Abstracts

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Cardiovascular Repair and Regeneration: Structural and Molecular Approaches in the Cellular Era

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Conference Program Committee
Conference Co-Chairs: Maurizio C. Capogrossi, Keiichi Fukuda, Sumanth D. Prabhu, and Mark Sussman

This annual meeting is the fourth for this premier research conference sponsored by the American Heart Association Council on Basic Cardiovascular Sciences, the world’s leading organization of cardiovascular scientists. The conference focused on how cellular-based approaches are being manipulated to enhance the repair and regeneration capabilities of the cardiovascular system with the goal of therapeutic-based interventions. The meeting featured both invited presentations and poster abstract presentations, with participants from around the world.

Abstracts for the poster presentations are provided in this special online supplement available at http://circres.ahajournals.org.
Small Molecule Targeting of Gβγ Reduces β-Adrenergic Receptor Desensitization and Normalizes Cardiac Dysfunction in an Acute Heart Failure Model

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Heart failure (HF) is a debilitating disease with poor prognosis, despite substantial therapeutic advances in the past two decades. Excess signaling through cardiac G-protein Gβγ subunits is an important component of HF pathophysiology. They recruit elevated levels of cytosolic G-protein-coupled receptor kinase 2 (GRK2, a.k.a., PKR2) to agonist-stimulated β-ARs in HF, leading to chronic β-AR desensitization and down-regulation; these events are hallmark of HF. Previous data has suggested that inhibiting Gβγ signaling and its interaction with GRK2 could be of therapeutic value in HF. We recently developed a novel small molecule targeting strategy to selectively inhibit Gβγ-binding interactions, and identified several Gβγ inhibitors small molecules (Bonacci et al., Science, 2006). In particular, we identified compound M119, which blocked the interaction of purified Gβγ and GRK2 in vitro. To validate this activity in a cellular setting, we isolated adult mouse cardiomyocytes, where M119 significantly reduced the inhibitory component of the Wnt pathway, in regulating cardiomyocyte differentiation from adult MSCs.

Control of Phenotypic Plasticity of Smooth Muscle Cells by BMP Signaling Through Myocardin-Related Transcription Factors

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Vascular smooth muscle cells (VSMCs), unlike other muscle cells, do not terminally differentiate. In response to injury, VSMCs change phenotype, proliferate and migrate as part of the repair process. Dysregulation of this plasticity program contributes to the pathogenesis of several vascular disorders, such as atherosclerosis, restenosis and hypertension. The discovery of mutations in the gene encoding BMPR2, the type II receptor for the Bone Morphogenetic Proteins (BMPs), in idiopathic pulmonary arterial hypertension (IPAH) provided a clue that BMP signaling may affect the homeostasis of VSMCs and their phenotype modulation. Here we report that BMP signaling potently induce SMG genes in pluripotent cells, and prevents de-differentiation of pulmonary artery SMCs (PASMCs). The BMP-induced phenotype switch requires intact Rhα/ROCK signaling, but is not blocked by inhibitors of the TGFβ and PI-3K/Akt pathways. Furthermore, cellular localization and recruitment of the myocardin-related transcription factors (MRTF-A and MRTF-B) to a SMα-actin promoter was observed in response to BMP treatment in vivo. Thus, we conclude that BMP signaling modulates the phenotype of VSMCs via cross-talk with the Rhα/MRTF/B pathway, and may contribute to the development of the pathological characteristics observed in patients with IPAH and other obliterative vascular diseases.

2007 BCVS Symposium Abstracts

Transendocardial Autologous Bone Marrow in Chronic Myocardial Infarction Using Helical Needle Catheter: 1-Year Follow-up in an Open-Label, Nonrandomized, Single-Center Pilot Study (the TABMM Study)

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Aims: Cell therapy has shown benefit in preclinical and clinical studies, although debate continues on the mechanism of action and the most appropriate methods for performing such therapies. We assessed the hypothesis that helical needle transendocardial delivery of autologous bone marrow (ABM) cells around regions of hypo or akinesis in chronic post-MI patients would be safe and possibly improve ejection fraction. Methods and results: 10 stable post-MI Patients with an ejection fraction (EF) <40% were enrolled. ABM cells were aspirated from the iliac crest and delivered percutaneously with a transendocardial helical needle catheter. 86x10^6 cells were injected into 7.1 ± 3.1 sites around the infarct to the target the pen-infarct zones. 2D echo left ventricle EF measurements, 24 hour Holter, and exercise tolerance testing were performed at baseline, day of procedure, 1 and 12 weeks, 6 and 12 months. There were no adverse events associated with the catheter based cell transplantation procedure. At 6 and 12 months, all patients showed an improvement in left ventricular EF over baseline (30 ± 4.6 to 40 ± 4.5, p < 0.003 at 6 months, 35 ± 4.8 to 42 ± 3 ± 5, p < 0.001 at 12 months). Conclusion: ABM cells delivered with the helical needle transendocardial catheter was safe in this small uncontrolled study in patients with chronic MI. Increased ejection fraction and other positive data trends support continued development of this therapeutic strategy in larger controlled trials.

GSK-3β Induces Cardiomyocyte Markers in Bone Marrow-Derived Mesenchymal Stem Cells (MSCs)

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Although adult MSCs have cardiomyogenic properties, the underlying signaling mechanisms are not well understood. The Wnt pathway significantly affects cardiomyocyte differentiation from various stem cell populations, but its effects vary substantially depending upon experimental conditions. The goal of this study was to clarify the role of GSK-3β, a major inhibitory component of the Wnt pathway, in regulating cardiomyocyte differentiation from adult MSCs. Either human or mouse bone marrow-derived MSCs were treated with 5 μM of 5-azacytidine (5-Aza) without serum for 24hrs, which induced mRNA expression of cardiomyocyte markers, including Nkx2.5 and myosin heavy chain (β-MHC), within 3 days. 5-Aza treatment increases β-MHC expression 5 days after treatment. We examined protein expression of GSK-3β and β-catenin, a downstream target of GSK-3β, during 5-Aza treatments. GSK-3β was increased by 5-Aza in a time-dependent manner, reaching a peak (~4 fold) on 5A day. This was paralleled by decreases in β-catenin (~50%), suggesting that the activity of GSK-3β was increased, whereas the Wnt pathway was suppressed by 5-Aza. To test the effect of GSK-3β upon cardiomyocyte differentiation, MSCs were transduced with GSK-3β-adenovirus, which increased 10–15 fold expression of GSK-3β and 80–95% downregulation of β-catenin on Days 3 – 12. Stimulation of GSK-3β caused induction of Nkx2.5 and ANF mRNA, peaking on Days 4 – 5, whereas LacZ-virus did not. In order to stimulate GSK-3β by alternative methods, MSCs were isolated from conditional GSK-3β transgenic mice. Isolated MSCs were transduced with 5-Aza to induce GSK-3β expression regulated by the tetacycline (tet-off) and -on systems, respectively. GSK-3β expression increased by the tet-off (4.7 fold) or tet-on system (3 fold) induced mRNA expression of Nkx2.5, α-NHC and GATA4, as well as protein expression of sarcomeric α-actin and troponin I in MSCs. In contrast, MSCs treated with LGI (100μM), an inhibitor of GSK-3β failed to induce cardiomyocyte markers such as troponin I. In summary, 5-Aza-induced increases in cardiomyocyte markers in MSCs were accompanied by activation of GSK-3β, and stimulation of GSK-3β induces expression of cardiomyocyte markers in adult MSCs.
No Evidence of Transdifferentiation of Human Mesenchymal or Hematopoietic Stem Cells into Cardiomyocytes Following Coculture with Neonatal Rat Cardiomyocytes

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Background: Several clinical trials have shown that stem cell based therapy may improve heart function. Both bone marrow derived mesenchymal (MSC) as well as hematopoietic stem cells (HSC) are reported to be multipotent. This study investigates whether neonatal rat cardiomyocytes (NRCM), when co-cultured, can induce transdifferentiation of either MSC or HSC into cardiomyocytes (CM). Methods and results: Ex vivo expanded human bone marrow derived MSC showed expression of CD44, CD73, CD90, CD105 but not of CD34, CD45, CD106 and CD184. The expanded MSC kept their multipotent characteristics. In contrast, HSC were freshly isolated by flow-sorting based on their expression of CD133+/CD45−. Co-cultures, using cell tracker green (5-chloromethylfluorescein diacetate labeled MSC and red labeled CM) and cell tracker red (5-(and 6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamin) labelled NRCM, performed coronary ligation lasting for 7 days. Cellular composition of the hearts was analyzed by flow cytometry, immunohistochemistry and Western blotting. Spatial distribution of this activity, excluded from hypoxic zones (also marked by increased lactate dehydrogenase activity), excluded from areas of differentiation and neovascularization, we found a slight hyperoxia. Many mature cardiomyocytes in the infarct penumbra also expressed LacZ at lower intensity.

Results: Co-culture of MSC with adult rat heart cells showed no evidence of transdifferentiation of MSC into CM. Despite the induction of CM specific lacZ reporter gene expression, no CM marker (TnT, TnI, MHC) or troponin I (TnI) could be detected. Furthermore, co-culturing HSC, could only detect a decrease in expression of TnI in HSC, but no expression of u-actin, myosin heavy chain (MHC) or troponin 1 (TnI) could be detected. Therefore, the reported functional improvement following cell based therapy for myocardial infarction may be due to other mechanisms than transdifferentiation of MSC or HSC.

Conclusion: Our results indicate the role of tissue oxygenation as a limiting factor in the myocardial repair after MI. In this respect, MSC and HSC have a different behavior, with MSC being more sensitive to low oxygenation and HSC more resistant.
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Epigenetic Reprogramming During SS-Dependent Differentiation of Mouse Embryonic Stem Cells: Role of Nitric Oxide and Histone Deacetylases
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Introduction: Stress shear (SS) reprograms mouse embryonic stem cells (ES) to express cardiovascular markers at least in part through the occurrence of epigenetic modifications. Nitric oxide (NO) is involved in SS signalling in vascular cells and facilitates cardiomyogenesis in ES. Here we show the results of experiments performed to assess the molecular mechanism activated by NO in the SS-dependent cardiovascular differentiation of ES. Methods and Results: Exposure of ES to the NO synthase inhibitor S-methyl-thiosourea (SMT), inhibited the SS-dependent expression of cardiovascular markers in ES, while nitric oxide donors anticipated cardiovascular differentiation in ES deprived from leukemia inhibitory factor (LIF). This result paralleled with a marked decrease in Oct4 expression, indicating a role for NO in the cardiovascular commitment of ES cells. The expression of developmentally regulated genes requires the fine tuning of the chromatin condensation/decondensation process in a temporally and spatially regulated manner, a phenomenon strictly dependent on chromatin modifying enzymes’ activity such as Histone Acetyltransferases (HATs) and Histone Deacetylases (HDACs). We found that after 1 hour of LIF deprivation, class II HDACs shuttled from the cytoplasm to the nuclear periphery of the cells, confirming the role of these chromatin modifiers in the epigenetic reprogramming of ES cells’ fate. This phenomenon was transient as these molecules returned to the cytoplasm in 6 hours. Intriguingly, the direct exposure of ES to NO donors allowed the nuclear retention of these enzymes beyond the 6 hours timepoint and significantly histone H3 acetylation, indicating the presence of a prolonged histone deacetylase activity in the ES nuclei upon NO treatment. The HDAC inhibitor Trichostatin A (TSA) blocked ES differentiation towards the endothelial lineage, while class I and II HDACs specific inhibitors induced the expression of endothelial and neuronal markers respectively. Conclusions: These data suggest a direct role of NO in the regulation of class II HDACs function and in the chromatin remodelling of ES cells and may envisage new epigenetic strategies to reprogram stem cells’ fate which may be useful for cardiovascular cell-based therapies.

Correlation Analysis of Endothelial Colony Forming Units and Endothelial Microparticles in Patients with Cerebrovascular Risks
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Endothelial progenitor cell (EPC) is a surrogate biologic marker for vascular regeneration and thus a potential therapeutic option for patients with active endothelial damage. This phenotype supports the contention that CD133+ cells are a hemangioblast population, and thus warrant further study as a therapeutic agent.

AMP-Kinase Activates Ubiquitin Ligases in Cardiomyocytes
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Left ventricular hypertrophy (LVH) is an independent risk factor for cardiovascular morbidity and mortality. Studies have shown that regression of LVH improves cardiac function. Conventional strategies used to reverse cardiac hypertrophy currently focus on decreasing pro-hypertrophic signaling. However, this approach has often been unsuccessful because of the vast redundancy within the pro-hypertrophic signaling network. Cardiomyocyte size, whether hypertrophic or atrophic, is determined by the balance of protein synthesis and degradation. Here we propose a new approach to reverse cardiac hypertrophy through the activation of pro-atrophic signaling pathways. Studies in skeletal muscle have already shown that the Ubiquitin Proteasome Pathway (UPP) is the major signaling pathway responsible for skeletal muscle atrophy. The activation of the two muscle-specific ubiquitin ligases, Muscle and atrophy F-box protein (Mafbx) and MuRF-1, inhibits protein degradation in vivo and in vitro. The UPP breaks down proteins in an ATP-dependent manner. Because the central determinant of the levels of ATP in the cell is 5’ Adenosine Monophosphate Kinase (AMPK), we propose that AMPK plays a role in the regulation of Mafbx and MuRF-1, thereby reversing cardiac hypertrophy through the UPP. In order to investigate the role of AMPK in the activation of Mafbx and MuRF-1 in vitro and in vivo, we used two model systems: neonatal rat ventricular myocytes (NRVM) and Matfix and MuRF1- knockout mice. In both models, AMPK was activated by treatment with the anti-diabetic drug Metformin (1,1-dimethyl biguanide HCl) or AICAR (5’-phosphoribosyl-5-aminoimidazole-4-carboxamid). Our data show that Metformin and AICAR both increase transcript levels of Mafbx/Atrogin1 and MuRF-1 in vitro. We conclude that AMP kinase potentially activates the UPP in cardiomyocytes. One of the consequences may be enhanced availability of amino acids for energy provision.

Int6: New Target for Angiogenesis (1) e03f3/Int6 Specifically Targets HIF-2α for Degradation by Hypoxia- and pHL-Independent Regulation
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Abstract: Hypoxia-inducible factors (HIFs) -1α and -2α are structurally similar as regards their DNA-binding and dimerization domains, but differ in their activation targets. In order to address this discrepancy, we performed yeast two-hybrid analysis and identified Int6/eIF3e/p48 as a novel target gene product involved in HIF-2α regulation. The Int6 gene was previously identified as a frequent integration site of the mouse mammary tumor virus (MVT). Here, by using two-hybrid analysis, immunoprecipitation in mammalian cells and HRE-reporter assays, we report the specific interaction of HIF-2α (but not HIF-1α or HIF-3α) with Int6. The results indicate that the direct interaction of Int6 induces HIF-2α degradation. This degradation was found to be both hypoxia- and pH-independent. Furthermore, Int6-siRNA increased endogenous HIF-2α expression, and followed by inducing sets of critical angiogenic factors comprising VEGF and bFGF mRNA in HeLa cells. Moreover, HIF-2α and the related factors are also expressed constitutively in human endothelial cells. We proved that Int6-siRNA induced mRNA of HIF-2α, bFGF, IL6, and IL8 in HUVEC cells, and these induction triggered to mediated the cord of formation on matrigels. These results indicate that Int6 is a novel and critical determinant of HIF-2α-dependent angiogenesis not only in HeLa cells, but also in human endothelial cells. Thus, Int6-siRNA transfer may be an effective therapeutic strategy in pathologic conditions such as heart and brain ischemia, and obstructive vessel diseases.
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Implant and Cellular Therapy: A New Option of Treatment for Patients with Chronic Coronary Disease with Intractable Angina or Cardiac Failure Without Possibility of Surgical Revascularization

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Objectives: Adult stem cells derived from bone marrow are being used in the treatment of chronic myocardial ischemia and myocardial infarction. Method 69 patients with refractory angina or cardiac failure that had no possibility of surgical revascularization-rization were included. The age of patients (66 men and 3 women) oscillated between 55 and 71 years old. Results: Hemodynamic parameters showed: 1) improvement of left ventricular ejection fraction (LVEF) by 19 points, 2) improvement of right ventricular ejection fraction (RVEF) by 22 points and 3) improvement of survival time for 3 years. The Cell implantation was made by retrograde injection through the Sinus Coronary Vein. The average of cells implanted (CD34+ and CD38) was of 22 × 10⁶. Follow up: After a period of one month a progressive increase of sectors contractility was observed in the echocardiograms. After 120 days it was observed that FE had improved between 38% and 43%. Scintigraphy controls revealed improved of the perfusion in the 53 patients in the perinectric and diffuse ischemic areas, 48 patients were subjected to Ventriculography after 90 to 120 days and it was observed that the FE improved up to 38%. Conclusions: The cellular implants have demonstrated a marked improvement of left ventricular function, surgery was avoided in 55 patients, and FE increase, observed by echocardiograms, and Ventriculography. 78% of the patients, showed an improvement of the contractility of periphery of scars. It was not observed any progression of coronary occlusive disease after a period of 2 years. 43 patients have achieved 3 year of evolution and they are asymptomatic or in functional class I.

P17

Cardiac Progenitors During Zebrafish Heart Regeneration

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Recent studies have identified populations of cardiac stem cells, or cardiac progenitors (CPs), in the adult mammalian heart. Some CPs are reported to have the potential to restore muscle when transplanted into infarcted hearts. Although these progenitors show little or no natural regenerative ability, the discoveries indicate that the mammalian heart has the potential for myocardial regeneration through CPs. In contrast with mammals, teleost zebrafish naturally regenerate cardiac muscle. In a recently published study, we found evidence that undifferentiated tropogenic CPs in the zebrafish have a primary role in the regeneration of the mechanically injured heart. However, the origin and developmental contributions of these regenerative progenitors are unknown. Here, we hypothesize that CPs arise from a resident pool in the adult zebrafish heart that are mobilized upon injury; injury can also elicit loss. Aim of the present study was to determine the presence of cells capable of giving rise to myocardial characteristics in the adult human and mouse epicardium and, eventually, the effect of myocardial infarction on these cells. Methods and Results: Candidate CPs in the zebrafish were identified by expression analyses and generate transgenic reporter lines to visualize the activity of CPs during regeneration. In addition, we will examine the differentiation potential of candidate CPs in vivo during regeneration using genetic fate mapping. This study will provide unique insights into vertebrate CPs and their participation in natural regeneration. Results from this study may have implications for establishment of regenerative therapies of myocardial infarction.

P18

Cardiomyocytes Grafts Contract, Alter the Mechanical Properties of Cardiac Progenitors During Zebrafish Heart Regeneration

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Here, we hypothesize that CPs arise from a resident pool in the adult zebrafish heart that are mobilized upon injury; injury can also elicit loss. Aim of the present study was to determine the presence of cells capable of giving rise to myocardial characteristics in the adult human and mouse epicardium and, eventually, the effect of myocardial infarction on these cells. Methods and Results: Candidate CPs in the zebrafish were identified by expression analyses and generate transgenic reporter lines to visualize the activity of CPs during regeneration. In addition, we will examine the differentiation potential of candidate CPs in vivo during regeneration using genetic fate mapping. This study will provide unique insights into vertebrate CPs and their participation in natural regeneration. Results from this study may have implications for establishment of regenerative therapies of myocardial infarction.

