammalian cDNAs for receptor-independent G regulators. Bioactive entities isolated by this platform were termed Activators of G-protein Signaling (AGS). A cDNA library was genereted from rat hearts subjected to repetitive transient ischemia induced by inflation of an implanteable balloon in anesthetized rats. The library contained around the proximal LAD (40 sec occlusion, 20 min interval, 8 hrs/day) that induced coronary collateral growth in 2 weeks. We report the identification of a new AGS protein, AGS8, which encodes an uncharacterized 1730 amino acid protein with four fibronectin-type III domains. Epiispsis analysis indicated that the activation of AGS8 by AGS-activating stimuli such as adenosine triphosphate was increased in the ischemic area of LV 3.5 fold compared to non-ischemic area, but was not induced in cardiac hypertrophy or heart failure suggesting a specific role of AGS88 in the signaling processes underlying ischemia. Indeed, hypoxic treatment of adult cardiomyocyte up-regulated AGS8 mRNA by 8-fold. This increase was not observed in cultures of cardiac fibroblasts, aortic smooth muscle cells or cardiac fibroblasts. The bioactivity of AGS8 in the yeast-based assay was independent of guanine nucleotide exchange by Gαq suggesting an impact on subunit interactions. Protein interaction assays with GST-AGS8 and purified G-protein subunits or brain lysates indicated direct binding of AGS8 to Gjγ. Subsequent studies suggested that the interaction of AGS8 with Gjγ occurred in a manner that did not alter the regulation of the effector PLC-β2 by Gjγ. These data indicate that AGS8 serves as an ischemia-inducible binding partner for Gjγ in the cardiomyocyte providing an unexpected mode of signal input to G. Such mechanisms of signal regulation may provide intrinsic cues for the adaptation of the heart to ischemia.

Enhancement of Cardiac Function through PKC-α Inhibition

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Cardiovascular disease is the leading cause of death in the western world, with heart failure representing an increasing patient population. Recent evidence suggests that enhancing cardiac contractility is of potential therapeutic benefit for the treatment of heart failure. We have previously shown that PKC-α is a critical regulator of cardiac contractility using genetically modified mice that either over-express or lack PKC-α (Brax et al., Nat Med 2004:10:248–254). Genetic disruption of PKC-α activity significantly enhances cardiac contractility and prevents heart failure in three different mouse models of disease, while overexpression of PKC-α in wild-type mice attenuates cardiac hypertrophy and promotes cardiac repair. In order to identify a more clinically relevant approach for the treatment of heart failure, selective PKC-α/beta phospho
glycoprotein inhibitors (Ro-31–8220 or Ro-32–0432) were employed. In an ex vivo isolated heart preparation, treatment of wild-type mice with Ro-32–0432 significantly increased a measure of cardiac contractility and left ventricular pressure. To examine the effects of this drug on vivo, heart function was measured using invasive hemodynamics in wild-type control mice, MPL null mice as a model of heart failure and G-αq-α q transgenic mice as another model of heart failure. In each case, infusion of Ro-31–8220 significantly increased contractility, even in G-αq-α q mice that were relatively insensitive to dobutamine, further indicating that the pharmacologic inhibition of PKC-α in a failing heart may represent a novel treatment for human heart failure.

A Novel Role for TLR4 in Postischemic Cardiac Inflammatory Response: Reactive Oxygen Species as Potential TLR4 Activator

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Desregulated cardiac inflammatory response augments myocardial ischemia/reperfusion (I/R) injury. While modulation of cardiac inflammatory response is important for myocardial protection, the signaling mechanism underlying this response remains to be elucidated. Recent studies suggest that Toll-like receptor 4 (TLR4), an immunoresponsive receptor for microbial products, is also involved in signaling for endogenous agents. We hypothesized that TLR4 signaling regulates post-ischemic cardiac inflammatory response. The purpose of this study was to examine whether defective TLR4 signaling affects myocardial production of inflammatory mediators following I/R and to determine the role of reactive oxygen species (ROS) in activation of TLR4. Hearts isolated from TLR4 defective (C3H/HeJ) and C3H/HeJ mice were perfused and subjected to normothermic global I/R (20 min/60 min). Macrophage inflammatory protein-2 (MIP-2) and tumor necrosis factor-alpha (TNF-α), analyzed by ELISA, were elevated following I/R in hearts from control mice. However, myocardial levels of MIP-2 and TNF-α were markedly reduced following I/R in hearts with defective TLR4. To determine the role of ROS in TLR4-mediated inflammatory response, hearts were stimulated with hydrogen peroxide (0.25 mM). While hydrogen peroxide induced the production of MIP-2 and TNF-α in hearts isolated from control mice, its effects were greatly attenuated in hearts with defective TLR4. Further experiments using isolated macrophages confirmed that hydrogen peroxide induces NF-κB and p38 MAPK activation and inflammatory mediator production in a TLR4-dependent manner. We conclude that TLR4 signaling plays a novel role in the regulation of post-ischemic cardiac inflammatory response and that ROS may mediate this response through activation of TLR4.

Role of Dual-Specificity Phosphatases in the Heart as Revealed by Gene Targeting

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The mitogen-activated protein kinases (MAPKs) have been shown to participate in diverse biological processes in the heart including cardiac hypertrophy, apoptosis, and heart failure. MAPKs are tightly regulated through the addition or removal of phosphate groups in threonine and tyrosine residues located in the activation loop domain. Inactivation of MAPKs is controlled by removal of these phosphate groups and is mediated through a family of proteins known as dual-specificity phosphatases (DSPs), which have
a high degree of specificity for individual MAPK substrates. DSPs directly bind to their MAPK substrate and remove phosphate groups from both threonine and tyrosine residues located in the activation loop domain, allowing MAPKs to be recycled. The potential role of the downstream counter-acting DSPs has not been explored in the heart. Therefore, in order to evaluate the function of DSPs in the heart as well as the consequences of increased MAPK signaling, we have systematically inactivated by gene-targeting several of the Drosophila heat-shock responsive MAPK pathway components. This approach will provide the first "physiologic" assessment of sustained and/or prolonged MAPK activation and its consequences on the heart. Initial observations show that DSP-targeted mice display an overall increased MAPK activation in the heart following agonist and pressure overload stimulation. Moreover, analysis of MKP-1 and MKP-2 expression in double mutant mice showed a significant increase in cardiac hypertrophy following chronic isoproterenol infusion and myocardial infarction. However, these double mutant mice subjected to chronic angiotensin II / phenylephrine infusion or 14 days of pressure overload showed no increased MAPK activation in the heart following agonist and pressure overload stimulation. These preliminary results suggest that in DSP-deficient mice, the nature of the hypertrophic stimulus will determine the levels of activity in each of the MAPK branches and eventually establish the resulting phenotype.

Genome-Wide Expression Profiling of NFAT Transcriptional Activity in a Ventricular Muscle Cell Line

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Cardiac hypertrophy is dependent upon activation of calcineurin-Nuclear Factor of Activated T cells (NFAT) signaling, but limited information is available on the nature and number of downstream NFAT target genes. Recently, cell lines were isolated from ventricular sarcomas from juvenile transgenic mice harboring a Nkx2.5-fused SV40 T antigen (Tag) construct. Nkx2.5-Tag cells were isolated from sarcomas, which actively proliferate, and withdraw from the cell cycle and adopt a cardiac muscle cell fate upon Cre mediatedloxP excision of the SV40 Tag (Ryokin et al. J Biol Chem 2003). We established a cellular system to allow inducible NFAT activation by stably transfecting the Nkx2.5-Tag cell line with the tetracycline-sensitive transcriptional repressor (TetR) by hygromycin selection, allowing a doxycyclin (DOX)-on system in culture. Individual clones were tested by transient transfection with a luciferase reporter under control of two tetracycline operators (tetO). Stimulation with DOX for 24 hrs resulted in derepression of the reporter gene and over 100-fold activation of luciferase expression in four distinct TetR stable clones. Using transient transfection with a luciferase reporter under control of two tetracycline operators (tetO). Stimulation with DOX for 24 hrs resulted in derepression of the reporter gene and over 100-fold activation of luciferase expression in four distinct TetR stable clones. Using double stable TetR clones, a construct harboring an active form of NFAT under control of tetO operators was stably transacted in TetR cells using zeocin selection. Double stable clones were chosen using NFAT-reporter genes and western blots before and after DOX stimulation as selection criteria. Two double-stable TetR-NFAT clones were selected to control for potential cell based variations, AdCre infected to mortalize and adopt a cardiac muscle fate, and subjected to the Agilent microarray system and 44k mouse whole genome arrays. After DOX-stimulation for 24 hrs, 175 genes were differentially expressed in both clones with a 3 fold-change in expression. Gene ontology analysis revealed that NFAT target genes profoundly affect the cardiac transcription, signal transduction, translation efficiency and extracellular matrix. Conclusively, this novel cellular model allows for rapid genome wide screening of target genes of transcription factors in ventricular muscle, and will provide more entry points to our fundamental understanding of the calcineurin/NFAT transcriptional pathway in cardiac hypertrophy.

Differential Transcriptional Regulation of Cardiac Metabolic Genes by Myocyte Enhancer Factor-2 and Peroxisome Proliferator-Activated Receptors

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During the progression to heart failure, changes in energy utilization occur in the heart, with the failing heart being more reliant on glucose utilization to the expense of fatty acid oxidation. Peroxisome proliferators-activated receptor (PPAR) family members are key regulators of cardiac fatty acid oxidation. In the regulatory region of the the PPAR target gene carnitine palmitoyl transferase-I (CPT-I), conserved PPAR response elements (PPRE) and myocyte enhancer-factor-2 (MEF2) binding sites are present. We analyzed the transcriptional relationship between different PPAR isoforms (alpha, beta and gamma) and MEF2A by combinatorial site directed mutagenesis of the PPRE and MEF2 binding sites, and demonstrate a preferential relationship between PPARbeta and gamma with MEF2A, and that disruption of the PPRE increases activation of the CPT-I gene dramatically after stimulation with beta and gamma specific agonists. Furthermore, disruption of the MEF2 binding site completely abolished the activation of the reporter gene, irrespective whether the PPRE site was intact or not, suggesting that PPAR activity strongly depends on MEF2 transcriptional activity. Finally, a micro array analysis on cardiac muscle cell lines, which inducibly express a constitutively active form of MEF2, revealed that 20% of the differentially expressed genes were involved in mitochondrial metabolism/fatty acid oxidation. Remarkably, only a minority of these genes had PPRE sites and were responsive to PPAR agonists. Using RNAi-mediated downregulation of each PPAR isoform and a dominant negative negative MEF2 antagonist, we show that disruption of MEF2 function increases the transcriptional regulation of endogenous metabolic genes. The combined observations suggest that for a select number of genes PPAR and MEF2 differentially converge to regulate adaptive fatty acid oxidation gene expression, with a dominant role for the bona fide pro-hypertrophic factor MEF2. Further analysis is also capable of altering the metabolic gene profile in cardiac muscle independent of PPAR activity. These data also suggest that the early hypertrophic phase is associated with an increased rather than decreased fatty acid utilization.

Reduced-Energy Diet Improves the Survival of Viral Myocarditis in Obese Mice: Relation to Cardiac Adiponectin Expression

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Obesity is an important risk factor for heart diseases. Whether weight loss affects the extent of viral myocarditis is a matter of debate. We hypothesized that weight loss could improve cardiac function and reduce viral myocarditis. We therefore used C57BL mice infected with Adenovirus 5 expressing the encephalomyocarditis virus (500 plaque-forming units/mouse) for KKAy feed ad libitum (100F) or 60% of the food intake (60F) and WT (n = 30 for each). Ten-day survival rate was 0% in 100F, whereas it was 17% in 60F and 40% in WT. Body weight in 60F was lower than in 100F (21.1 ± 0.3 vs. 29.1 ± 0.24 g, P < 0.05, n = 4 for each) on Day 0 and continuously lower on Day 8 (22.7 ± 0.8 vs. 30.7 ± 0.7, P < 0.03 vs. 1). Heart weight/body weight ratio in 60F was lower than in 100F (5.4 ± 0.2 vs. 7.1 ± 0.4, P < 0.05, 4 for each). Histological scores (0 to 5) for myocardial necrosis and inflammation on Day 5 were significantly lower in 60F than in 100F (nectosis; 1.8 ± 0.3 vs. 3.2 ± 0.7, P < 0.05, inflammation; 1.4 ± 0.5 vs. 2.2 ± 0.4, P < 0.05, n = 4 for each). Circulating adiponectin levels on Day 0 were significantly elevated in 60F compared with 100F (32 ± 9 vs. 22 ± 2 micro/mL, P < 0.05), and those in 60F on Day 5 were also higher than in 100F. Circumferential wall thickness in 60F was significantly decreased compared with in 100F on Day 5 (0.23 ± 0.03 vs. 1.0, P < 0.05, n = 4 for each). Cardiac adiponectin mRNA was negatively correlated with cardiac TNF-alpha mRNA (r = −0.53, P = 0.01, n = 12). On Day 0, thymus weight/ body weight and spleen weight/body weight in 60F were significantly (P < 0.05) lower than in 100F and WT. Weight loss improved the survival and myocardial damages in obese mice with viral myocarditis and also induced the cardiac expression of adiponectin. Therapeutic modulation of cardiac adiponectin might provide benefit to the cardioprotective effect against acute heart failure due to viral myocarditis in obese subjects.

PPARα Activation Upregulates the Fatty Acid Oxidation Pathway and Reduces Cardiac Ceramide Content in Heart Failure but Does Not Affect LV Dysfunction

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Peroxisome proliferator activated receptor α (PPARα) overexpression in transgenic mice increases the lipotoxic intermediate ceramide and causes LV remodeling and dysfunction. Furthermore, in advanced heart failure (HF), the expression of genes regulated by PPARα is down-regulated causing decreased fatty acid oxidation (FAO), which may function as a positive compensatory response. We therefore hypothesized that cap-coupled administration of the PPARα pathway with a direct PPARα agonist would exacerbate left ventricular (LV) dysfunction and dilatation in HF. Rats underwent left coronary artery ligation or sham surgery (n = 10), and 8 weeks later were assigned treatment with the PPARα agonist fenofibrate (inf + Feno, 150 mg/kg/day; n = 13) or vehicle (inf + Veh, n = 13) untreated (inf + Untr, n = 10). Twenty weeks post ligation, LV function was assessed by echocardiography and catherization. LV systolic function was decreased and end diastolic area increased to a similar extent in both infarcted groups. Treatment with the PPARα agonist up-regulated the mRNA expression of the PPARα regulated genes, and medium chain acyl-CoA dehydrogenase (MCAD) protein expression and activity were increased in the Inf + Feno group compared to sham and Inf + Veh groups (Table). However, the mRNA and protein expression of PPARα and retinol X receptor α were unchanged. Treatment with fenofibrate significantly increased LV mass/body mass ratio compared to sham and Inf + Veh. Although cardiac ceramide content was increased in the infarcted groups, PPARα activation reduced ceramide content. In conclusion, PPARα activation increased mRNA expression of FAO enzymes, increased MCAD protein expression and activity and reduced myocardial ceramide content. These results suggest that PPARα activation of the FAO pathway and ceramide content do not contribute to LV dysfunction and remodeling in a rat model of coronary artery ligation-induced HF.
Increased levels of O-linked N-acetylglucosamine (O-GlcNAc) on nucleoplasmin proteins are associated with decreased calcium entry into cardiomyocytes and improved tolerance of mammalian cells to stress. Therefore, we hypothesized that in the heart glucosamine (GlcN) treatment would increase hexosamine biosynthesis pathway (HBP) flux and protein O-GlcNAc levels, resulting in improved recovery following ischemia/reperfusion injury. To test this hypothesis, we isolated ventricular myocytes from normal, adult rats and cultured them in normal glucose (NG) or high glucose (HG) media. We used both normoxic and hypoxic conditions to induce cell death. GlcN treatment increased hexosamine biosynthesis and protein O-GlcNAc levels in both normoxic and hypoxic conditions.

To further investigate the role of O-GlcNAc in cardiac function, we performed a series of functional assays. GlcN treatment increased the rate of glucose uptake and improved contractile function. We also measured the levels of HBP metabolites, such as UDP-GlcNAc and O-GlcNAc, which are key markers of the HBP.

In conclusion, our results demonstrate that GlcN treatment can increase hexosamine biosynthesis and improve cardiac function. These findings support the potential use of GlcN as a therapeutic agent for the treatment of ischemic heart disease.
1, 2, and 4 months duration. Using the isolated working heart preparation, cardiac power and substrate metabolism were assessed at baseline and in response to an acute increase in work (adrenergic-stimulation). At four months, rats fed high fat and “western” diets weighed more than those fed control diet (728 ± 10 and 732 ± 14 g, p < 0.05). Increasing age, in all groups, was associated with an increasing cardiac power (p < 0.05). However, cardiac power at 1 month was significantly higher in the high fat group compared to “western” groups compared to control group (p < 0.01). The increase in cardiac power was not associated with an increase in glucose oxidation either before or after inotropic stimulation. Those fed a “western” diet have similar cardiac power to those fed a control diet, but exhibit impaired metabolic flexibility in response to an acute increase in work. Thus, in spite of impaired metabolic flexibility the functional reserve of the heart remains preserved in the early stages of diet induced obesity.

Isolation, Expansion, and Characterization of Cardiac Stem Cells from Endomyocardial Biopsies

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Recent discoveries identifying the heart as a source of stem cells have opened up new prospects for autologous cellular cardiomyoplasty. Injection of cardiac stem cells (CSCs) isolated from rodent hearts and human surgical specimens into the infarct borderzone in rodents has been shown to result in CSC engraftment and improved ventricular function. We have made substantial progress in isolating, expanding and characterizing CSCs from right ventricular endomyocardial biopsies derived from humans and pigs. When biopsies are plated on fibronectin, a lawn of flat cells spreads from the biopsy and covers the bottom of the dish in 14 – 20 days (versus 2 – 3 days in pigs). Upon this lawn, small phase-bright cells appear; these can be seen budding off from the main explant and then seeding the dish, a process which is robust by 12 – 16 days (versus 5 – 7 days in pigs). The cells are expanding exponentially, initially after 5 – 7 days, and then every 4 – 5 days (for a total of 4 harvests). When grown in suspension, the cells form cardiospheres (CSPs) within 12 – 20 days in human samples (4 – 6 days in pig). CSPs are then expanded by growing them as monolayers on culture flasks coated with fibronectin; human CSCs have a doubling time of 48 hours (versus 24 hours in pig). Differences in growth characteristics between CSCs derived from humans and pigs may be attributable to species differences or to the fact that the pigs were young and healthy, while patients (n = 30) undergoing endomyocardial biopsies had congestive heart failure or were post-transplant with varying degrees of rejection on therapy. An important finding is that all biopsies exhibit proliferation of CSCs in vitro, albeit with variable growth rates yielding 7 – 70 million cells in 30 – 45 days. Hence the time of transplantation after biopsy harvesting may have to be tailored to each patient’s CSC growth characteristics, but our preliminary data indicate that all patients will eventually have sufficient cells for cell transplantation. These data demonstrate the feasibility of obtaining sufficient numbers of stem cells from human endomyocardial biopsies for cellular cardiomyoplasty.

Withdrawn

Improvement of Cardiac Function after Sole Therapy with ACC 133+ Bone Marrow-Derived Stem Cells

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Objectives: To restore tissue viability in ischemic myocardium not amenable to CABG, transplantation of bone marrow derived stem cells (BMSCs) has been used in combination with conventional revascularisation. Growing evidence suggests that transplantation of autologous BMSCs can improve the perfusion and contractile function of ischemic myocardium. This procedure could potentially benefit transplant candidates awaiting a donor heart. Methods: We performed a prospective, nonrandomized, open-label study in 8 heart transplant candidates with ischemic heart failure. All patients were not amenable to CABG. Each patient underwent baseline ramp treadmill protocol, echocardiography, 24-hour Holter monitoring, and cardiac MRI, which were repeated at 2 and 6 months. Up to 300 ml bone marrow was harvested from the iliac crest and processed into 5 – 10 cell suspensions (7 – 10 x 106 cells; purity 90 – 99%) cells were injected in predefined region within hibernating myocardium after thoracotomy. Each patient received 10 to 15 injections of 0.5 ml (total amount: 10 ml). Results: Improvement of cardiac function, as assessed by cardiac MRI, could be documented 3 month postoperatively (mean pre- and postoperative LVEF 18.3% and 29% / LVEDD 79.2 mm and 57.4 mm). A dose dependent improvement of the ejection fraction would be determined in 5 patients. Conclusions: In 6 of the 8 cases we observed significant improvement that the patients were no longer eligible for transplantation. In conclusion ACC 133+ injections were performed safely and resulted in improved exercise capacity. This technique may hold promise as an alternative to medical management in patients with ischemic heart failure who are ineligible for conventional revascularisation.

Autologous Myoblast Transplantation after Myocardial Infarction Induces Ventricular Electrical Stability

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Small scale clinical trials suggested the feasibility and the efficacy of autologous myoblast transplantation to improve ventricular function after myocardial infarction. However, these trials were hampered by unexpected episodes of life-threatening ventricular tachyarrhythmias (VT). We investigated cardiac electrical stability after myoblast or other cell transplantation to the myocardium. Wistar rats were subjected to left coronary artery ligation to induce myocardial infarction. At day 7 post-ligation, animals were randomized into 3 groups: a control group receiving no further treatment, a vehicle group injected with culture medium into the infarction zone, and a group injected with autologous myoblasts. Holter monitoring, performed at day 7 to day 35, did not discriminate the myoblast group (n = 5, 733 hr) from the vehicle group (n = 5, 597 hr). Programmed Electrical Stimulation (PES) was performed at day 14, 21, 28 and 35 post-ligation, to evaluate further a cardiac substrate for arrhythmia susceptibility. The occurrence of sustained VT during PES was similar in control and vehicle groups (5/17 and 4/18 rats respectively; p = 0.50). In contrast, 13/20 rats (65% from the myoblast group showed at least one episode of sustained VT during PES (p < 0.05 and p < 0.005 versus control and vehicle groups). As a further control group, rats injected with autologous bone marrow mononuclear cells into the infarcted myocardium did not show increased susceptibility to PES, in an infarcted rat model, (1) medium or cell injection does not facilitate per se the occurrence of tachyarrhythmias, (2) myoblast transplantation into injured myocardium induces electrical instability with triggering of sustained ventricular tachycardia. Because ventricular arrhythmias are life-threatening diseases, we suggest that such preclinical evaluation should be conducted for any new source of cells to be injected into the myocardium.