P19

Identification of Myocardial and Vascular Precursor Cells in Human and Mouse Epicardium

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Background: During development, the epicardium is the source of multipotent mesenchymal cells which give rise to coronary vessels, including endothelial cells, smooth muscle cells and, possibly, cardiac myocytes. Cell transplantation studies on infarcted myocardium have shown improved cardiac function and, possibly, cardiac myocytes. However, little is known about the underlying cellular/molecular mechanisms that lead to these improvements. The goals of this study were to determine if neonatal rat cardiomyocytes can improve global myocardial function. Thin tissue strips were dissected from infarcted hearts and chemically de-endothelialized to study the cellular effects of cardiomyocyte grafts. The mechanical properties of remote myocardium far from the infarct, and 4) improve global myocardial function. The mechanical properties of remote myocardium. Supported by HL64387 to C. E. Murry. Supported by HL64387 to C. E. Murry.

P20

Efficacy of Intramyocardial Autologous Angiogenic Cell Precursors Injection for Ischemic Cardiomyopathy

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Introduction: The objective is to determine efficacy of intramyocardial angiogenic cell precursors (ACP) injection in ischemic cardiomyopathy (ICM). Methods: Twenty-three patients (cell group) underwent intramyocardial ACPs injection. Twenty-three case-matched patients from heart failure database treated by medical means were served as controls. There was no statistically significant different between the cell and the control groups in relation to preoperative left ventricular ejection fraction (LVEF) and co-morbidities. In the cell group, mean age was 61.0 ± 10.5 years. Mean LVEF was 25.8 ± 10.6. NYHA Class was 3.0 ± 0.6. Thirteen underwent cell injection alone and 10 underwent combined Off-pump CABG (OPCAB) and cell injection. ACPs expressed CD34, CD133, KDR, Tie-2, CD44, von Willbrand factor, CD31Bright, concomitant binding of Ulex-Lectin and uptake of acetylated low density lipoprotein (Ac-LDL), secreted interleukin-8, vascular endothelial growth factor and angiogenin were injected into the non-viable myocardium and hypokinetic segments in the cell group. The number of cells prior to injection was 25.2 ± 17.9 million cells. Results: There was no new ventricular arrhythmia. NYHA was improved by 1.1 ± 0.8 (P = 0.001) at 198 ± 105.4 days and six-months walk test improved in the cell group. The quality of life evaluated by Short-Form 36 demonstrated improving of physical function, role-physical, role-emotion, general health, and vitality domains in cell group at 6 months’ follow up. The LVEF was improved in 82.6% of patients (19/23). The LVEF improved from 25.8 ± 7.5 to 32.2 ± 10.9% (P < 0.001) at 145 ± 7 to 106.5 days in the cell group. There was no significant different in changes of LVEF in the cell injection alone and the combined OPCAB with cell injection (6.5 ± 5.9 vs. 7.2 ± 8.8 point %, P = 0.8). The change in the LVEF did not differ between the cell group and controls (6.8 ± 7.1 vs. 6.8 ± 15.1 point %). Conclusions: Intramyocardial ACPs injection is safe in the ICM patients. The NYHA class and quality of life were improved. The LVEF was significantly improved in the cell group even though the change of LVEF was not significant different from the controls. Large-scale placebo-controlled studies are in progress.
Gender Differences in Cardiac Remodeling and Inflammatory Cytokine Expression Induced by Volume Overload in Rats

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In previous studies we have described gender differences in myocardial remodeling (i.e., eccentric versus concentric) induced in response to an infrarenal aorticaval (AV) fistula. Accordingly, this study sought to determine if this gender dimorphism was the result of differences in cardiac remodeling. To this end, we studied cardiac myocyte size, and the number of nuclei per cardiomyocyte were determined in hearts from rats that had an AV fistula or sham surgery. Evaluation of isolated, unstressed myocytes (fistula versus sham) at 3, 5, 7 and 14 days post-surgery revealed that male rats experienced a significant decrease in cardiomyocyte length (117.6 ± 4.5 μm vs. 93.8 ± 2.3 μm) as well as an increase in the percentage of mononucleated cardiomyocytes (11% vs. 18%) at 5 days post-fistula. This decrease was transient, with restoration of normal average lengths by 14 days post-fistula. In contrast, females had progressive increases in cardiomyocyte length (sham 95.6 ± 6.7 μm; 7-days fistula 108.1 ± 3.8 μm; 14-days fistula 113.7 ± 9.9 μm) and the percentage of mononucleated cardiomyocytes decreased (sham 10.5 ± 3.4%; 7-days fistula 5.4 ± 0.4%; 14-days fistula 3.25 ± 1%). These findings, together with negative staining for proliferating cell nuclear antigen (PCNA) in cardiomyocytes isolated from male rats at 5 days post-fistula, suggest cytokinesis is impaired in male hearts. Concurrent with the divergent remodeling of the cardiomyocyte, tumor necrosis factor-α was increased in the myocardium of male hearts, together with degradation of myocardial collagen and impaired cardiomyocyte adhesion to the extracellular matrix. These changes were not present in female hearts. Therefore we conclude that TNF-α, which is known to activate matrix metalloproteinases, is a key factor contributing to the gender differences seen in cardiomyocyte hypertrophy and myocardial remodeling.

Use of a Novel Technology to Identify N-Linked Glycoproteins on the Cell Surface of Cardiac Progenitor Cells: Discovering Markers of Differentiation

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To enhance discovery of specific biomarkers of differentiation, a new, directed proteomic approach is being employed to identify cell surface N-linked glycoproteins present at discrete stages during in vitro differentiation of stem cell-derived cardiac and skeletal mesodermal lineages. In this initial study, the cell surface glycoprotein capture (CSCG) method was used to covalently label the extracellular domain of glycoproteins present on intact C2C12 cells (murine myogenic, skeletal lineage) with a biotin tag. Following enzymatic digestion, all biotin-labeled glycopeptides were captured using streptavidin beads and the N-linked glycopeptides, specifically, were subsequently released using N-Glycosidase F. The resulting peptides were analyzed using high mass accuracy MS to identify the proteins and confirm the site of glycosylation. Further analyses verified the presence of a known or predicted transmembrane domain, the presence of the conserved N-linked glycosylation sequence motif (AsnXaaS/T) at each glycosylation site, and finally that the glycosylation sites mapped to the extracellular domain. The two biological replicates had greater than 80% specificity for the N-linked cell surface glycoproteins (specificity is defined as number of N-linked glycopeptides captured / total number of peptides captured) and 67 non-redundant cell surface N-linked glycoproteins had been identified. Examples include cell surface receptors (macrophage mannose receptor, basic fibrilostat growth factor receptor 1, ephrin type A and B receptors), cell adhesion proteins (M-cadherin, N-cadherin), and channels (calcium channels, solute carrier family proteins). One particularly interesting protein found by the current study is N-cadherin. As the mRNA expression of N-cadherin is known to be elevated in cardiac tissue when compared to other cell types, it could potentially be specific for early detection of the cardiac lineage. The expression of N-cadherin on C2C12 cells has been validated by immunohistochemistry, thus confirming the CSCG results. It is predicted that this targeted proteomics approach will be useful for identifying cell surface markers that could be used for monitoring differentiation and selecting pure cell populations.
P26
Transcriptional Variability in Stem Cell Populations Isolated from Adult Mouse Myocardium
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Although the mammalian heart has low regenerative potential, a number of research groups have reported isolation of cardiac stem/progenitor cells from adult myocardium based on the expression of surface markers. One interesting aspect of these studies is the number of different “marker” patterns used to identify these cell populations. It is not known whether these differences reflect the presence of multiple stem cell types, or distinct stages of development of a single pluripotent progenitor. We have isolated a clonogenic cell population from adult mouse myocardium that is c-kit+, sca-1+ positive. Different cell morphologies were observed within the initial isolates and have been described by other groups. Individual clones were therefore isolated for the purpose of analyzing differences in gene expression. We have now isolated 65 clones from which 42 have been maintained in culture for at least 20–30 passages. We have selected 6 clones based on their different morphology for transcriptional analysis using a focused stem cell array. About 44% of the transcripts (C7 in 94) related to stem cell maintenance and differentiation were highly abundant (Cts~30) in all 6 clones. Using unsupervised clustering based on Euclidean Distance measure, we used the transcriptional profiles to present the 6 clones in a hierarchical clustering image. This algorithm was able to group clones based on similarity in expression profile. We have calculated absolute transcript levels from Cts~35. The most abundant shared transcripts included those involved in cell proliferation; c-myc, Ccn1 and p300. Clone 42 was the most divergent and has a unique star-like morphology compared to the others. The most diverently expressed genes among the different clones were CXCL12, involved in chemoattraction, the growth factor FGF1, the Notch1 signaling axis in surviving cardiomyocytes following infarction is critical to understanding myocardial stem cell based signaling. Hypothesis: Stimulation of Notch activity by HGF mediated through c-Met and Akt survival signaling pathways, and Notch1 signaling in turn enhanced Akt activity. This suggests a positive and feedback mechanism between Notch and Akt signaling in adult myocardium following infarction.

P27
The Hematopoietic Actions of G-CSF Improve Cardiac Function and Repair After Myocardial Ischemic Injury
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Granulocyte-colony stimulating factor (G-CSF) administered after myocardial infarction (AMI) improves heart function and repair. Although initial studies suggested that improved function/repair were due to differentiation of hematopoietic cells into cardiomyocytes and blood vessels, more recent data indicate that the hematopoietic cells remain in the hematopoietic phenotype and that direct effects of G-CSF on cardiomyocytes may be responsible for its beneficial effects. We tested this hypothesis using G-CSF receptor deficient mice in a mouse model of AMI. Wild type mice were reconstituted with either wild type bone marrow cells (control) or G-CSF receptor deficient bone marrow (G-CSFR BM-/-). Injury was induced by occlusion of the coronary artery, followed by treatment with saline or G-CSF for 5 days. Twenty eight days later cardiac function was assessed after placement of a Miller catheter into the left ventricle as was cardiac repair. Effects on cardiac repair were assessed immunohistochemically, measuring CD34+ vessels and small alpha-actinin+ and GATA-4+ cardiomyocytes in the infarct area. G-CSF BM-/- culture mice had 20% to 50% better cardiac function compared to G-CSFR control mice (P<0.05). G-CSF also increased vessels in the infarct zone (1.9-fold, P<0.05) and immature cardiomyocyte (alpha-actinin+, GATA-4+) numbers (1.8-fold, P<0.05). G-CSF did not improve cardiac function in G-CSFR BM-/- mice (P>0.05), nor did it stimulate new vessels or immature cardiomyocyte numbers in the infarct zone. In contrast, treatment of G-CSFR BM-/- mice with a single dose of the CXCR4 antagonist AMD3100 not only improved indexed cardiac function (P<0.05) but also stimulated increases in new blood vessels (2.3-fold, P<0.05) and immature cardiomyocyte numbers (1.5-fold, P<0.05). Thus any direct effects of G-CSF on cardiomyocytes in the infarcted heart are insufficient to improve cardiac function or augment repair. We conclude that mobilisation of hematopoietic progenitors is a critical component of the therapeutic effect of G-CSF for improving cardiac function and stimulating repair after myocardial infarction via paracrine mechanisms.

P28
Notch and P13K Signaling in Cardiac Myocytes in Vitro and in Vivo
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Introduction: The Notch network regulates multiple cellular processes, including cell fate determination, development, differentiation, proliferation, apoptosis and regeneration. Notch receptor activation generates the Notch Intracellular Domain (NICD), which translocates to the nucleus and turns on target genes, including Hes1. Notch activity influences HGF/c-Met receptor and PI3K/Akt signaling cascades. Delineating connections within the Notch/c-Met/Akt signaling pathway is essential in understanding how Notch-mediated transcriptional effects in surviving cardiomyocytes following infarction is critical to understanding myoccardial stem cell based signaling. Hypothesis: Stimulation of Notch activity by HGF increases activation of Akt, which enhances Notch activation, implicating a bidirectional feedback mechanism between Notch and Akt in border zone cardiomyocytes. The impact of HGF on Notch activity and signaling was examined in 3.3D cells immortalized cardiac fibroblast cell line with characteristics resembling parental cardiac fibroblasts. The cell line remained proliferating for 14 days in serum free medium. The 3.3D cell line is the first telomerase-immortalized cardiac fibroblast cell line with characteristics resembling parental cardiac fibroblasts, and can serve as a novel tool for research on infarct healing.

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Candesartan Improves Myocardial Damage in Obese Mice with Viral Myocarditis and Induces Cardiac Adiponectin
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To clarify the mechanism of the effects of angiotensin II receptor type 1 antagonist, candesartan, upon cardiac adiponectin in the combination of myocarditis with obesity, we examined whether KKAy mice, a model for acute viral myocarditis treated by candesartan and investigated cardiac adiponectin regulation. Mice were divided into candesartan early treatment group (Can-early) receiving orally candesartan at daily dose of 10mg/kg 7 days before starting viral inoculation and then 7 days; candesartan late treatment group (Can-late) or vehicle (Vehicle) receiving candesartan starting simultaneously with viral inoculation and then 7 days. Enzyme-homoleciumerditis virus was used to induce the acute viral myocarditis. Differences in myocardial damages, serum adiponectin and myocardial expression of adiponectin, tumor necrosis factor-alpha (TNF-a), CCAT/enhancer binding protein alpha (C/EBPa) and peroxisome proliferator-activated receptor gamma (PPARG) and nuclear factor-kappaB (NF-kB) mRNA among these groups were detected. Acute viral myocarditis treated in Can-early and Can-late groups showed reduced myocardial necrosis and cellular infarction as compared with those in the Vehicle. On day 4 the circulating adiponectin levels were significantly higher in Can-early than those in Vehicle. Mice in Vehicle had significantly reduced in myocardial adiponectin mRNA after viral myocarditis. Cardiac adiponectin mRNA was significantly higher in Can-early andCan-late than in Vehicle on days 4 and 7. Cardiac C/EBPa in Can-early andCan-late groups were significantly increases on day 4. Myocardial NF-kB and TNF-a mRNA in Can-early and Can-late groups were significantly reduced on day 7. Candesartan treatment improved myocardial injury in obese mice with acute viral myocarditis and induced expression of adiponectin with the induction of C/EBPa as well as the reduction of cardiac NF-kB and TNF-a.

P30
Characterization of a Telomerase-Immortalized Cardiac Fibroblast Cell Line
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The wound healing response after myocardial infarction includes replacement of damaged tissue with scar. In this process, specialized fibroblasts called myofibroblasts appear in the infarct area and prevent dilatation of the heart by active contraction followed by deposition of extracellular matrix proteins. However, the role of primary fibroblasts for in vitro studies on cardiac wound healing has been limited by poor proliferation, low transfection efficiency and spontaneous differentiation into myofibroblasts. Therefore, we developed a telomerase-immortalized cardiac fibroblast cell line, called 3.3D. The cell growth and differentiation characteristics as well as gene expression patterns were determined in these cells. We also analyzed specific responses to transforming growth factor β1 (TGFβ1) and interferon-γ (IFNγ), two cytokines affecting myofibroblast differentiation. Telomerase activity was significantly higher in the 3.3D cells, and transfection efficiency was >50% vs. <1% in the parental cardiac fibroblasts. The cell line remained proliferating for >40 passages, as compared to <5 passages for the parental fibroblasts. The differentiation of the 3.3D cells was determined by assessing the (myofibroblast markers α-smooth muscle actin (α-SMA), collagen type I (ColI), fibronectin-1 (FN1) and the EDA splice variant of fibronectin (EDA-FN), by quantitative PCR. Exposure to TGFβ1 (10 ng/ml, 24h) increased the expression of α-SMA by 3.6-fold (P<0.05). The induction of α-SMA was confirmed by Western blotting and immunocytochemistry. Exposure to IFNγ (10 ng/ml, 24h) failed to induce expression of α-SMA in 3.3D cells. In contrast to TGFβ1, exposure to IFNγ (10 ng/ml, 24h) failed to induce α-SMA expression but increased collagen I by 1.7-fold (P<0.05), FN1 by 2.0-fold (P<0.01) and EDA-FN by 1.7-fold (P<0.05). Both TGFβ1 (10 ng/ml) and IFNγ (10 ng/ml) significantly increased the proliferation of 3.3D cells by 25% and by 20%, respectively. The migration of the 3.3D cells was not altered by TGFβ1 or IFNγ. The 3.3D cell line is the first telomerase-immortalized cardiac fibroblast cell line with characteristics resembling parenteral cardiac fibroblasts, and can serve as a novel tool for research on infarct healing.
Soluble Natriuretic Peptide Receptor-Related Fragment (sNRF) Inhibits NP Action and Is Increased in the Failing Heart

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In heart failure, the beneficial effects of natriuretic peptides (NPs) are blunted, in part, due to NP receptor (NPRA) unresponsiveness caused by an unknown mechanism. We initially identified sNRF in a yeast 2 hybrid screen for a human heart library. The sNRF cDNA transcript encodes the C terminus of NPRA (NPRA52-1381). It extends 215 nt into intron 15 of the NPRA gene, followed by exons 16–22 of the fully spliced NPRA mRNA sequence. Another group subsequently reported (GenBank BX329044) a similar cDNA derived from placenta. In primary cultures of neonatal rat cardiac fibroblasts (CF), sNRF expression blocks NP's inhibitory effects on α-smooth muscle actin expression, a key marker of CF differentiation. In other experiments sNRF appears to potentiate TGF-β action independently of NPRA. To determine if sNRF mRNA is differentially expressed in human heart disease, total mRNA was extracted from LVs of 13 hearts explanted from patients undergoing cardiac transplantation and was compared to normal LV (n=4). Quantitative real-time RT-PCR using primers corresponding to the intron 15 sequence and the 3’ end of NPRA exon 17 revealed sNRF mRNA expression in the failing hearts was higher than any of the normal hearts. Relative sNRF expression was 26-fold higher compared to the mean control value (range 2.8- to 91-fold, p<0.0001). In the same experiment, we determined the levels of full-length NPRA mRNA using a TaqMan probe that spans exons 7 and 8 of the full-length NPRA transcript that are upstream of the presumed sNRF transcription initiation start site and, as such, would not recognize sNRF. There was no difference between the expression levels of full-length NPRA in the normal versus diseased hearts (0.8-fold compared to the mean control value, range 0.4- to 2.2-fold) indicating that regulation of sNRF from its putative promoter in intron 15 is independent of NPRA expression initiated at the NPRA promoter flanking exon 1. We conclude that sNRF expression inhibits critical NPRA functions and may have NP-independent effects. sNRF mRNA is increased in the clinical setting of heart failure. Whether sNRF inhibition reverses NP resistance or blocks TGF-β effects in heart failure remains to be determined.

Cardiac Proteasome Complexity: Subpopulations with Distinct Molecular Compositions

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The ubiquitin-proteasome system plays a key role in protein degradation in mammalian cells. At the proteasomal core, the proteasomal subunit complexes exhibit the activity of the 26S proteasome-, trypti- and chymotrypsin-like. Therapeutic studies target the 20S by inhibitors in diseases with deregulated protein quality control, proliferation or apoptosis. In cardiovascular research, results from these studies are ambiguous, which might be related to 20S heterogeneity. Previously, we developed novel protocols for accurate isoelectric focusing of multi-protein complexes. Using these, 20S complexes were separated into subpopulations with distinct isoelectric points (pI; Mol Cell Proteomics. 2006; 5: S22). The separated complexes retained their structural integrity and proteolytic activities. To provide insights in the functionality of cardiac proteasomes, proteasome subpopulations and proteolytic activities of the cardiac proteasome were compared. The active sites of the 20S are located on subunits β1, β2 and β5 and confer altered substrate specificities and turnover. Immunodetection of these subunits in proteasome complexes with distinct pI's demonstrated variations in assembly. Submit β1 was 47% higher expressed in complexes with pI 5.21 than in those with pI 5.28. Since β2 was significantly lower in complexes with pI 5.21, the analyzed complexes present at least in part hitherto unreported intermediate proteasomes in cardiac tissue. Complexes with different pI's exhibited distinct proteolytic activities as well. Proteasome subpopulations potentially play different roles in cellular events similar to the immunoprotea- some in the immune response. In cardiovascular systems, they might be involved in modulating cardiac function. Our studies provide the foundation to analyze proteasome subpopulations in the heart.

Distinct Functional Complexes of 20S Proteasomes in Murine Heart and Liver

Aldrin Gomes, Glen Young, Oliver Drews, Xiaohai Li, Chenggong Zong, Peipei Ping; UCLA, Los Angeles, CA

The proteasome system represents a key component in the intracellular protein degradation machinery. Despite advancements in proteasome research which suggests its involvement in over 20 diseases, mechanisms pertaining to its molecular regulation remain poorly understood. An organelle targeted proteomic effort was undertaken to comprehensively characterize highly purified heart and liver 20S proteasomes. Blue-native gel electrophoresis combined with LC/MS/MS was used to delineate the molecular composition of this organelle. Heterogeneity was observed with respect to the components of the three inducible β subunits in the 20S proteasomes: the β2 expression in the purified 20S complexes was found to be 41% higher in the liver compared to the heart by immunoblotting. Corroborating this finding was the large difference in the average peak area of the two most intense peaks from the heart β2 mass spectra (47%) compared that to the liver 20S (100%). Immunoblotting showed that the liver 20S proteasomes contained higher relative amounts of phosphorylated Ser, Thr and Tyr residues than the heart 20S. A difference in proteasome function of these two organs was also observed: the chymotrypsin like proteolytic activity of the cardiac 20S was significantly greater than that of liver 20S; whereas the caspase like proteolytic activity of the liver 20S was greater than that of cardiac 20S. The 20S proteasomes from both heart and liver contained at least 10 different associating partners. One binding partner, protein phosphatase 1 (PP1), which was associated with both heart and liver 20S complexes, showed distinct functional roles in the 20S complexes from the different tissues. Addition of PP1 to cardiac 20S significantly enhanced the βi activity (50% ± 2% increase when compared to heat-inactivated PP1); whereas PP1 had no detectable effect on the βi activity of the liver 20S. These results demonstrate an organ specific heterogeneity of 20S proteasomes in the heart and liver, with respect to their molecular composition, complex assembly, post-translational modification and proteolytic function. Understanding these differences is critical for the development of compounds that would selectively target heart proteasomes for future treatment of cardiac diseases.

Increased Expression of Heat Shock Protein 25 in Doxorubicin-Treated Failing Hearts

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Doxorubicin (DOX) is known to cause cardiotoxicity in cancer patients, who are treated with this drug or its cocktails, for various malignancies. Recent studies have shown that loss of cardiac Hsp25 expression due to the DOX induced oxidative stress is associated with cardiac dysfunction in DOX-treated failing hearts. We thus have developed a hypothesis that over expression of small Hsps may serve as marker of oxidative stress in DOX-treated failing hearts. In the present work we investigate the level of Hsp25 and its phosphorylated isoforms in DOX-treated failing hearts. Mice (n=10) were injected (IP) with 6mg/kg body weight for 8 weeks. Control groups were treated with equal volume of saline for the same period of time. The hemodynamic and contractility of the heart in these mice were followed by echocardiography and electrocardiogram. At the eighth week, development of heart failure was confirmed by reduced ejection fraction and contractility by echocardiography. ECG also showed S-T segment elongation consistently. After confirming the heart failure, the animals were sacrificed and their hearts isolated. The isolated hearts were

Table 1.

<table>
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<th>Group</th>
<th>pg Luciferase / mg tissue</th>
<th>S.D.</th>
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<tr>
<td>P+E+</td>
<td>431.47</td>
<td>247.84</td>
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<td>P+E+ (200 V/cm, 150 ms)</td>
<td>4,895.67</td>
<td>2,138.78</td>
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<tr>
<td>P+E+ (100 V/cm, 250 ms)</td>
<td>3,729.78</td>
<td>1,705.31</td>
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<tr>
<td>P+E+ (100 V/cm, 250 ms)</td>
<td>10,913.76</td>
<td>3,101.52</td>
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<tr>
<td>P+E+ (100 V/cm, 250 ms)</td>
<td>7,364.55</td>
<td>5,002.98</td>
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Table 2.

<table>
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<th>Group</th>
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<tr>
<td>P+E+</td>
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<td>P+E+ (100 V/cm, 250 ms)</td>
<td>266.62</td>
<td>91.73</td>
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then minced to obtain the heart lysates. We determined the level of total, s-15, and s-48 phosphorylated Hsp25 in control and DOX treated group. In DOX treated group, we found three times increased expression of Hsp25 as well as the phosphorylated isofoms. The 2D western blotting showed increased number of spots in DOX treated cases. Three major spots were noticed in control, while 6 different spots were found in DOX treated. In addition to this, we carried out MS/MS analysis of these proteins and confirmed that they indeed correspond to the Hsp25 proteins. We conclude from these studies that, in doxorubicin treated failing hearts, there is increased expression of Hsp25 due to oxidative stress.

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Gradual Reoxygenation After Prolonged Myocardial Ischemia Improves Cardiac Function and Reduces Oxidative Stress in a Porcine Model: An Optimizing Postconditioning Strategy

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Objective: Hyperoxic reoxygenation of the ischemic myocardium after aortic declamping may aggravate myocardial reperfusion injury in cardiac surgery. The study assessed a new gradual reoxygenation protocol for postconditioning and its effects on cardiac function and oxidative stress.

Methods: Twenty-one adult pigs were put on cardiopulmonary bypass (CPB) with prolonged aortic cross-clamping (60min). After aortic declamping, pigs underwent hyperoxic reoxygenation (paO2, 250–350mmHg) for the initial 10min of reperfusion in group I (n=10). In group II (n=11), gradual reoxygenation started for two minutes at low (paO2, 40–50mmHg), and continued for another 8 minutes at a higher oxygen levels (paO2, 50–90mmHg). Animals were weaned from CPB after further 20min of normoxic reperfusion. Cardiac function was measured by conductance catheter technique during 60min post CPB. Oxidative stress was assessed by detection of oxygen radicals using electron paramagnetic resonance (EPR) spectroscopy as well as by neutrophil oxidative burst assays within the coronary sinus blood (CS) after aortic declamping. Results: End-systolic pressure volume relationship (ESPVR) and peak left ventricular pressure rise (dp/dtmax) were significantly less decreased in group II after CPB end. Correspondingly, early myocardial oxygen production and oxygen consumption, as well as oxidative burst assays in CS were significantly reduced in group II compared to group I during and after reperfusion. Conclusion: Gradual reoxygenation in the initial reperfusion period leads to significantly less reduced cardiac function and oxidative stress after prolonged myocardial ischemia, and serves to optimize postconditioning strategies.

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The Myocardial Protective Effect of Pioglitazone Is eNOS- and iNOS-Independent

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Background: Endothelial nitric oxide synthase (eNOS) activation with subsequent inducible NOS (iNOS) and cyclooxygenase-2 (COX2) activation are essential for mediating the myocardial infarct size (IS) limiting effects of statins. In the rat, the peroxisome proliferator-activated receptor γ (PPARγ) agonist pioglitazone (PIO) limits IS and increases myocardial 6-keto-PGF1α levels without activating eNOS and iNOS. The protective effect is blocked by COX2 inhibitor. Hypothesis: PIO limits IS in the eNOS-/- and iNOS-/- mice. Methods: Male C57BL/6 wild-type (WT), eNOS-/- and iNOS-/- mice received PIO 10 mg/kg/d (PIO+) or water alone (PIO-) for 3 days. Mice underwent 30min coronary arterial occlusion and 4h of reperfusion, or hearts were harvested and subjected to ELISA. Results: PIO reduced IS in the WT, as well as in the eNOS-/- and iNOS-/- mice (Figure). Myocardial 6-keto-PGF1α levels were increased by PIO in the WT (40.2±0.8 vs. 26.9±0.7 pg/mg; p<0.001), eNOS(−/−) (41.8±0.5 vs. 23.4±0.4 pg/mg; p<0.001), and iNOS(−/−) mice (40.6±0.8 vs. 24.3±0.4 pg/mg; p<0.001). Conclusions: The myocardial protective effect of PIO is eNOS and iNOS independent. As eNOS activity decreases with age, diabetes and advanced atherosclerosis, this effect may be relevant in the clinical setting.

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Inhibition of Ischemic Cardiomyocyte Apoptosis Through Targeted Ablation of Bnip3 Restraints Postinfarction Remodeling

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Cardiomyocyte apoptosis contributes to early infarct expansion and late global ventricular dysfunction after myocardial infarction. Bnip3, a proapoptotic BH3-only Bcl2 family protein, is transcriptionally upregulated in cardiomyocytes by hypoxia. We hypothesized that Bnip3 mediated cardiomyocyte apoptosis contributes to post-infarction LV remodeling. We used gene targeting and conditional overexpression to evaluate the effects of Bnip3 on in vivo cardiomyocyte apoptosis, and determine the consequences of ablatting murine Bnip3(ΔKO) on cardiac function, infarct size, and ventricular remodeling after surgical ischemia-reperfusion (IR) injury induced by reversible LAD artery occlusion for 60 minutes. Bnip3ΔKO did not affect hearts of unstressed mice. After IR, Bnip3ΔKO mice had no differences in mortality, early infarct size (at 24 hours by gadosmin enhanced MRI) or late infarct size (at 3 weeks by MRI and pathology) as compared to wild type (WT). Two days after IR, apoptosis was significantly diminished in Bnip3 KO peri-infarct myocardium (6.7±0.8% vs 6–11.5±1.3% in WT, P<0.01), at remote myocardium (3.7±0.7% vs 6.0±0.4% in WT, P<0.01). Three weeks after IR, Bnip3ΔKO mice exhibited preserved global left ventricular systolic performance as compared with systolic dysfunction in WT (change in LVEF from 24 hours to 3 weeks: 11±6% vs −20.7±7% in WT, n=6–9, P=0.015) and significantly diminished left ventricular dilatation compared with WT (change from 24 hours to 3 weeks in left ventricular end-diastolic volume: 46±11% vs 108±17% in WT, P<0.007; and LV end-systolic volume: 35±20% vs 181±41% in WT, P=0.005). Also, the myocardial scar was significantly thicker in Bnip3ΔKO (0.91±0.12mm vs 0.61±0.55mm in WT, P<0.047). These observations suggest myocardial salvage by apoptosis inhibition following IR injury in Bnip3ΔKO mice. Forced cardiac expression of Bnip3 increased cardiomyocyte apoptosis in unstressed mice (1.21±0.14% vs 0.17±0.02% in controls; n=4, P=0.029), causing progressive left ventricular dilatation and diminished systolic function that recapitulates Bnip3-mediated post-IR remodeling. In conclusion, post-ischemic cardiomyocyte apoptosis mediated by Bnip3 is a major determinant of ventricular remodeling in the infarcted heart.

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Glucose Phosphorylation and Mitochondrial Binding Are Required for the Protective Effects of Hexokinase I and II

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Alterations in glucose metabolism have been demonstrated in diverse disorders, ranging from heart disease to cancer. The first step in glucose metabolism is carried out by the hexokinase (HK) family of enzymes. Overexpression of HKI and HKII in tissue culture has been shown to protect against oxidant-induced cell death. The protective effects of these enzymes are thought to be due to either an increase in glucose phosphorylation or closure of the mitochondrial permeability transition pore (mPTP) as a result of HK binding to the voltage dependent anion channel (VDAC) on the mitochondrial membrane. VDAC is believed to form part of mPTP, opening of which is responsible for cell death. In order to determine the relative contribution of mitochondrial binding and glucose phosphorylating activities of Hks to their overall protective effects, we expressed full length HKI and HKII, their truncated proteins lacking the mitochondrial targeting and conditional overexpression to evaluate the effects of Bnip3 on heart function. Bnip3 appears to be through PKCδ, as inhibitors of this enzyme led to a reversal of this process. These results suggest that both glucose phosphorylation and inhibition of mPTP contribute to the protective effects of HKI and HKII. Furthermore, overexpression of HKII and HKI lead to VDAC phosphorylation in a PKCe dependent pathway. These findings bear implications of HK...
overexpression and binding to the mitochondria as a potential clinical treatment strategy for various forms of human disease.

ErbB2 Blockade and Downregulation Lead to Cardiomyocyte Cell Death Through Distinct Pathways

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Leo I Gordon, Amareshwar Singh, Sheila Prachand, Elliot Lieberman, Lin Sun, Michael A Burton, Tejalresha J Namdev, Owen Lyon, Abayomi L Prausnitz, Cleveland Clinic, Cleveland, OH; Hossein Ardeshil; Northwestern Univ, Chicago, IL

The tyrosine kinase receptor erbB2 (or Her2 in humans) is a member of the epidermal growth factor receptor family. It is highly expressed in many cancer types, and its overexpression is correlated with a poor prognosis in breast and ovarian cancer. Treatment with Herceptin (a monoclonal antibody against the extracellular domain of erbB2) has resulted in a significant improvement in survival of breast cancer patients overexpressing erbB2. However, this agent also causes cardiomyopathy and exacerbates anthracycline-induced cardiotoxicity. Treatment of isolated cardiomyocytes with anti-erbB2 induces apoptosis through modulation of Bcl-xl and -xS. In this report, we studied the mechanism for the deleterious effects of erbB2 antibody (Ab) or erbB2 protein downregulation in cardiomyocytes. We hypothesized that the deleterious effects of erbB2 Ab in cardiomyocytes are mediated through an increase in the levels of reactive oxygen species (ROS). Treatment of neonatal rat cardiomyocytes with erbB2 Ab resulted in a dose dependent increase in ROS production and cell death compared to control IgG treated cells. Reduction of erbB2 protein using RNA interference led to an increase in cell death but did not alter the levels of ROS. Furthermore, dog hearts subjected to ischemia displayed lower levels of erbB2 protein, suggesting a role for this protein in ischemic injury of the hearts. Similar results were obtained in explanted hearts of patients with ischemic cardiomyopathy. Finally, we also show that erbB2 signaling in cardiomyocytes is through a PKC-dependent pathway. These results provide evidence for a role for erbB2 in cardiomyocyte survival at baseline and ROS production as a possible mechanism of the deleterious effects of Herceptin on the heart. Thus, therapies targeting oxidative stress may have beneficial effects in cancer patients receiving this form of therapy.

B-Type Natriuretic Peptide Decreases Gastric Emptying and Intestinal Absorption

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Symptoms of impaired gastrointestinal (GI) function such as nausea and malabsorption occur in people with heart failure. Prior studies have shown that one of the natriuretic peptides, CNP, inhibits human and animal isolated gastric smooth muscle cells. We aimed to determine if BNP affects gastric emptying and/or intestinal absorption. Conscious C57BL/6J (WT) and Natriuretic Peptide Receptor B (NRP-B)-/- knockout (KO) mice (n=5 in each group) were given a 10ng/g of [11005]/H11005/[H11005]H25.9 for control (p<0.05). To test for absorption, conscious WT and KO mice were gavaged with equal amount of 70-kDa FITC chex and test the rate of gastric emptying. Half hour later, the animals were sacrificed and the stomach and the intestine were isolated. Gastric emptying was measured as the percentage of fluorescence that has emptied from the stomach as compared to the total fluorescent counts. Peroxidase, a gastric emptying, half hour after gavage in WT mice was 78.8 + 0.2% for BNP treated vs. 97.3 + 0.3% for control (p<0.05). In KO mice emptying was 96.2 + 0.1% vs. 91.3 + 0.3% (p>0.05). For absorption, the use of 384 well plates and an automated tracking program was explored to study the distribution of natriuretic peptides. Three-day treatment with oral atorvastatin (ATV) increases the expression and absorption of gastric emptying and intestinal absorption. This effect is mediated via the NRP-B receptor. The BNP dose used in this study raises the plasma level to approximately 500pg/ml, a level consistent with a 90% decrease in gastric emptying and 100% decrease in intestinal absorption. In KO mice emptying was 96.2 + 0.1% for control (p<0.05). To test for absorption, conscious WT and KO mice were gavaged with equal amount of 4-kDa FITC chex and test the rate of gastric emptying. Half hour later, blood was collected and the plasma fluorescence measured and compared. The absorption rate, measured in relative fluorescence units in WT mice was 28.2 + 7.8 for BNP treated vs. 98.1 + 25.9 for control (p<0.05). No significant difference was seen between BNP treated vs. control KO mice. The results show that BNP decreases gastric emptying and intestinal absorption. This effect is mediated via the NRP-B receptor. The BNP dose used in this study raises the plasma level to approximately 500pg/ml, a level consistent with a 90% decrease in gastric emptying and 100% decrease in intestinal absorption.
pioglitazone (PIO) activates COX2 without upregulating eNOS and iNOS. We studied whether PIO upregulates COX2 expression and activity in eNOS- and iNOS- mice. Methods: WT, eNOS- and iNOS- mice received PIO (10 mg/kg/d) or water alone by oral gavage for 3 days. On the fourth day, heart were harvested and assayed for eNOS, iNOS, and COX2 mRNA levels (rt-PCR) and COX2 activity (ELISA). Results: PIO caused a small increase in eNOS mRNA in the WT and iNOS- mice of contrast, while the hypothesis that RGS S5 may regulate Ang II in the WT and iNOS- mice. COX2 mRNA levels were significantly increased by PIO in the WT, as well as the eNOS- and iNOS- mice. Similarly, COX2 activity was increased by PIO in the WT (13.8±0.33 vs. 3.36±0.12 mmol/min/mg; p<0.001), eNOS (6.65±0.21 vs. 3.24±0.10 mmol/min/mg; p<0.001) and iNOS (12.5±0.6 vs. 5.3±0.4 mmol/min/mg; p<0.001), respectively. Cultured carotid arterial SMCs were treated with Ang II (100 nM) for various time period (0 –24hrs) after serum withdrawal. Expressions of all Kv1.2, Kv1.5, and Kv2.1 mRNA tend to be increased by Ang II in SMCs transfected with EGFP only and treated with Ang II for 24 hr. In conclusion, RGS 5 may regulate Kv, thereby regulating contraction of SMCs. We tested the hypothesis that RGS 5 may regulate Kv, thereby regulating Ang II-mediated force generation. Expressions of three Kv (Kv1.2, Kv1.5, and Kv2.1) transcripts were increased by Ang II in Sprague-Dawley rats. And, consistent with this observation, angiotensin II (Ang II)-mediated arterial thickening of peripheral arteries caused by uncontrolled division and possible dedifferentiation of vascular Smooth Muscle cells (vSMCs). Here we report that TGFs-like protein 3(TF3β) is a binding partner of BMPRII tail domain (BMPRII_TD). Upon BMP stimulation, Trb3 associates with BMPRIA and facilitates the phosphorylation and activation of Smurf1. Smurf1 is a E3 ubiquitin ligase which targets both BMPRI and Smad1/5, and leads to repression of Smad dependent signaling. Therefore, the degradation of Smurf1 by Trb3 leads to a stabilization of Smurf1 targets, and ultimately potentiation of BMP Smad-dependent signaling. In isolated vSMCs, downregulation of Trb3 inhibits BMP-mediated cellular responses. This leads to an increase in both proliferation and de-differentiation, both characteristic pathological features of pulmonary arteries from IPAH patients. This work therefore identifies Trb3 as a critical component in a novel mechanism of regulation of the BMP pathway by BMPRIA. Furthermore, this work allows for a better understanding of the molecular mechanism behind PH, an important step in developing novel strategies of treating this disease.