Bone Marrow-Derived Cardiomyocytes in the Heart after Heart and Bone Marrow Transplantation

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Introduction: Several studies report the generation of cardiomyocytes from bone marrow derived stem cells. In this study we analyzed the presence of cardiomyocytes of recipient or donor origin after heart transplantation (HTx) or of donor origin after Bone Marrow Transplantation (BMT). Patients and materials: Six male HTx patients transplanted with a female heart and after BMT 6 female patients transplanted with male BMT were analyzed. Heart tissues were obtained at autopsy between 6 and 949 days after HTx or BMT. In 10 out of 12 patients both after HTx and BMT 0.1 – 1.3% Y-chromosome positive cardiomyocytes were observed, with a tendency of higher numbers in HTx compared to BMT. The % of Y-chromosome positive endothelial cells was also small. The main hybridization and with one of the following mAb (immunohistochemistry): anti-myosin (cardiomyocytes), anti-CD34 (endothelial cells) and CD45 (leukocytes). The % Y-chromosome positivity of 1000 cardiomyocytes was determined. Results and conclusion: In 10 out of 12 patients both after HTx and BMT 0.1 - 1.3% Y-chromosome positive cardiomyocytes were observed, with a tendency of higher numbers in HTx compared to BMT. The % of Y-chromosome positive endothelial cells was also small. The main population of Y-chromosome positive cells were CD45 positive and were heart infiltrating BM derived cells, being higher in HTx than in BMT. These findings indicate a small but significant percentage of BM derived cardiomyocytes after HTx or BMT. The percentage of culture mediated myocardial cardiomyocytes, and a myoblast group injecting with autologous myoblasts was not discriminative, the patients were likely transplant for indicating that either the turn-over of these cells is high or that only a brief period after transplantation these cells enter the heart and become cardiomyocytes.

Ex Vivo Expanded Human Bone Marrow-Derived CD133+ Cells Improve Cardiac Function in Rats with Acute Myocardial Infarction

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We have examined whether ex-vivo expanded human bone marrow (BM)-derived CD133+ cells (EC-CD133+) in presence of the copper chelator tetraethylthiophosphonate (TETP) may
participate in post myocardial infarction (MI) healing. CD133+ cells isolated from human BM, were examined in the presence of IL-6, TPO, FL-3 ligand, and SCF with or without TPE for 2 weeks. After 3 weeks the total nuclear cell expanded by 210 ± 27 fold. The increase in the CD34+, CD133+ and CD34+/CD38+ cell populations was 28 ± 8, 19 ± 4 and 263 ± 91, respectively. 3 weeks EE-CD133c expressed VEGF and VEGF receptor RNA as examined by RT-PCR and secreted VEGF at levels of 121 ± 41 pg/10^6 cells as demonstrated by ELISA analysis. The biological activity of conditioned medium derived from EE-CD133c induced endothelial and vascular smooth muscle cell proliferation. CXCR4 was expressed on the surface of 40% of EE-CD133c as demonstrated by FACS analysis. An MI model was established in atrophic myocyte rats. EE-CD133c (1x10^7) or saline (control) were injected into the centre of the infarct myocardium 6 days post MI. Each animal was compared 4 weeks post-treatment with those observed prior to treatment. EE-CD133c treatment, attenuated left ventricle (LV) systolic dilation by 89% and improved LV contractility by 72% as compared to control animals (p < 0.017). Interestingly, injection of the unexpanded BM cell fraction not bound to the CD133 column upon isolation with EE-CD133c, greatly diminished the improving benefit observed for EE-CD133c. Clinical trials examining the safety and feasibility of intracoronary injection of autologous EE-CD133c to patients with ischemic heart disease are currently in preparation. 

Conclusions: A TPE allows BM-derived CD133+ cells to self renew by permitting their ex-vivo proliferation while hindering their differentiation. B. The resulting EE-CD133c express receptors (VEGF-R, CXCR4) involved in homing to hypoxic/inflamed zones and secret factors relevant to cardiac regeneration. C. Injection of EE-CD133c into infarcted myocardium improves left ventricular function.

Mechanism of Human Skeletal Myoblast Survival in Porcine Heart: Fusion Between Myoblasts and Porcine Cardiomyocytes

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Introduction: Little information is available about the in vivo behavior of human skeletal myoblasts due to the ethical issues and difficulties of using human subjects as models. We employed a xenotransplantation animal heart model to investigate the differentiation of human skeletal myoblasts. Methods: Human skeletal myoblasts labeled with Lac-z reporter gene and transplanted into pig heart. Pig heart model of myocardial infarction was developed by coronary artery ligation and grouped as DMEM injected (group-1 n = 10), human skeletal myoblast transplanted (group-2 n = 15). After 3 weeks, 3ml DMEM with or without 3x10^6 myoblasts were injected into the center and perifaction. Animals were maintained on immunosuppression for 6 weeks after cell transplantation. Pigs were euthanized and hearts were explanted at 2, 6 and 12 weeks post treatment and processed for histological studies. Results: Extensive survival of myoblasts as revealed by Lac-z expression was only found in pig heart from group-2. Most of Lac-z positive tissue had the typical phenotypic expression of pig cardiomyocytes. Counterstaining Lac-z positive tissue with specific anti-pig IgG antibody demonstrated that the Lac-z positive tissue was pig heart tissue. Triple counterstaining with DAPI revealed that Lac-z expression nuclei were centrally located in the pig cardiomyocytes suggesting that myoblasts integrated into host pig cardiomyocytes to form mosaic muscle fibers. Flowcytometric analysis of pig sera revealed that anti human skeletal myoblast antibody was transiently present (< 2 weeks after cell transplantation) at very low level. Conclusion: Human skeletal myoblasts do not differentiate into cardiomyocytes. Fusion between human skeletal myoblasts and pig cardiomyocytes is the mechanism of xenotransplanted human skeletal myoblasts survival in pig heart.

Heart-Restricted NF-κB Inhibition Allogrates Left Ventricular Remodeling after Transaortic Banding

Christian Freund, Max Delbrueck Ctr for Molecular Med, Berlin, Germany; Roel van der Kooi, Chuanfu Li; East Tennessee State Univ, Johnson City, TN

We have previously shown that NF-κB activation is needed for the development of cardiac hypertrophy in vivo. We have also observed reduced cardiac hypertrophy in TLR4-deficient mice following pressure overload. Myeloid differentiation factor 88 (MyD88) is an important adapter protein for transduction of signaling from TLR4 to NF-κB, therefore, we proposed that NF-κB might be an important intermediary contributing to the development of cardiac hypertrophy by mediating TLR4 signaling leading to NF-κB activation. To evaluate this hypothesis, we constructed adenovirus expressing dominant negative MyD88 (Ad5-dmMyD88) and transfected the Ad5-dmMyD88 into rat hearts immediately followed by aortic banding for 3 weeks. Transfection of Ad5-GFP served as control. The ratio of heart weight/body weight (HW/BW) was calculated as an index of cardiac hypertrophy and cardiac myocyte size was examined by FITC labeled wheat germ agglutinin staining of membranes. Cardiac myocyte apoptosis was determined by TUNEL assay and myocardial interstitial fibrosis was stained by Masson’s Trichrome. Following aortic banding for three weeks, the ratio of HW/BW was significantly increased by 38.8% in untreated rats (0.47 ± 0.01 vs 0.34 ± 0.01) and the cardiac myocyte size from the left ventricle was increased by 49.6 % compared with sham control. Cardiac myocyte apoptosis was increased by 11.5% and cardiac fibrosis also significantly increased in aortic banded hearts compared with sham control. Transfection of Ad5-dmMyD88 into the myocardium significantly reduced aortic-banding-increased the ratio of HW/BW by 46.2% and decreased cardiac myocyte size by 58.6% compared with aortic banded rats. Transfection of Ad5-dmMyD88 also significantly decreased aortic banding-increased cardiac myocyte apoptosis and fibrosis in the myocardium. Transfection of Ad5-GFP did not affect aortic banding-induced cardiac hypertrophy. Transfection of Ad5-dmMyD88 significantly reduced the aortic banding-increased NF-κB binding activity by 41.8%, levels of phospho-iκBα by 26.7%, phospho-IκBα by 52.8%, respectively. Our results suggest that MyD88 is an important component in TLR4 mediated NF-κB activation pathway that contributes to the development of cardiac hypertrophy in vivo.

Blocking MyD88-Dependent Signaling Pathway Reduces Pressure-Overload Induced Cardiac Hypertrophy In Vivo

Tuanzhu Ha, Yehua Li, Fang Huang, Jim Kelley, David L Williams, William Browder, Race L Kao, Chuanfu Li; East Tennessee State Univ, Johnson City, TN

We have previously shown that NF-κB activation is needed for the development of cardiac hypertrophy in vivo. We have also observed reduced cardiac hypertrophy in TLR4-deficient mice following pressure overload. Myeloid differentiation factor 88 (MyD88) is an important adapter protein for transduction of signaling from TLR4 to NF-κB, therefore, we proposed that NF-κB might be an important intermediary contributing to the development of cardiac hypertrophy by mediating TLR4 signaling leading to NF-κB activation. To evaluate this hypothesis, we constructed adenovirus expressing dominant negative MyD88 (Ad5-dmMyD88) and transfected the Ad5-dmMyD88 into rat hearts immediately followed by aortic banding for 3 weeks. Transfection of Ad5-GFP served as control. The ratio of heart weight/body weight (HW/BW) was calculated as an index of cardiac hypertrophy and cardiac myocyte size was examined by FITC labeled wheat germ agglutinin staining of membranes. Cardiac myocyte apoptosis was determined by TUNEL assay and myocardial interstitial fibrosis was stained by Masson’s Trichrome. Following aortic banding for three weeks, the ratio of HW/BW was significantly increased by 38.8% in untreated rats (0.47 ± 0.01 vs 0.34 ± 0.01) and the cardiac myocyte size from the left ventricle was increased by 49.6 % compared with sham control. Cardiac myocyte apoptosis was increased by 11.5% and cardiac fibrosis also significantly increased in aortic banded hearts compared with sham control. Transfection of Ad5-dmMyD88 into the myocardium significantly reduced aortic-banding-increased the ratio of HW/BW by 46.2% and decreased cardiac myocyte size by 58.6% compared with aortic banded rats. Transfection of Ad5-dmMyD88 also significantly decreased aortic banding-increased cardiac myocyte apoptosis and fibrosis in the myocardium. Transfection of Ad5-GFP did not affect aortic banding-induced cardiac hypertrophy. Transfection of Ad5-dmMyD88 significantly reduced the aortic banding-increased NF-κB binding activity by 41.8%, levels of phospho-iκBα by 26.7%, phospho-IκBα by 52.8%, respectively. Our results suggest that MyD88 is an important component in TLR4 mediated NF-κB activation pathway that contributes to the development of cardiac hypertrophy in vivo.

Heart-Restricted NF-κB Inhibition Allogrates Left Ventricular Remodeling after Transaortic Banding

Sandra Dunger, Franz-Volhard Clinic, HELIOS Klinikum, Berlin, Germany; Rainer Dietz, Franz-Volhard Clinic, HELIOS Klinikum, Berlin, Germany; Leon J de Windt, Hubrecht Lab and Interuniv Cardiology Inst Netherlands, Utrecht, Netherlands Antilles; Christian Freund, Max Delbrueck Ctr for Molecular Med, Berlin, Germany; Roel van der Kooi, Chuanfu Li; East Tennessee State Univ, Johnson City, TN

On a cellular basis, left ventricular (LV) remodeling occurs by hypertrophy and apoptosis of cardiomyocytes. NF-κB is an ubiquitous transcription factor which has been shown to mediate hypertrophy in vitro and to protect many cell types from apoptosis. Recently we reported that cardiomyocyte-restricted inhibition of NF-κB in vivo attenuated hypertrophy induced by short term stimulation with β-adrenergic agonists or angiotensin II (7 or 14 days, respectively). Here we tested whether heart-restricted inhibition of NF-κB attenuates long-term hypertrophy induced by transgenic expression of the cardiac myocyte-specific inhibitor of NF-κB (TNF-α). The latter, in turn, causes an increased in the amount and transactivation activities of β-catenin, a zona fide UPS substrate regulating cardiac growth. The UPS plays a central role in proteolysis, regulating critical cellular processes. Thus, UPS impairment by aberrant protein aggregation represents an important pathogenic mechanism in cardiac remodeling and failure.
**Decreased GSK-3 Signaling Activity Promotes Hypertrophy and Apoptosis in Cardiac Myocytes**

Shinichi Horioto, Hidehara Tomita, Chul Hong, Jing Liu, Junichi Sadoshima; UMDNJ, Newark, NJ

Glycogen synthase kinase-3 (GSK-3) negatively regulates cardiac hypertrophy. However, it remains to be elucidated whether GSK-3 is salutary or detrimental during heart failure (HF). In order to address this issue, HF was induced by rapid pacing superimposed upon chronic left ventricular (LV) hypertrophy caused by thoracic aortic constriction (TAC) in double transgenic murine models expressing a significantly increased (15%) in heart weight/body weight compared with non-TG (NTG) littermates, while cardiac function was normal at baseline. In order to clarify the role of reduced GSK-3β kinase activity during hypertrophy and HF, we subjected to TAC. Although pressure overload caused significant levels of cardiac hypertrophy in both NTG and R92Q/beta mice, increases in LV weight/body weight were greater in TG mice than in NTG (TG 55% vs. NTG 34%).

**Switching the Cardiac MyHC Composition from Alpha to Beta in a Mouse Model of cTnT-Related FHC Leads to Significant Alterations in Cardiac Remodeling and Mortality**

Ron Rice, Albert Einstein College of Med, Bronx, NY; Huamei He, Kirsten Hoyer, Avery Buchholz, Liisa Valppu, Lin Xu; Med Univ of South Carolina, Charleston, SC

Heart failure (HF) is a major clinical problem, contributing to the progression of HF. A feline pressure overloaded right ventricular (RV) hypertrophy model was used to generate an independent double transgenic murine model expressing the R92Q mutation on an 80% beta MyHC background in the heart. All of the expected genotypes were identified and activities of the splicing factors, resulting in subsequent exon skipping and an alteration of the splicing patterns of cardiac proteins that contribute to contractile dysfunction in cardiac hypertrophy and failure. A feline pressure overloaded right ventricular hypertrophy was used as an in vivo model and a-adrenergic stimulated adult cardiomyocytes were used as an in vitro model to test this hypothesis. Here we show that the previously described “small-heart” phenotype. These findings suggest that the expression of the R92Q cTnT mutation on the more “human” beta MyHC background causes alterations in the pathogenic cardiac remodeling process and leads to a significant decrease in ventricular mass. In contrast, HW/BW ratio was increased by 18% in R92Q/beta mice exhibiting an 18% increase in early mortality (consistent with the human disease) in transgenic mice. 

**Regression of Left Ventricular Hypertrophy by Inhibition of Mammalian Target of Rapamycin**

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Left ventricular hypertrophy (LVH) is a key risk factor for cardiac morbidity and mortality. The mammalian target of rapamycin (mTOR) is known to play a critical role in the determination of cell size. In order to elucidate the effects of this switch on cardiac contractile performance and reserve.
heterogeneous ribonuclease protein A (hnRNP A1), which increases exon skipping, was upregulated 2 to 5 fold in the feline pressure-overload hypertrophic right ventricle. The upregulation was seen as early as 24 hours after banding and lasted up to 1 week, with peak expression at 48 hours. The expression of alternative splicing factor (ASF), which is an antagonist of hnRNP A1, was downregulated more than 2 fold in the pressure-overload hypertrophic ventricle at protein level. Therefore, the ratio of hnRNP A1 to ASF was dramatically increased in the pressure-overload right ventricle. Similar results were obtained when we further tested the ratio changes of the splicing antagonists in isolated adult cardiocytes subjected to α-adrenergic stimulation. In conclusion, these data support a novel paradigm that pressure overload induced hypertrophy alters cardiac protein splicing isoforms by regulating the activity of splicing factors.

FKBP12, which binds the immunosuppressants FK506 and rapamycin, was reported to interact with skeletal muscle ryanodine receptor (RYRtype2). In contrast, the cardiac ryanodine receptor (RYR type1), appears to bind selectively the FKBP12 homologue, FKBP12.6. However, FKBP12-deficient mice had normal skeletal muscle but had severe dilated cardiomyopathy and ventricular septal defects, which suggested that FKBP12 may also participate in the pathogenesis of heart diseases. To define the functions of FKBP12 in vitro, we constructed adenovirus (Ad)-mediated gene transfer to overexpress FKBP12 in neonatal rat cardiomyocytes. The area and perimeter of cardiomyocyte were measured by image analysis system. Protein and mRNA expression were detected by western blot and RT-PCR. Cytosolic free calcium level was measured using fluorescence spectrometers. Compared with control cardiomyocytes, Ad-FKBP12 infected cells displayed a smaller myocyte size (area: 3906±124 um2vs 5199±106 um2, perimeter: 289.6±6 um vs 314.6±6 um, P<0.01) and reducing β-MHC mRNA expression (OD ratio: 0.77±0.01 vs 1.00±0.00, P<0.01), and inhibited ERK1/2 protein expression (OD ratio for p42ERK: 0.34±0.03 vs 1.00±0.01, P<0.05; p44ERK: 0.49±0.02 vs 1.00±0.03, P<0.01). Compared with control cardiomyocytes, a lower basal cytosolic free calcium level (ratio: 0.63±0.03 vs 0.78±0.04, P<0.05) and inhibited 1 mM ryanodine induced intracellular calcium increase (ratio%:2.4±0.07 vs 2.76±0.05, P<0.05) were observed in Ad-FKBP12 infected cardiomyocytes. FK506 could further reduce both the size of Ad-FKBP12 infected cardiomyocytes and the expressions of β-MHC and ERK1/2. This study indicated that overexpression of FKBP12 suppressed cardiomyocyte hypertrophy by inhibiting ERK1/2 and cytosolic free calcium signal, the effects of which could be further enhanced by FK506. It suggested that FKBP12 may participate in the modulation of cardiac structure and function (supported by NSF grant No. 53070537).

Role of Neutrophil-Derived Protease Cathepsin G in Cardiac Remodeling During Early Volume Overload Induced Cardiac Hypertrophy in the Rat


In hearts with hemodynamic overload, there is a complex sequence of compensatory events resulting in a continual state of remodeling mediated by changes in extracellular matrix turnover and in cardiac myocyte function, growth and survival. One of the earliest events during cardiac remodeling is thought to involve an inflammatory reaction. This study examined the role of neutrophil derived protease cathepsin G (CG) in left ventricular (LV) remodeling in volume overloaded (VO) hearts. Rats were studied at 6, 12, 24 and 48 hrs and 5 days post-aorticaval fistula (ACF) and compared to sham-operated controls (n=8, all groups). ACF induced cardiac hypertrophy and diastolic and decreased LV interstitial collagen volume at 12 and 48 hrs after ACF. This collagen degradation was associated with a significant increase in neutrophil infiltration at 12 hrs and in CG activity at 12, 24 and 48 hrs after ACF (2.1, 1.8 and 1.25 fold over sham, respectively). ACF also increased caspase 3 activity at 24 and 48 hrs after ACF and this increase strongly correlated with the increase in CG activity (r=0.82 at 24 hrs and r=0.88 at 48 hrs after ACF). To determine whether CG is involved in LV remodeling, rat hearts were injected with a bolus of CG (0.020) or its vehicle via catheter based technique. After 5 days of CG injection, LV wall thickness decreased and wall stress increased significantly. Collagen volume decreased by ~50% (4 of 2; P<0.01 vs. control) and caspase-3 activity increased by ~25% (260 of 208 RFU/min/mg; P<0.05 vs. control). These data showed that early induction of VO induces an increase in neutrophil infiltration and CG activity that may promote extracellular matrix degradation and subsequent myocardium apoptosis.

The Ras Interacting Protein Rf Differentially Regulates Hypertrophic Signaling Pathways in Cardiomyocytes

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Cardiomyocyte hypertrophy results from the activation of multiple signaling pathways that regulate cell morphology, gene expression, and survival. Studies in both isolated cardiomyocytes and in animals demonstrate an important role for the monomeric G-protein Ras in coordinating the activity of multiple intracellular signaling pathways involved in the hypertrophic response. Ras selectively regulates multiple kinase cascades via interactions with its effectors MEK1, Raf, and PI3-kinase. In isolated cardiomyocytes, Ras interaction with MEK1 promotes JNK activation and apoptosis whereas interaction with Raf, which leads to ERK1/2 activation, and PI3-kinase are thought to promote cardiomyocyte protection. Previous studies identified the Ras guanine nucleotide dissociation stimulator like factor (Rfl) as a protein that interacts with Ras in cardiomyocytes. Endogenous Rfl expression was detected in neonatal rat ventricular myocytes (NRVMs) and adult mouse cardiomyocytes. To begin to understand the role of Rfl in regulating cardiomyocyte function, an adenoviral-mediated gene delivery approach was used to increase Rfl expression in NRVMs and changes in gene-expression examined using DNA microarrays. Overexpressing Rfl altered the expression of 296 genes, including many known to regulate cardiac function. There was an upregulation of genes associated with an adaptive cardiomyocyte response (e.g., early growth response-1 and PKC epsilon) and downregulation of genes associated with maladaptive cardiomyocyte responses (e.g., MEK1 and PKC delta). To determine if Rfl differentially regulated Ras signaling, activated Ras (V12Ras) was expressed alone or together with Rfl in NRVMs. Expressing V12Ras in NRVMs resulted in activation of ERK1/2, JNK, and PI3-kinase. Increasing Rfl expression alone had no effect on the activity of these kinases; however, Rfl attenuated Ras-mediated JNK activation without affecting ERK1/2 or PI3-kinase activation. These data indicate that Rfl may serve a cardioprotective role by inhibiting Ras-mediated activation of maladaptive signaling pathways (e.g., MEK1/JNK) while preserving signals that are thought to be adaptive (e.g., ERK1/2 and PI3-kinase).