Regulators of G-Protein Signaling 5 Proteins May Upregulate Expression of Voltage-Gated K⁺ Channels in Response to Angiotensin II, Thereby Inhibiting Angiotensin II-Mediated Arterial Contraction

Regulators of G-Protein signaling (RGS) proteins inhibit signal transduction of G-protein-coupled receptors by increasing GTPase activity. We previously observed differential expression pattern of the R4 subfamily of RGS proteins in various vascular segments in Sprague-Dawley (SD) rats. And, consistent with this observation, angiotensin II (Ang II)-mediated arterial reactivity was significantly less in the abdominal aorta (AA), expressing RGS 5 transcript 10 folds as much as thoracic aorta (TA), compared with TA. Ang II is known to inhibit voltage gated K⁺ channels (Kc) in vascular smooth muscle cells (SMCs), leading to depolarization and contraction of SMCs. We tested the hypothesis that RGS S5 may regulate Kc, thereby regulating Ang II-mediated force generation. Expressions of three Kc (K1.2, K1.5, and K2.1) transcripts were measured by real-time reverse transcription Q-PCR in arterial SMCs from SD rats. Expressions of K1.5 and K1.2 mRNA, normalized to GAPDH, were greater in AA SMCs versus TA SMCs (3.4±0.32 vs. 2.34±0.12 mmol/min/mg; p<0.001), respectively. Cultured carotid arterial SMCs from SD rats were transfected with pREP2-EGFP vector expressing either 1) human RGS 5/EGFP or 2) EGFP only, as a negative control, using Human Ad5SMC Nucleofector™ kit. Successful transfection of human RGS 5 was verified by Western blot assay. Then transfected SMCs were treated with Ang II (100 nM) for various time period (0–24hrs) after serum starvation. Expressions of all K1.2, K1.5, and K2.1 mRNA tend to be increased by Ang II treatment (1–24 hrs), compared with baseline (0 hr), in human RGS S5/EGFP transfected SMCs. SMCs transfected with human RGS 5/EGFP and Ang II-treated for 24 hrs had an increased expressions of transcripts of all K1.2, K1.5, and K2.1 mRNA, compared with SMCs transfected with EGFP only and treated with Ang II for 24 hr. In conclusion, RGS 5 may upregulate expression of Kc in arterial SMCs in response to Ang II, thereby inhibiting Ang II-mediated vascular contraction.

Targeting Focal Adhesion Kinase with siRNA Prevents and Regresses Load-Induced Cardiac Hypertrophy in Mice

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Hypertrophy is a critical event in the onset of failure in chronically overloaded hearts. FAK (Focal Adhesion Kinase) has attracted particular attention as a mediator of load and agonists-induced hypertrophic growth of left ventricle (LV). We demonstrated here that myocardial FAK expression and activity are induced in chronic hypertrophy but not in resting pressure overload. We also showed that the activity coincident with the LV chamber dilatation and impaired function. To elucidate the role of FAK in the pathophysiology of LV hypertrophy and failure induced by chronic pressure overload we used specific small interference RNA (siRNA). Myocardial FAK expression was reduced to ~20% at one and to ~50% at seven and fifteen days after treatment with 0.12 mmol siRNA targeted to FAK. Transient FAK silencing prevented as well as reversed the hypertrophy and the fibrosis of chronically overloaded LV, while preserving the chamber function, despite the persistent pressure overload. However, FAK depletion did not influence basal cardiac function or structure in mice that underwent short operation. As an attempt to elucidate the mechanism by which FAK silencing prevented the structural and functional deterioration of chronically overloaded LV we examined the expression and activity of metalloproteinase-2 (MMP-2). We found that FAK silencing canceled the increased activity of MMP-2 in overloaded LV. These effects were accompanied by a higher survival rate in mice that underwent FAK silencing. We therefore conclude that FAK signaling contributes to the genesis of the hypertrophy and that its persistent activation may adversely affects the structure and function of chronically overloaded LV.

Characterization of a Novel, Cardiac-Specific Isoform of the Cell Cycle-Related Kinase That Is Regulated During Heart Failure

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Myocardial infarction (MI) is often followed by heart failure (HF) but the mechanisms precipitating the transition to HF remain largely unknown. A genomic profile was performed by DNA microarray in a monkey model of MI, from the myocardium adjacent to chronic (2 month) MI followed by three weeks pacing to develop HF. The transcript of the gene encoding the cell cycle-related kinase (CCKR) was down-regulated 2-fold in HF heart compared with control (P<0.05), which was confirmed by quantitative PCR. Upon sequencing, the cardiac CCKR showed a conservation of the N-terminal kinase domain, but differed in its C-terminal half from the isoform cloned previously in other tissues, due to an alternative splicing with frame shift. The nucleotide sequence of the variant is 100% identical between mouse and human. Expression of the corresponding protein was confirmed by western blot upon generation of a specific antibody, and was found to be limited to heart, liver and kidney. Protein expression also decreased in HF heart compared to control (P<0.05). Adeno-mediated over-expression of the cardiac-specific CCKR in cardiac myocytes induced a 36% increase in protein/DNA content (P<0.05) and a 50% decrease in apoptosis upon chelerythrine treatment (P<0.05) compared to control. In addition, the substrate specificity of cardiac CCKR differs from the generic isoform. A well known substrate for the generic CCKR is the cyclin-dependent kinase cdk2. Phosphorylation of cdk2 significantly increased upon adeno-mediated overexpression of the "generic" CCKR in isolated cardiac myocytes, which was accompanied by a higher percentage of myocytes in the S and G2 phases of the cell cycle, as measured by flow cytometry. Reciprocally, the overexpression of the cardiac CCKR did not increase cdk2 phosphorylation and did not affect the cell cycle. We conclude that the heart expresses a splice variant of CCKR, which promotes cardiac cell growth and survival but does not affect the cell cycle, which may be linked to the progression of HF.
vivo as a COX inhibitor activates TFE-1 dependent promoters. They propose that the TFE-1/COX association may regulate transcription by three mechanisms: (1) COX phosphorylation directly regulates MCT-ADP binding by TFE-1; (2) TFE-1 can recruit COXbeta and associated proteins to DNA, regulating the activity of nearby transcription factors or chromatin structure; and (3) TFE-1 can recruit the COX2 holoenzyme to muscle promoters, where it can regulate the activity of other transcription factors that are COX2 targets, such as NFκB.

**Unfolded Protein Response and Hypoxia-Inducible Factor-1α Stability in Primary Culture of Rat Adult Cardiomyocytes**

Ekaterina Fomicheva, Terri G Edwards, Joseph M Metzger; Univ of Michigan, Ann Arbor, MI

It was recently published that activation of the Unfolded Protein Response (UPR) occurs in inflammatory diseases and hypoxic cardiac myocytes. Interestingly, it was also noticed that a stabilization of Hypoxia Inducible Factor 1 alpha (HIF-1α) occurs under the same conditions. We hypothesized that UPR might exert an effect on HIF-1α level in adult rat cardiac myocytes.

**Methods:** We developed a double viral system (DVS) as a tool for tracking HIF-1α abundance in myocytes. The DVS consisted of a sensor and an effector virus: the sensor virus has 6xREs in the promoter and an oxygen dependent domain (ODD) in fusion with the yeast GAL4-p65 protein and activated by the presence of HIF-1α. The effector consisted of six GAL4 binding sites upstream of a minimal viral promoter, and is able to amplify signal by the binding of the GAL4-ODD-p65 protein to activate the reporter luciferase (Luc) gene.

**Results:** We transduced freshly isolated myocytes with the sensor 6xRE-p65GAL4-ODD-p65 and the 6xUS-Luc effector viruses. Virus encoding Renilla luc was used as an internal control and CMV-Luc virus was used as a control for adenoviral efficiency. Hypoxia was induced by exposure to a gas mixture (0.5% O2, 5% CO2, 95% N2) compared to normoxia (85% air and 5% O2). Under normoxic conditions, two different treatments were applied after 24 hours following viral transduction: calcium ionophore A23187 was added to the medium to induce UPR and DMOG was used to inhibit prolyl hydroxylase activity. After the next 24 hours, myocytes were lysed and Dual Luc assay was performed (firefly Luc activity was normalized to Renilla Luc). Dual luc assay (n=6–8) displayed the highest amplification of expression for group under hypoxia 1.18x10^4 ± 1.2x10^3 RLU vs normoxia 1.81 ± 0.2 RLU (P<0.0001). An elevated level of expression was observed in myocytes treated with A23187 3.8x10^5 ± 8.6x10^4 RLU and with DMOG 3.0x10^5 ± 3.3x10^4 RLU compared to myocytes kept in normoxia 1.81 ± 0.2 RLU (P<0.0001). The expression in myocytes transduced with CMV-Luc 9.31x10^5 ± 1.6x10^5 RLU was also elevated compared to normoxia 1.81 ± 0.2 RLU (P<0.0001).

**Conclusions:** Our data showed UPR has an effect on HIF-1α abundance in adult rat myocytes. These results indicate that UPR and HIF-1α may contribute to protecting the myocardiun during hypoxic stress.

**Gender-Specific Role of Transcription Factor NF-κB in Left Ventricular Remodeling on Pressure Overload**

Christina Gehrke, Laura Zelaraya, Claudia Noack, Anke Renger, Rainer Dietz, Claus Ekaterina Fomicheva, Terri G Edwards, Joseph M Metzger; Univ of Michigan, Ann Arbor, MI

We have shown that in vivo cardiac specific inhibition of nuclear factor κ B (NFκB) leads to an abrogation of Angiotensin II induced hypertrophy. To clarify the role of NFκB in left ventricular (LV) remodeling, we analyzed whether myocardiun inhibition of NFκB preserves left ventricular function after transaortic constriction (TAC). To obtain NFκB− mice, we bred mice expressing a stabilized mutant of the κBκB with mice expressing Cre recombinase under the control of cardiomyocyte specific alpha myosin heavy chain (TG). TAC with low gradient (21±1±3/mmHg) induced cardiac hypertrophy in control male and female mice after 4 weeks demonstrated by echocardiography analysis (table 1). Attenuation of hypertrophy was observed only in TG male mice. After 8 weeks fractional shortening (FS) was decreased in TG and CT irrespective of gender. Similar results were obtained, when TAC was performed with higher gradients (48±1±4/mmHg). After 2 weeks controls and mutants showed decreased FS. TG mice had even worse LV remodeling (FS sham: 29±1±1% (mean±SEM), control: 13±3±3%, TG: TAC: 9±1±5%). Gene expression analysis revealed no upregulation of hypertrophy markers betatubA, alpha sarcomeric actin, αN and SNAP in TG mice while this was readily detectable in CT mice. To elucidate the gender specific effects of NFκB inhibition, we analyzed the effect of dihydrotestosterone (DHT) on NFκB activity. We observed a dose dependent activation of NFκB reporter gene construct by DHT in isolated neonatal rat cardiomyocytes (NFκB: 1±0±07 ind. fold, NFκB+DHT (100nM/kM) 1.9±0.18). In summary, inhibition of cardiac hypertrophy through attenuation of NFκB activity was not able to prevent functional LV deterioration upon pressure overload. The observed gender differences concerning cardiac hypertrophy with an attenuated response in female mice might in part be explained by the signalling axis DHT/NFκB.

**Low Gradient TAC Echo Data**

<table>
<thead>
<tr>
<th>Control male sham</th>
<th>Control female sham</th>
<th>Control male + TAC</th>
<th>Control female + TAC</th>
<th>TG male + TAC</th>
<th>TG female + TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV septum (mm)</td>
<td>7.8±0.5</td>
<td>6.9±0.5</td>
<td>5.8±0.5</td>
<td>8.1±0.2</td>
<td>7.1±0.3</td>
</tr>
<tr>
<td>LV posterior wall (mm x 0.1)</td>
<td>6.3±0.2</td>
<td>6.3±0.2</td>
<td>6.9±0.5</td>
<td>6.3±0.2</td>
<td>6.6±0.5</td>
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**Effects of Graded Levels of Thyroid Activity on Cardiac Function and Arteriolar Density in Rats**

Yingheng Liu, Rebecca A Redetzke, Suleman Said, Cardiovascular Inst, Sioux Falls, SD; Gabriella M de Escobar, Instituto de Investigaciones Biomedicas Albertos Solis, Madrid, Spain; A Martin Gerdes, Cardiovascular Inst, Sioux Falls, SD

The link between thyroid dysfunction and cardiovascular diseases has been recognized for more than a hundred years. While overt hypothyroidism leads to impaired cardiac function and possibly heart failure, the cardiovascular consequences of borderline low thyroid function are not clear. Establishment of a suitable animal model would be helpful. In this study, we characterized a rat model to study the relationship between cardiovascular function and graded levels of thyroid activity. We used rats with surgical thyroidecmytomy and subcutaneous implantation of various T4 doses (1.5, 2.5, 4.5, and 6.5μg/100/g/d) for three weeks. In terminal experiments, cardiac function was evaluated by echodocardiograms and hemodynamics. Myocardial arteriolar density was also quantified morphometrically. Thyroid hormone levels in serum and heart tissue were determined by RIA assays. Histomorphology alone is insufficient to determine low thyroid conditions. Results suggest that gender functional changes may be helpful in decisions regarding treatment of borderline thyroid conditions.

**Activation/Phosphorylation of Protective Protein Kinase PKCε Attenuates the Degradation of This Kinase: A Novel Mechanism for Cardioprotection**

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Recent investigations suggest an important role of new protein synthesis in the manifestation of a cardioprotective phenotype. However, very little is known regarding whether protein degradation plays a role in cardioprotection, and so what specific mechanisms are involved in this process. To address this issue, we conducted experiments to comprehensively examine the role of the ubiquitin-proteasome system in modulating degradation of a previously well-characterized cardioprotective kinase, protein kinase C ε (PKCe). The rate of PKCe degradation was found to be tightly coupled with its ubiquitination and was significantly inhibited by three structurally independent inhibitors of the proteasome system, epoxomicin, clasto-lactacystin β-lactone and MG132. Selective activation of PKCe in cardiac cells enhanced PKCe phosphorylation and reduced PKCe protein degradation, demonstrating that phosphorylation/activation of PKCe renders this kinase less susceptible to degradation. Further, phosphorylation of proteasome dependent degradation was also confirmed by phosphorylation site mutagenesis studies on PKCe. The half-life of PKCe T566E and PKCe T566A (a mutation which prevents phosphorylation of this residue) in Cos7 cells was determined by pulse-chase experiments to be ~11 and ~8 hours respectively. Furthermore, cardiac tissues from PKCe transgenic mice exhibiting a cardioprotective phenotype showed increased PKCe phosphorylation, elevated PKCe phosphorylation, decreased PKCe ubiquitination, and reduced PKCe degradation compared with those in wild type control mice. Taken together, these data demonstrate (i) that the ubiquitin-proteasome system modulates PKCe degradation in cardiac cells and (ii) that activation/phosphorylation of PKCe in cardioprotection attenuates the degradation of this kinase, supporting a possible critical role of the ubiquitin-proteasome system in sustained activation of protective proteins and thereby cardioprotection.

**Exclusive Signaling of α1-Adrenergic Receptors via 1 Splice Variant of Phospholipase Cβ1 (b) in Rat Cardiomyocytes**

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Activation of Gq in cardiomyocytes can lead to cardiomyocyte protection, hypertrophy or apoptosis. For death in vivo and in vitro, Subtypes of P2Cg, the immediate downstream effectors of Gq, mediate these responses. Neonatal rat cardiomyocytes (NRVM) express PLCβ3 and the two splice variants of PLCβ1, PLCβ1a and PLCβ1b, which differ only in their extreme C-terminus. PLCβ1a has a C-terminal PDZ interacting domain, and PLCβ1b has a C-terminal proline rich sequence, suggesting the possibility of differential localisation and thus different functional roles. We addressed the question: (i) Are PLCβ1 splice variants selectively stimulated one of these two splice variants. In NRVM, GFP-PLCβ1a localised to the cytosol, and was largely excluded from the nucleus, whereas GFP-PLCβ1b was concentrated at the sarcolemma, demonstrating a pronounced selectivity in the intracellular localisation of the two splice variants. Degradation of both PLCβ1a and PLCβ1b containing a nuclear localisation sequence, and localizing to nuclei in non-excitable cells, was not observed in NRVM. GFP-PLCβ1a was also excluded from the nucleus, and localised to the cytosol. IPWestern blotting showed that PLCβ1a was pre-coupled with Gαq, whereas neither PLCβ1a nor PLCβ1b was found to be pre-coupled with Gαq, demonstrating an unexpected level of specificity in downstream Gα signaling. As RNAi was not successful in knocking down PLCβ1 expression, myristilated peptides corresponding to the C-terminal regions of PLCβ1a and PLCβ1b were used. When added to NRVM,
the PLCβ1-B C-terminal peptide eliminated the PLC response to α,α-AR agonists, and induced the rapid dissociation of PLCβ1 from the sarcoplasm into the cytoplasm. The PLCβ1-B C-terminal peptide did not affect PLCβ2 or PLCβ3. The PLCβ2-A C-terminal peptide had no effect on the α,α-AR agonist response or PLCβ1-B, PLCβ2 or PLCβ3 intracellular localization. This implies that a sarcomeral protein specifically binds the unique C-terminal region of PLCβ1-B. Such a protein could target PLCβ1-B to the sarcoplasm and to the caveolae where it associates with Gqα. This provides the potential to selectively inhibit PLC activation via Gqα in heart by inhibiting the sarcomeral targeting of PLCβ1-B.

SERCA2a Overexpression Induces eNOS Activation in Human Coronary Artery Endothelial Cells

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Endothelial dysfunction, characterized mainly by an impairment of nitric oxide (NO)-dependent vasodilatation, plays an important role in the pathophysiology of coronary artery disease. Recently, we have shown that adenosine overexpression of SERCA2a increases coronary flow in a rat model of diabetic cardiomyopathy. However, the importance of SERCA2a therapy and the mechanisms by which it affects the regulation of vascular tone and NO production in endothelial cells is currently unknown. The aim of this study is to investigate the role of SERCA2a gene transfer in the regulation of eNOS expression and activity, NO production and proliferation of cultured Human Coronary Artery Endothelial Cells (HCAEC). To confer long term stable expression adeno-associated-virus (AAV-1) vector was used. HCAEC were infected with AAV1-β-galactosidase (as a control) or AAV1-human SERCA2a for 7 days. The eNOS expression was then analyzed by RT-PCR and Western-blot and activation was monitored in HCAEC-infected cells by measuring eNOS phosphorylation at Ser 1177. The proliferation of HCAEC was performed with colorimetric immunoassay for the quantification of cell proliferation, based on measurement of BrdU incorporation during DNA synthesis. Control non-infected cells or cells infected with AAV1-β-galactosidase expressed low levels of eNOS, however, infection with AAV1-human SERCA2a exhibited a significant increase in eNOS protein and mRNA levels. Immunoblot analysis also showed that stimulation with histamine (1μM, 10 min) resulted in a robust increase in phosphorylated eNOS at Ser 1177 with AAV1-human SERCA2a compared to control cells. eNOS-Ser 1177 phosphorylation is calcium dependent. HCAEC, infected with AAV1-1-SERCA2a for 7 days, and then infected with an adenovirus overexpressing parvalbumin, a protein that buffers intracellular calcium ions, for 24 hours, partially decreased eNOS phosphorylation. In addition, overexpression of SERCA2a did not inhibit HACEC proliferation compared to the control cells. Since SERCA2a overexpression leads to an increase of the reticulum endoplasmic calcium load, our data suggest that SERCA2a may be involved in the modulation of eNOS expression and activity through regulation of temporal kinetics of Ca2+ transient.

The Calcium Binding Protein Calmyrin Modifies the Myocardial Stress Response in Vivo

Joerg Heineke, Jian Xu, Michelle Sargent, Allen York, Jeffery D Molkentin; Children’s Hosp Med Ctr, Cincinnati, OH

We identified the gene calmyrin as being upregulated in cardiomyocytes undergoing hypertrophy. Calmyrin is a 22kDa Ca2+-/magnesium switch protein, which changes configuration and translocates to the membrane upon Ca2+ binding. Calmyrin is highly expressed in the neonatal mouse heart, and in the adult myocardium its expression and localization to the sarcolemma were protected against ischemia/reperfusion injury and had a significantly smaller infarct size (p<0.001). Furthermore, the TG (HW/BW ratio in mg/g: TG 7.5±2.4; control, 5.0±1.5; p<0.05) mice had a robust myocardial overexpression of calmyrin: Aortic constriction in inducible transgenic mice (TG). We focused our analysis on lines 26.6 and 27.5, which had a robust myocardial overexpression of calmyrin:

- mRNA levels. Immunoblot analysis also showed that stimulation with histamine (1μM, 10 min) resulted in a robust increase in phosphorylated eNOS at Ser 1177 with AAV1-human SERCA2a compared to control cells. eNOS-Ser 1177 phosphorylation is calcium dependent. HCAEC, infected with AAV1-1-SERCA2a for 7 days, and then infected with an adenovirus overexpressing parvalbumin, a protein that buffers intracellular calcium ions, for 24 hours, partially decreased eNOS phosphorylation. In addition, overexpression of SERCA2a did not inhibit HACEC proliferation compared to the control cells. Since SERCA2a overexpression leads to an increase of the reticulum endoplasmic calcium load, our data suggest that SERCA2a may be involved in the modulation of eNOS expression and activity through regulation of temporal kinetics of Ca2+ transient.

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GATA4 Prevents Death in Cardiac Myocytes Lacking α1-Adrenergic Receptors but Is Not Required for α1A-Mediated Survival Signaling

Yuan Huang, Sanford Rech/USD, Sioux Falls, SD; Megan Egletneth, Sanford Sch of Medicine/USD, Vermillion, SD; Casey D Wright, Chase T Markman, Quan-hai Chen, Nicole L Baye, Giangrong Liang, Timothy D O’Connell; Sanford Rech/USD, Sioux Falls, SD

Recently, we demonstrated a direct protective effect of the α1A-adrenergic receptor subtype (α1A-AR) in cardiomyocytes and defined an α1A-ERK signaling pathway that is required for myocardocyte survival signaling. Previous studies suggested that the cardiac-specific transcription factor GATA4 is activated by ERK and mediates survival signaling in cardiac myocytes. Here, we examined GATA4 as a downstream regulator of α1A-ERK survival signaling in cardiac myocytes. As with our previous studies, we used cultured α1β2AβKCOcardiomyocytes, which lack α1-ARs and are susceptible to cell death induced by a variety of stimuli, including norepinephrine (NE), doxorubicin and H2O2. In the current study, myocyte death was induced by 1 μM (NE) and measured by annexin V/propidium iodide staining. Our results show that overexpression of GATA4 is sufficient to protect α1β2AβKCOcardiomyocytes from NE-induced cell death (control, 6.1±0.4, NE, 17.2±0.6, GATA4 control, 7.8±0.5; GATA4 NE, 11.0±0.4%, n=4–5, P<0.05). However, in α1β2AβKCOcardiomyocytes expressing the α1A-subtype, phenylephrine (PE, α1-agonist) did not increase the phosphorylation of GATA4, although PE increased ERK phosphorylation and a constitutively active MEK1 (upstream activator of ERK) increased GATA4 phosphorylation. Furthermore, aortic constriction increased GATA4 phosphorylation, while decreasing ERK phosphorylation in α1β2AβKCO mice. In α1β2AβKCOcardiomyocytes, which express the α1A-subtype and are protected from NE-induced cell death relative to α1β2AβKCOcardiomyocytes, we found that siRNA-mediated knockdown of GATA4 did not reverse the protective effects of the α1A-subtype in response to NE (control siRNA, 6.6±1.2, NE, 8.2±1.6%, α1β2AβKCO mice, 6.9±0.7; NE, 5.6±0.2%, n=2). The failure of the α1A-subtype to phosphorylate GATA4, the increased phosphorylation of GATA4 following aortic constriction in α1β2AβKCO mice, and the failure of GATA4 knockdown to reverse α1A-ERK-mediated survival signaling all indicate that GATA4 is not required for α1A-mediated survival signaling. In summary, these results demonstrate that whereas overexpression of GATA4 is sufficient to rescue α1β2AβKCOmyocytes from NE-induced cell death, GATA4 is not required for α1A-mediated survival signaling in cardiac myocytes.

Regulation of Akt/PKB Activity by P21-Activated Kinase in Cardiomyocytes

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Akt/PKB is a critical regulator of cardiac function and morphology, and its activity is governed by dual phosphorylation at active loop loop (Thr380) by phosphoinositide-dependent protein kinase-1 (PDK1) and at carboxyl-terminal hydrophobic motif Ser473 by a putative PDK2. P21-activated kinase-1 (Pak1) is a serine/threonine protein kinase implicated in cardiac hypertrophy, and was shown previously to activate Akt through an undefined mechanism. Here we report Pak1 as a potential PDK2 that is essential for Akt activity in cardiomyocytes. Both Pak1 and Akt can be activated by multiple hypertrophic stimuli or growth factors in a phosphatidylinositol-3-kinase (PI3K)-dependent manner. Pak1 overexpression induces Akt phosphorylation at both Ser473 and Thr380 in cultured cardiomyocytes and in transgenic heart. Conversely, silencing or inactivating Pak1 gene diminishes Akt phosphorylation in cultured cardiomyocytes and in gene targeted mouse heart. Purified Pak1 can directly phosphorylate Akt at Ser473 but not at Thr380 (Figure), suggesting that Pak1 may be a relevant PDK2 responsible for Akt activation in cardiomyocytes. Our results connect two important regulators of cellular physiological functions and provide a potential mechanism for Pak1 signaling in cardiomyocytes.

Plant Estrogens in the Rodent Diet Negatively Impact Genetic Heart Disease in Males

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The perception that soy food products and dietary supplements will lead to healthy hearts has been recently challenged by our demonstration that cardiac disease in male mice with hypertrophic cardiomyopathy (HCM) is worsened by the traditional soy-based rodent chow.
Background: Cardiovascular repair and tissue engineering has emerged as one way for treatment of end-stage heart failure. However, to date it is not clearly defined which one is the preferred route for stem cell delivery. Methods: We briefly reviewed the trials that had applied different routes for the stem cell (SC) delivery into the injured cardiac muscle. Results: There are different routes for SC delivery into cardiac muscle including intravenous, transcoronary, transendocardial, and transcapsular injections. The best for administering SCs highly depends on the patients’ revascularization plan; whether they are going to undergo PTCA, CABG, and even medical therapy alone. Additionally it is necessary to notify that beside some advantages all the routes have their specific limitations. For instance, perfect transendocardial injection is impossible unless the NOGA mapping is available; consequently in centers that are not equipped with this technology, like many third-world countries, it is limited to utilize. Also, larger cells like mesenchymal SCs and skeletal myoblasts are not suitable candidate for intravenous injection because a large number of them are trapped into capillary systems. Conclusion: Transcoronary injection of SCs is the most common route to date. Translating intramyocardial injection of SCs, during CABG are rare and include low number of SCs. However, it seems that as a result of the better access to most of injured regions and direct vision of the surgeon, it is suitable for patients undergoing CABG. Taken together, it seems that a large-scale study is inevitable to compare different routes of SC delivery and discover the best.

P66 Human Mesenchymal Precursor Cells Secrete Soluble Factors That Promote Proliferation of Endothelial Cells and Vascular Smooth Muscle Cells
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Background: We have previously shown that human bone marrow-derived mesenchymal precursor cell (hMPC) therapy following myocardial infarction (MI) improves LV dysfunction and remodeling. These beneficial effects were associated with increased myocardial arteriogenesis and angiogenesis, which were at least partly due to the actions of hMPC-derived paracrine factors. To examine the mechanisms by which soluble factors secreted by hMPCs promote neovascularization, we sought to examine the effects of hMPC-conditioned medium (CM) on human aortic endothelial cells (EC) and vascular smooth muscle cells (SMCs). Methods: CM from 106 hMPCs was generated by culturing cells in serum-free medium for 48h. hMPC-secreted cytokines were profiled by antibody capture arrays. Concentrated CM was added to cultures of EC and SMC with or without neutralizing monoclonal antibodies (MAbs) and cellular proliferation was examined by WST-1 assay, flow analysis of BrdU incorporation, and Western blotting for cyclin D1 and intracellular signaling pathways. Results: IL-6, MCP-1 and TNF-alpha were among the most prominent signals detected in hMPC CM. hMPC-derived CM induced a 51.7% increase in EC proliferation as assessed by WST-1 assay above unstimulated (p<0.05). Elevated cyclin D expression and PI3K phosphorylation in EC in response to CM-treatment were blocked by TNF-alpha MAb. CM also had proliferative effects on SMCs, which were inhibited by IL-6 MAb and by MCP-1 MAb. SMC proliferation was associated with phosphorylation of STAT5 and PI3K. MAbs against IL-6 and MCP-1 each inhibited IL-6, but only MCP-1 blockade reduced PI3K phosphorylation. Conclusions: HMSCs secrete a number of soluble factors which may contribute to neovascularization by differentially promoting proliferation of EC and SMC cells. These data support a paracrine mechanism for the proangiogenic effects of hMPC therapy in the post-MI setting.

P68 Local Delivery of Statin with Biodegradable Polymorphic Nanoparticle Improves Therapeutic Efficacy of Ischemic Neovascularization
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[Background] It has been shown that “systemic” administration of statins (HMG-CoA reductase inhibitor), (1) increases the number and function of endothelial progenitor cells (EPCs); (2) stimulates incorporation of EPCs to ischemic/injured tissues; and (3) accelerates regeneration/healing of damaged tissues. However, these beneficial effects have been exclusively noted at doses extremely higher than those used in the clinical settings. Therefore, “local” nanoparticle-mediated delivery should be a clinically feasible approach with minimal systemic side effects. In this study, we aimed to develop a novel local delivery system using biodegradable polynorphic nanotechnology, and to examine whether the nanoparticle-mediated local delivery of statin at clinical dose range is useful to improve ischemic neovascularization. [Methods and Results] To develop a novel nanotechnology-based system, we prepared biodegradable poly-lactide-glycolide copolymer nanoparticles (NPs) with a mean diameter of 200 nm by the emulsion solvent diffusion method. We then produced unilateral hindlimb ischemia in mice, which were injected with FITC-loaded NPs (0.18 mg/100 μl), or pitavastatin [0.01 mg/100 μl (0.4 mg/kg)], or statin-loaded NPs (0.18 mg/100 μl containing 0.01 mg of pitavastatin) into ischemic muscles immediately after induction of hindlimb ischemia (n=8 to 12 each). Trace experiments showed that FITC was uptaken by mainly interstitial endothelial cells in the injected ischemic sites. Laser Doppler and morphometric analyses showed that single injection of statin-loaded NPs, but not FITC-loaded NPs or pitavastatin, significantly enhanced recovery of blood perfusion to the ischemic limb and increased capillary and arteriolar density. Neither group of mice showed the signs of rhabdomyolysis, such as an elevation of myoglobin. [Conclusion] We have shown the successful intracutaneous delivery of statin in ischemic tissues using our nanotechnology, and the local effects on tissue perfusion and neovascularization in ischemic tissues with potential side effects. The present data demonstrates the potential therapeutic benefits of our nanotechnology-based strategy for therapeutic neovascularization.

P64 Different Routes of Stem Cell Delivery: Which One Is the Preferred Way?
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Introduction: Mesenchymal stem cells (MSC) represent an attractive cellular population for cell transplantation and tissue engineering purpose. Bone marrow-derived MSC (BMSC) have special differentiation and immunomodulatory properties and might be available for allogeneic cell therapy. Aim of the present study was to search for mesenchymal stem cells in the neonatal thymus and further characterize the differentiation and immunomodulatory properties of these cells. Methods: Shz and Shz-“primed” human HSCs in the repair of damaged myocardium, and to discover the molecular and cellular mechanisms underlying cardiac regeneration. We have taken a small molecule approach by screening a synthetic organic chemical library for pharmacologic activators of Nck-2, a signature gene of cardiac fate, using a luciferase knock-in bacterial artificial chromosome (BAC) integrated into the genome of mouse P19CL6 pluripotent stem cells and transgenic mice. We describe a family of sulfonyl hydrazone (Shz)-based small-molecules that could trigger cardiac regeneration by promoting the cardiac marker gene Shz expression in vivo. These compounds do not lead to stimulation. On activated PBMNC, the nTMSC suppress proliferation. Conclusion: Our results confirm that the neonatal thymus contains mesenchymal stem cells (nMSC) with great differentiation potential and immune modulatory properties.

P65 Synthetic Small Molecules that Initiate Cardiac Lineage Commitment and Enhance the Cardiogenic Potential of Human Peripheral Blood Stem/Progenitor Cells
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New strategies are needed to enhance the effectiveness of hematopoietic stem/progenitor cells (HSCs) in the repair of damaged myocardium, and to discover the molecular and cellular mechanisms underlying cardiac regeneration. We have taken a small molecule approach by screening a synthetic organic chemical library for pharmacologic activators of Nck-2, a signature gene of cardiac fate, using a luciferase knock-in bacterial artificial chromosome (BAC) integrated into the genome of mouse P19CL6 pluripotent stem cells and transgenic mice. The screen yielded a promising collection of structurally diverse and chemically interesting small-molecule activators of early cardiac lineage genes, like Nck-2 and myocardin. Here, we describe small-molecules that activate Shz-dependent signaling and show that they induce miRNA and protein expression in embryonic stem and adult stem/progenitor cells, including granulocyte colony stimulating factor (GCSF)-mobilized circulating human HSCs. When transplanted into experimentally cryo-injured rat myocardium, Shz-“primed” human HSCs restored contractile function to normal, correlating with persistence of human cardiac mRNAs and proteins in cells of the chimeric myocardium, up to one month after injection. Shz small-molecules have also been successfully injected directly into rat myocardium. We conclude that small-molecules like Shz, identified in a stem cell-based high throughput chemical screen, might be the starting points for potential new drugs to enhance the repair of damaged myocardium by pharmacologically regulating the cardiac gene and cell fate program in human HSCs.

E66 Circulation Research Vol 101, No 5 August 31, 2007 compared to a milk-based diet. We provide evidence here that the dietary phytoestrogens, genistein and daidzein (in the soy-based chow) are sufficient to cause the poor phenotypes seen in soy-fed HCM male mice including ventricular dysfunction, myocardial fibrosis, induction of beta-myosin heavy chain, and activation of caspase-3. We show that soy and phytoestrogens differentially activate genes in males compared to females. We also show that diet has the greatest impact on the genetic landscape of the healthy heart, more so than gender or HCM alone, and that this dietary effect is magnified by disease.

P67 Differentiation Characteristics and Immunomodulatory Properties of Neontal Thymus-Derived Mesenchymal Stem Cells (nMSC)
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Int6 for Angiogenesis (3): Small Interfering RNA (siRNA) for Therapeutic
Normal Induction of Angiogenesis
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The hypoxia-inducing factor-2α (HIF-2α) has been reported to be a key factor in the targeted induction of angiogenesis and regulated by Int6. It is also demonstrated that suppression of endogenous Int6 induced normal angiogenesis in mouse skin, and has led to the further development of a siRNA targeting Int6 for therapeutic use. Here, we used two distinct technologies, siDirect™ and siChimera™ to develop a therapeutic siRNA targeting Int6. siDirect™ is a sequence design tool used to minimize the possibility of off-target effects, and siChimera™ offers a DNA/RNA chimera oligonucleotide, in which part of the RNA in the siRNA is substituted with DNA to increase stability against RNase. The ability of this therapeutic modified siRNA to suppress Int6 expression was evaluated using RT-PCR in vitro. We selected suitable siRNA sequences targeting rat and human Int6 with significant suppression of Int6 expression and relative stability in serum by siChimera™ technology. This result suggested a possibility of siChimera targeting Int6 as a therapeutic agent in the treatment of peripheral arterial diseases such as arteriosclerosis obliterans, even without any chemical modifications. Animal studies are being conducted to further explore the therapeutic potential and safety of this new drug agent, and therapeutic effects have been confirmed in A549 model animals. We are now preparing for pre-clinical studies in anticipation of human clinical trials.

Critical Role of Muscle Regeneration in Therapeutic Neovascularization
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Therapeutic neovascularization by implantation of mononuclear cells (MNC) is now prevalent as a method to treat patients with critical limb ischemia. It had been believed that MNC contain vascular progenitors and also secrete angiogenic factors, and that the cells thereby induce neovascularization of the recipient ischemic tissue. More recent studies demonstrated the priority of angiogenic growth factors, rather than the processes of post natal vasculogenesis, in the mechanism of the cell therapy. Lately, we reported that angiogenic growth factors secreted from ischemic skeletal muscle cells, but not from the implanted MNC cells themselves, plays the major role in the therapeutic neovascularization. However, mechanisms by which implanted MNC induce muscle cells to secrete angiogenic factors remain unknown. Here we show the precise mechanism how MNC transplantation induces production of growth factors by skeletal muscle. First, we noticed that myocytes which produced growth factors were regenerating myoblasts, indicating a close relationship between MNC transplantation and skeletal muscle regeneration. To test this in vitro, we co-cultured myoblast cell line C2C12 with MNC, and found that this strongly induced myoblasts to proliferate, and to produce various kinds of growth factors and cytokines. In vivo, MNC enhanced activation and proliferation of myoblasts, which were accompanied by significant enhancement in the production of angiogenic growth factors. These processes were observed within 24 hours of MNC transplantation, which was prior to the growth of collateral arteries into the ischemic muscle. After 9 days, animals treated with MNC showed increased number of regenerating myoblasts, enhanced production of growth factors, and marked development of collateral arteries that supplied blood flow into ischemic muscle. These effects by MNC were abolished in the aged mice with diabetes and mice. This result supports our hypothesis that MNC transplantation directly enhances muscle regeneration by inducing myoblast activation and proliferation, and that this process is inevitable for the mechanism of therapeutic neovascularization.