Differential Proteomic Analysis of the Pressure Overloaded Right Ventricle of Young Rats over Time Reveals Specific Changes in Heat Shock and Antioxidant Proteins

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Background: RV hypertrophy and failure are important problems in congenital heart disease. Previously we reported alterations in metabolic and stress proteins in RV hypertrophy after 6 wks of pulmonary artery banding (PAB). To study the protein expression changes over time, differential proteomic analysis was extended to 12 and 20 wks of PAB. Methods: Male Wistar rats (hvk) underwent PAB or sham operation. Three time groups were analyzed: 6, 12, 20 wks (n=6 per group). For proteomics, individual RV homogenates were subfractionated and the cytoplasmic fraction was subjected to 2-DE (pI 3–10, Mw 10–250 kDa). Coomassie blue stained gels of individual animals were compared using PDQUEST. Significantly up- or downregulated spots were in gel identified and analysed by MALDI-TOF-MS linked to Mascot Results. In total, 200 spots were identified. A time-dependent increase of Prx-6 expression suggest its role in antioxidant defense. Conclusion: The progression of RV hypertrophy and failure are important problems in congenital heart disease. Previously we reported alterations in metabolic and stress proteins in RV hypertrophy after 6 wks of pulmonary artery banding (PAB). To study the protein expression changes over time, differential proteomic analysis was extended to 12 and 20 wks of PAB. Methods: Male Wistar rats (hvk) underwent PAB or sham operation. Three time groups were analyzed: 6, 12, 20 wks (n=6 per group). For proteomics, individual RV homogenates were subfractionated and the cytoplasmic fraction was subjected to 2-DE (pI 3–10, Mw 10–250 kDa). Coomassie blue stained gels of individual animals were compared using PDQUEST. Significantly up- or downregulated spots were in gel identified and analysed by MALDI-TOF-MS linked to Mascot Results. In total, 200 spots were identified. A time-dependent increase of Prx-6 expression suggest its role in antioxidant defense.

Proteolytic Cleavage of β-Dystroglycan and Heart Failure

Stephen C Armstrong; Cardiovascular Research Inst, Sioux Falls, SD

Background: Beta dystroglycan (βDG) is a trans-membrane glycoprotein that is central-ized within the dystrophin protein complex and stabilizes the myocardial sarcolemma during osmotic and mechanical stresses. We have reported the production of a 30k proteolytic fragment of βDG (30k-βDG) during myocardial ischemia in an in vitro adult rabbit cardiomyocyte model. The production of this fragment was directly related to the

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onset of irreversible ischemic injury and it was localized to the sarcolemma. This fragment does not bind to the extracellular, laminin binding glycoprotein, alpha-dystroglycan.

Methods/Results: We examined the ß-sarcoglycan (ß-SG) deficient, heart failure model T0-2 hamsters - which reportedly produce myocardial 30K-JDG. This corresponds with the presence of 30K-JDG in sarcoglycanopathy patients. High levels of myocardial ß-SG were observed by Western blotting in the F1B control hamsters. In contrast, T0-2 hamsters demonstrated a marked reduction in myocardial ß-SG, which did not observe 30K-JDG at 8 weeks of age in T0-2 or F1B control hamsters. However, at 10–15 weeks of age, 30K-JDG is produced in high levels in the myocardium of T0-2 animals, relative to the amount of full-length (43Kd) JDG. This contrasts with the low levels of 30K-JDG in F1B hamsters at 10–15 weeks. This production is accompanied by a progressive loss of cardiac function (ejection fraction-EF and fractional shortening-FS) and chamber dilatation (left ventricular inner diameter, diastolic-LVIDd and left ventricular inner diameter, systolic-LVIDs) as determined by M-mode echocardiography. Discussion: Our results suggest that the absence of ß-sarcoglycan disrupts the sarcoglycan complex and renders ß-SG deficient susceptible to proteolytic cleavage. This proteolytic cleavage might predispose the heart to the cardiac dysfunction that is observed in ß-sarcoglycan deficient hamsters. Matrix metalloproteinases are a potential mediator of this proteolytic event.

<table>
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<tr>
<th>LVId(mm)</th>
<th>LVPd(mm)</th>
<th>EF(%)</th>
<th>FS(%)</th>
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<td>F1B-8 wks</td>
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Cardiac Hypertrophy Caused by Deficiency in Cytosolic or Mitochondrial Creatine Kinase
Alexander H Maass, Christoph Zechner, Veronika Posch, Matthias Spindler, Sebastian K G Maier; Univ of Wuerzburg, Wuerzburg, Germany

Background: The creatine kinase (CK) system is an important component of cellular energy homeostasis and therefore critical for normal muscle function. Mice deficient in cytosolic CK (M-CK-/-) have impaired skeletal muscle function, while mice deficient in mitochondrial CK (mito-CK-/-) have normal muscle function. We have previously shown a differential deficiency for endurance vs. high intensity exercise in these mice associated with differential induction of cardiac hypertrophy. The current study was aimed at identifying the cellular basis of cardiac hypertrophy in these mice. Methods and results: Mice were housed in cages with or without access to a cage wheel for 3 weeks and times and distances of wheel running were recorded. M-CK-/- ran slower and shorter than wild type mice whereas mito-CK -/- showed no significant difference. Mito-CK -/- exhibited baseline line hypertrophy that did not increase with exercise. Cellular basis of hypertrophy in mito-CK-/- was an increase in length of cardiac myocytes by 50% vs. wild type animals. This hypertrophy was not associated with an increase in the fetal gene program as commonly observed with disease models of cardiac hypertrophy. M-CK-/- had normal heart size with aggravated hypertrophy vs. wild type when mice were subjected to endurance exercise. This aggravated hypertrophy was associated with an increase in the fetal gene program, most notably increased expression of atrial natriuretic peptide and ß-myosin heavy chain mRNAs. Conclusions: Mito-CK-/- mice perform well, whereas M-CK -/- have a defect when subjected to endurance exercise. The basal hypertrophy observed in mito-CK-/- cannot be further increased by exercise and hearts exhibit no markers of pathologic cardiac hypertrophy. Hearts of M-CK-/- mice respond with aggravated pathologic hypertrophy to additional stress. Current experiments are aimed at functional studies in vivo and signal transduction causing the hypertrophic phenotypes.

High-Fat Diet Prevents Cardiac Hypertrophy and Improves Contractile Function in the Hypertensive Dahl Salt-Sensitive Rat
William Stanley, Isidore C Okere, David J Chess, Paul Ensminger, Brian D Hoyt, Margaret P Chandler; Case Western Reserve Univ, Cleveland, OH

The role that dietary lipid and plasma fatty acid concentration play in the development of cardiac hypertrophy in response to hypertension is not clear. We treated Dahl salt sensitive rats for six weeks with either normal chow (NC; n=7), normal chow with added salt (NC-salt; n=7), or a diet high in long chain saturated fatty acids (60% of calories from fat) with added salt (HFD -salt; n=9). Cardiac function was assessed by echocardiography and systolic blood pressure was measured. Systolic blood pressure measurement was made via: a block in cell cycle progression, prevention of increased myocyte size and inhibition of the induction of atrial natriuretic factor (ANF) mRNA expression. Thus, sizes of neonatal myocytes treated with serum and 2.5 or 5 µM NSC672121 were reduced significantly (39.3% and 62.3%, resp.), compared to cultures stimulated with serum alone (100±3%). Similarly, ANF mRNA expression in neonatal myocytes treated with serum and 2.5 µM/L NSC672121 was significantly reduced to levels equivalent to that of serum-starved cultures. Moreover, NSC672121 did not induce hyperphosphorylation of ERK1/2 in adult myocytes at doses of 10µmol/L or less, although these doses were sufficient to inhibit significantly the induction of ANF expression, consistent with an abrogation of cardiomyocyte hypertrophy. Taken together, NSC672121 abrogates the activation of the Raf-MEK-ERK signalling pathway that occurs during hypertrophy and blocks the induction of hypertrophy in both neonatal and adult cardiomyocytes. Thus, phosphatases inhibited by NSC672121, such as CD25, might prove to be suitable pharmacotherapeutic targets for the treatment of detrimental hypertrophy.

The Protein Tyrosine Phosphatase Inhibitor NSC672121 Prevents the Induction of Hypertrophy in Cardiomyocytes
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Mitogen-activated protein kinase (MAPK) signalling pathways have been associated with the induction of cardiac hypertrophy in response to different hypertrophic stimuli. In this study, we have investigated the role that protein tyrosine phosphatases (PTP) play in modulating cardiac hypertrophy, using the PTP inhibitor, 2-(3-mercapto-1-hydroxyethyl)-3-methyl-1,4-naphthoquinone (NSC672121; also known as CpdD5). A potent inhibitor of cell growth, NSC672121 is thought to mediate cell cycle arrest by inhibiting CDC25 phosphatases. Recently, it has been shown that NSC672121 treatment also can lead to hyperphosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2). The mechanism of this hyperphosphorylation has been suggested to involve inhibition of the dephosphorylation of Raf and/or ERK1/2 by CDC25 or MAPK phosphatases (MKPs), respectively. In this study, we have shown that co-treatment of neonatal or adult cardiomyocytes with 20% serum and various concentrations of NSC672121 (0.5–5µmol/L) for 24h prevented the induction of hypertrophy in a dose-dependent manner as shown by: a block in cell cycle progression, prevention of increased myocyte size and inhibition of the induction of atrial natriuretic factor (ANF) mRNA expression. Thus, sizes of neonatal myocytes treated with serum and 2.5 or 5 µmol/L NSC672121 were reduced significantly (39.3% and 62.3%, resp.), compared to cultures stimulated with serum alone (100±3%). Similarly, ANF mRNA expression in neonatal myocytes treated with serum and 2.5 µmol/L NSC672121 was significantly reduced to levels equivalent to that of serum-starved cultures. Moreover, NSC672121 did not induce hyperphosphorylation of ERK1/2 in adult myocytes at doses of 10µmol/L or less, although these doses were sufficient to inhibit significantly the induction of ANF expression, consistent with an abrogation of cardiomyocyte hypertrophy. Taken together, NSC672121 abrogates the activation of the Raf-MEK-ERK signalling pathway that occurs during hypertrophy and blocks the induction of hypertrophy in both neonatal and adult cardiomyocytes. Thus, phosphatases inhibited by NSC672121, such as CD25, might prove to be suitable pharmacotherapeutic targets for the treatment of detrimental hypertrophy.

High-Salt Diet Exacerbates Isoproterenol-Induced Cardiac Hypertrophy and Myosin Isoform Switching
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Isoproterenol (ISO) exerts positive chronotropic and inotropic effects via ß-adrenergic receptors. Sustained ISO administration produces myocardial hypertrophy and myosin isoform switching; manifested as increased heart weight:body weight (HWI), downregulation of ß- myosin heavy chain (ß-MHC), and upregulation of ß- myosin heavy chain (ß-MHC), respectively. Since ISO activates CHKATPase activity greater than that of ß-MHC, the isoform switch results in reduced myocardial shortening velocity and eventual contractile dysfunction. We hypothesized that a high-salt (HS) diet would enhance ISO-induced hypertrophy and potentiate induction of fetal genes. Male Sprague-Dawley rats (175 - 225g) were placed into weight-matched groups and fed a normal salt (NS) diet. Prior to surgery (3d), rats were placed on a HS (8.0% NaCl) diet or maintained on NS (0.3% NaCl). Rats then continuously received either ISO (4 mg/kg/d c.s.) or vehicle (VEH: 0.5 mg/ml sodium bisulfite in 0.9% NaCl) via implanted osmotic pumps. After 2 or 4 days, sustained ISO stimulation differed by measuring heart rate (HR). Rats were then sacrificed and subjected to morphological and biochemical analysis. HR did not differ between HS and NS fed VEH rats. ISO produced greater HR increases in HS than NS after 2 (HS: 25.3±2.9% vs. NS: 16.8±3.5%) and 4 days (HS: 33.3±3.9% vs. NS: 16.6±2.2%) relative to same-diet VEH rats. Although myocardial hypertrophy was induced in all ISO rats, HS
Development and Validation of a Whole-Cell Immunodetection Assay for the Identification of Natriuretic Peptide Signaling Pathway Modulators in Neonatal Rat Ventricular Myocytes


The natriuretic peptide (NP) signaling pathway exhibits a critical capacity to negatively modulate the signaling mechanisms of several known pro-hypertrophic stimuli in cardiomyocytes (e.g. AT2, ET1, PE etc.). One of the key components of this signaling pathway is atrial natriuretic peptide (ANP), which binds to the extracellular surface of the receptor. The ANP-dependent increase in intracellular cGMP levels subsequently causes the allosteric activation of protein kinase G (PKG), which is the predominant mediator of the anti-hypertrophic signaling events of the NP signaling pathway. Importantly, small molecule therapeutics that positively modulate the NP signaling pathway in cardiomyocytes may provide a unique means of prevention and/or reversal of pathological cardiomyocyte hypertrophy; a condition that precedes dilated cardiomyopathy in the failing human heart. Here we describe the development and validation of a cell-based assay for NP signaling pathway modulators using the immunodetection-based cytoblot technology. This assay is based on the detection of a PKG specific phosphorylation event in neonatal rat ventricular myocytes and is sensitive to molecules that augment the NP signaling pathway by either elevating cGMP levels or by directly activating PKG. We have assessed the responsiveness of this assay to NP signaling pathway modulators using ANP, a soluble guanylate cyclase activator (SNAP), phosphodiesterase inhibitors (e.g. ZAP/RINAV) and a direct PKG activator (B-8CPT-cGMP). We have also validated the performance of the assay under high throughput assaying conditions by assaying a limited collection of chemically diverse small molecules. The results of our assay development and validation are presented.

CXCR4: A New Functional Receptor on the Myocardium that Modulates Cardiac Contractility

Robert Pyo, Jinling Sui, Diomedes Logothetis, Mount Sinai Sch of Med, New York, NY; Burns Blaxall Univ, Rochester, Rochester, NY; Roger Hajjar, Massachusetts General Hosp, Boston, MA; Alison Schecter; Mount Sinai Sch of Med, New York, NY

Inflammatory mediators are important in the development of heart failure (HF). Chemo- kines, small glycoproteins that elicit directed migration of leukocytes to sites of inflammation, have been implicated in the pathogenesis of HF, by mechanisms underlying chemokine modulation of cardiac function are unknown. To evaluate whether CXCL12 (a.k.a. stromal cell-derived factor-1) can directly function as a positive inotrope, we measured papillary muscle (PM) contractility. Isometric tension generated by increasing [Ca2+]i concentrations of extracellular calcium [Ca2+]o was calculated. Log ED50 was calculated. (Log ED 50 Control: 1.5 ± 0.04, Log ED 50 AS: 2.0 ± 0.30) and field stimulated (0.5 Hz, 25°C) to measure sarcomere shortening (SS). Thiol quantification was performed by two-photon microscopy while visualization of spontaneous Ca2+ sparks was obtained by optical imaging. Purified cardiac rymodine receptors (RyR2) were reconstituted in planar lipid bilayers to test HNO effect on RyR2 function. The HNO donor Anger’s Salt (AS) increased SS (n = 117.±25% at 1.0 mM, n13; p < 0.01 vs base) without changes in Ca2+ transients. This effect was not reproduced by equimolar NO donated by DEA/NO. In contrast to DEA/NO, inhibition of guanylyl-cyclase n13; p < 0.01). Pre-treatment with GSH abrogated the increase in ss. Thus, HNO may improve cardiac function in a manner that could be therapeutically useful in patients with heart failure.

Nitroxy Anion: A Novel Thiol-Sensitive Positive Inotrope that Enhances SR Ca2+ Release

Carlo G Tocchetti, Johns Hopkins Med Inst, Baltimore, MD; Wang Wang, Lab of Cardiovascular Science, National Inst on Aging, NIH, Baltimore, MD; Hector H Valdivia, Univ of Wisconsin Med Sch, Madison, WI; Miguel Aon, Johns Hopkins Med Inst, Baltimore, MD; Giulietta Di Benedetto, Venetian Inst of Molecular Med, Padua, Italy; Brian O'Rourke, Johns Hopkins Med Inst, Baltimore, MD; Jeffrey P Froehlich, Univ of Maryland Sch of Med, Baltimore, MD; Marcello Zaccari, BIOCHEMA, Carlo Mazzoni, M.D. Sci, Med, Padua, Italy, Heping Cheng, Lab of Cardiovascular Science, National Inst on Aging, NIH, Baltimore, MD; David A Kass, Nazzareno Paolocci, Johns Hopkins Med Inst, Baltimore, MD

Nitroxy (HNO), the one-electron reduced form of nitric oxide (NO), is a redox-sensitive positive inotrope in vivo, although mechanism of action has remained unclear. Here we show that HNO directly stimulates sarcoplasmic reticular (SR) Ca2+ release and this effect is sensitive to cellular thiol content. Isolated cardiac myocyte myocytes were suspended in Tyrode’s solution (1mM Ca2+) and field stimulated (65 Hz, 2°C) to measure sarcomere shortening (SS). Thiol quantification was performed by two-photon microscopy while visualization of spontaneous Ca2+ sparks was obtained by optical imaging. Purified cardiac rymodine receptors (RyR2) were reconstituted in planar lipid bilayers to test HNO effect on RyR2 function. The HNO donor Anger’s Salt (AS) increased SS (n = 117.±25% at 1.0 mM, n13; p < 0.01 vs base) without changes in Ca2+ transients. This effect was not reproduced by equimolar NO donated by DEA/NO. In contrast to DEA/NO, inhibition of guanylyl-cyclase n13; p < 0.01). Pre-treatment with GSH abrogated the increase in ss. Thus, HNO may improve cardiac function in a manner that could be therapeutically useful in patients with heart failure.

Direct Activation of Cardiac Myosin, a Novel Mechanism for Improving Cardiac Function

Fady Mallak, Cytokinetics, Inc, South San Francisco, CA; You-Tang Shen, Univ of Med and Dentistry of New Jersey, Newark, NJ; Tatsu Katori, Johns Hopkins Univ, Baltimore, MD; Sandra H Suenou, Robert Anderson, David Cox, Marc Garard, James Hartman, Cytokinetics, Inc, South San Francisco, CA; Song-Jung Kim, Univ of Med and Dentistry of New Jersey, Newark, NJ; Erica Krzywack, Cytokinetics, Inc, South San Francisco, CA; Alex Kukov, Univ of Med and Dentistry of New Jersey, Newark, NJ; Kenneth H Lee, Pu-Ping Lu, Alex Muci, Congrong Niu, Hector Rodriguez, Ion Sustehiro, Sheila Sylvester, Todd Tochimoto, Kathleen A Elias, Bradley P Rosen, Robert M Myerowitz, Stephen F Patner, Univ of Med and Dentistry of New Jersey, Newark, NJ; David J Morgans; Cytokinetics, Inc, South San Francisco, CA

Current inotropes act upstream of the sarcosome to increase intracellular calcium and secondarily increase cardiac contraction. In addition to effects on contractility, these agents increase heart rate, oxygen consumption, and the incidence of arrhythmias, as well as reduce blood pressure. A more direct approach to improve cardiac contractility that may addresse these liabilities is activation of the force generating protein, cardiac myosin itself. Utilizing a reconstituted version of the cardiac sarcosome, we screened a small molecule library and identified several chemical classes that activate the cardiac myosin ATPase. One compound class has been optimized extensively using an iterative process guided by biochemical and cellular activity. OK-1213296 is an exemplar of this class. Transcript kinetic analysis of the mechanism of action demonstrates that OK-1213296 accelerates the release of phosphate by 2 fold (EC50 = 2.0 ± 0.7 μM) without affecting the ADP release rate, suggesting that OK-1213296 accelerates transition of myosin into the force-generating state without affecting its exit rate. Using Fura-2 loaded primary rat cardiac myocytes, OK-1213296 (0.5 μM) increased cellular contractility by 30 ± 1.3% but did not alter peak systolic calcium (Fura-2 ratio − 1.24 ± 0.02 after treatment vs. 1.25 ± 0.01 at baseline, n = 12, p > 0.05). In anesthetized normal rats, infusion (6.3 mg/kg bolus followed by 9.0 mg/kg/h) of OK-1213296 increased echocardiographic fractional shortening from 47.1 ± 5.5% at baseline to 59.2 ± 4.0% at 30 minutes (n = 6, p < 0.01) while vehicle controls did not change significantly (p > 0.05). In a chronically instrumented conscious dog heart failure model produced by myocardial infarction plus tachycardia pacing, infusion of OK-1213296 (8 mg/kg bolus followed by 8 mg/kg/h, n = 7) rapidly increased (< 0.05) systolic elastance, regional wall thickness, and fractional shortening by 20±6%, 38±11% and 34±8% respectively. In addition, heart rate and left atrial pressure reduced by 14±2% and 4±2% mmHg while mean arterial pressure was not significantly altered and diastolic function was not impaired. Thus direct activation of cardiac myosin improves cardiac function in a manner that could be therapeutically useful in patients with heart failure.
Nitroxylic Stimulation ATP-Dependent Ca2+ Uptake in Sarcoplasmic Reticulum Membranes Isolated from Mouse Heart

Jeffrey P. Froehlich, Gerald Wilson, Univ of Maryland Sch of Med, Baltimore, MD; Carlo G. Tocchetti, David A. Kass, Nazareno Paolocci, Johns Hopkins Med Inst, Baltimore, MD

Nitroxylic anion (HNO/NO) generated from Angel's salt (AS) exerts positive inotropy/lusitropy on canine (in vivo) and murine (in vitro) hearts. These findings suggest that HNO stimulates ATP-dependent Ca2+ accumulation by the sarcoplasmic reticulum (SR) Ca2+ pump (SERCA2a). These effects are independent of p2-receptor activation, implying that they involve elements of the Ca2+ signaling mechanism downstream from the receptor or a direct action on the pump itself. To test if AS/HNO can directly modify SERCA2a, we isolated SR vesicles from mouse hearts and studied ATP-dependent Ca2+ uptake by stop-flow stopped mixing arsenazo III as Ca2+ indicator. The free [Ca2+]i in the incubation medium was adjusted to ~2 μM calculated from the Ca2+ binding constant of arszenazo III (κd = 30 μM). Mixing murine SR vesicles with 0.5 mM ATP at pH 7.4 and 21°C resulted in a biphasic time course of Ca2+ accumulation that was ~75% complete in 1 sec and reached a plateau by 6–10 sec. Ca2+ uptake in the slow phase was sensitive to the Ca2+ ionophore, A23187, consistent with it representing Ca2+ transport into the vesicle lumen. Exposure of the SR vesicles to 250 μM AS for 20 min increased the velocity of Ca2+ uptake in the slow phase, resulting in a monophasic time course that equilibrated in less than 2 sec. Total Ca2+ uptake remained unchanged in the presence of AS. These findings support the hypothesis that AS stimulates the catalytic efficiency of SERCA2a (Vmax) by relieving the back inhibition from the Ca2+ transport gradient. Quenched-flow experiments with canine cardiac SR vesicles, which increase Ca2+ uptake activity in response to AS, revealed that AS activates E2P hydrolysis, which competes with Ca2+ re-binding at the luminal transport sites (on E2P). E2P hydrolysis is also activated by phosphorylation of phospholamban (PLB) and by the depletion of PLB from the SR membrane, both of which stimulate Ca2+ pump activity. We propose that AS/HNO acts by chemical modification of SERCA2a, PLB or both, and that this stimulates Vmax by relieving the PLB-dependent inhibition of SERCA2a and, secondarily, by reducing the affinity of the luminal transport sites for Ca2+. These findings combined with the fact that HNO enhances intracellular Ca2+ release explain HNO in vivo positive inotropy/lusitropy.