Protein Kinase b3 (akt1)-Mediated Extracellular Matrix Remodeling is Essential for Vascular Responses
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Extracellular matrix (ECM) such as collagens and fibronectins controls a number of cell functions, including adhesion and migration and, in turn, determine the course of ischemic injury, wound healing and angiogenesis. ECM maintains the integrity of the blood vessels and its secretion and assembly by the fibroblasts is important for the repair of cardiovascular tissues followed by myocardial infarction. Previous study in the lab using Akt1-/- mice showed that Akt1 is the major Akt isoform in vascular cells and accounts for ~70% of the total Akt activity. Absence of Akt1 results in a series of ECM defects in mouse tumor models and in vivo, including reduced levels of collagen in skin and impaired laminin content within the vascular basement membrane (Nat. Med. 2005; 11(7):788–796). Matrix reorganization is dependent on its secretion and assembly, which, in turn, is a result of integrin activation in fibroblasts. Thus, modulation of Akt1 activation in fibroblasts will have effects on tissue remodeling in skin wounds and ischemic heart. In this study we sought to investigate the role of Akt1 in the regulation of integrin activation, ECM recognition, migration and matrix assembly by the fibroblasts and to understand the effect of Akt deficiency on injury-induced vascularization and healing in vivo. To do this, we have used a multifaceted approach employing Akt1-/- mice and fibroblasts, cell lines with a range of constructs expressing various forms of Akt1 such as WT-Akt1, constitutively active (myrAkt1) and dominant negative (DN-Akt1), specific inhibitors, antibodies that stimulate or block β3 integrins and antibodies that detect the active forms of integrins β1, β3 and β6. We demonstrated a novel function of Akt1 in fibroblast regulated ECM secretion and assembly and its effect on remodeling of wound tissues. Our study provides the first direct evidence that Akt1 is necessary for the inside-out activation of β3 integrins that regulate ECM secretion and migration of fibroblasts on matrix proteins such as fibronectin and collagen. This result suggests that in impaired secretion and assembly of ECM proteins such as collagen and fibronectin in vitro, ultimately leading to impaired vascular maturation and tissue remodeling in skin wounds.

β-Catenin Downregulation Improves Cardiac Function After Myocardial Infarct
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We have recently reported that downregulation of β-catenin is required for adaptive cardiac hypertrophy, Here, we aimed to clarify the in vivo role of β-catenin in LV-remodeling after myocardial infarction (MI). We previously reported that downregulation of β-catenin is required for adaptive cardiac hypertrophy, and that the direct interaction of Int6 with HIF-2α protein. Int6-siRNA or the dominant negative mutant, Int6-DN, was injected an int6-siRNA expression vector into subcutaneous region of mouse skin. The knockdown of endogenous Int6 in mouse fibroblasts induced strong suppression of Int6 expression even under normoxic conditions. Moreover, by using small interfering RNA (siRNA) against HIF-2α, we found that the silencing of endogenous Int6 was sufficient to induce HIF-2α expression, even under normoxia, and it enhanced the expression of angiogenic factors such as angiopeptin, fetal liver kinase-1, Tie-2, and VEGF. To elucidate the function of Int6/HIF-2α, we injected an int6-siRNA expression vector into subcutaneous region of mouse skin. The knockdown of endogenous Int6 in mouse fibroblasts induced strong normal angiogenesis through stabilization of HIF-2α. Pathological analysis indicated that the newly synthesized blood vessels were normal arteries with smooth muscle and elastic fibres, and normal veins were also observed. These results indicated that int6 is a novel and critical determinant of HIF-2α-dependent angiogenesis, and suggested that int6-siRNA transfer may be an effective therapeutic strategy for heart and brain ischaemia, hepatic cirrhosis and obstructed vessel diseases.

Withdrawn

Transendocardial Cather Delivery of Biopolymer Matrices for Enhanced Cell Retention in Cardiovascular Cell-Based Therapy
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Background Cardiac regeneration strategies using stem cells have repeatedly had inefficient cell retention in the target zone. Biopolymers can enhance local cell retention by two theoretical means: First, the entrapment of cells within an in-situ cross linked or gelled biopolymer matrix providing a matrix to support tissue integration. Secondly, by acting as a scaffold the lymphatic and venous drainage routes by which cells can be flushed away from the targeted delivery site. However, life threatening embolic events can be caused by release of cell aggregates, cells suspended in viscous biopolymer gel, or controlled release biotherapeutic formulations within the left ventricular chamber during transendocardial delivery. This safety issue has not been described or addressed previously. Methods and results A new tri-lumen bipolar helical needle transendocardial catheter system has been developed which enables the delivery of advanced biotherapeutic strategies for myocardial regeneration and repair, and presents a number of design features to address embolic safety concerns. This catheter has been built on the footprint of a helical needle catheter currently in use in clinical trials. Commercially available two part biopolymers have been delivered through the catheter at various flow rates and volumes in-vitro, and into swine myocardium ex-vivo. Transendocardial injection was shown to be feasible, with acceptable generated pressures. The critical issue of potential leakage into the ventricular chamber was assessed with no back leak observed. This delivery system provides a means to confirm engagement to the tissue before delivery, assesses myocardial venous and lymphatic drainage at the selected delivery site prior to delivery, and inhibit back-bleed of delivered agents. Theoretical considerations suggest that optimal mixing of two part biopolymers in tissue can be achieved with viscosity matching. Conclusions Catheter based tissue matrix delivery approaches have significant potential as safe and effective tools providing broad utility for cardiac tissue engineering strategies.
P77 STAT3-Dependent Cross-Talk Signaling Mechanism of LIF and BMP-2 in Mouse Embryonic Stem Cell Differentiation into Cardiomyocytes and Its Therapeutic Efficacy in Mouse Model of Myocardial Infarction

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Background: Pluripotent stem cell therapy may be an attractive source for post-infarction myocardial repair and regeneration. However, the specific stimuli and signal pathways that may control embryonic stem (ES) cell-mediated cardiomyogenesis remains to be completely defined. The aim of the present study was to investigate (a) the effect and underlying signal transduction pathways of leukemia inhibitory factor (LIF) and bone-morphogenic protein-2 (BMP-2)-induced mouse ES cell (mES-D3 line) differentiation into cardiomyocytes (CMC) and (b) the efficacy of mES cells, pre-treated with LIF + BMP2, for functional and anatomical cardiac repair in surgically induced mouse acute myocardial infarction (AMI) model. Methods and Results: Various doses of LIF and BMP-2 and their inhibitors or blocking antibodies were tested for mES differentiation to CMC, in vitro. CMC differentiation was assessed by mRNA and protein expression of CMC-specific markers, Connexin-43, CTI, CT, Mef2c, Mdr2c, Nkx2.5, GATA-4 and αMHC. LIF and BMP2 synergistically induced the expression of CMC markers as early as 4 days in culture. Signaling studies identified STAT3 and MAP kinase (ERK1/2) as specific signaling components of LIF + BMP2 mediated CMC differentiation. Inhibition of either STAT3 or MAP kinase blocked CMC differentiation. Furthermore, the addition of a specific inhibitor dramatically suppressed LIF + BMP2 mediated CMC differentiation. Moreover, in mouse AMI model, transplantation of lentivirus-GFP-transduced, LIF + BMP2 pre-treated mES cells, improved post-MI left ventricular function and enhanced capillary density. Transplanted cells engrafted in the injured myocardium and differentiated into CMC and endothelial cells. Conclusion: Our data suggest that LIF and BMP2-2 may synergistically enhance CMC differentiation of transplanted stem cells. Thus the LIF/BMP2 cocktail or augmentation of their downstream signaling components may facilitate the effects of stem cell based therapy for post-MI myocardial repair and regeneration.

P78 Dedifferentiation and Epigenetic Reprogramming of Somatic Cells into Pluripotent Stem-like Cells and Its Therapeutic Efficacy in Mouse Model of Acute Myocardial Ischemia

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Background: De-differentiation or reverse lineage-commitment of adult somatic cells into multipotent progenitor cells might provide an attractive, oocyte-independent alternate source for therapeutic cloning to generate pluripotent, autologous stem cells for regenerative medicine. We tested the hypothesis that exposure of NIH3T3 fibroblasts cytoplasm to mouse embryonic stem cell (mES) extracts may provide regulatory molecules required for de-differentiation. Methods: NIH3T3 control cells and NIH3T3 cells treated with mES extracts only, mES extracts pre-treated with inhibitor of BMP2 mediated CMC differentiation. Histone modifications were determined by ChIP and ChIP-chip analyses. Results: Various doses of LIF and BMP-2 and their inhibitors or blocking antibodies were tested for mES differentiation to CMC, in vitro. CMC differentiation was assessed by mRNA and protein expression of CMC-specific markers, Connexin-43, CTI, CT, Mef2c, Nkx2.5, GATA-4 and αMHC. LIF and BMP2 synergistically induced the expression of CMC markers as early as 4 days in culture. Signaling studies identified STAT3 and MAP kinase (ERK1/2) as specific signaling components of LIF + BMP2 mediated CMC differentiation. Inhibition of either STAT3 or MAP kinase blocked CMC differentiation. Furthermore, the addition of a specific inhibitor dramatically suppressed LIF + BMP2 mediated CMC differentiation. Moreover, in mouse AMI model, transplantation of lentivirus-GFP-transduced, LIF + BMP2 pre-treated mES cells, improved post-MI left ventricular function and enhanced capillary density. Transplanted cells engrafted in the injured myocardium and differentiated into CMC and endothelial cells. Conclusion: Our data suggest that LIF and BMP2-2 may synergistically enhance CMC differentiation of transplanted stem cells. Thus the LIF/BMP2 cocktail or augmentation of their downstream signaling components may facilitate the effects of stem cell based therapy for post-MI myocardial repair and regeneration.

P79 In Vivo Tracking of Stem Cell Distribution Following Intracoronary Delivery in the Setting of Myocardial Infarction

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Background: Cell-based therapy for ischemic heart disease has emerged as a promising novel therapeutic strategy. Little is known about the fate of different populations of stem cells (SC) delivered by the intracoronary (IC) route. Methods: Using a clinically-relevant swine model (a 60-minute balloon occlusion MI model) and near-infrared fluorescence to provide high sensitivity in vivo tracking of SC after IC delivery, the intramyocardial distribution patterns of mesenchymal (MSC), bone marrow-mononuclear (BMMNC) and peripheral blood-mononuclear (PBMMNC) SC were quantified in the presence and absence of myocardial injury. Quantification consisted of fluorescence signal intensity (SI) analysis. Results: Major findings from comparisons were (Figure): (1) within-cell groups: whereas all 3 cell fractions maintained optimal SI in non-infarcted hearts, only SI from MSC remained unaltered in infarcted hearts; (2) between-cell groups: in normal hearts, non-significant differences in terms of SI were found among all cell fractions; but in infarcted hearts MSC SI not only remained elevated but were significantly different relative to the other 2 cell fractions; (3) differences were maintained throughout the 60-min period. Conclusion: This study provides the first in vivo evidence of different intramyocardial distribution patterns of SC delivered by IC route following an MI. These findings suggest that stem cell distribution during the first 60 min post-injection could potentially impact the clinical efficacy of cardiac cell-based therapy.
Influence of Redox Processes in the Progression of Aortic Valve Calcification

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Pathogenesis of degenerative aortic valve (AV) stenosis is analogous to atherosclerosis, for which oxidative stress is relevant. We hypothesized that oxidative stress contributes to progression of AV calcification/stenosis. In a rabbit AV calcification model, we investigated topography of reactive oxygen species (ROS) production and effects of antioxidants tempol and lipic acid in calcification progression. Male rabbits were fed VitD$_3$ 12.5 IU/kg +/− cholesterol 0.5% for 12 weeks in the absence (HCD, n=34) or presence of tempol 100 μmol/kg/day (HCDT, n=11). Cholesterol 0.5% only (HC, n=9) or regular chow (C, n=32). HCDL and HEDT rabbits developed morphocyte ingrowth and calcification. Superoxide and H$_2$O$_2$, detected respectively by hydrothoxygen and dichlorofluo- rescein microtopography, and 3-nitrotyrosine immunoreactivity, were increased not only in inflammatory cells but preferentially around calcifying nuclei. Tempol increased, while lipic acid decreased H$_2$O$_2$ signals. This subpopulation of calcifying vascular cells, which express osteopontin and Cbfa-1, had increased expression of NADPH oxidase subunits p22phox and Nox2, and of oxidase regulator protein diudse isomerase, which were co-localized. Real-time PCR showed switch from Nox1 to Nox4 mRNA expression in HCD vs C rabbits. Importantly, AV/aorta calcification, assessed by echocardiography, EBIT and histology, decreased with lipic acid but increased with tempol (p<0.05). Tempol further enhanced apoptosis and decreased proliferation/osteopontin expression vs HCD. Human AV were collected from autopsied young individuals with normal valve (n=6), elderly with valve sclerosis (n=4) and patients undergoing replacement due to stenosis (n=5). We found analogous increases in ROS production and protein expression around calcifying nuclei. An in vitro model of vascular smooth muscle cell calcification showed increased Ca$^{2+}$ deposit with tempol but not with lipic acid. Thus, ROS production is markedly increased around calcifying nuclei in human or experimental AV calcification, associated with at least Nox2 and Nox4 expression. However, the specific ROS involvement may possibly dictate the effect in calcification progression.

The Heme Oxygenase System Protects Cardiac Tissue by Suppressing Angiotensin II

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Uregulating the heme oxygenase (HO) system with HO inducers like hemin removes prooxidant heme and thus cytoprotective. Importantly, the products from the HO pathway are also rate limiting for superoxide production which feeds a positive feedback loop amplifying the inflammatory/oxidative insults.

The Contribution of Second Heart Field Cells to the Jaw Muscle Reveals the Multipotential Nature of the Cardio-Craniofacial Mesoderm

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Recent studies demonstrated that common progenitor populations of mesoderm cells in the head contribute to both cardiac and skeletal muscle lineages, suggesting that developmental multipotency is more pervasive than we previously expected. In this study, we utilized several fate-mapping techniques, along with gene expression analyses in chick embryos, to systematically track both cardiac and skeletal muscle precursors. We demonstrate that the cardiac progenitor population, known as the second heart field (SHF), lies between the cranial paraxial mesoderm (CPM) and the lateral splanchnic mesoderm (first heart field or FH) at cardiac crest stages. During gastrulation, these cells are segregated with the lateral mesoderm, and marked by the expression of Isll, Nkx2.5, Tbx20 and Fgfd1. We further provide cellular and molecular insights into the regionalization of the pharyngeal mesoderm within the branchial arches by both CPM and SHF cells; CPM cells contribute to the proximal region of the myoseptum, whereas SHF cells populate the distal portion of this arch. Moreover, our findings reveal regional developmental programs for CPM and SHF-derived branchiomyocardial precursors. While both muscle types express Myf5 and Myod1, SHF-derived muscle anlagen (e.g., the Mandibular Adductor complex) express Pax7 and SHF-derived muscles (e.g., the Intermandibular) express Isll. Furthermore, the late differentiation marker Myhc, was significantly delayed in the SHF-derived/Isll+ myoblasts. In addition, ectopic activation of the Wnt/β-catenin pathway resulted in a cardiac looping phenotype, along with inhibition of Nkx2.5 and Isll expression in the SHF, suggesting a role for this signaling pathway in the regulation of the second heart field. These experiments provide the first insights into the processes underlying the development of the cardiac-craniofacial mesoderm by embryogenesis.
High-Mobility Box 1 Protein Induces Cardiac Stem Cell Activation in a Paracrine Manner

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Introduction. High Mobility Box 1 Protein (HMGB1) is a non-histone chromatin protein which modulates gene transcription. HMGB1 is released into the extracellular space by necrotic cells and activated macrophages in response to injury; under these conditions HMGB1 acts as a cytokine. We recently demonstrated that HMGB1-1 administration into the mouse heart during acute myocardial infarction resulted in cardiac tissue regeneration by stimulating proliferation and differentiation of resident Cardiac Stem Cells (CSCs) and significantly improved ventricular function. In the present study we analyzed the mechanism involved in HMGB1-mediated effect on CSCs. Specifically, we analyzed the possibility that human cardiac fibroblasts (cFbs) exposed to HMGB1 may exert a paracrine effect on CSCs.

Methods and Results. Human cFbs were exposed to HMGB1 (10 ng/ml); the conditioned medium (CM) was collected after 72 hr and analyzed for the presence of growth factors, cytokines and chemokines using a multiplex-27 bead assay and LumineX technology. It was found that HMGB1 enhanced cFbs secretion of VEGF, Mip-1α, IFN-γ, GM-CSF, IL-10, IL-1β, IL-6, IL-1ra, TNF-α. CMs obtained from untreated- and HMGB1-treated cFbs were then used to evaluate their effects on CSC function. CSCs were isolated from the non-myocyte population of the mouse and human hearts by magnetic selection for the antigen c-kit. CM from HMGB1-treated cFbs enhanced CSC migration 2 fold (p=0.01), and induced CSC differentiation towards the endothelial lineage as detected by ActD-Di incorporation (23.7%±2.5% vs 14.5%±1.8%, n=6; p<0.01). Finally, CM from HMGB1-treated cFbs stimulated CSC proliferation, evaluated by BrdU incorporation assay (6.9%±1.5% vs 2.1%±0.2%, n=3, p<0.05). Importantly, HMGB1 administration directly to CSCs did not affect their proliferation and differentiation into endothelial cells. Conclusions. HMGB1 stimulates growth factor cytokine and chemokine release by cFbs which, in turn, enhances CSC proliferation, migration and differentiation. This paracrine effect could account, at least in part, for the ability of HMGB1 to induce regeneration after myocardial infarction.

Compositional Changes in Glycosaminoglycan Content with Age in the Heart Valve

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While the need for a tissue-engineered heart valve for treatment of pediatric valve disease is well established, the creation of an age-specific valve has been precluded by limited characterization of pediatric valve composition. The objective of this study was to analyze the composition of the valve in several age groups in a bovine calf model of valve degeneration. We hypothesized that the glycosaminoglycans (GAGs) that are critical to tissue differentiation, structural assembly, and mechanical properties. To this end, we used fluorophore assisted carbohydrate electrophoresis to analyze the concentration and sulfation of the GAGs within various porcine midsystolic mitral (MV) and aortic (AV) valves. The mitral valve was divided into the central cusp zone (MCZ) and a leaflet zone, and the valve was heated. The AV contained significantly more GAG content compared to the MV, especially at older age. The AV contained high proportions of CS, particularly C0S, which increased with age (p<0.001). The MCZ/MV contained significantly more GAGs but proportionally more than the dermal sulfate (DS) and chondroitin sulfate (CS) (p<0.001). This region also had the most CS and unsulfated CS (COs) (p<0.05). As age increased, the MV showed a decrease in overall hydration but an increase in GAGs, particularly 4-sulfated DS (p=0.03). This indicates the possibility of a different GAG composition with age. Furthermore, the AV contained significantly more GAG content compared to the MV and MFV, confirming the decreases in GAG content. The results indicate that the composition and sulfation of GAGs change throughout the life cycle of the AV, and are critical to valve function.

HSC Contribution to the Cardiac Intimal and Valvular Fibroblast Populations

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Background: During embryonic heart morphogenesis, cardiac fibroblasts are believed to originate from the pro-epicardial organ and endocardial cells that undergo an epithelial-to-mesenchymal transformation. It is generally thought that new fibroblasts in the adult are derived from proliferation of those developmentally derived fibroblasts. Reports of the potential of post-natal circulating cells of bone marrow origin to contribute to adult fibroblast populations suggest that this may be a mechanism for contribution to adult cardiac fibroblast populations. Methods: To investigate the contribution of circulating cells of bone marrow hematopoietic stem cell (HSC) origin to the adult cardiac fibroblast population, we transplanted clones derived from a single EGFP + Lin-, Sca-1 +, CD34- bone marrow HSC into lethally irradiated congenic non-EGFP mice. The amount of EGFP + cells in the peripheral blood was monitored using flow cytometry for 4 months post-POI. Results: No EGFP + cells were observed in any animals through 4 months post-POI.