Isoform-Specific Differential Roles of the Sarcolemmal Calcium Pump in the Heart

Delvace Oceandy, Sukhyal Prehar, Florence M Baudoin, Min Zi, Elizabeth J Cartwright, Ludwig Neyens; Univ of Manchester, Manchester, United Kingdom

The role of the plasma membrane calcium ATPase (PMCA), an enzyme extruding calcium from the cytosol, in cardiac physiology is incompletely understood. Two isoforms of PMCA, PMCA1 and 4, are expressed in the cardiomyocytes. We have previously reported that PMCA4b is involved in cellular signalling rather than excitation-contraction coupling, but the differential role of the isoforms 1 and 4 remains enigmatic. To address this question we generated transgenic mice overexpressing PMCA1b or 4b in the myocardium under the control of the MLC2v promoter and performed invasive haemodynamics at baseline and in response to isoproterenol using a pressure-volume catheter system. Transgenic mice overexpressing PMCA1b had significantly increased isotropic response to isoproteoroten compared to wildtype as reflected by the percentage change in end systolic elastance (Ees). These findings combined with the fact that HNO enhances intracellular Ca2+ release explain HNO in vivo positive inotropy/lusitropy.
Association of Improvements in Parameters of Idiopathic Dilated Cardiomyopathy with Treatment of Insulin Resistance Using Thiazolidinediones and Metformin

Paul B Berez; Anne Arundel Med Ctr, Annapolis, MD

This retrospective, observational research was instigated by the observation of several dramatically improved idiopathic dilated cardiomyopathy (ICD) cases in our clinic associated with treatment of insulin resistance in both diabetic and non-diabetic patients. The clinical data of all patients with a diagnosis of ICD in our clinical setting, and numbered 16 cases. All were either diabetics or underwent a 50 gram 1 hour glucose challenge test with post challenge glucose and insulin levels showing insensitivity of insulin resistance. Only data unassociated within 6 months of acute ischemic events was counted. All patients received standard therapy for ICD, although over the time of observation, some new treatments were added to accepted standard therapy. The majority of cases had no change in ICD-active medications beginning to end. When T2D medications were used, generally extra diuretic dosage was empirically prescribed, and electrolytes followed closely. Often, with insulin treatment, the diuretic dosage was later decreased. The benefits and risks of using metformin and T2D medication in these patients was discussed, and no adverse events occurred. One patient was counted as a 2 cases as the T2D medication was withdrawn by other physicians at another hospital during a critical illness, with regression back to severe clinical ICD. This patient was retreated with a T2D medication and dramatic improvement again observed. Echocardiographic data was obtained, but certain parameters were sometimes missing from the data due to technical limitations of the studies.

RESULTS: Ejection Fraction beginning 35.2%, and 45.8% (n=14, P= 0.007–paired t-test). Left Ventricular Systolic beginning 4.85cm, and 3.82 cm (n=11, P not significant). Left Atrium beginning 4.39 cm, and 4.46 cm (n=12, P not significant). In the case described above, a 71 year old AA woman with metabolic syndrome, a dose-response relationship of the T2D to the echocardiographic parameters was observed.

CONCLUSION: This observational study provides support for the hypothesis that insulin resistance medications may lead to clinical improvement in ICD, and we are now planning a prospective study to test this hypothesis.

Infusion of Inotropes During Myocardial Reperfusion Does Not Affect the Extent of the No-Reflow Phenomenon: Experimental Study

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Background: Inotropic support is often necessary in the setting of acute myocardial infarction, especially when complications arise. We sought to investigate the effect of levosimendan and dobutamine (agents with positive inotropic properties used in clinical practice) on the extent of the no-reflow phenomenon (NRP) and coronary blood flow (CBF) in a porcine experimental model of ischemia-reperfusion. Methods: In 18 animals after mid-thoracotomy, the left anterior descending coronary artery was ligated for 60 min, followed by 120 min of reperfusion. At 50 min of the ischemia period, the animals were randomly assigned to three groups. Group A (n=6) animals received no medication, whereas in group B (n=6), levosimendan (24 μg/kg/min infusion) and in group C (n=6), dobutamine (10 μg/kg/min) were administered during reperfusion, started 10 min before the end of the ischemia period. The no-reflow area was assessed by thioflavine staining and was expressed as a percentage of the area of the left ventricle at risk. Coronary flow was recorded by a probe of an ultrasound transit time flowmeter, placed at the site of the ligation and CBF during reperfusion was expressed as a percentage of baseline CBF. Results: Neither levosimendan nor dobutamine infusion affected NRP compared to controls (55±5.2% versus 45.7±10.7 versus 49.7±15.9%, respectively, P=ns between groups). There was a tendency for higher CBF in group A, in comparison to groups B and C, but this difference was not statistically significant. Conclusions: Levosimendan and dobutamine infusions had an insignificant effect on NRP and CBF when administered during the reperfusion period in a porcine experimental model of acute myocardial infarction. According to these results, levosimendan and dobutamine can be used safely in the setting of acute myocardial infarction, subjected to 20 minutes of global ischemia at 37°C followed by 2 hours of reperfusion, during which time we monitored high-energy phosphates using 31P-NMR spectroscopy (n = 5), and left ventricular developed pressure (LVDP). In another group of hearts (n = 5), we monitored intracellular Na+ using 23Na-NMR spectroscopy. Consistent with Ca2+ entry via NCX during ischemia, we found that hearts lacking NCX had significantly less decline in ATP during ischemia (NCX: 39 ± 6% of pre-ischemic ATP level vs. WT: 17 ± 5%; P<0.05), a delay in time to ischemic contracture (NCX: 17 min vs. WT: 12 min; P<0.05), and reduced maximum contracture. Furthermore, on reperfusion following ischemia, NCX KO hearts showed better recovery of LVDP (36 ± 4% of pre-ischemic level in NCX vs. 9 ± 2% in WT), improved PCR recovery, and reduced Na+ overload. Furthermore, NCX KO hearts had reduced infarct size (7.0 ± 2.5% in NCX vs. 49.5 ± 8.7% in WT). Thus, NCX-KO hearts exhibited significantly less ischemia-reperfusion injury, suggest that Ca2+ entry via reverse mode NCX is a major cause of ischemia-reperfusion injury and that the inhibition of NCX during angioplasty, cardiac surgery, and ischemia is a promising therapeutic target.

Biochemical and Proteomic Characterization of the Murine Cardiac 26S Proteasome

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The 26S proteasome system is the major intracellular quality control system that targets misfolded proteins for refolding or degradation. By maintaining proper intracellular protein homeostasis, a well organized and well regulated 26S proteasome system is essential to the viability of eukaryotic cells. Characterization of the proteasome in murine heart is an important step towards understanding the role of the proteasome system in cardiac pathophysiology. The 26S proteasome is composed of a 20S core complex and one or two 19S regulatory complexes. Biochemical studies showed that the proteolytic activities as well as the amount of 20S proteasome in the heart are significantly less than those of the 20S in the kidney, liver and lung. Purification and characterization of the murine cardiac 26S proteasome identified the expression of at least 37 distinct subunits in this vital cardiac organelle. Western immunoblotting confirms the expression of key subunits in the isolated cardiac cells. Among the subunits detected in the cytosolic fraction of the normal cardiac tissue are the three inducible 20S subunits, β1i, β2i, and β5i. Importantly, an alternative splicing isoform of a 19S subunit was found together with its dominant isoform. The co-expression of the three inducible 20S isoforms as well as that of the alternatively spliced subunits in the 19S proteasome suggest a complex level of regulation on this protein degradation machinery in the heart. In addition, LC/MS/MS characterized that a subset of these 37 subunits were N-terminally acetylated (e.g., RPT3); separately, a subset of these subunits were N-terminally myristoylated. After undergoing ischemia/reperfusion the amount of 26S proteasome present in the heart (determined by western blotting of both 19S and 20S subunits) showed no significant change compared to the sham heart. However, the β1j and β5j proteolytic activities, but not the βj2 activity, of the 20S proteasome were selectively reduced in the ischemic heart, indicating regulation of the cardiac 26S proteasome system by ischemia/reperfusion injury.

Overexpression of Cyclic GMP-Dependent Protein Kinases Attenuates Necrosis and Apoptosis following Ischemia/Reoxygenation in Adult Rat Cardiomyocytes via Generation of ROS and Opening of the Mitochondrial KATP Channel

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Background: Cyclic GMP-dependent protein kinases (PKG) are major mediators of cGMP signaling in the cardiovascular system. Depending on the type of cells, PKGs are involved in the proapoptotic/necrotic cell death as well as antiapoptotic/prosurvival pathways in the cardiovascular and nervous systems. We designed the present study to determine the direct role of these kinases in protection of cardiomyocytes against necrosis and apoptosis following ischemia/reoxygenation injury. In addition, we investigated the involvement of K<sub>ATP</sub> channels and ROS in myocyte protection with PKGs. Methods & Results: Adult rat cardiomyocytes were infected with adenoviral vectors containing PKGβIIIa or PKGβ II beta coding regions. After 24 hours, the cells were subjected to 90 minutes of simulated ischemia (SI) and 1 hr of reoxygenation (RO). To evaluate the role of K<sub>ATP</sub> channels, sub-group of cells were treated with 5-hydroxydecanoate (5-HD, 100 μM), HMR11098 (30 μM) or gliclazide (50 μM), the respective blockers of mitochondrial, sarcolemmal or both types of K<sub>ATP</sub> channels prior to simulated ischemia. Necrosis was determined by trypan blue exclusion assay and apoptosis was assessed by TUNEL staining. Overexpression of PKG I alpha and I beta significantly reduced necrosis, i.e. decreased the number of trypan blue positive cells (from 34.1±2.9% to 0% of total cells in SI-R0 control group to 19.6±1.8% in Ad-PKGα and 15.48±0.53% in Ad-PKGβII group respectively, P<0.001). The number of TUNEL positive cells after 90 min of SI and 18 hours of RO was also reduced from 15.69±1.01% of total cells in SI-R0 control group to 6.72±0.54% in Ad-PKGα and 6.53±0.87% in Ad-PKGβII groups (n=3; P<0.001). Both gliclazide and 5-HD completely abolished the protection against necrosis and apoptosis in myocytes overexpressing Ad-PKGα and I beta while...
HMR1098 had no effect on protection. In addition, N-(2-mercaptopyrrolopropanoylglycine (MPG, 1mM), a putative ROS scavenger also blocked protection against necrosis and apoptosis in cardiomyocytes overexpressing PKG-I alpha and I beta. **Conclusion:** Overexpression of PKG-I alpha and I beta protect cardiomyocytes against necrosis and apoptosis through opening of mitochondrial K_{ATP} channels and release of ROS.

**Differential Regulation of Mitochondrial and Cytosolic Redox States by Substrate Supply and Oxygen Delivery During Ischemia**

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During the initial period of myocardial ischemia oxygen consumption is reduced and glycolysis is activated, resulting in increased NADH/NAD^{+} in the cytosol and mitochondria that effect lactate metabolism and pyruvate and fatty acids oxidation. It is not possible to assess metabolic fluxes and metabolites in cytosolic and mitochondria compartments in the intact heart, however it can be predicted using in silico computational models. We recently developed a model of human myocardium that incorporates cytosolic and mitochondrial compartments and key metabolic species and reactions, (e.g. glycolysis, proton gradient, oxidative phosphorylation, malate/aspartate shuttle, fatty acid oxidation, and TCA cycle). The model was composed of 51 nonlinear differential equations and was solved by DLSODE (a robust implicit integrator for stiff and sparse systems). Initial state conditions with normal coronary flow (1 ml/g/min) approximated values obtained on pigs, dogs and humans. Flow reductions to 0.7, 0.4 and 0.1 ml/g/min were simulated, and closely matched experimental data for MVO_{2} and lactate production observed in our experiments. We investigated the changes in cytosolic and mitochondrial NAD^{+} and [NADH] over the initial hour of ischemia (see Fig.). Mitochondrial redox was rapidly reset to higher values in proportion to the reduction in oxygen delivery while cytosolic redox showed a biphasic response, with a sharp initial increase followed by an exponential decrease to new steady state. In conclusion, model simulations demonstrate that mitochondrial redox is controlled by oxygen delivery, while cytosolic NADH/NAD^{+} is controlled both by glycolytic flux and oxygen delivery.

**Cardiomyocyte-Restricted Overexpression of the Human eNOS Gene Significantly Attenuates Myocardial Ischemia Reperfusion Injury**

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We examined the effects of cardiomyocyte-specific transgenic overexpression of the human eNOS gene (CS-eNOSTg) in an in vivo murine model of myocardial ischemia reperfusion injury (MI/R). Specifically, we compared CS-eNOSTg mice against mice with systemic overexpression of the human eNOS gene (SYS-eNOSTg) to wild-type (WT) controls. Mice were subjected to 30 min. of left coronary artery occlusion and 24 h reperfusion. Myocardial infarct size was measured using Evans Blue and TTC staining. In addition, cardiac function was assessed at baseline and 72 hr. following MI/R using 2-D echocardiography. Systemic eNOS overexpression reduced myocardial infarct size (INF) per area-at-risk (AAR) by 42%. In contrast, cardiac specific eNOS overexpression attenuated INF/AAR by 63%. Postischemic myocardial fractional shortening (%FS) was significantly improved (p < 0.05) in the CS-eNOSTg (33.1 ± 4.3) vs. wild-type (24.9 ± 2.3) but not in SYS-eNOSTg mice (24.6 ± 3.3). These results suggest that targeted genetic overexpression of eNOS in the myocardium may be superior to global overexpression of eNOS in attenuating ischemia reperfusion injury.

**Myocardial Infarct Size**

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<th>Time (min)</th>
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***p < 0.001 vs. WT***

**Reversible Blockade of Electron Transport During Ischemia Protects Myocardium by Preserving Mitochondrial Function**

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Mitochondrial dysfunction contributes to myocardial injury during ischemia (ISC) and reperfusion (REP). ISC damages electron transport complexes I, II, and III leading to a decrease in the rate of oxidative phosphorylation (OXPHOS). Reversible blockade of electron transport with amytal before ISC protects OXPHOS during ISC. We proposed that protection of OXPHOS during ISC will preserve OXPHOS and decrease myocardial damage during REP. Langendorff perfused rat hearts were treated with amytal (2.5 mM bolus for 1 min immediately before ISC) or vehicle and underwent 25 min global ISC (37°C) and 30 min REP without additional treatment. Subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria were isolated at end of REP to measure OXPHOS with glutamate as substrate. Left ventricular developed pressure (LVDP), diastolic pressure (DP) and lactate dehydrogenase (LDH) release were measured. Amytal protected OXPHOS in SSM and IFM following ISC-REP with preserved state 3 and the decreased state 4 rates leading to improved respiratory control ratio (RCR). Amytal also prevented ischemic contracture (DP-ISc) and improved functional recovery during REP with increased left ventricular developed pressure (LVDP-REP) and decreased diastolic pressure (DP-REP). Amytal attenuated LDH release during REP, indicating decreased myocyte cell death. Thus, reversible blockade of electron transport during ISC preserves mitochondrial OXPHOS and mitigates myocardial damage during REP.
implantation alone or simultaneous cell/angiogenesis treatment. These data suggest the AdVEGF pre-treatment enhances left ventricular function to an extent greater than either cell/H11006 alone (38/H11006 animals (1.9/H11006 per-fusion (Evans Blue and 15/H11006 ligation and injection of an adenovirus encoding VEGF 121(AdVEGF, n/H11006 application of either strategy alone endogenous TNF signaling is cytoprotective in the setting of myocardial ischemia/survival pathways. Accordingly, the purpose of this study was to determine whether protein, TNF receptor associated factor 2 (TRAF2) which may lead to activation of cell In response to myocardial injury, the proinflammatory cytokine, tumor iNOS activity without affecting the AT-mediated increased expression. AT also increases myocardial We have shown that Atorvastatin (AT) reduced infarct size (IS) by increasing expression and activity of cyclooxygenase-2 (COX2). AT also increases myocardial concentration of iNOS. Co-administration of oral valdecoxib, a COX2 inhibitor, abrogated the protective effect of AT without affecting the iNOS expression. We investigated the effects of COX1, COX2 and iNOS on IS and whether iNOS is mediating the AT-induced increased COX2 activity. Methods: Rats received 3-day pretreatment with AT 10 mg/kg added to the drinking water or water alone. In addition, rats received IV SC58125 (COX2 inhibitor), SC560 (COX1 inhibitor), 1400W (iNOS inhibitor) or vehicle alone. 15 min later rats underwent 30 min ischemia followed by 4h reperfusion (I/R protocol), or the hearts were explanted without being subjected to ischemia (ELISA and immunoblotting), COX2 activity was measured by ELISA for tissue 6-keto-PGF1α after adding arachidonic acid with and without SC58125. iNOS activity was measured by L-arginine to L-citrulline conversion assay. Results: Body weight, left ventricular weight and the area at risk were comparable among groups. AT reduced IS. SC58125 and 1400W attenuated this effect without affecting IS in the non-A/I treated rats (Fig. 1). AT increased COX2 activity. Both SC58125 and 1400W attenuated this effect (Fig. 2). SC560 did not affect IS or COX2 activity. SC58125 did not affect iNOS expression and activity, whereas 1400W blocked iNOS activity without affecting the AT-mediated increased expression. Conclusions: AT reduced IS by increasing the expression and activity of both IS and COX2. iNOS is upstream to COX2 and is needed for COX2 activation.
157 Cardioprotective Effects of a Selective β Estrogen Receptor Agonist in a Mouse Ischemia-Reperfusion Injury Model

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Compared to female hearts, perfused male mouse hearts show increased ischemia-reperfusion injury under hypercontractile conditions. Studies of transgenic β estrogen receptor (ER) and βER knock out mice suggest that the estrogen-mediated cardioprotective effects are βER dependent. This study was designed to investigate whether overactivity abolishes the cardioprotection observed in female mouse hearts and whether cardioprotection can be restored by treatment with a selective βER agonist (2,3-bis(4-hydroxyphenyl)-propionitrile, DPN). Overactivity in female mice were treated with 17β-estradiol, a selective βER agonist, or vehicle for two weeks using subcutaneously implanted Azeb mini-implants. Isolated Langendorff-perfused hearts were perfused for 25 minutes prior to a one minute treatment with isoproterenol, followed by 20 minutes of normothermic global ischemia and 40 minutes of reperfusion. Left ventricular developed pressure (LVPD) and heart rate were measured using a latex balloon inserted into the left ventricle and connected to a pressure transducer. Recovery of cardiac function at the end of the 40 minutes of reperfusion was expressed as the percentage of the pre-ischemic rate pressure product (RPP = LVPD x heart rate). We observed that the hearts from overactivity mouse mice had a significantly lower functional recovery than the hearts from intact female mouse hearts. Furthermore, hearts from male mice treated with a selective βER agonist exhibited significantly better functional recovery than hearts from either vehicle treated overactivity male mice or wild type male mice. These findings suggest that under hypercontractile conditions induced by overactivity, selective βER agonist has a cardioprotective effect in the mouse model.

158 Glucose-Dependent Insulinotropic Polypeptide Treatment Eliminates Gender Differences in Ischemia-Reperfusion Injury by Affecting Substrate Selection

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Under conditions of hypercontractile function, female hearts exhibit less ischemia/reperfusion injury compared to males. Peptides such as glucagon like peptide and glucose-dependent insulinotropic polypeptide (GIP) have been shown to reduce injury. We examined whether agonists such as GIP would be equally protective in males and females. We treated Langendorff perfused male and female mouse hearts with 10 mM GIP for 25 min prior to 1 min treatment with 10mM isoproterenol followed by 20 min of global ischemia. In untreated hearts, females had significantly better recovery (35±5%) and significantly less necrosis (52±7%) than males (17±2% recovery; 73±5% infarct). Treatment with GIP resulted in little change in functional recovery or infarct size in the female group, whereas the male group showed significantly better recovery of function (56±8%) and significantly less necrosis (41±8%). We tested whether the protection is related to glucose utilization by using 13C nuclear magnetic resonance spectroscopy to measure the ratio of glucose/acetate incorporated into glutamate in the heart. Untreated and GIP treated male and female hearts were perfused with a mixture of 13C-labeled glucose (10mM) and acetate (0.25mM) to measure whether GIP had any affect on substrate selectivity. GIP treatment in female hearts had higher basal glucose/acetate ratios than males. GIP treatment resulted in no change in the glucose/acetate ratio in females, whereas the ratio in males increased from 0.8 to 1.4 (p<0.05). Since glycogen synthase kinase-3 (GSK) has been shown to phosphorylate pyruvate dehydrogenase, we investigated whether treatment with GIP might be effective at balancing glucose and acetate oxidation through a GSK-mediated mechanism. In untreated animals, males had P-GSK levels which were 3.5 fold lower than females (P<0.05). After treatment with GIP, P-GSK levels in the females remained similar, whereas the males exhibited an eight-fold increase in P-GSK (P<0.05). These data suggest that protection in females may involve an increase in phosphorylated GSK which results in enhanced oxidation of glucose. GIP enhances protection in males by increasing GSK phosphorylation and glucose oxidation to levels observed in females.

159 Protective Effect of Oxo-Bridged Dinuclear Ruthenium Amine Complex(ru360) in Reperfused Rat Myocardium

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Ischemic preconditioning (IPC) is one of the most efficient strategies for reducing ischemia/reperfusion injury. The precise mechanism by which IPC exerts its protective effect is still under consideration. One hypothesis proposes, that the opening of mitochondrial K+-ATP channels dissipate the inner mitochondrial membrane potential established by the proton pumps reflecting the driving force for Ca2+ influx, in mitochondria. Mitochondrial calcium uniporter (mCaU), thus attenuating mitochondrial calcium overload. Mitochondrial calcium overload has been closely related to myocardial damage and cell death. Current work in our laboratory, shows that ru360 exerts protection against reperfusion injury by direct regulation of mCaU in isolated hearts. To analyze the cardioprotective effect of ru360 in a whole animal model, we perfused the compound after 5 minutes of coronary occlusion, followed by 5 minutes reperfusion in rat hearts. ECG tracings from rats treated with an i.v. injection of 55 nmol/kg of ru360 showed a total absence of post-reperfusion arrhythmias. This finding was correlated with an improved blood pressure recovery (96% Ru360 vs 25% control). Oxidative phosphorylation was maintained in post-ischemic rat hearts perfused with ru360 and calcium uptake was partially inhibited. We conclude that reperfusion injury is mediated mainly by mitochondria and that ru360 exerts its protective effect by interacting with the mCaU in an in vivo model.

160 Folic Acid Prevents Ischemia/Reperfusion-Induced Ros Generation

An L Moens, Hunter C Champion, David A Kass, Johns Hopkins Univ, Baltimore, MD; Floris L Wuyts, Jean-Pierre Timmermans, Christiaan J Vrints; Univ of Antwerp, Antwerp, Belgium

Background: Folic acid (FA) enhances the bioavailability of tetrathydrobiopterin, a key cofactor for normal endothelial nitric oxide synthase activity. Abnormal NOS function is thought to play a role in ischemia/reperfusion (IR) injury. We therefore investigated the effect of folic acid on IR-induced myocardial necrosis, and identified mechanisms underlying its benefit. Materials and Methods: 170 rats underwent in vivo ischemia for 30 min. by ligation of the main descending branch of the left coronary artery, followed by reperfusion for 60 min. FA was administered as a 7-day pretreatment (1, 4 and 10 mg/day) by gastric gavage or as a single N-bolus of 2.5 mg just after the onset of ischemia. Control rats underwent IR, but received no FA. Myocardial necrosis was evaluated by TTC/EB-staining (infarct size), modified Trichrome-staining (contract band necrosis) and TUNEL (apoptosis). ROS generation was evaluated using 2’7’-dichlorodihydrofluorescein diacetate (DCF- reflecting hydrogen peroxide formation) and dihydroethidium (DH- reflecting superoxide formation)-staining and scored (0 –5: 0 = no staining, 5 = markedly present). Results: FA dramatically reduced infarct size (expressed as % of risk region, control: 60.3±4.1%, treatment 2.5 mg: 3.0±2.3%, pretreatment 1mg: 3.8±1.2%, with similar percent reduction at higher doses; p<0.001 vs control). Folic acid treatment also significantly prevented the formation of early contraction bands (26.7± 2.6% in LV of control IR hearts vs. 4.6± 1.2% in folate pretreated hearts, P<0.001). Mitochondrial calcium overload has been closely related to myocardial damage and cell death. Current work in our laboratory, shows that ru360 exerts protection against reperfusion injury by direct regulation of mCaU in isolated hearts. To analyze the cardioprotective effect of ru360 in a whole animal model, we perfused the compound after 5 minutes of coronary occlusion, followed by 5 minutes reperfusion in rat hearts. ECG tracings from rats treated with an i.v. injection of 55 nmol/kg of ru360 showed a total absence of post-reperfusion arrhythmias. This finding was correlated with an improved blood pressure recovery (96% Ru360 vs 25% control). Oxidative phosphorylation was maintained in post-ischemic rat hearts perfused with ru360 and calcium uptake was partially inhibited. We conclude that reperfusion injury is mediated mainly by mitochondria and that ru360 exerts its protective effect by interacting with the mCaU in an in vivo model.

161 Modulation of Cardiac Proteasomes in PKC Transgenic Mice Exhibiting a Cardioprotective Phenotype

Chengzong Zong, Aldrin V Gomes, G W Wang, Steven Le, Glen Young, Beniam T Berhan, Dawn M Pantaleon, Joseph A Loo, William Wead, Peipei Peng; David Geffen Sch of Med at UCLA, Los Angeles, CA

Cardiac proteasomes serve as the essential protein degradation machinery in the heart, however, the molecular composition, the function, and the regulatory processes of this vital cardiac organelle remain virtually unknown. Recent studies from our laboratory revealed that this rare-studied organelle consists of a collection of multiprotein complexes, which can be separated by native electrophoresis as distinct mixtures of protein species including the 19S and the 20S proteasomes. The 20S proteasome, composed of 14 subunits, manifest at least three independent proteolytic activities exhibited by the β1, β2, and β5 subunits, respectively. In this investigation, we seek to determine if activation of PKC β, a process that engenders cardioprotection against ischemic injury, modulates expression and activity of the 20S proteasome. We purified and analyzed the 20S proteasome complexes from the murine heart using two-dimensional electrophoresis and western blotting combined with LC/MS/MS. The purified 20S proteasome from non-transgenic mice (Tg) was found to contain 14 constitutive subunits and the three inducible subunits (β1, β2, and β5). In addition, we analyzed the PKCγ Tg exhibiting a cardioprotective phenotype. Our data showed that the expression levels of the 20S proteasome subunits (β3, β7 and β5) in the PKCγ Tg were significantly increased (e.g., β3 increased by 169 ± 130% about control) whilst the expression of 19S subunits (RP1, RP1, RP4) were not altered. Importantly, despite an up-regulation of the β5 subunits, the collective 20S proteolytic activities including that of the β1, β2, and β5, exhibited no demonstrable changes. Co-immunoprecipitation assays showed that that with the 20S (20S), an association that was enhanced in the PKCγ Tg. In contrast, the changes of β3 were not observed in PKCγ mice expressing a kinase dead isoform. Taken together, these data illustrate that PKCγ activation modulates cardiac 20S proteasomes, a process that involves an enhanced association of PKCγ to the 20S complexes and altered expression of specific 20S subunits, providing the first piece

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of evidence documenting a kinase dependent regulation of the essential protein degradation system in the heart.

Phosphorylation is a Major Regulator of the Proteasome Activity

Chengong Zong, Aldrin V Gomes, Joe Ilias, Beniam T Berhane, Dawn M Pantaleon, Joseph A Loo, Peipei Ping; Univ of California, Los Angeles, Los Angeles, CA

The cardiac 26S proteasome is an organelle composed of a multiprotein complex, serving as the essential protein degradation system in the heart. We investigated whether phosphorylation of the proteasome has a functional role in regulating the proteolytic activity in the heart. Studies using electrophoresis combined with liquid chromatography tandem mass spectrometry revealed distinct types of post-translational modifications on a single molecule of the proteasome, including N-terminal myristylation, N-terminal acetylation. Purified murine cardiac 20S proteasomes were separated by two-dimensional gel electrophoresis (2DE) and immunoblotting with anti-Ser, anti-Thr and anti-Tyr antibodies. These experiments demonstrated distinct phosphorylation 20S patterns, documenting serine, or threonine, or tyrosine phosphorylation of a subset of cardiac proteasome subunits. These experiments also confirmed the phosphorylation of two subunits previously reported in non cardiac proteasomes (α6 and α7) as well as the phosphorylation on two subunits that are characterized by the cardiac proteasome subunits (α1 and β3). Results obtained from the 2DE analysis with Pro-O dye (a fluorescent dye which specifically binds phosphorylated proteins) further confirm the phosphorylation of these proteasome subunits. We next examined the effect of phosphorylation on the proteolytic activity of distinct 20S proteasome subunits. Dephosphorylation of the 20S proteasome (confirmed by Pro-O staining of the 20S subunits) with Calb intestinal alkaline phosphatase (CIAP) resulted in no change in the proteolytic activity of the β1 proteasome subunit; in contrast, dephosphorylation induced a 3 fold increase in the proteolytic activity of the β2 proteasome subunit, and a more than 10 fold increase in the proteolytic activity of the β5 proteasome subunit, when compared to the phosphorylated proteasome. These data provide the first direct evidence demonstrating regulation of the proteolytic activity in a proteasome subunit specific fashion, documenting phosphorylation as a critical regulatory mechanism to modulate this vital organelle in the heart.

ATP-Loaded Liposomes: Effective Protection of Myocardium in Isolated Rat Heart and Rabbits with Experimental Myocardial Infarction

Tatyana S Levcenko, Daya D Verma, William C Hartner, Eugene A Bernstein, Vladimir P Torchilin; Northeastern Univ, Boston, MA

ATP-loaded liposomes (ATP-L) protect myocardium against ischemia-reperfusion damage in isolated rat hearts and in rabbits with experimental myocardial infarction. ATP-L were infused into isolated rat hearts before and during 25 min of global ischemia followed by 30 min reperfusion. Left ventricular developed pressure (LVDP) at the end of reperfusion significantly recovered to 72% of the baseline with ATP-L compared to 26%, 46%, and 51% in groups treated with the Kreb's-Henseleit (KH) buffer, empty liposomes (EL), and free ATP (F-ATP). The ATP-L-treated group showed a lower left ventricular end diastolic pressure (LVDP) after ischemia-reperfusion: LVDP was 23 ± 3 (± SE) mmHg with ATP-L compared to 56 ± 6, 43 ± 6, and 31 ± 2 in KH buffer, EL, and F-ATP controls. Incubation of F-ATP and ATP-L with ATPase, eliminated the protective effect of F-ATP, while the effect of ATP-L was unchanged. Preservation was proportional to ATP-L infusion time and was ATP concentration-dependent. A 10-fold lower dose of ATP reduced recovery of LVDP to 47%. To demonstrate an in vivo cardio-protective effect, NZW rabbits were anesthetized, intubated, ventilated and the thorax opened to expose the heart. Approx. 3 ml of ATP-L, EL, or KH buffer were infused through the coronary arteries during brief clamping of the aorta and a regional occluding coronary arterial snare was tightened. The snare was released after 30 min of ischemia and re-established 3 hrs. Uninterrupted perfusion demarcated the area at risk. The anesthetized animal was sacrificed, and the left ventricle (LV) was isolated, sliced horizontally into 5 approx. equal thickness pieces, and photographed to estimate the area at risk. Nitroblue tetrazolium incubation outlined the area at risk. Staining of the 20S subunits) with Calf intestinal alkaline phosphatase (CIAP) resulted in no evidence demonstrating regulation of the proteolytic activity in a proteasome subunit specific fashion, documenting phosphorylation as a critical regulatory mechanism to modulate this vital organelle in the heart.

AMP-Activated Protein Kinase Protects Cardiomyocytes against Hypoxic Injury through Attenuation of ER Stress

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Oxygen deprivation leads to the accumulation of misfolded proteins in endoplasmic reticulum (ER), causing ER stress. Under conditions of ER stress, three protective mechanisms are activated: the attenuation of protein synthesis, the up-regulation of genes encoding protein chaperone and degradation. These mechanisms serve to reduce the accrual of misfolded proteins within the ER. AMP-activated protein kinase (AMPK) is a key regulatory enzyme involved in cellular energy homeostasis. In hypoxic cardiomyocytes, it has been shown that AMPK activation is associated with an inhibition of protein synthesis. Therefore, we tested the hypothesis that AMPK could attenuate hypoxia-induced ER stress through the inhibition of protein synthesis in heart. Hypoxia-induced Bip and CHOP were significantly inhibited by pretreatment of 5-aminoimidazole-4-carboxamide riboside (AICAR), chemical activator of AMPK, in a dose- and time-dependent manner. In parallel, adenosine expressing dominant-negative AMPK (DN-AMPK) significantly attenuated the effect of AICAR. Furthermore, we examined whether AMPK regulated the activation of caspase12, ER stress-dependent caspase, under hypoxia in cardiomyocyte. Pretreatment with AICAR inhibited activation of caspase12 under hypoxia. DN-AMPK blocked it. We concluded that activation of AMPK contributes to cardiac protection against hypoxic injury through attenuation of ER stress and that attenuation of protein synthesis may be the mechanism of cytoprotection of AMPK.

Glucocorticoid Administration Improves Calcium Handling by Cardiomyocytes after Ischemia and Reperfusion

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Objective: Repair of congenital heart disease often requires cardiopulmonary bypass (CPB) and a period of myocardial ischemia with the potential for reperfusion injury. We previously demonstrated that pre- and intraoperative glucocorticoid administration protects myocardium during CPB and reperfusion. The hypothesis was that glucocorticoid therapy could improve calcium handling in cardiomyocytes isolated from pigs after ischemia and reperfusion (I/R).

Methods: Cardiomyocytes were isolated from piglets undergoing CPB and 2 hr of circulation with or without glucocorticoids (n=3-4/pig). Methylprednisolone (30 mg/kg, IV) was administered 6 hr before and at the time of CPB. Cardiomyocytes were isolated from mature myocardium. Preload recruitable stroke work 2 hr after reperfusion decreased from baseline without glucocorticoids (41±14 vs 28±4, p<.05) but was not different from baseline in animals receiving glucocorticoids (47±12 vs 28±4, p=.05). I/R prolonged total calcium transient time to peak after I/R (57±14 vs 48±2, p<.05). Calcium transient amplitude was blunted after I/R (757±169 mV) compared to controls (832±233 mV, p<.05). Glucocorticoids restored the range of calcium transient time-to-peak after I/R (57±14 vs 48±2, p<.05). Phosphorylation of phospholamban, an intracellular mediator of sarcoplasmic reticulum calcium transfer in cardiomyocytes, was not different with glucocorticoid treatment. Conclusion: One mechanism of glucocorticoid protection for the myocardium during I/R may be through maintenance of intracellular calcium handling, although this mechanism does not appear to be regulated through phospholamban.

Does PI3-Kinase Play a Role in Arachidonic Acid-Induced Protection of Neonatal Cardiac Myocytes?

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Although there is evidence for a role for arachidonic acid (ARA) as a modulator in signal transduction cascades, the significance of its participation in the PI-3 pathway remains to be elucidated. Therefore the aims of this study were to i) characterize the phosphorylation status of two phosphorylation sites on PKB, namely Ser473 and Thr389 during simulated ischemia (SI) and reperfusion; ii) determine the effect of ARA on PKB phosphorylation during SI and reperfusion; iii) establish whether the activation/inhibition of PKB is harmful or beneficial to the myocyte. Methods: Cultured neonatal cardiomyocytes were exposed to 20 μM ARA complexed to BSA for an hour before the onset of 60 minutes of SI (induced with desoxycorticosterone and KCN) followed by 30 minutes of reoxygenation. Samples were analysed by Western blotting with phospho-specific antibodies recognizing phosphorylated Ser473 and Thr389 of PKB. Apatosis was determined by polyADP-ribosepolymerase cleavage and caspase-3 activation. Hoechst 33342 stain was used to view the morphological features of apoptosis by fluorescence microscopy. Cell viability was measured by the MTT assay. Results: ARA significantly increased cell viability and attenuated apoptosis during PI3-K inhibition; PI3-K phosphorylation was inhibited by ARA during SI/R, while Thr389 remained phosphorylated. Wortmannin (PK3-inhibitor) significantly decreased cell viability and increased caspase-3 activation as well as the apoptotic index, when added to ARA-induced protected cells. Conclusion: We therefore propose that one of the ways through which ARA induces its protection, is through PI3-kinase and speculate that, in contrast to former belief that both sites should be phosphorylated for increased PKB activity, sustained Thr389 phosphorylation is adequate for PKB activation and subsequent inhibition of apoptosis.
Hydrogen Peroxide Production by Myocardial Mitochondria is Reduced by Exercise Training

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Reactive oxygen species (ROS) are a component of ischemia-reperfusion injury, are elevated during exercise, and are thought to contribute to the aging process. Mitochondria are a major source of ROS and an increase in mitochondrial Ca2+ accumulation augments their production. Exercise is known to provide cardioprotection against ischemia/reperfusion injury, but the mechanisms for the protection are unclear. The purpose of this study was to test the hypothesis that exercise results in a decrease of mitochondrial ROS in the heart. Adult male Fisher 344 rats were randomly assigned to one of two treatment groups: sedentary control (SED) (n = 8) or exercised trained (ET) (n = 11). Trained rats ran on a motorized treadmill for 10 weeks (5-days/week, 60 min/day, 25m/min, and 6″ grade). Heart mitochondria were isolated and oxidative phosphorylation measures determined to assure that high quality preparations were used. H2O2 production, an indicator of ROS production, was detected by fluorescence (Amplex Red) with succinate as substrate. Mitochondrial antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx)) were also determined. No differences were observed between SED and ET for oxidative phosphorylation parameters including State 3 rate, respiratory control ratio (State 3/State 4), and ADP:O ratio. Likewise, mitochondrial SOD and Gpx activities were not affected by exercise. CAT activity was very low compared to SOD and Gpx, but its activity was increased 49% (from 0.59±0.03 to 0.88±0.11 Units/mg protein; P<0.05) after exercise training. H2O2 production by ET was lower than SED in the absence of calcium (323±12 vs 362±11 pmol H2O2 (mining protein, P<0.05) and in the presence of 50 μM CaCl2 (154±3 vs 197±7, P<0.05). Addition of rotenone, which blocks reverse flow of electrons to complex 1 in the electron transport chain, greatly reduced H2O2 production and eliminated differences between ET and SED. We conclude that exercise training reduces ROS production in myocardial mitochondria primarily through adaptations specific to complex 1.

Metropolot and Captoproplt After Apoptotic Genetic Expression in the Murine Heart After Myocardial Infarction

David Rosenbaum, Michael White, Wayne Fisher, Harold Garner, J Michael DiMaio; UT Southwestern Med Ctr, Dallas, TX

Objectives: Beta blockers and ACE inhibitors revolutionized the care of the CHF patient. Multi-institutional studies demonstrate improved cardiac function with their use; however, the molecular basis for their action remains incompletely understood. DNA microarray technology provides a resource for the examination of the effects of these drugs on a molecular basis. We hypothesize that we can alter post myocardial infarction cardiac function in mice with pharmacological intervention and then determine if this change in cardiac function stems from differential gene expression between the treatment groups. Methods: We ligate the left anterior descending coronary artery in mice (C57/Bl6) to induce myocardial infarction. Post-operatively, we treat the animals with either metropolot alone (n = 15), captoproplt alone (n = 15), metropolot/captoproplt combination (n = 5), or no treatment (n = 5). On post-operative day 7, we perform echocardiography to determine the fractional shortening (FS) as a surrogate for cardiac function. Finally, we identify differentially regulated genes with microarray analysis. Results: As expected, the mice treated with metropolot alone and metropolot/captoproplt combination demonstrated improved FS when compared with no pharmacological treatment (42% and 44% vs. 30%; respectively; P<0.001). The microarray results demonstrated 89 genes upregulated and 60 genes downregulated when comparing murine hearts that had undergone MI to hearts that had undergone MI and treatment with 7 days of metropolot and captoproplt. Two of the upregulated genes, Birc5 (survivin) and Bifar, are involved in apoptotic regulatory mechanisms. Conclusion: This study begins to identify the genetic mechanisms through which pharmacological interventions improve cardiac function. The two genes identified above are responsible for regulation of apoptosis. The combination of metropolot and captoproplt appears to prevent cardiomyocytes from undergoing cell death through regulation of these genes. With further work, the knowledge of other genes responsible for functional cardiac improvement will allow the development of other drugs and genetic interventions for improving the outcome of patients with CHF.

Intra-Aortic Balloon Counterpulsation Facilitates Coronary Microcirculation During Myocardial Reperfusion: Experimental Study

Elias Tosilas, Argois Makrianis, Stavros Drakos, Paraskevi Savvari, Stratos Charitos, Charalampos Pierrakos, Mihalis Argiriou, John Nanas; Univ of Athens, Athens, Greece

Background: Studies of the effects of the intra-aortic balloon pump (IABP) have reported an increase or no change in coronary blood flow (CBF). We sought to determine the changes in CBF produced by the IABP in intact coronary arteries and during reperfusion in an ischemia/reperfusion experimental model. Methods: A 30-mI IABP was placed in the descending aorta of 7 open-chest pigs. Each pig underwent occlusion of the mid left anterior descending (LAD) coronary artery for 1 h, followed by reperfusion for 1.5 h. The effect of IABP support on systolic aortic pressure (SAP) and peak diastolic aortic pressure (pDAP) was recorded. The mean CBF, distal to the LAD occlusion site, was measured with a transit-time ultrasound flowmeter, at baseline and during reperfusion, with and without the IABP. Results: The results of IABP support on aortic pressures and CBF, both in the intact heart (baseline) and during reperfusion, are shown in the table. Conclusions: In the intact heart, IABP caused a decrease in CBF, probably because of a lesser myocardial O2 demand from a decrease in afterload produced by counterpulsation. During reperfusion, when the O2 demand of ischemic tissue is increased, IABP produced an increase in pDAP and CBF and, therefore, in O2 supply, suggesting that implementation of the IABP during reperfusion may be an effective intervention to reduce infarct size.

Depression of Sarcoplasmic Reticulum in Ischemia and Reperfusion Is Species Dependent: A Study in Animals and Humans

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In the mammalian cardiac myocyte, sarcoplasmic reticulum (SR) regulates Ca release via the L-type Ca receptors ( RyRs ) and Ca uptake by the Ca-ATPase ( SERCA ). The contractilility of SR in excitation and contraction coupling is known to vary with species or development. During ischemia/reperfusion, myocardial contracture and Ca overload are often attributed to depressed SR function, but the precise changes are not well defined. In this study, we have investigated potential SR changes in acute ischemia/reperfusion in animals and in chronic ischemic cardiomyopathies (ICM) in humans. Isolated perfused hearts from the adult rat, guinea pigs and neonatal rabbits (10 days old) were subjected to global ischemia (~30 min) and reperfusion (~30–60 min). Western blot of SDS-solubilized cardiac homogenates showed that ischemia/reperfusion caused significant degradation of SERCA (~35%, P<0.001) in rats, but not in guinea pigs or neonatal rabbits. RyRs were depressed in rats as well as guinea pigs after ischemia and reperfusion (Figure). Phospholamban (PL) was not significantly degraded during ischemia and reperfusion, but underwent phosphorylation during ischemia and dephosphorylation during reperfusion. Human ICM showed no depression of SERCA, RyRs and PL compared with nonfailing donors, but PKA-mediated PL phosphorylation was depressed. These data suggest that, in ischemia/reperfusion, SR depression is species-dependent and Ca uptake and release can be differentially affected.