Induction of Type I Arginase by IL-4 and IL-13 Through PI3K and cAMP in Bovine Coronary Artery Endothelial Cells

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Introduction: Arginase is the enzyme that hydrolyzes L-arginine to produce L-ornithine, a precursor molecule for the biosynthesis of polyamines, which are implicated in tissue repair during inflammation mediated by various chemical factors including arachidonic acid metabolites and cytokines such as interleukin 4 (IL-4) and interleukin 13 (IL-13). However, the effects of IL-4 and IL-13 on arginase remain unclear in cells playing important roles in inflammation. Since endothelial cells are one of major cells for inflammatory process, we examined the possibility that IL-4 and IL-13 could up-regulate arginase in bovine coronary artery endothelial cells (BACE) and the underlying signal transduction pathway in this regulation. Methods: For arginase activity assay, the conversion of [guanido-C14] arginine to [14C]urea was measured with scintillation counting. The amount of prostacyclin (PGI2) released into the culture media and cAMP and cAMP activity were measured with colorimetric method. Results and conclusion: IL-4 and IL-13 significantly induced both cAMP and cAMP activity, prostacyclin (PGI2) synthesis and the arginase activity and protein expression in bACE. In addition, the treatment of arachidonoylfluoromethyl ketone (ACDFC), an inhibitor of cPLA2, and indomethacin, an inhibitor of cyclooxygenase (COX), suppressed arginase activity elevated by IL-4 and IL-13. Both SC-560, a COX-1 selective inhibitor, and NS-398, a COX-2 selective inhibitor, also attenuated the arginase activity increased by those cytokines. In contrast, administration of AA-861 and MK-886, ioxynigene inhibitors, had no effect on the cytokine-elevated arginase activity. The arginase activity and protein expression were also augmented by PGD2 and dibutyryl-cAMP, and H-8 and KT-5920, which are specific inhibitors, hint that IL-4 and IL-13 may act by increasing the activity increased by those cytokines. In contrast, administration of AA-861 and MK-886, ioxynigene inhibitors, had no effect on the cytokine-elevated arginase activity. The arginase activity and protein expression were also augmented by PGD2 and dibutyryl-cAMP, and H-8 and KT-5920, which are specific inhibitors, hint that IL-4 and IL-13 may act by increasing the activity increased by those cytokines.

Induction of Type I Arginase by IL-4 and IL-13 Through PI3K and cAMP in Bovine Coronary Artery Endothelial Cells

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P94 Effect of Chronic Insulin Therapy on Mitochondrial Function in an Ex Vivo Animal Model of Diabetes and Hypercaloric Diet Submitted to Global Myocardial Ischemia-Reperfusion

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Introduction: Obese diabetics have more and worse ischemic heart disease; this may be improved by insulin (INS) therapy. Mitochondria play an important role in cardiac ischemia. If these subjects are treated with INS do they have better mitochondrial function? Aim: To evaluate, in a model of diabetes + hypercaloric diet + ischemia-reperfusion (IR), if chronic therapy with insulin improves cardiac mitochondrial function. Material and methods: Goto-Kakizaki (GK) diabetic rats (fed with an hypercaloric diet between 2 and 6 months) were divided in 4 groups (n=10/group): A-DHDK control (no medication) [IR];B-INS control (insulin -as needed- between month 5 and 6); C-DHDK IR (as DHDK control and then IR); D-B2S IR (as INS control and then IR). At 6 months, hearts were removed and submitted to 165 min perfusion (control) or 10 min perfusion + 35 min ischemia + 120 min reperfusion (IR). Mitochondrial parameters assessed were: oxidative stress (colorimetric thiobarbituric acid colourimetry test -TBARS), mitochondrial swelling and calcium uptake (fluorimetry). Results: INS-treated rats had significantly lower oxidative stress levels, both in control (0.72±0.01 vs. 0.81±0.04 nmol TBARS/mg protein;p<0.05) and IR (0.93±0.02 vs. 1.05±0.02 nmol TBARS/mg protein;p<0.05). Figure 1. INS-treated animals also showed a significant decrease in mitochondrial swelling, both in IR (2.2±1.6 vs. 68.1±1.8 arbitrary units;AU;p<0.05) and in control (30.0±2.1 vs. 37.0±3.0 AU;p<0.05). INS therapy significantly improved calcium uptake in IR (63.0±2.8 vs. 53.9±0.8 nmol/mg protein;p<0.05). Conclusion: In our model of diabetes and IR, INS improves cardiac mitochondrial function, due to less oxidative stress and better ischemia tolerance (higher calcium uptake and lower mitochondrial swelling).

P95 Proteomic Analysis of Mitochondrial Proteins in Preconditioned Mouse Myocardium: Effects of Endosomal Trafficking

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The mechanisms by which ischemic preconditioning (PC) decreases tissue injury during a subsequent sustained ischemic insult are yet to be understood. It has been proposed that PC initiates signaling cascades that converge on mitochondrial end-effectors and result in cardioprotection. We have previously observed that inhibition of endosomal recycling by addition of bafilomycin blocks the protection afforded by PC. We propose that endosomal signaling may result in the translocation of signaling proteins to the mitochondria. To test this hypothesis, we utilized two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) to detect differences in protein expression levels and post-translational modifications of mitochondrial fractions from Langendorff mouse hearts preconditioned (+/- bafilomycin) for 4 cycles of 5 min ischemia and 5 min reflow and time matched control perfused hearts. Using comparative gel analysis, we focused on proteins that were altered (> 1.5-fold difference in visible abundance) in PC hearts but not in control or PC + bafilomycin treated hearts. Spots corresponding to the NADH dehydrogenase (Complex I) were downregulated in control PC hearts and upregulated in PC treated in the presence of bafilomycin. A number of key proteins involved in the PC response were then analyzed in more detail. Future studies will involve cross-species comparison to assess similarities/differences in PC and IR responses.

P92 Interruption of Transforming Growth Factor-β Signaling Attenuates Cardiac Remodeling and Fibrosis

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Introduction: Transforming growth factor-β (TGF-β) is a profibrogenic/proliferative factor that is overexpressed in the heart under stressed conditions. Transgenic mice overexpressing TGF-β have cardiac enlargement accompanied by interstitial fibrosis. Pressure overload stress induces hypertrophic reverse aortic constriction (TAC) is associated with enhanced cardiac expression of TGF-β. Objective: To test the hypothesis that interruption of TGF-β signaling attenuates cardiac remodeling and fibrosis in the pressure overload stressed heart. Methods: Our experimental model was a novel transgenic mouse that expresses an inducible dominant negative form of the TGF-β type II (DnTGFRII) receptor gene and thus lacks functional TGF-β signaling. Eight to 10 wk old male DnTGFRIIfl mice and nontransgenic (NTG) control mice were given 25 mg M2ZStO4 to drink 1 wk prior to TAC to induce the expression of DnTGFRII gene. One wk after TAC, hearts were excised, weighed, and processed for histological and immunohistochemical analyses. Quantitative assessment of cell proliferation and interstitial collagen content was performed in Ki67 and picrosirious red stained sections of left ventricle (LV) by light microscopy with a Qimaging QiCam Fast Cooled Color CCD 12-bit camera interfaced with a computer system running MetaMorph 6.2v se software. Results: TAC induced significant increases in number of Ki67 labeled nonmyocyte cells in LV of NTG mice (> 25 fold, or 62.6/1.5 Ki67 positive cells per field 40x) and H2O control DnTGFRIIfl (> 12 fold, or 51.8/4.2) mice. This effect was dramatically reduced (to 2 fold, or 8.1/3.6) in mice in which the DnTGFRII gene was induced with ZnSO4 (p<0.05 vs respective controls). TAC increased interstitial collagen content (% in NTG mice only (4.76/2.03 and 5.64/1.36 for H2O and Zn++ groups, respectively, p<0.05). TAC increased LV weight equally in all 4 experimental groups. Conclusion: TGF-β signaling stimulates proliferation of non-myocyte cells and increases interstitial collagen content in the mouse heart in response to pressure overload stress. These pro-fibrotic effects of TGF-β were not reflected in LV weight, which likely was dependent cardiac myocyte hypertrophy.

P93 Molecular Tracking of Bone Marrow Progenitor Differentiation in Vitro

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Background: While indirect immunohistochemical evidence suggests bone marrow cells home to sites of vascular injury, incorporate to vessel walls and differentiate, this methodology is limited by antibody specificity, signal loss and cross reactivity issues. Lentiviral vectors encoding for identifiable marker gene controlled by lineage specific promoters can be used to track differentiation bone marrow progenitors into endothelial and/or smooth muscle like cells. Methods: Minimal human VE-Cadherin promoter and human Smoothelin-B promoter was cloned into a self-inactivating (SIN) second generation lentiviral vector (VECad-eGFP and SMTHB-eGFP). Results: BMPCs were treated with the angiogenic growth factors VEGF (25 ng/mL), bFGF (25 ng/mL) or Angiopoietin 1 (20 ng/mL). BMPCs were activated, and can block apoptosis through multiple manners. Nevertheless, cardiomyocytes

P96 Screening Nuclear Factor of Activated T-Cells Gene in Lebanese Patients with Valvular Congenital Heart Diseases

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Most forms of congenital heart diseases (CHD) result from aberration in cardiac morphogenesis including errors in septation, valve formation, and proper patterning of the great vessels. Transcription factors are key proteins that dictate the mRNA synthesis rate and subsequently protein translation. Nuclear factor of activated T-cells (NFATc) belongs to the Rel family of transcription factors. In mice, it is expressed in the embryonic heart and is restricted to the endocardium where it plays a major role in valve formation. In order to establish role of NFATc1 in patients with CHD, we started screening for possible mutations in patients and their families (parents and siblings) registered in the Children’s Cardiac Registry Center (COCR) at the American University of Beirut Medical Center (AUB-MC). DNA was extracted from 38 patients with pulmonary stenosis, tricuspid atresia and ventricular septal defects. PCR amplification and DNA sequencing were done on the patients and their parents/sibling. Our findings revealed different single nucleotide polymorphisms (SNPs) that were not previously reported. Moreover, in exon 2 we have found a novel missense mutation in a patient with tricuspid atresia, leading to a change in amino acid at position 66 from proline to leucine. None of the 80 control individuals included in the study did show this mutation suggesting it might be disease-causing. In addition, we have found a duplicated segment in exon 7 in 57% of the patients enrolled in our study. This duplication may play a role in RNA splicing and could be a predisposing factor for a sub category of congenital heart diseases. All together our results point to a role for NFATc1 in heart malformations in humans.

P97 A Novel Cardiac Apoptotic Pathway: The Dephosphorylation of Apoptosis Repressor with Caspase Recruitment Domain by Calcineurin

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Apoptosis repressor with caspase recruitment domain (ARC) is cardiac abundant, constitutively activated, and can block apoptosis through multiple manners. Nevertheless, cardiomyocytes still undergo apoptosis that is related to cardiac diseases such as myocardial infarction, cardiomyopathy and heart failure. The occurrence of apoptosis indicates that the interplay between pro- and anti-apoptotic factors is imbalanced. It remains enigmatic as to whether this imbalance can be contributed by ARC when it loses its anti-apoptotic function under pathological conditions. Both isoprostrenol (Ilo) and aldosterone (Aldo) promoted cardiomyocyte apoptosis with a decrease in the phosphorylation levels of ARC. Direct incubation or co-culture of ARC with cardiomyocytes resulted in the reduction of ARC phosphorylation levels. Inhibition of calcineurin could attenuate the decrease in ARC phosphorylation levels. These data indicate that the decrease in ARC phosphorylation levels induced by Ilo and aldosterone was due to
its dephosphorylation by calcineurin. Further study revealed that ARC could prevent iso- and Aldo-induced apoptosis. However, such an effect depended on its phosphorylation status. While ARC was dephosphorylated and lost its anti-apoptotic function, the phosphorylation levels of FOXO3a, a transcriptional factor, were reduced upon stimulation with iso and Aldo. Concomitantly, Fox ligand was upregulated, and the death-inducing signaling complex (DISC) was formed. Inhibition of FOXO3a by its RNAi could attenuate both Fox ligand upregulation and cardiomyocyte apoptosis. ARC could block DISC formation depending on its phosphorylation. Our study identifies a novel cardiac apoptotic pathway in which ARC is dephosphorylated by calcineurin. This pathway may constitute a component in the cardiac apoptotic cascades.  

Diminished GATA4 Protein Levels Contribute to Hyperglycemia-Induced Cardiomyocyte Injury

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Hyperglycemia is an independent risk factor for diabetic cardiomyopathy and heart failure. However, the mechanisms that mediate hyperglycemia-induced cardiac damage remain poorly understood. The transcription factor GATA4 is essential for cardiac homeostasis, and its protein levels are dramatically reduced in the heart in response to diverse pathologic stresses. In this study, we investigated if hyperglycemia affects GATA4 expression in cardiomyocytes and if enhancing GATA4 signaling could attenuate hyperglycemia-induced cardiomyocyte injury. In cultured rat cardiomyocytes, high glucose (HG, 25 or 40 mM) markedly reduced GATA4 protein levels as compared with normal glucose (NG, 5.5 mM). However, the HG-induced GATA4 mRNA content, either steady-state or polysome associated, remained unchanged. Increased culture medium osmolarity did not explain the reduction in GATA4, because normalizing osmolarity with mannitol (Man) did not alter GATA4 protein levels (NG 100±12%, Man 97±8% vs HG 43±16%; p<0.05). HG-induced GATA4 reduction was reversed by MG262, a specific proteasome inhibitor, suggesting that the ubiquitin protein system (UPS) is likely responsible for HG-induced GATA4 depletion. Nevertheless, HG did not activate the UPS in cardiomyocytes as indicated by a GFP UPS reporter, nor did it increase the peptide activities or protein expression of the proteasomal subunits. However, the mRNA levels of E3 ubiquitin ligase CHIP (Carboxyl terminus of Hsp70-interacting protein) were markedly increased in or protein expression of the proteasomal subunits. However, the mRNA levels of E3 ubiquitin ligase CHIP (Carboxyl terminus of Hsp70-interacting protein) were markedly increased in cardiomyocytes injured by HG. CHIP inhibition partially restored GATA4 protein levels, suggesting that increased CHIP expression may contribute to HG-induced GATA4 reduction. In conclusion, our findings indicate that HG-induced cardiomyocyte injury is associated with impaired GATA4 expression, and this effect is at least partially mediated by increased CHIP expression.

Study of Protection by Salicylaldehyde Isonicotinoyl Hydrazine Against Hydrogen Peroxide- and Anthracycline-Induced Toxicity to Cardiac Cells

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Anthracine antibiotics rank among the most effective and frequently used antineoplastic agents. However, repeated treatment with anthracyclines is accompanied by the risk of cardiomyopathy and heart failure development. Pivotal pathophysiological role has traditionally been attributed to the iron-catalyzed intramycarial production of reactive oxygen species (ROS). In this study we aimed to compare and contrast the protective effects of salicylaldehyde isonicotinoyl hydrazine (SIH) - a strong, cell permeable iron chelator - against the hydrogen peroxide (H2O2)- and anthracycline-induced toxicity to isolated ventricular cardiomyocytes from neonatal rats. Cardiomyocytes treated with 10 μM daunorubicin initially increased beating rate, which was followed by irregular activity, disintegration of cellular monolayer and eventually the arrest of their contractility. Daunorubicin exposure resulted in progressive and significant release of lactate dehydrogenase (LDH) to the culture medium. Co-incubation with SIH, tested at concentrations 3 - 100 μM, partially but significantly reduced the daunorubicin-induced LDH release. However, no apparent dose-dependency was observed, as the LDH activity reduction achieved with 100 μM SIH (25%) was comparable to that of 3 μM (22%, n.s.). Only partial protection of cardiomyocytes by SIH against daunorubicin was in contrast with our observations that SIH rapidly enters myocytes and efficiently displaces iron from a fluorescence-quenched intracellular iron-catalyzed compound. H2O2-induced alterations in cell morphology, dissipation of mitochondrial inner membrane potential, as well as to abolish both apoptosis (annexin-V staining, nuclear chromatin shrinking, TUNEL positivity) and necrosis (propidium iodide staining). In conclusion, we have shown that intracellular iron chelation with SIH is able to fully protect cardiac myocytes from H2O2-induced toxicity. However, only partial (max. 25%) protection from daunorubicin cardiotoxicity suggests that additional, Fenton-type ROS-independent, mechanisms play an important role in this pathology.

Endotoxin-Induced Cardiac Dysfunction in Mice

Overexpression of Heat Shock Protein 27 in the Myocardium Attenuated Hydrogen Peroxide- and Anthracycline-Induced Toxicity to Cardiac Cells

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Cardiac protection of heat shock protein 27 (Hsp27) has been observed in myocardial ischemia/reperfusion injury and doxorubicin-induced heart failure. However, the role of Hsp27 in endotoxin–induced cardiac dysfunction, a lethal complication during sepsis/septic shock, has not been investigated. We hypothesized that overexpression of Hsp27 will attenuate endotoxin–induced cardiac dysfunction. To evaluate our hypothesis, transgenic mice overexpressing Hsp27 solely in heart (TG) and wild type littermate controls (WT) were challenged with LPS. Circulation Research Vol 101, No 5 August 31, 2007

Crosstalk Between MCIPI-Calcineurin and TAK1 Signaling Pathways Regulate Cardiomyocyte Hyper trophy

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The calcium-activated protein phosphatase calcineurin is controlled by the regulator of calcineurin (RCAN), also known as modulatory calcineurin-interacting protein (MCIPI), in yeast up through mammals. The physiologic function of MCIPI remains an area of ongoing investigation, because both positive and negative calcineurin regulatory effects have been reported. Here, we performed a yeast two-hybrid screen with MCIPI as bait, which identified TAK1 associated protein 2 (TAB2) as an interacting partner. The C-terminal domain of TAB2 was sufficient to mediate interaction with MCIPI in yeast, as well as in mammalian cell lysates. Importantly, TAB1, TAK1 and TAB2 interacted with MCIPI and calcineurin in cardiomyocytes by forming a macromolecular complex, suggesting the potential for reciprocal regulation between a phosphatase and a kinase. Indeed, overexpression of TAK1 and TAB1, or TGFβ treatment, lead to phosphorylation of MCIPI in cardiomyocytes, as well as in vitro with purified proteins. Analysis of TAK1 phosphorylation sites within MCIPI by mass spectrometry revealed two novel sites, S94 and 136. Mechanistically, TAK1 overexpression induced FTAC1 nuclear translocation, NFAT transcriptional activation and hypertrophic growth in cardiomyocytes that was blocked by the calcineurin inhibitor CAIN. In addition, TAK1-induced NFAT transcriptional activity was almost completely blocked in mouse embryonic fibroblasts (MEFs) deficient in mctp1. Adenovirus mediated expression of MCIPI restored TAK1-induced NFAT transcriptional activity in mctp1-deficient MEFs. Interestingly, we also observed that calcineurin negatively regulated TAK1 signaling through dephosphorylation of the TAK1/TAB1/TAB2 complex. Thus, TAK1 and calcineurin–MCIPI constitute a feedback regulatory circuit in which TAK1 positively regulates calcineurin–NFAT signaling through phosphorylation of MCIPI, while calcineurin negatively regulates TAK1 signaling through dephosphorylation of components within the TAK1/TAB1/TAB2 complex.
Activation of Transient Receptor Potential Vanilloid Receptor Type 1 Improves Vasodilation by Inhibition of RhoA/Rho Kinase Pathway

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The RhoA/Rho-kinase (ROCK) pathway plays an important role in the pathophysiology of many diseases, including hypertension, myocardiopathy and heart failure. Activation of the RhoA/ROCK pathway has been demonstrated in vascular tissue from type 2 diabetic db/db mice. Transient receptor potential (TRP) channels were found to be involved in the regulation of vascular function. It is unclear whether activation of vanilloid receptor type 1 (TRPV1) can influence the vascular function. Objective: aim of this study was to determine whether TRPV1 agonist, capsaicin, has a beneficial effect on vascular function in mice on high fat diet/HD.