Abstract Presentations

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Molecular Characterization of a Murine Model of Dilated Cardiomyopathy

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Genetic dilated cardiomyopathy (DCM), caused by myosin point mutations, leads to depressed cardiac function and heart failure, but the link between primary disorder and clinical phenotype is poorly understood. Using a murine model, the effects of two DCM
misense mutations on α-cardiac myosin molecular mechanics were characterized. One mutation was located in the actin binding domain (SS52P) and another in the coiled region (F764L). All mutant mice developed normally and survived for more than a year, but demonstrated phenotypic changes characteristic of DOM. Both maximal left ventricular pressure and fractional shortening were reduced (shortening: 47 ± 4% SS52P, 54 ± 7% F764L) compared to wild-type mice (73 ± 11% WT). At the molecular level, both mutant myosins exhibited reduced ability to move actin filaments in vitro (mean values for V0, the force generating capacity of the LA for 0.3 μM NTPs: SS52P, 3.4 ± 0.4 μM FT; 4.2 ± 0.7 μM FT (W) and a reduced actomyosin ATPase rate (0.7 s–1 head–1 SS52P, 0.5 s–1 head–1 F764L vs. 1.0 s–1 head–1 WT)). Assuming V0p is proportional to the myosin step size (dp,1) divided by the duration that myosin strings remain attached to F-actin (tact,0) (i.e. V0 = dp,1 / tact,0), a change in either dp,1 and/or tact,0 might account for the decreased V0p. Single-molecule, laser trap experiments revealed that for the SS52P mutant, dp,1 was decreased (SS52P: 5.8 ± 2.4 nm; WT: 7.6 ± 2.2 nm) while tact,0 increased (SS52P: 36 ± 11 ms; WT: 29 ± 6 ms). An in vitro mobility assay measured similar values for both generating capacities of the mutants relative to WT; however the product of V0p and tact,0 force suggests that the power producing capability of both mutant myosins may be compromised relative to WT myosin. A newly developed a load-clamped laser-trap assay will allow force-velocity relationships to be determined on isolated mutant cardiac myosins, enabling comparisons of the power generating capabilities. A lower power generating capacity in the DOM mutants would contrast with the proposed enhanced power output for the R403Q mutant myosin associated with familial hypertrophic cardiomyopathy (FHC). Thus an altered power generating capacity, and not force or velocity alone, may be the determining factor that predisposes an individual to either FHC or DOM.

172 Porcine Cardiac Muscle Contractility is Enhanced In Vitro by Increasing Ratios of 2-Deoxy-Adenosine Triphosphate/Adenosine Triphosphate

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Contractile parameters of porcine cardiac muscle were examined after replacing ATP with varying ratios of 2-deoxy-ATP/ATP (constant total MgNTP). Previous studies indicated that because there was a substantial enhancement of contractility at both maximal and submaximal Ca2+ when 2-deoxy-ATP (dATP) completely replaced ATP as the substrate in rat cardiac preparations, dATP or dTTP in an ATP-free buffer in cardiac cells may be beneficial as a treatment of the symptoms of heart failure. We chose to study porcine cardiac muscle because it consists of a μ-MHC myosin isoform kinetically similar to that of human cardiac myofilament. If the affinities of dATP and ATP for cardiac myosin are similar, a linear increase in contractile parameters is expected as the ratio of dATP/ATP is increased to at least 1:1 by dATP. We find that dATP enhances isotropic force (Fiso) in porcine cardiac muscle (~9%, pCA 4.5; ~4%; pCA 5.5), although not as markedly as in rat μ-MHC cardiac muscle (~39%, pCA 4.5; ~160%, pCA 5.5). At maximal Ca2+ (pCA 4.5) Fiso is increased linearly with dATP/ATP ratios. At submaximal Ca2+ (pCA 5.5), this relationship is nonlinear, with the greatest effect at 2–20% dATP, suggesting a >-fold higher affinity of dATP for cardiac myosin than ATP. The minimum dATP/ATP ratio for significant increases in Fiso was determined to be 10% dATP (< 0.01). We found higher Ca2+ sensitivity of force in the porcine preparation (pCA 50 ~ 5.9 ± 0.2) as compared to previous rat data (pCA 50 ~ 5.4 ± 0.1). The rate of tension redevelopment (kTR) data indicate that dATP causes an elevation of the kTR over ATP at those activation levels that result in greater Fiso than Fiso for ATP, and the kTR increases linearly as the ratio of dATP/ATP is increased at high Ca2+. NTP hydrolysis and F-actin sliding speed both increased linearly as the dATP/ATP ratio was increased. These results suggest that low dATP/ATP ratios increase the number of strongly bound dATP, as well as ATP binding to cardiac myosin, that low dATP/ATP ratios increase the number of strongly bound dATP, as well as ATP binding to cardiac myosin. That is, PKA treatment increased the amplitude of stretch activation and the rates of decay and delayed force transient were significantly faster in MyBP-C–/– myofibrils than in WT myofibrils. Following PKA treatment, there were significant changes in the stretch activation of WT but not MyBP-C–/– myofibrils. That is, PKA treatment increased the amplitude of stretch activation and the rates of decay and delayed force transient were significantly faster in WT but not MyBP-C–/– myofibrils. These results suggest that MyBP-C plays an important role in defining both the number of attached cross-bridges and the kinetics of cycling cross-bridges at sub-maximal activation in the absence and presence of PKA catalyzed phosphorylation of myofilibrin proteins.

175 Myocyte-Specific Excision of the Murine Vinculin Gene Leads to Cardiac Fibrosis and Premature Death

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Vinculin is a ubiquitously expressed membrane-associating protein that links the actin cytoskeleton to the cell membrane. In cardiac myocytes Vinculin is found at cell- to- cell contact sites termed intercalated discs (ICDs) as well as in cell-to- matrix adhesions. Global Vinculin knock-out caused embryonic lethality by E10. We have shown that global heterozygous inactivation of the Vinculin (Vinc) gene caused stress-induced cardiomyopathy, yet Vinc's precise role in cardiac myocytes (CM) is still unknown. Accordingly, we generated cre/lox mice to allow CM specific inactivation of the Vinc gene. Homologous recombination of the floxed Vinc exon3 was tested by Southern blotting as well as by PCR analysis. PCR analysis of isolated CM from heterozygous Vinc KO showed successful Cre excision by ML2cv-Cre. Preliminary evaluation of homozygous ventricular-myocyte specific Vinc KO mice showed no embryonic lethality. Four of these Vinc KO mice were used for further mating (three females and one male). One female mouse died soon after labor and one died in mid-gestation without any prior signs of sickness. The two remaining mice (1 f/1 m) never mated and survived till age 16 weeks. Echocardiographic analysis showed mild concentric hypertrophy of Vinc KO hearts compared to controls. Preliminary analysis, determined by real-time PCR, was reduced by 0.7% compared to sham controls (p < 0.01). To determine the effects of TM downregulation on endocardial thromboresistance, we measured in situ generation of activated protein C (APC) and quantified microthrombus formation by using green fluorescent and red fluorescent thrombogenic latex beads. The in vivo experiments demonstrated that the LA of banded rats was reduced by 35% compared to sham controls (2.9 ± 0.2 μm mg−1 g−1 vs. 1.7 ± 0.18 μm mg−1 g−1, p < 0.005) and local thrombin activity was increased by 60% (2.3 ± 0.23 μm mg−1 g−1 vs. 1.45 ± 0.38 μm mg−1 g−1, p < 0.003). To determine if restoring TM expression would reduce local thrombin activity, four days prior to aortic banding rat LA were transduced with an adenoviral vector expressing human TM (AdTM5Hs) or a control vector expressing no transgene (AdNull), (n=5/group). APC generating capacity was significantly higher in AdTM5Hs banded compared to Adnull banded controls (2.9 ± 0.2 μm mg−1 g−1 vs. 1.2 ± 0.1 μm mg−1 g−1, p < 0.0001) and caused a significant reduction in local thrombin activity (1.5 ± 0.1 μmol mg−1 g−1 vs. 2.8 ± 0.1 μmol mg−1 g−1, p < 0.0001). Conclusion: TM expression by the atrial endocardium is markedly reduced by acute elevations in filling pressures and leads to increased local thrombin generation. Targeted restoration of LA TM expression restores APC generation and normalizes bound thrombin activity in acute heart failure.

174 Effects of Protein Kinase A Phosphorylation on Stretch Activation Kinetics of Wild Type and Myosin Binding Protein C Null Mouse Myocardium

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It is well recognized that protein kinase A (PKA) catalyzed phosphorylation of troponin I (Tnl) and myosin binding protein C (MyBP-C) decreases Ca2+ sensitivity of force and accelerates cross-bridge cycling kinetics in murine myocardium. However, the relative contribution of phosphorylation of Tnl and MyBP-C in altering myocardial properties and mechanical properties of mouse myocardium is not yet clear. In the present study, we examined the effect of PKA catalyzed phosphorylation of myofilibrillar proteins on mechanical properties of skinned myocardial preparations, in particular stretch activation, isolated from hearts of wild-type (WT) and mice that do not express MyBP-C (MyBP-C–/–). Following a rapid stretch of 0.5–2.5% of initial length of muscle skinned myocardial preparations at sub-maximal (~50%) of maximal forces, the force first increased rapidly, then decayed rapidly to a minimum value, and finally rose progressively to a maximum value (delayed force transient). The rate at which the delayed force transient reached a maximum value was determined by fitting the force transient to a single exponential function and the amplitude of the stretch activation response was normalized to the total force generated by the skinned myocardial preparations. In the absence of PKA-treatment, the amplitude of the stretch activation was significantly lower than both the rates of decay and delayed force transient were significantly faster in MyBP-C–/– mycardium. These results suggest that MyBP-C plays an important role in defining both the number of attached cross-bridges and the kinetics of cycling cross-bridges at sub-maximal activation in the absence and presence of PKA catalyzed phosphorylation of myofilibrin proteins.
The β-1 Integrin Couples α-Adrenergic Receptors to Angiotsinogen Gene Expression in Rat Ventricular Myocytes

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Integrins are cell surface receptors consisting of α/β heterodimers that bind extracellular matrix molecules and mediate cell adhesion. Studies in major integrin-deficient mouse models and in patients with orocecal malrotation suggest that αvβ3 in cardiomyocytes is p52αv, which adheres to collagen and laminin. We have demonstrated that αv-adenosine stimulation (phenylephrine, PE) increases angiotsinogen (Ao) gene expression (195 vs. control, 63.63 vs. 729/.08) did not differ between treatment groups. Pulmonary hypertension was reduced (1.04 ± 0.7 vs. 11.79 ± 3.38, p=0.02) and FE (2.40 ± 0.8 vs. 11.79 ± 4.43, p=0.02) respectively. The expression of Ao gene protein was increased after CPB and CP in both age groups (younger 1.07 ± 0.1 vs. 0.81 ± 0.12, p=0.01) and in elderly 1.12 ± 0.15 vs 0.82 ± 0.14 vs. p=0.03). The same pattern could be found with RNA analysis (both age groups combined 1.04 ± 0.07 vs. 0.87 ± 0.06, p=0.002). The protein expression of VEGF was 10% lower in the elderly group when compared to the younger group. The mechanism(s) responsible for this benefit is not fully understood. We tested the hypothesis that cytochrome c oxiade degradation is a limiting factor for mitochondrial function in failing cardiomyocytes and that inhibition of MPTP opening may be useful in preventing degradation of cytochrome c. Cytochrome c. The latter is partially due to opening of mitochondrial permeability transition pores (MPTP) in heart failure leading to exposure of cytochrome c oxidase to cytosolic components that are normally excluded and subsequently to protein degradation. In this study, we tested the hypothesis that cytochrome c oxiade degradation is a limiting factor for mitochondrial function in failing cardiomyocytes and that inhibition of MPTP opening may be useful in preventing degradation of cytochrome c. Oxidase were isolated from LV myocardium of 7 dogs with heart failure produced by intracardiac microembolizations. Cardiomyocytes isolated from LV tissue of 7 normal dogs were used for comparison. Expression of subunit-II of cytochrome c oxidase was evaluated by homogenate of both normal and failing cardiomyocytes. In a second set of experiments, failing cardiomyocytes were incubated in the presence (0.2μM) and absence of the specific inhibitor of MPTP cyclosporine A (CsA) at 37°C for 24 hours. In all instances, measurements were made using Western blots and bands quantified in densitometric units. Results: Expression of subunit-II of cytochrome c oxidase was significantly lower compared to normal cardiomyocytes (7.0 ± 0.6 vs 12.4 ± 0.5, p<0.0001). Incubation of failing cardiomyocytes with the MPTP inhibitor CsA significantly increased expression of subunit-II of cytochrome c oxidase compared to incubation in the absence of CsA (13.1 ± 1.1 vs. 6.3 ± 0.2, p<0.0001). Conclusions: Observations made in this study indicate that in failing cardiomyocytes protein expression of subunit-II of cytochrome c is decreased. This degradation can be reversed by inhibition of MPTP opening. The latter may be an approach that could be useful in preserving mitochondrial function in heart failure.

Effect of Aging on the Cardiopulmonary-Induced Change in Growth Factor Response

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Background: Angiogenic growth factors have been used to treat high risk patients with severe myocardial ischemia not amenable to conventional therapeutic modalities. The response of human atrial microvessels and atrial expression of VEGF and FGF-2 were examined in younger (less than 65 years of age) and elderly (more than 65 years of age) patients. Methods: At the time of cardiac surgery, human atrial biopsies were harvested before and after cardiopulmonary bypass (CPB) and cardiopulmonary (CP). Protein and RNA expression of VEGF and FGF-2 were quantified using Western and Northern blots respectively. Atrial microvascular responses were examined using video microscopy. Results: Micro-vessel reactivity analysis studies showed less relaxation in the elderly patients compared to the younger patients for both VEGF (5.80 ± 1.70 vs. 11.90 ± 3.38, p=0.02) and FGF-2 (4.20 ± 0.86 vs. 11.79 ± 4.43, p=0.02) respectively. The expression of VEGF protein was increased after CPB and CP in both age groups (younger: 1.07 ± 0.11 vs. 0.81 ± 0.12, p=0.01) and in elderly (1.12 ± 0.15 vs. 0.82 ± 0.14 vs. p=0.03). The same pattern could be found with RNA analysis [both age groups combined (1.04 ± 0.07 vs. 0.87 ± 0.06, p=0.002]. The protein expression of VEGF was 10% less in the elderly group when compared to the younger group. Conclusions: Microvessels from elderly patients showed less ability to relax in response to angogenic growth factors and thereby may have less potential to elicit an angiogenic response. The ischemic insult of CPB and CP leads to increased expression of VEGF protein. Surgical intervention may diminish the angiogenic response to growth factor proteins, which needs to be taken into consideration during surgically based trials.

Ranolazine Increases Cytochrome C Oxidase Activity and Rate of ATP Synthesis in Failing Cardiomyocytes

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Background: We showed that acute administration of the partial fatty acid oxidation inhibitor ranolazine (RAN) improves LV function in dogs with heart failure (HF). The mechanism(s) responsible for this benefit is not fully understood. We tested the hypothesis that, in failing cardiomyocytes, RAN improves myocardial energetics. Methods: Studies were performed in cardiomyocytes isolated from the LV of 7 dogs with HF produced by coronary microembolizations. Cytochrome c oxidase dependent respiration of cardiomyocytes was measured in the presence of 1 μM rotenone and 0.4 mM N,N,N',N'-tetramethyl-p-phenylenediamine using an oxygraph and a Clark electrode. The electron flow was blocked at complex III (CoQH2→c reductase) with antimycin (2μg/mL). Measurements were made in the absence of RAN (HF-Control) and after the addition of 1 μM, 5 μM, and 25 μM of RAN. Mitochondrial respiration was quantified in ng atoms of O/min/2x10^6 cardiomyocytes. Maximal rate of ATP synthesis (ATPmax) was measured using luciferase and a luminometer and expressed in relative light unit/sec/10^6 cardiomyocytes (RLU/s). Results: RAN significantly increased CODR irrespectively of dose (Table). This improvement was also irrespective of dose. Conclusions: In failing cardiomyocytes, RAN increases rate ATP of synthesis and improves activity of cytochrome c oxidase. The mechanism(s) by which RAN elicits this stimulation remains to be elucidated.
HRC: A Novel Regulator of Sarcolipin Calcium Sequestration and Cardiac Function

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Defects in the pathways that regulate cardiac sarcolipin calcium release (SR Ca uptake and release represent prime targets for driving the deterioration of Ca homeostasis and progression to heart failure. A recently identified histidine-rich Ca binding protein (HRC) has been suggested to be part of the Ca release complex and may thus play an important role in the impaired SR Ca cycling. To elucidate the physiological function of HRC, we generated transgenic mice that overexpressed (3-fold) this protein in the heart. Increased HRC levels were associated with impaired SR Ca uptake rates (35%) and delayed cardiac contraction and Ca transients in isolated ischemic heart or heart disease patient. The increased HRC expression was associated with a 4 fold increased protein expression of triadin and the Na-Ca exchanger, yet the Vmax of the Na-Ca exchanger was significantly depressed. There were no alterations in L-type Ca channel protein levels, although L-type Ca current density was significantly increased (19%), with no change in the channel’s inactivation kinetics. This impaired Ca homeostasis and cardiac dysfunction compromised the heart’s responses to increased stress by either hemodynamic load or the aging process. After 18 months of age, cardiac remodeling deteriorated to congestive heart failure in transgenic mice. Thus, HRC is an integral regulatory protein in cardiac SR Ca cycling, and targeting HRC to increase SERCA activity may represent a new adjunctive therapy in end-stage heart failure.

G-CSF Mobilized CD34+ Cells Augment Functional Potential via High Telomerase Activity

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[Objectives] Endothelial Progenitor Cells (EPCs) were initially defined as CD34+ cells circulating in adult peripheral blood. Recently, our group is applying G-CSF mobilized CD34+ cells for vascular regeneration in ischemic heart or leg disease patients. We investigated CD34+ cell function derived from each peripheral blood for more effective cell therapy. [Methods] CD34+ cells were isolated from mononuclear cells derived from patient peripheral blood (PAD: Peripheral Artery Disease with G-CSF [age 68–78], Buerger disease with G-CSF [age 20–41], Buerger disease without G-CSF [age 20–40]) using CD34 antibody-magnetic beads conjugated system. TERT mRNA and protein expression were detected by RT-PCR and Western blot analysis, respectively. Telomerase activity was evaluated following PCR-based TRAP assay. Migratory effect was assessed in response to fibronectin. Moreover, CD34+ cells were expanded and VEGF induced activation in MAPK or Akt pathway was evaluated by Western blot analysis. [Results] TERT mRNA, protein expression, and telomerase activity were abundant in CD34+ cells derived from healthy volunteer with G-CSF and Buerger disease with G-CSF, however the level was low in CD34+ cells derived from PAD with G-CSF and healthy volunteer without G-CSF. Mean Telomere Length was 10.2kb in healthy volunteer with G-CSF, 8.6kb in Buerger disease, and 6.5kb in PAD with G-CSF, respectively. Migratory potential was also superior in CD34+ cells from Buerger disease with G-CSF and healthy volunteer with G-CSF to the sample from healthy volunteer without G-CSF or PAD with G-CSF. VEGF induced activation in ERK pathway was enhanced (2–3fold) by the sample from Buerger disease with G-CSF and healthy volunteer with G-CSF. [Conclusions] G-CSF augmented high potential CD34+ fraction from bone marrow. Aging factor impaired the cell function via telomere dependent mechanism. The impaired Ca homeostasis and cardiac dysfunction compromised the heart’s responses to increased stress by either hemodynamic load or the aging process. After 18 months of age, cardiac remodeling deteriorated to congestive heart failure in transgenic mice. Thus, HRC is an integral regulatory protein in cardiac SR Ca cycling, and targeting HRC to increase SERCA activity may represent a new adjunctive therapy in end-stage heart failure.

PKC Sensitizes Cardiac Myofilaments to Ca2+ by Phosphorylation of Troponin I

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Responsiveness of cardiac myofilaments to Ca2+ is one parameter regulated by activation of G-protein coupled receptors in cardiac ventricular tissues. Heart failure is associated with an increase of myofilament Ca2+ sensitivity that may contribute to impaired cardiac muscle function in diastole (slowed or incomplete relaxation), but the underlying molecular mechanisms are poorly understood. Mechanisms of altered myofilament Ca2+ sensitivity are best characterized for phosphorylation of cardiac troponin I (cTnI) on serines23,24, which has the opposite effects to those observed in failure including desensitization of myofilaments to Ca2+ and acceleration of relaxation. Here we show that PKCδ-δ, a PKC isoform thought to be up-regulated in heart failure, induced a robust (0.11 pC) Ca2+ sensitizing effect on cardiac myofilaments if serines23,24 on cTnI were replaced with alanine. Incorporation of radiolabeled phosphate from 32P-γ-ATP showed that cTnI rather than myosin regulatory light chain was the principal protein phosphorylated under these conditions. The phosphorylation stoichiometry was 0.2–0.4 moles phosphate per cTnI. Removal of three additional sites on cTnI, serines20,23 and threonine26, by conversion to alanine or to aspartate diminished both Ca2+ sensitization and 32P incorporation into cTnI. Phosphorylation of cTnI on sites other than serines23,24 may enhance myofilament Ca2+ sensitivity following physiological stimulation of Gq coupled receptors on normal hearts, and an over-reliance on this mechanism may contribute to diastolic dysfunctioning failing hearts.

Closed-Chest Cell Injections into Mouse Myocardium Guided by High-Resolution Echocardiography

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The mouse and the rat are important models for the development of therapeutic stem cell implantation to treat ischemic myocardium. However, their small size hampers accurate implantation into the left ventricular (LV) wall. Precise positioning of injections requires surgical visualization of the heart, subject to complications and impractical for repeated injections. Furthermore, the thickness of the myocardium is comparable to the length of a needle bevel; so surgical exposure does not preclude inadvertent injection into the LV cavity. We describe the use of high resolution echocardiography to guide transcoronary injections accurately into the myocardial wall. Mice (n = 10) were anesthetized with isoflurane and imaged in a supine position using a VisualSonics Vevo660 echocardiography system in B mode. After obtaining a long axis view, 5–10 μL of a mixture of echocardiography contrast (Optison®) and 0.2 μm fluorescent microspheres was injected subdiaphragmatically through a 30 gauge needle. The needle, myocardial wall, and contrast were all clearly visible. Injections were performed in different mice into the myocardial wall and the LV and RV cavities. Hearts were immediately harvested and frozen, and cryosections were visualized with a fluorescence microscope to identify implantation sites. We were thus able to discern the position of the needle tip during injection and determine its position within the myocardium or lumen. Importantly, injection into the LV cavity resulted in an accumulation of microspheres along the needle track, suggesting that non-guided injections into rodent hearts in vivo have not been successful even if the majority of the injection was lost in the chamber, a potential explanation for inconsistency of published results. Conclusions: Use of this system will allow precise cellular implantation into mouse myocardium by accurately guiding injections to the desired location, and confirm successful implantation of cells.

Withdrawn

Differentiation of Mouse and Monkey ES Cells into Cardiac Myocytes and Cell Implantation for Myocardial Regeneration Therapy

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Embryonic stem (ES) cell transplantation has been expected as a tool for novel regenerative therapy of severe heart failure. Therefore, it is of importance to enhance differentiation efficiency of ES cells into cardiac myocytes. Myocardial cell differentiation requires activation of a cardiac-specific gene program. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) govern cardiac-specific gene expression patterns by being recruited to target genes through association with specific transcription factors such as HAT-4 or MEF2. We previously reported that one of HATs, p300 can activate DNA-binding and DNA-binding by GATA-4 as well as promote a transcriptionally active chromatin configuration. In addition, HAT activity of p300 is required for GATA-4-mediated transactivation of cardiac specific genes. Here we show that inhibition of HDACs by trichostatin A (TSA), induces acetylation of cardiac zinc finger protein GATA-4 as well as histone-3/4, and enhances myoblast cardiac cell differentiation in mouse ES cells. Furthermore, in order to perform pre-clinical studies in primate, we have established a system of monkey ES cell differentiation into cardiac myocytes and delivered these cells to the monkey myocardium. Embryonic bodies (EBs) produced by culturing ES cell aggregates, were transferred into gelatinized dishes, and then stimulated with TSA. TSA increased the percentage of beating EBs and expression of cardiac myosin heavy chain (MHC) α–actinin, suggesting TSA-enhanced differentiation of monkey ES cells into cardiac myocytes. Finally, to examine the monkey ES cells differentiation into cardiac myocytes in vivo, we delivered monkey ES cells constitutively expressing yellow fluorescence protein (YFP) through the central lumen of PTCA balloon catheter following balloon occlusion for 60 minutes. At 10 days after the delivery without any immunosuppressants, we observed YFP-positive cells around the infarcted area. Some of them...
Insoluble Deposits in Cardiac Myocytes from Human Dilated Cardiomyopathy

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Heart failure (HF) is a chronic debilitating condition where the progressive loss of contractile function results in disability and death. While HF is often a result of ischemic injury to the myocardium, in a significant subset of cases, classified as “idiopathic dilated cardiomyopathy” (DCM), the pathogenetic mechanisms that give rise to HF remain speculative. We propose that protein misfolding may be involved in the origin of the DCM and that its development may be related to the deposition of insoluble aggregates inside cardiomyocytes. Myosin light chain-2 (MLC-2) obtained from hearts explanted from patients with heart DCM 5 patients with amyloidosis and 12 non-failing “donor” hearts were stained by Congo Red and Thryoflavin S. The failing hearts explanted from patients with cardiac amyloidosis were used as positive control. DCM and amyloid hearts showed congophilic staining suggestive of protein aggregates. Whereas the amyloid hearts demonstrated the typical pattern of interstitial deposition, the DCM hearts showed congophilic staining mainly within the cardiac cells. Inconsistent changes in the expression and/or translation of proteins commonly related to some of the known protein misfolding neurodegenerative diseases were identified by RT-PCR, western blotting or immunohistochemistry. An important fraction of protein synthesis, folding and post translational modifications occurs inside the sarco-endoplasmic reticulum (SR). In cardiomyocytes, the SR has the dual role of modulating contractility while maintaining constant Ca²⁺ levels for the control of protein synthesis and processing. Abnormal SR Ca²⁺ homeostasis is a well characterized feature in failing hearts. We propose that abnormal Ca²⁺ homeostasis might result or determine protein misfolding. In addition, proteins belonging to the chaperone family are abundant in the SR allowing nascent polyepitopic chains to assume their correct three-dimensional conformation. We found an increase in expression of chaperone proteins GRP 78 (114.4%) and GRP 94 (58.9%) in failing human hearts suggesting a condition of SR stress and protein misfolding. In conclusion preliminary data suggest that protein misfolding can be an important pathogenetic mechanism leading to cardiac dysfunction and HF.

Reduction in Plasma Homocysteine Levels in Essential Hypertensive Patients in Response to Folic Acid Supplementation is not Associated with MTHFR C677T Gene Polymorphism

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Hyperhomocysteinemia has been regarded as an independent risk factor for cardiovascular disease. The vascular risk associated with hyperhomocysteinemia (HHcy) has been found to be stronger in hypertensive individuals. The water-soluble B vitamins (especially folate) are essential on an enzyme that catalyzes homocysteine conversion to methionine. A genetic variation, and are present every several-hundred bases, on average, throughout the human genome. A MTHFR C677T gene polymorphism may predict the response to ACE inhibitor therapy

Apo E Genotypes Modulate the Plasma Lipid Response to Atorvastatin in Indian Patients with Coronary Artery Disease

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Considerable variability exists in the plasma lipid and lipoprotein response to statin treatment due, in part, to genetic factors. The gene for apolipoprotein E (ApoE) is polymorphic and the different genotypes modulate baseline lipid levels. The objective of the present study was to evaluate the effect of the apoE genotypes on the lipid response to atorvastatin treatment in patients with coronary artery disease (CAD), followed-up in a cardiology clinic at Post Graduate Institute of Medical education and Research, India. Subjects and methods. 100 patients who were clinically confirmed with CAD and treated according to NCEP-ATPIII guidelines were included in the study. Plasma lipids were measured before and after 16 weeks of treatment with 10 mg /day of atorvastatin. Polymorphisms of ApoE were determined by restriction fragment length polymorphism (RFLP). RESULTS: ApoE genotype distributions were 4.28% with epsilon 2/2, 3.5% with epsilon 2/3, 17.41% with epsilon3/3 and 30.0% with epsilon 2/4, 21.4% with epsilon 3/4 and 23.57% with epsilon 4/4 respectively. Apo E allele frequencies for epsilon 2, 3 & 4 were 0.51, 0.296 and 0.492 respectively. Apo E genotype did not have any effect on baseline lipid levels after adjustment for age, gender and body mass index (BMI) (P > 0.05). The reduction in total cholesterol level was significantly higher in patients carrying epsilon 4 allele than in those with epsilon 2 or 3 alleles following treatment (-28.4% vs. -13.1%, P < 0.05). Compared with patients carrying epsilon 3 or epsilon 4 allele, those with epsilon 2 allele showed a significantly higher percentage reduction of LDL-C level after treatment (P < 0.05). CONCLUSION The reduction in total cholesterol level was more prominent in patients carrying epsilon 4 allele, and those with epsilon 2 allele showed a significantly higher percentage in reduction of LDL-C level after treatment. Apo E gene polymorphism appears to influence the response to atorvastatin in Indian patients with CAD.

Association of Antihypertensive Response of Angiotensin I Converting Enzyme Inhibitor to a New ACE SNP (rs4295) Genotype in Essential Hypertension

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The blood pressure (BP) response to any single antihypertensive drug is characterized by marked interindividual variation. Genetic factors may influence the response to antihypertensive medication. Single-nucleotide polymorphisms (SNPs) are the most frequent type of genetic variation, and are present every several-hundred bases, on average, throughout the human genome. A SNP (rs4295) of Angiotensin 1 converting enzyme gene (ACE) was selected from SNP (rs 4295) in Indian patients with essential hypertension attending Hypertension clinic at a Post Graduate Institute of Medical Education and Research, India. Subjects and Methods. The study population consisted of 71 essential hypertensive patients who were on ACE inhibitor antihypertensive therapy. Their blood pressure was measured every 2 weeks for 6 weeks. The patients whose diastolic blood pressure decreased by at least 10 mmHg were phenotyped as responders to ACE inhibitor therapy. Based on the Snp genotypes of the patients, we identified 36 responders and 35 non-responders to ACE inhibitor therapy in the study. The SNP (rs4295) of Angiotensin 1 converting enzyme gene (ACE) was selected from SNP databases of NCBI. Genotyping of SNP rs4295 was done using an in house developed restriction digestion based nested PCR assay. Upon restriction digestion of the nested PCR product we successfully identified all the three genotypes of these SNPs.

Prediction and Testing of Noncardiovascular Drugs Affecting Cardiovascular Aliments

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RIDESCENT, a computational method of knowledge discovery, was applied to the prediction of effects of existing, non-cardiovascular drugs on cardiovascular diseases. RIDESCENT (implicit Relationship IDentification by in-Silico Construction of an Entity-based Network from Text) is a computer program that predicts novel relationships by
identifying and scoring objects sharing large sets of relationships with an object of interest. The average cost to develop a new prescription drug is $802 million, and it takes 10–15 years to develop a new treatment, medicine, and to win approval to market it in the United States. The application of IRIDESCENT to drug discovery should increase speed of discovery and reduce discovery costs and should also reduce the time and cost of Phase II trials. IRIDESCENT was applied to the Medline database to find implicit relationships between small molecules and the following objects of interest: “cardiac hypertrophy” and “myocardial infarction”. A short-list of predictions was generated by manual analysis of the IRIDESCENT output and studies were initiated in mouse models of these indications.

Of 10 drugs tested thus far for cardiac hypertrophy, one has been established as having an effect. Of five drugs tested for myocardial infarction, one appears to have a slight effect and further studies are being conducted.

Co-supplementation of Cucumber, Zinc, and Iron Differentially Influence Antioxidant Status and Lipid Peroxidation in Hypercholesterolemic Rabbis

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The aim of this study was to investigate the effects of single or combined supplementation of Cu, zinc (Zn), iron (Fe) on serum level of malondialdehyde (MDA), as an index of lipid peroxidation, antioxidant enzymes activities (superoxide dismutase, catalase, and glutathione peroxidase), and plasma vitamin E concentration in hypercholesterolemic rabbits. For this purpose, 54 juvenile male New Zealand white rabbits were allocated to nine dietary groups including chow fed rabbits without mineral supplementation, HC-Cu, HC-Zn, HC-Fe, HC-Cu + Zn, HC-Cu + Fe, HC-Zn + Fe, and HC-Cu + Zn + Fe. The lowest antioxidant enzymes activities were shown in rabbits given HC-Cu + Fe. Serum MDA level of rabbits co-supplemented with Cu and Fe were found to be significantly higher than that of rabbits supplemented with Cu and Zn, singly and together (P<0.05), but it did not show significant difference as compared with rabbits given HC-Cu + Fe without mineral supplementation (P>0.05). Zn supplementation by itself significantly increased antioxidant enzymes activities in comparison with other groups (P<0.01), whereas the difference of superoxide dismutase activity between rabbits given HC-Cu + Zn and HC-Cu + Zn + Fe were found to be insignificant (P>0.05). None of supplementary regimens affected the plasma level of standardized vitamin E. Collectively, our results are consistent with Zn as a predictor of oxidative stress, but co-supplementation with Cu and Fe reduces Zn antioxidant effects. These results warrant further studies to investigate the interaction between these trace elements in the regulation of molecular mechanisms of cellular antioxidant capacities.

A Loss of Function Gqα Promoter Polymorphism is Associated with Accelerated Progression of Heart Failure

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Background: Gqα is a critical regulator of cardiac hypertrophy, which is an independent risk factor for death. Transgenic mouse studies have demonstrated that Gqα is necessary for pressure-induced hypertrophy, sufficient to cause load-independent hypertrophy, and can mediate the transition of compensated hypertrophy to congestive heart failure (CHF). The effects of modulated Gqα signaling in the human condition are unknown. We hypothesized that promoter polymorphisms of the Gqα gene that affect Gqα transcriptional activity due to ablation of a Sp-1 binding site. Patients from the Cincinnati Heart Failure Study (CHF Ctr, Tabriz, Iran (Islamic Republic of); Jamal Gaemmagami; Tabriz Univ of Med Sciences, Tabriz, Iran (Islamic Republic of)

The aim of this study was to investigate the effects of single or combined supplementation of Cu, zinc (Zn), iron (Fe) on serum level of malondialdehyde (MDA), as an index of lipid peroxidation, antioxidant enzymes activities (superoxide dismutase, catalase, and glutathione peroxidase), and plasma vitamin E concentration in hypercholesterolemic rabbits. For this purpose, 54 juvenile male New Zealand white rabbits were allocated to nine dietary groups including chow fed rabbits without mineral supplementation, HC-Cu, HC-Zn, HC-Fe, HC-Cu + Zn, HC-Cu + Fe, HC-Zn + Fe, and HC-Cu + Zn + Fe. The lowest antioxidant enzymes activities were shown in rabbits given HC-Cu + Fe. Serum MDA level of rabbits co-supplemented with Cu and Fe were found to be significantly higher than that of rabbits supplemented with Cu and Zn, singly and together (P<0.05), but it did not show significant difference as compared with rabbits given HC-Cu + Fe without mineral supplementation (P>0.05). Zn supplementation by itself significantly increased antioxidant enzymes activities in comparison with other groups (P<0.01), whereas the difference of superoxide dismutase activity between rabbits given HC-Cu + Zn and HC-Cu + Zn + Fe were found to be insignificant (P>0.05). None of supplementary regimens affected the plasma level of standardized vitamin E. Collectively, our results are consistent with Zn as a predictor of oxidative stress, but co-supplementation with Cu and Fe reduces Zn antioxidant effects. These results warrant further studies to investigate the interaction between these trace elements in the regulation of molecular mechanisms of cellular antioxidant capacities.

Hemodynamic and Humoral Effects of Atrial Natriuretic Peptide on Pulmonary Circulation after Cardiac Surgery

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Background: Atrial natriuretic peptide (ANP) has biological effects such as natriuresis, diuresis, vasodilation and inhibition of RAA system. And it has the effect of cardiac and renal protection. However, the effect of ANP on pulmonary circulation is unclear. Methods: A total of 12 consecutive adult patients scheduled to undergo cardiac surgery with cadiopulmonary bypass were prospectively selected for this study. ANP was infused from right atrium through S-G catheter at 0.1\mu g/kg/min for 60minutes at 6 hours after the operation. Blood samples for measurement ANP and cyclic GMP, second messenger of ANP, were drawn from pulmonary artery (PA) through S-G catheter and left atrium (LA) through left atrial pressure line before and after infusion of ANP. And hemodynamic data (and the other humoral factors of RAA system and brain natriuretic peptide) were measured at the same time. Results (Table): Plasma level of ANP in LA significantly decreased more in PA, markedly decreased in LA pressure, cyclic GMP in PA significantly increased vs in PA. Conclusion: ANP infusion produced significant decreases in mean PA pressure, and markedly systemic PA pressure. Conclusion: Infusion of ANP after cardiac surgery affects pulmonary circulation, markedly pulmonary artery, ANP has an effect of dilation pulmonary artery and decreases pulmonary artery pressure.

Identification, Regulation, and Effects of Sprouty1, an Intrinsic Inhibitor of the ERK Signaling Cascade, in the Human Heart

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Accumulating evidence suggests an increasing role for the Ras/Raf/MAPK/ERK signaling cascade in the regulation of myocardial function and remodeling. We screened a comprehensive human microarray expression profiles for novel genes regulating the ERK cascade. From paired human heart samples harvested at the time of implant and explant with a left ventricular assist device (LVAD), we identified Sprouty1, an evolutionarily conserved gene defined as a negative regulator of the Ras/Raf/MAPK/ERK signaling pathway, to be significantly up-regulated in response to mechanical unloading. Real-time quantitative PCR, immunoblotting, and immunohistochemistry confirmed that Sprouty1 was up-regulated in human cardiac tissue following LVAD therapy with a concordant decrease in ERK activation. To our knowledge, these findings were the first to identify Sprouty1 expression in the human heart and suggest that Sprouty1 may serve as an
intrinsic inhibitor governing Ras/Raf/MEK/ERK signaling and ventricular remodeling in response to alterations in workload and wall stress. In vitro studies demonstrated that direct up-regulation of Sprouty1 in HL-1 murine cardiac myocytes leads to a temporally-mediated decrease in the activation of ERK by fibroblast growth factor. Adenoviral up-regulation of Sprouty1 in culture also led to a significant decrease in serum- and VEGF-stimulated endothelial cell proliferation. We have identified a novel regulatory mechanism controlling Sprouty1 expression in cardiac myocytes. Parallel growth of HL-1 murine cardiac myocytes in normoxic (RA) versus hypoxic (1% O2) conditions has demonstrated significantly increased levels of cellular Sprouty1 and decreased ERK activation for up to 12 hours in an oxygen limiting environment. The in vivo effects of Sprouty1 on the heart began with the development of transgenic mice whose overexpression of Sprouty1 is limited to cardiac tissue as driven by the alpha-myosin heavy chain promoter. Heart specific overexpression of Sprouty1 is increased with respect to wild-type beginning at 1 week of age and increases throughout development until maturity. Echocardiographic comparisons of heart function between wild-type and transgenic mice show no significant differences under baseline conditions.

**Discovery of a Heart-Specific and Failure-Associated Gene in Human Heart Failure**

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In order to identify differentially expressed genes/pathways involved in the development of human heart failure, we profiled 10 human hearts, 5 non-failing and 5 failing with the diagnosis of diastolic cardiomyopathy using the Affymetrix oligonucleotide HG-U133 array. In this study, we report the identification and characterization of a novel gene, HSFA (Heart-Specific Failure-Associated). HSFA was shown by Affymetrix microarray analysis to be up-regulated 2-fold in failing versus non-failing hearts, with expression levels in failing hearts similar to ANF. This 2-fold increase in expression of HSFA in failing hearts was confirmed by Northern blot analysis and quantitative real-time PCR. Expression of HSFA was highest in the left ventricle, limited in other tissues and absent in the brain. Chromogenic mRNA in-situ hybridization using a riboprobe for HSFA in failing hearts revealed high gene expression in cardiomyocytes and endothelial cells of blood vessels. Interestingly, Northern blot analysis also identified the presence of an alternatively spliced-put out of 100bp located in the 3-UTR. This was found exclusively in the myocardium. Furthermore, the HSFA isoform containing the extra 3'-UTR 100bp sequence was up-regulated in failing human hearts. This is of particular interest since 3'-UTRs are involved in post-transcriptional regulation of gene expression, including regulation of mRNA subcellular localization and mRNA stability. This additional 100bp in the 3'-UTR region may represent a mechanism to increase stability of HSFA mRNA stability by post-transcriptional regulation, and thus increase HSFA protein levels in failing myocardium. The complete cDNA sequence of HSFA (3111 bp) was obtained by 5' and 3' RACE (GenBank AF217967) and a protein of 157 amino acids was deduced from the nucleotide sequence. HSFA is localized to chromosome 12q23. We have identified phosho-regulating proteins using polyhistidine pull-down of recombinant HSFA with human heart lysates. Altered post-transcriptional regulation as a result of altered mRNA stability is likely to be an additional mechanism of regulation of protein expression in heart failure.

**Regulation of Cardiomyocyte Stress Signaling by Protein Kinase D1**

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Class II histone deacetylases (HDACs) 5 and 9 are transcriptional repressors of cardiomyocyte hypertrophy that are subject to phosphorylation-dependent neutralization in response to stress signaling. Recent studies have suggested a role for protein kinase C (PKC) and its downstream effector protein kinase D (PKD), in the control of HDACs phosphorylation. While PKCs are well-documented regulators of cardiac signaling, the ability to phosphorylate and neutralize class II HDACs is poorly understood. In this study we used an inducible cardiac myocyte-specific \( \beta \)1 integrin knockout (\( \beta \)1KO) mouse model for investigating integrin function. We found that disruption of the \( \beta \)1 integrin gene in the adult cardiac myocyte caused 90% reduction of \( \beta \)1 protein and altered the physiological response to profuse transverse aortic constriction (TAC) (aortic gradient 109±22 mmHg). When examined in mice subjected to a more moderate TAC (aortic gradient 8.5±4.5 mmHg) the pattern of multiple growth response signaling pathways was detected. Basally the \( \beta \)1KO showed significant reduction in p42/44 ERK activity vs. controls (n=4 each) while 4 weeks following the moderate TAC, significant reduction in activity of ERK67%, Akt (72%) and P-38 (89%) were detected in the \( \beta \)1KO. Since integrin and G-protein-coupled receptor (GPCR) signaling pathways have been suggested to have common nodal points in non-myocytes, we assessed crosstalk between these two pathways in myocytes from the \( \beta \)1KO mice. Basally the \( \beta \)1KO cardiac myocytes showed P-FAK and Akt activities decreased by 25% and 80% respectively vs. control myocytes. Following isoproterenol stimulation, FAK, AKT and ERK activities increased 77%, 90% and 70% respectively vs. unstimulated cells in the control mice, while no significant change was detected in the \( \beta \)1KO animals. Conclusion: 1) 90% reduction of \( \beta \)1 integrin protein expression in the cardiac myocyte could be achieved in the mature murine heart in vivo. 2) Following death following high-grade aortic constriction. 2) With lesser degrees of aortic constriction, the KO mice survived but had decreased activities of Akt, ERK, and p-38 kinases compared to control, 3) In isolated myocytes from the KO mice, FAK and AKT activities were basally reduced and 4) Isolated stimulation caused increased in FAK, AKT, P-38 and ERK activity in control cells, but not in KO cells. Cardiac myocyte integrins are thus essential for normal mechanotransduction, integrin and non-integrin (adrenergic) signaling events in the murine heart. The mecha- nistic basis for these events is undergoing additional investigation.

**G-Protein-Coupled Receptor Signaling Pathways Are Disrupted in \( \beta \)1 Integrin Cardiac-Specific Knockout Mice**

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The role of \( \beta \)1 integrin in modulating signaling transduction pathways in the intact myocardium is poorly understood. In this study we used an inducible cardiac myocyte-specific \( \beta \)1 integrin knockout (\( \beta \)1KO) mouse model for investigating integrin function. We found that disruption of the \( \beta \)1 integrin gene in the adult cardiac myocyte caused 90% reduction of \( \beta \)1 protein and altered the physiological response to profuse transverse aortic constriction (TAC) (aortic gradient 109±22 mmHg). When examined in mice subjected to a more moderate TAC (aortic gradient 8.5±4.5 mmHg) the pattern of multiple growth response signaling pathways was detected. Basally the \( \beta \)1KO showed significant reduction in p42/44 ERK activity vs. controls (n=4 each) while 4 weeks following the moderate TAC, significant reduction in activity of ERK67%, Akt (72%) and P-38 (89%) were detected in the \( \beta \)1KO. Since integrin and G-protein-coupled receptor (GPCR) signaling pathways have been suggested to have common nodal points in non-myocytes, we assessed crosstalk between these two pathways in myocytes from the \( \beta \)1KO mice. Basally the \( \beta \)1KO cardiac myocytes showed P-FAK and Akt activities decreased by 25% and 80% respectively vs. control myocytes. Following isoproterenol stimulation, FAK, AKT and ERK activities increased 77%, 90% and 70% respectively vs. unstimulated cells in the control mice, while no significant change was detected in the \( \beta \)1KO animals. Conclusion: 1) 90% reduction of \( \beta \)1 integrin protein expression in the cardiac myocyte could be achieved in the mature murine heart in vivo. 2) Following death following high-grade aortic constriction. 2) With lesser degrees of aortic constriction, the KO mice survived but had decreased activities of Akt, ERK, and p-38 kinases compared to control, 3) In isolated myocytes from the KO mice, FAK and AKT activities were basally reduced and 4)Iso stimulation caused increased in FAK, AKT, P-38 and ERK activity in control cells, but not in KO cells. Cardiac myocyte integrins are thus essential for normal mechanotransduction, integrin and non-integrin (adrenergic) signaling events in the murine heart. The mecha- nistic basis for these events is undergoing additional investigation.
with ME2FA overexpression in the heart elicited severe thinning of the cardiac walls and chamber dilation. Using NKI-Tag cells, an immortalized cardiac cell line, we generated double whole-chromosome transgenic mice expressing ME2FA under a doxycycline (Dox)-inducible manner. Upon Cre-mediated mortalization and Dox-stimulation, these cells demonstrated an elongated morphology. In order to investigate the change in gene expression underlying this cellular remodeling, we performed micro-array analysis on two independent inducible ME2F20NP16 clones. After Dox-stimulation for 24 hrs, 75 genes were found to be differentially expressed in both clones with a 2 fold-change in expression. Gene ontology classifications revealed an over-representation of genes involved in cell-matrix adhesion and cytoskeletal remodelling. These data suggest that ME2F2 transcriptional activity induces (maladaptive-) remodelling of cardiomyocytes, and further investigation of the affected genes could provide novel insights in the underlying mechanisms of eccentric remodelling and dilated cardiomyopathy.

Crosstalk Between ASK1 and Calcineurin Signaling Pathways in Cardiomyocytes

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Apoptosis signal-regulating kinase 1 (ASK1) is a pivotal component in cytokine and stress induced apoptosis. Here we describe a novel functional interaction between ASK1 and calcineurin in cardiomyocytes, first identified by yeast-2-hybrid screening with calcineurin B1 as a bait. Purified calcineurin could effectively dephosphorylate ASK1 at Ser-967 in vitro, and overexpression of constitutively active calcineurin lead to dephosphorylation of ASK1 in cardiomyocytes. Dephosphorylation of ASK1 at Ser-967 by calcineurin results in dissociation of the ASK1/14–3-3 complex with a concomitant increase of ASK1 kinase activity. Moreover, overexpression of constitutively active calcineurin or treatment with the calcium mobilizer ionomycin dramatically increased ASK1-induced cell death in cardiomyocytes. Thus, calcineurin is an ASK1 phosphatase in vivo and in vitro and plays a critical role in ASK1 regulation. On the other hand, calcineurin expression also suppresses calcineurin-inactivated NFAT nuclear translocation, NFAT transcriptional activity and the hypertrophic response in cardiomyocytes, partially through activation of p38 and JNK. Taken together, we describe a novel molecular interaction between calcineurin and ASK1 which may be important in the regulation of hypertrophic and apoptotic responses in cardiomyocytes.

Development of Novel Methods for Delivery of siRNA to Cardiomyocytes to Assess the Contribution of Phospholipase C β to Hypertrophic Responses in Neonatal Rat Cardiomyocytes

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Activation of receptors coupled to the Gq family of heterotrimeric G proteins causes hypertrophy of neonatal rat ventricular myocytes (NRVMs) as indicated by increased cell size as well as increased expression of ‘hypertrophic markers’ such as ANP and MLC2. All of these responses can be sustained by direct activation of Gq and depletion of this family prevents hypertrophy. Phospholipase Cβ3 (PLCβ3) isoforms are currently the only known effectors of Gq signaling, but to date their role in hypertrophic responses have not been evaluated. We have subcloned two subtypes of PLCβ3 are expressed in the myocardium, β1 and β2. We assessed the importance of these two subtypes in cardiomyocyte growth, we designed siRNA sequences to target the two selectively. Sequences were prepared as hairpin cDNA copies and expressed under a polymerase III promoter. Effective sequences were selected by co-transfection with luciferase expressed under a protein kinase Cα-response element and luciferase responses to PLC activation measured. The selected PLCβ3-αi and PLCβ3-βi sequences reduced expression by 80% and 60%, respectively. We next developed an adenoviral vector to efficiently express these selected hairpins in cardiomyocytes. We constructed a novel bi-cistronic vector expressing the RNA hairpin under a pol III promoter as well as GFP under a CMV promoter to allow assessment of transfection efficiency. NRVMs were treated with phenylephrine (50 μM plus propranolol 1 mM) (PE) for 48h to initiate Gq-dependent hypertrophy. Scrambled hairpin sequences did not alter hypertrophic responses to PE. However, PLCβ3-αi sequences reduced both ANP and MLC2 responses by 52% siRNA to PLCβ3-αβi resulted in reduction of MLC2 expression by 50%. The ability to express siRNA from an adenoviral vector will facilitate the usefulness of this technology for cardiac research.

Functional Characterization of the Voltage-Dependent Anion Channel Family of Proteins and Regulation by Protein Kinase C ε

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Mitochondrial permeability transition (MPT) is an organellar impairment that plays a causative role in cell death following stress, including ischemia/reperfusion injury in the myocardium. MPT involves permeabilization of the inner and outer mitochondrial membranes—leading to cytochrome c release—and is thought to be modulated by MPT pores, multiprotein complexes that span this double membrane system. In the outer membrane, the voltage-dependent anion channel (VDAC) has been implicated as a central component of this pore, however, the fundamental regulation of the 3 known mammalian isoforms of VDAC is unknown. We generated recombinant, C-terminally His-tagged VDAC1, VDAC2 and VDAC3 proteins by subcloning the 3 mouse genes into an E. coli expression vector, rVDAC proteins were expressed in inclusion bodies as detected by western blotting. Using a 6M guanidine-HCl denaturation/renaturation protocol, we purified rVDAC and reconstituted it into synthetic liposomes (2% cardiolipin in phosphatidylcholine) viaovernight dialysis. To assess MPT functionality as a large non-specific pore, liposomes were loaded with FITC-conjugated cytochrome (cyt c). After washing of VDAC-reconstituted liposomes to remove extra-vesicular cyt c, pore formation was induced by reactive oxygen species (ROS; xanthine [100 μM] and xanthine oxidase [20 μM/mll]) and measured by cyt c release as a percentage of total release after liposome destruction (boiling). The individual reconstituted VDAC isoforms exhibited distinct pore-forming properties, in particular, rVDAC3 appeared to be less prone to form a pore in response to ROS in vitro as compared to rVDAC1 and 2. To determine whether these isoforms are regulated by the signaling kinase PKC (a cardioprotective kinase previously shown to be present with an MPT pore), liposomes reconstituted VDAC isoforms were preincubated with PMA+protein (molar ratio 1:50). PKC+VDAC3. PKCe was sufficient to significantly reduce the ROS-induced cyt-c release via both rVDAC1 and rVDAC2. These data provide the first insight into functional regulation of VDAC by a cardiosprotective signaling kinase, and support PKCε-dependent modulation of this protein as a potential mechanism to prevent MPT.

A Novel Role for Protein Phosphatase 2A in the Regulation of the Sarcolemmal Na+/H+ Exchanger via Adenosine A1, Receptors

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Stimulation of myocardial α1-adrenoceptors increases sarcolemmal Na+/H+ exchanger (NHE1) activity by an ERK/p90RSK-dependent mechanism, most likely via p90RSK-mediated phosphorylation of the NHE1 regulatory domain. Prior stimulation of adenosine A1 receptors inhibits this response through a Gβγ-mediated pathway; the distal signaling mechanisms of such inhibition are unclear and were investigated in cultured adult rat ventricular myocytes (ARVM). Stimulation of A1 receptors by cyclopentyl adenosine (CPA, 1 μM) had no effect on the activation of the ERK/p90RSK pathway by phenylephrine (100 μM). However, in combination with CPA, antibodies for activated ERK1/2 and p90RSK, or (2) p90RSK in vitro kinase (WK) assays utilising βIP incorporation into a fusion protein (GST-NHE1) containing residues 625–747 of the NHE1 regulatory domain. Exposure of ARVM to CPA induced translocation of the catalytic subunit of protein phosphatase 2A (PPP2Ac) from the cytosol to the particulate fraction. Such translocation was abolished by either pre-treatment with pertussis toxin or adenosine A1-receptor activation. Phosphorylation of transducin Gβγ subunit (as a Gβγ scavenger), thereby suggesting a role for Gβγ subunits originating from G1 family heterotrimeric G proteins. Modified IF assays determined that purified PPP2Ac could dephosphorylate p90RSK- phosphorylated GST-NHE1, with such dephosphorylation inhibited by okadaic acid (1 μM) and endothall (100 μM). Sarcolemmal NHE1 activity in ARVM (n=12–14/group) was determined by microfluorimetry, using the pH fluor probe CNARF-1. Phosphorylation significantly increased NHE1 activity (2.1-fold) compared to control and this effect was abolished by pre-treatment with CPA. In the presence of the P2A inhibitor endothall (100 μM), phosphorylne again significantly increased NHE1 activity (3.4-fold) compared to control; however, under these conditions, pre-treatment with CPA no longer prevented the phenylephrine-induced increase in NHE1 activity (3.4-fold). These studies reveal a novel role for the PPP2A holoenzyme in A1 receptor-mediated regulation of NHE1 activity in ARVM, the mechanism of which may involve Gβγ-mediated translocation of the PPP2Ac subunit and NHE1 dephosphorylation.

Calcineurin and Protein Kinase C Regulate Cyclooxygenase-2 Expression in the Heart

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Upregulation of components of the acute inflammatory response, such as COX-2 and iNOS, is important for the adaptation of the myocardium to ischemia/reperfusion stress. The aim of this study was to determine (1) if the calcineurin signalling pathway regulates cyclooxygenase-2 (COX-2) expression in the heart, (2) if it interacts with the protein kinase C (PKC) pathway and, (3) if it is cell-type specific. Adenovirally mediated overexpression of an active mutant of calcineurin A (CA) in cultured neonatal rat ventricular cardiomyocytes (NRVMs) induced COX-2 expression. COX-2 induction by CA was potentiated by co-expression of the wild type α and ε isoforms of PKC. Reporter constructs comprising +104 to -1796 bp of the human COX-2 gene proximal promoter region containing the effect of CA and its response to CA was mediated by a region between -1796 and -1700 containing a number of NFAT and AP-1 sites. In contrast, no COX-2 induction was seen in isolated cultured adult cardiomyocytes under the same experimental conditions. Therefore, COX-2 expression in NRVMs was assessed by immunofluorescence confocal imaging in relation to the myocyte-specific marker α-actinin. COX-2 was basally
expressed in all cardiac cells but strong COX-2 induction was only observed in non-myocytes (fibroblasts) when cells are overexpressing Cnα2 + PKCα. This distribution of COX-2 expression was confirmed in vivo by studies using frozen mouse heart sections which showed higher COX-2 expression in integral interstitial non-myocytes located between the cardiomyocytes. In conclusion, these results demonstrate that the calcineurin signaling pathway mediates COX-2 induction in cardiac cells, that the effect is potentiated by PKCα, and that expression of interstitial non-myocytes play an important role in mediating the cardiac inflammatory response.

The TNFR Family Member, 4–1bb, Contributes to Doxorubicin-Induced Apoptosis Distal to Mitochondrial Membrane Potential Disruption

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In order to study the mechanism of doxorubicin (DOX)-induced cardiotoxicity, we have developed a mouse model of DOX-induced cardiomyopathy, using a single retro-orbital injection of DOX. Two to five weeks after DOX injection, reduced cardiac function, measured by echocardiography, and cardiac apoptosis were evident. In contrast to WT, 4–1BB-deficient mice have improved cardiac function, and reduced apoptosis. Here we report that DOX induced apoptosis of neonatal rat ventricular cardiomyocytes (NRVM) in a time and dose dependent manner. After 0.5uM or 1.0uM DOX treatment, hypodiploid DNA content increased from 1.00 + 0.36% to 2.68 + 0.53% (P < 0.01 N=6), and 33.75 + 4.84% (P < 0.01 N=6), respectively. At 1.0 uM DOX, hypodiploid DNA increased from 1.80 + 0.6% at time 0 to 7.91 ± 1.94% (p < 0.01 N=6), 10.52 ± 2.27% (p < 0.01 N=6), and 25.75 ± 2.22% (p < 0.01 N=6) at 6, 18, and 24 hours, respectively. In NRVM, DOX-induced the surface expression of 4–1BB and 4–1BL in 6 hours. Murine soluble 4–1BB Fc (1 ug/ml), which blocks the activation of 4–1BB by 4–1BL, significantly reduced DOX-induced apoptosis; hypodiploid DNA content was 10.52 ± 2.27% in controls vs 7.58 ± 0.66% in 4–1BB Fc NRVM. These results prompted us to determine whether G-CSF or BMSC could improve cardiac function in a DOX model of cardiomyopathy. Here we report that G-CSF did not substantially improve cardiac function, despite its ability to increase circulating c-Ki+ cells and white blood cell counts. DOX induced cardiac dysfunction is evident by day 7–10. Unfractionated BMSC did not delay the onset of disease and did not lower the penetrance of dysfunction in DOX-treated mice. Transplantation of individual bone marrow populations, including hematopoietic stem cells (HSC), c-Ki+ cells, or non-stem cells after DOX administration did not interfere with the progression of cardiac disease. Thus, G-CSF and bone marrow stem cell transplantation did not improve cardiac function in a DOX mouse model.
Histone Acetyltransferase Activity of p300 Is Required for the Promotion of Left Ventricular Remodeling Following Myocardial Infarction in Adult Mice In Vivo

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Left ventricular (LV) remodeling following myocardial infarction is associated with hypertrophy of surviving myocytes and represents a major process that leads to heart failure. A p300 protein is one of intrinsic histone acetyltransferases (HAT) and governs gene expression patterns by being recruited to target genes through association with specific transcription factors. In cardiac myocytes, p300 serves as a coactivator of hypertrophy-responsive transcriptional factors such as MEF-2 and GATA-4 and is involved in hypertrophic stimuli-induced acetylation and DNA binding of GATA-4. However, the role of p300 HAT activity in LV remodeling following myocardial infarction (MI) in vivo is unknown. To solve this problem, we have generated transgenic mice (TG) overexpressing intact p300 in the heart and TG overexpressing mutant p300 in the heart. This mutant, by its two-amino-acid substitution in the p300 HAT domain, lost its HAT activity and was unable to activate GATA-4-dependent transcription. Then, these two kinds of TG and their wild-type mice (WT) were subjected to MI or sham operation at the age of 12 weeks. Before MI, echocardiography revealed no difference in LV size and function among these mice. Five weeks later, in both WT and TG, MI mice exhibited LV remodeling exemplified by lower LV ejection fraction and by larger LV end-diastolic and systolic dimensions compared with sham-operated mice. However, intact p300-TG revealed significantly (p<0.05) more progressive LV remodeling than corresponding WT. In contrast, mutant p300-TG exhibited LV remodeling similar with WT. There were no differences in infarct size among these mice. In both WT and TG, LV levels of endothelin-1, a downstream target of p300/GATA-4 pathway as well as a marker of LV remodeling, were higher in the MI group than the sham-operated group. In intact p300-TG but not in mutant p300-TG, this increase was further exaggerated compared with corresponding WT. These findings demonstrate that cardiac overexpression of p300 promotes LV remodeling following MI in adult mice in vivo and that HAT activity of p300 is required for these processes.

Transcriptome-Based Models for Signaling Networks in the Heart Early Post-Myocardial Infarction

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We surveyed the mouse heart transcriptome for transcripts responsive to acute myocardial infarction (AMI) in three regions of the left ventricle, namely ischemic/infarcted tissue (IF), the surviving left ventricular free wall (FW) and the interventricular septum (IVS). At six time points from 15-min to 48 h post-AMI, 36,899 transcripts were assayed in both AMI and sham surgery mice. We compared AMI and sham temporal expression patterns for each gene expression patterns by being recruited to target genes through association with specific transcription factors. In cardiac myocytes, p300 serves as a coactivator of hypertrophy-responsive transcriptional factors such as MEF-2 and GATA-4 and is involved in hypertrophic stimuli-induced acetylation and DNA binding of GATA-4. However, the role of p300 HAT activity in LV remodeling following myocardial infarction (MI) in vivo is unknown. To solve this problem, we have generated transgenic mice (TG) overexpressing intact p300 in the heart and TG overexpressing mutant p300 in the heart. This mutant, by its two-amino-acid substitution in the p300 HAT domain, lost its HAT activity and was unable to activate GATA-4-dependent transcription. Then, these two kinds of TG and their wild-type mice (WT) were subjected to MI or sham operation at the age of 12 weeks. Before MI, echocardiography revealed no difference in LV size and function among these mice. Five weeks later, in both WT and TG, MI mice exhibited LV remodeling exemplified by lower LV ejection fraction and by larger LV end-diastolic and systolic dimensions compared with sham-operated mice. However, intact p300-TG revealed significantly (p<0.05) more progressive LV remodeling than corresponding WT. In contrast, mutant p300-TG exhibited LV remodeling similar with WT. There were no differences in infarct size among these mice. In both WT and TG, LV levels of endothelin-1, a downstream target of p300/GATA-4 pathway as well as a marker of LV remodeling, were higher in the MI group than the sham-operated group. In intact p300-TG but not in mutant p300-TG, this increase was further exaggerated compared with corresponding WT. These findings demonstrate that cardiac overexpression of p300 promotes LV remodeling following MI in adult mice in vivo and that HAT activity of p300 is required for these processes.

Reversible Heart Failure Caused by Inducible Gnaq Requires Activation of Phospholipase Cβ

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Receptors coupled to Gnaq, a signal transduction molecule in the heterotrimeric G protein superfamily, are thought to play a key role in the development of heart failure. Herein we demonstrate that transgenic mice that express a silent Gnaq protein (GnaqQ209L-hbER) in cardiac myocytes develop decompensated heart failure with severe edema following drug treatment to activate the recombinant Gnaq protein. This phenotype is associated with increased βMHC and ANF mRNA levels, a decrease in β-L type Ca2+ current density, dephosphorylation of phospholamban (PLB) and decreased sarcoplasmic reticulum Ca2+-ATPase-2 (SERCA-2) activity. In contrast, transgenic mice expressing a Gnaq mutant (GnaqQ209L-AA-hbER) that cannot activate one of its effectors, phospholipase Cβ (PLCβ), do not develop heart failure or changes in PLB phosphorylation, but do show changes in cardiac gene expression and Ca2+ currents similar to the GnaqQ209L-hbER mice. Heart failure, cardiac morphological changes and alterations in PLB phosphorylation are reversible upon termination of the Gnaq signal. These results demonstrate that activation of Gnaq in cardiac myocytes in adult mice causes a dilated cardiomyopathy and activation of PLCβ is required to induce this phenotype.

Prolonged Duration of Sepsis Causes Elevated Endothelin-1 and Myocardial Dysfunction Correlating with DNA Fragmentation

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We tested the hypothesis that elevated myocardial endothelin correlates with DNA fragmentation and myocardial dysfunction during sepsis. Male Sprague-Dawley rats (350-400g) were randomized into sham, 1-, 3- and 7-days sepsis groups. Sepsis and sham-sepsis was induced using 200 mg/kg cecal inoculum and 5% dextrose water i.p., respectively. Post-mortem analysis showed severely inflamed peritoneum with presence of pus in all septic animals which was directly proportional to the duration of sepsis. We observed 10,33 and 42% mortality in 1-, 3- and 7-days sepsis groups, respectively. Animals in 3-days and 7-days post sepsis exhibited pulmonary edema and increased heart weight. Plasma and myocardial endothelin (ET)-1 concentration in 7-days sepsis group was significantly elevated as compared to sham and 1-day sepsis groups. Sepsis produced a significant prolongation of left ventricular isovolumic relaxation rate constant, tau, in 1-day sepsis group as compared to sham. Three and 7-days septic groups showed a significant prolongation in tau as compared to sham as well as 1 day sepsis groups. To assess a correlation with myocardial performance and apoptosis, protein expression of Bax and TdT-FragEL™ staining was performed. Sepsis produced a significant upregulation of myocardial Bax in 3-days and 7-days sepsis groups. We observed increased number of apoptotic nuclei in 3- and 7-days septic myocardium, suggesting an increased DNA fragmentation. Sepsis-induced mortality, depressed myocardial performance and elevated ET-1 levels correlate with DNA fragmentation and elevated Bax expression. We concluded that sepsis-induced decrease in myocardial performance could be due to induction of apoptosis.
Abstract Presentations

Circ Res. 2005;97:e9-e50

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

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