Methods: four-week-old C57BL/6J mice were used with normal diet (ND) or HD for 4 months. Results: mice on HD had a markedly increase in blood pressure compared with mice on ND (32.5 ± 7 vs 16 ± 12, p < 0.001). Nitroglycerin-induced relaxation of mice aortic rings was measured using a force transducer connected with a polygraph. Results: mice on HD had a markedly increased blood pressure compared with mice on ND (16 ± 12 vs 32.5 ± 7, p < 0.001). Similarly, acetylcholine-induced relaxation of mice aorta rings was significantly decreased in mice on HD compared with mice on ND (12 ± 16 vs 32.5 ± 7, p < 0.001). However, administration of atenolol and nicorandil improved the relaxations of mice aortic rings (atenolol: 28 ± 9 vs 12 ± 16, p < 0.001; nicorandil: 32.5 ± 7 vs 12 ± 16, p < 0.001).

A Novel Nuclear Function for GRK5 and Its Potential Role in the Hypertrophic Response

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G-protein coupled receptor kinases (GRKs) are critical regulators of adrenergic signaling in the heart. During heart failure (HF) GRK2 and GRK5 protein levels are elevated, leading to a diminished cardiac function. Mice with cardiac-specific overexpression of GRK5 have a unique phenotype in response to overload hypertrophy. GRK5 mice, but not GRK2 overexpressors or non-transgenic control (NLC) mice, rapidly decompensate within 4 weeks after aortic banding (TAC) with signs of HF. GRK5, unlike GRK2 can reside in the nucleus and contains a nuclear localization (NLS) and export sequence (NES). Our hypothesis is that cardiac decompensation after TAC in GRK5 mice is due to its unique activity in the nucleus. Histone deacetylases (HDAC) reside in the nucleus and act as transcriptional repressors of hypertrophy at the level of MEF2. Phosphorylation of HDACs results in their nuclear export and activation of hypertrophic gene transcription. We found that nuclear GRK5 can phosphorylate HDAC5 and interacts with HDAC3 in myocytes. Further, we found nuclear export of HDAC5 when co-transfected with a nuclear form of GRK5 (GRK5 ΔNES). Pressure-overload hypertrophy is triggered by Gβ activation and we found that expression a constitutively active mutant Gβq in myocytes leads to significant GRK5 nuclear translocation and Gβq and GRK5 overexpression enhances ME2 activity in myocytes. Finally, significant HDAC activity in vivo can be found after immunoprecipitation of GRK5 from mouse hearts and this is increased after TAC. Our results indicate that GRK5 possess nuclear HDAC kinase activity and this novel non-G protein-coupled receptor activity of this GRK may play a key role in cardiac hypertrophy and HF.

RassF1A Inhibits Cardiac Hypertrophy Through Mst1

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RAS-association domain family 1, isoform A (RassF1A) negatively regulates cell growth in non-cardiac cells. Since RassF1A activates mammalian sterile-20-like kinase 1 (Mst1), a serine/threonine kinase, which promotes apoptosis and inhibits growth of cardiac myocytes, we examined whether RassF1A negatively regulates cardiac hypertrophy through Mst1. RassF1A is expressed in the heart and upregulated by transverse aortic constriction (TAC) (1.3 fold) in mice. In order to determine the function of endogenous RassF1A, we used RassF1A KO mice (KO). Although KO did not show any obvious cardiac hypertrophic phenotype at 3 months of age, left ventricular weight (LW) / tibial length (TL) (n = 7, 5, 6, 5) and myocyte size (1.5 fold, p < 0.05, n = 4, 3) in KO were significantly greater than those in wild type mice (WT) at ages of 7–10 months. LW ejection fraction (LVEF) in KO was significantly lower than that in WT (61 ± 2% vs 70 ± 1%, p < 0.05, n = 6, 6, 5) and the extent of LV fibrosis was greater in KO than in WT (4 ± 2% vs 7 ± 3%, p < 0.05, n = 6, 6, 8) and lung weight/TL (15 ± 3.3 vs 9.6 ± 0.7, p < 0.05, n = 6, 8) were significantly lower in WT than in KO at 3 months of age. These findings suggest that endogenous RassF1A negatively regulates cardiac hypertrophy at baseline and after TAC. Adenosine-mediated expression of RassF1A in cultured cardiac myocytes significantly activated Mst1, accompanied by phosphorylation of phenylephrine (PE)-induced increases in myocytes size. Expression of dominant negative Mst1 (DN Mst1) reversed the anti-hypertrophic effect of RassF1A (PE 1.5 fold, PE + RassF1A 0.9 fold, PE + RassF1A + DN Mst1 1.5 fold, p < 0.05, n = 6, 6, 5). Although RassF1A physically associates with Mst1, expression of RassF1A in the context of dominant negative Mst1 failed to bind Mst1. Expression of RassF1A L308F inhibited PE-induced cardiac hypertrophy (PE + RassF1A L308F 1.7 fold), suggesting that interaction with Mst1 is required for the
anti-hypertrophic effect of RassflA. These results suggest that RassflA acts as a negative regulator of cardiac hypertrophy. Furthermore, the anti-hypertrophic effect of RassflA is mediated through direct interaction with Mst1.

Oxidative Genotoxicity Reverts the Antiapoptotic Function of Laminar Flow: Opposite Roles for p21Wafl and p53

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Abstract: Laminar shear stress (SS) provides powerful antiapoptotic signals protecting endothelial cells from a large variety of damaging conditions/agents including oxygen peroxide, oxidized lipoproteins and serum starvation. The present work provides evidences that under specific circumstances SS signalling, surprisingly, activates an intense pro-apoptotic program in human umbilical vein endothelial cells (HUVEC) exposed to the DNA damaging agent Bleomycin, Adriamycin or to a cycle of hypoxia/re-oxygenation. In brief, cells were treated with a laminar SS of 12 dynes/cm2 for 4 to 16 hours in presence or absence of Bleomycin (10 ng/ml) or Adriamycin (10 ng/ml) or after 3 hours of hypoxia (1% O2) followed by 1 hour of reoxygenation. In all these conditions SS elicited a strong induction of reactive oxygen species (ROS) and cells underwent apoptosis. This process was characterized by an increased production of nitric oxide which correlated with an excited expression of the inducible nitric oxide synthase (iNOS). HUVEC treated with the iNOS inhibitor GW274150 reduced their intracellular levels of N and were protected from apoptosis in the presence of SS Bleomycin, Adriamycin or hypoxia/re-oxygenation. Biochemical analyses revealed that in presence of Bleomycin p53 was phosphorylated on serine-15 and acetylated on lysine-382 reflecting the transcriptional activation of this molecule along a pro-apoptotic pathway. Experiments with the antioxidant N-acetyl-cysteine (NAC) or with the small hairpin RNA interference of p53 completely abolished apoptosis in the presence of SS indicating that both ROS and p53 were important in this process. In addition, in in vitro experimental setting, SS failed to stimulate p21Wafl expression. However, in spite of the increased intracellular level of oxidative stress the adenovirus-mediated p21Wafl delivery restored the SS antiapoptotic effect. Taken altogether these results provide the evidence that oxidative stress may alter the function of laminar flow and suggest, under specific circumstances, a possible negative role of SS in the pathogenesis of cardiovascular diseases.

Large Tumor Suppressor 2 (Lats2), a Downstream Effector of Mammalian Steroid H11005

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Protein Expression of Sca1 and c-Kit in Explanted Left Ventricular Myocardium of Patients and Dogs with Heart Failure

Noritsugu Nakano, Yutaka Matsui, Junichi Sadoshima; Univ of Medicine and Dentistry of New Jersey, Newark, NJ

Mst1 is an evolutionarily conserved serine threonine kinase, which is a critical regulator of growth and death of cardiac myocytes. Mst1 forms a complex with HA45 and Lats2, thereby activating Lats2. Lats2 partially mediates the proapoptotic effect of Mst1 in cardiac myocytes in vitro. To elucidate the cardiac function of Lats2 in vivo, we generated transgenic mice with cardiac-specific expression of Lats2 (Tg-Lats2, line #9) and dominant negative Lats2 (Tg-DN-Lats2). At 5–6 months of age, Tg-Lats2 exhibited a greater left ventricular end-diastolic volume (25.8±8.1 vs 17.5±5.6 ml/g), a higher left ventricular weight/body weight (LVW/BW) ratio (1.4±0.2 vs 1.0±0.1 g/ml), a larger heart weight/body weight ratio (HW/BW) (1.2±0.2 vs 0.8±0.1 g/ml), and normal LV function. These differences reached statistical significance (p<0.05). LV myocyte cross sectional area (CSA) was significantly smaller in Tg-Lats2 than in WT (52 vs 58%, p<0.005) and RV myocyte CSA showed a similar tendency. Significantly reduced RWW/BW and LVEF were also observed in Tg-Lats2 line #20 (milder expression than line #9), Tg-DN-Lats2 exhibited greater levels of interstitial fibrosis (13.0±3.4%, p<0.05) at baseline. Although the number of TUNEL positive myocytes was not significantly different between Tg-Lats2 and WT at baseline, it was significantly greater in Tg-Lats2 after transverse aortic constriction for 4 weeks (0.12±0.02% vs 0.03%, p<0.05). In contrast, Tg-DN-Lats2 exhibited significantly greater LW/BW (3.8±3.27, n=10, p<0.05) and RWW/BW (0.96±0.73, p<0.05) and normal LV function. These results suggest that Lats2 is a critical negative regulator of ventricular mass and stimulation of Lats2 leads to increases in cell death, fibrosis and cardiac dysfunction. Thus, Lats2 has both similar and distinct cardiac functions compared to Mst1.

Four and a Half LIM Domains 2 (HFL2) Is a Negative Regulator of the Contractile Phenotype of Vascular Smooth Muscle Cells

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Vascular smooth muscle cells (VSMCs) retain phenotypic plasticity, which allows them to switch from a fully differentiated and quiescent “contractile” phenotype to a dedifferentiated and proliferative “synthetic” phenotype. This phenotypic change plays a role during vascular remodeling and development of vascular diseases, such as atherosclerosis and diabetic pulmonary arterial hypertension. The molecular mechanism of regulating this switch, however, is not fully understood. We investigated the role of HFL2 in the regulation of SM phenotype in VSMCs. It has been reported that overexpression of HFL2 suppresses RhoA-stimulated induction of SM-specific gene promoters using reporter assays. We show that HFL2 antagonizes both basal and BMP4-induced expression of SM-specific genes including a-smooth muscle actin, SM22, and calponin. Overexpression of HFL2 in pulmonary artery smooth muscle cells (PASMCs) by recombinant adenovirus reduced expression of SM genes and promoted a morphological change that mimics the “synthetic” phenotype. Consistently, downregulation of endogenous HFL2 by siRNA in PASMCs increased the basal expression of SM genes. We also found that HFL2 is, at least in part, required for suppression of SM genes by PDGF-BB treatment. These results suggest that HFL2 may play a critical role in dedifferentiation of SMCs.

Foxo Transcription Factors Activate Akt and Attenuate Insulin Signaling Through Inhibition of Protein Phosphatases

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Insulin resistance and metabolic syndrome are rapidly expanding public health problems. Acting through the PI3K/Akt pathway, insulin and insulin-like growth factor-1 (IGF-1) inactivate Foxo transcription factors, a class of highly conserved proteins important in numerous physiological functions. However, Foxo is a downstream target of insulin, Foxo also controls upstream signaling elements governing insulin sensitivity and glucose metabolism. Here, we report that sustained activation of either Foxo1 or Foxo3 in cardiac myocytes increases basal levels of Akt phosphorylation and kinase activity. Foxo-activated Akt directly interacts with and phosphorylates Foxo, providing feedback inhibition. We reported previously that Foxo factors attenuate cardiomyocyte calcineurin (PP2B) activity. We now show that calcineurin forms a complex with Akt, and inhibition of calcineurin enhances Akt phosphorylation. In addition, Foxo activity suppresses protein phosphatase 2A (PP2A) and disrupts Akt-PP2A and Akt-calcineurin interactions. The Foxo-mediated repression of Akt-PP2A interactions and phosphatase activities contributes, at least in part, to increased Akt phosphorylation and kinase activity. Importantly, Foxo-dependent increases in Akt activity diminish insulin signaling, as manifested by reduced Akt phosphorylation, reduced membrane translocation of Glut4, and decreased glucose uptake in response to insulin. Conversely, genetic inactivation of Foxo3 enhances insulin-dependent Akt phosphorylation. Finally, reverterant, an activator of Sirt1, increases nuclear localization of Foxo1 in cardiac myocytes, and thus increases its transcriptional activity. Taken together, this study demonstrates that changing Foxo activity have a dose-responsive inhibitory effect on insulin signaling in cardiomyocytes through altered basal Akt phosphorylation levels, culminating in reduced insulin sensitivity and impaired glucose metabolism.

Protein Expression of Stem Cell Markers Is Increased in Left Ventricular Myocardium of Patients and Dogs with Heart Failure

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Background: Sca1 and c-Kit are key stem cell markers that, when observed in tissue, provide evidence for the presence of bone marrow stem cells (BMSC). We previously showed that ongoing cardiomyocyte injury, degeneration and loss occur in the failing left ventricle (LV). This ongoing myocardial injury creates an ideal substrate for colonization of the myocardium by circulating BMSC; the latter are decreased in dogs with chronic heart failure. In this study, we tested the hypothesis that protein expression for Sca1 and c-Kit may be increased in the failing LV myocardium in response to ongoing injury, degeneration and loss of cardiomyocytes. Methods: Protein expression for Sca1 and c-Kit was measured in LV myocardium of explanted dogs with heart failure comparing data from normal donor hearts transplanted (NL-Dog, n=6), normal donor human hearts which were deemed not suitable for transplantation (NL-Human, n=6) and LV myocardium of patients with ICM and IDC compared to LV myocardium from NL-Dog (ICM, n=32, IDC, n=5). Results: Data are shown in the table. Protein expression for Sca1 and c-Kit was significantly increased in LV myocardium of patients with ICM and IDC compared to LV myocardium from NL-Dog and IDC hearts as well as in LV myocardium of HF-dogs compared to NL-Dogs. Conclusions: The results of this study indicate that in LV myocardium of explanted failed human hearts, regardless of etiology, as well in LV myocardium of dogs with experimentally-induced HF, protein expression for Sca1 and c-Kit is increased. This upregulation of key stem cell markers in HF suggest the possibility that colonization of the myocardium by circulating BMSC occurs in HF and may also explain the decrease in circulating BMSC observed in dogs with HF.

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<th>Protein Expression of Sca1 and c-Kit</th>
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<td>Sca1 (dil)</td>
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* p<0.05 vs NL-Human; † p<0.05 vs NL-Dog
PKCe Mediates Nonischemic Preconditioning-Dependent Bradykinin-2 Receptor Signaling

Xiaping Ren, Siyun Liao, Jo El J Schultz, Maria Brown, Walter K Jones; Univ of Cincinnati, Cincinnati, OH

Introduction: We previously demonstrated a cardioprotective effect of non-ischemic surgical trauma (abdominal incision) against I/R injury that has both a late and early phase, similar to ischemic preconditioning (IPC). We further showed that this novel phenomenon, remote preconditioning of trauma (RPCT), requires Bradykinin signaling. Hypothesis: The late phase of RPCT elicits a cardioprotective response by activating a signaling pathway that includes BK2R, PKCe and NF-kB. Methods: RPCT was initiated in vivo, 24 h (late RPCT) before 45 min I/R and infarct size determined. Blockade of NF-κB (IkBDN transgenic mice) and iNOS gene ablation were employed to determine the effect upon MI after RPCT. RPCT was performed with or without pretreatment with chelerythrine (Smg/kg), a non-selective PKC inhibitor. Translocation of PKCα-isoforms from cytosol to the particulate fraction were measured by quantitativeimmunoblotting in wild type and bradykinin 2 receptor knockout (BK2R KO) mice 24 h after RPCT. Results: Late RPCT significantly reduced infarct size (55.3% vs. 33.2% P<0.05). NF-κB blockade prevented development of late RPCT (40.7±1.8% vs. 36.8%, P>0.05). iNOS is not required for the protective effect of late RPCT (39.2±4.1% in sham vs. 15.4±2.7% in iNOS knockout mice, P<0.05). The cytosolic to particulate ratios were shifted for PKCe and PKCd 24 h after late RPCT (PKCe, 2.03±0.27 to 2.81±0.15, P<0.05; for PKCd, 2.3±0.5 vs. 0.9±0.17, P=0.05). Moreover, the effects of RPCT upon PKC isoform localization were abrogated in BK2R KO mice. PKCa activation was not affected by RPCT. Conclusions: The protective effect of RPCT against MI involves bradykinin signaling upstream of PKC modulation. Specifically, PKCe is activated and PKCd is repressed, 24h after a RPCT stimulus. This BK-dependent modulation of PKCe and PKCd is associated with activation of NF-κB, which is involved in the phenomena of late RPCT. Such a cardioprotective non-ischemic phenomenon may be clinically useful particularly peri-surgically and in patients at risk for MI.

Enantiomer-Specific β2-Adrenergic Receptor–G–Protein Coupling in Cardiomyocytes

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β2-Adrenoceptor (β2AR) couples dually to G and pertussis toxin (PTX)-sensitive G proteins, resulting in functionally opposing effects on cardiomyocyte contractility. A fundamental question regarding receptor-G protein interaction is, therefore, whether different agonists can traffic receptors to different G proteins. We have recently shown that activation of PKC-isoforms from cytosol to the particulate fraction were measured by quantitativeimmunoblotting in wild type and bradykinin 2 receptor knockout (BK2R KO) mice 24 h after RPCT. Results: Late RPCT significantly reduced infarct size (55.3% vs. 33.2% P<0.05). Moreover, the effects of RPCT upon PKC isoform localization were abrogated in BK2R KO mice. PKCa activation was not affected by RPCT. Conclusions: The protective effect of RPCT against MI involves bradykinin signaling upstream of PKC modulation. Specifically, PKCe is activated and PKCd is repressed, 24h after a RPCT stimulus. This BK-dependent modulation of PKCe and PKCd is associated with activation of NF-κB, which is involved in the phenomena of late RPCT. Such a cardioprotective non-ischemic phenomenon may be clinically useful particularly peri-surgically and in patients at risk for MI.
of Thr-phosphorylated cTnT. To explain the protoplastesis by H2O2 in wild-type PKCζ and Thr-phosphorylated cTnT, we examined caspase-3, a protease involved in apoptosis and oxidative stress. We found H2O2 activated caspase-3 as detected by the presence of the processed forms, and this explains the protoplastesis observed in wild-type PKCζ and Thr-phosphorylated cTnT. These data demonstrate that PKCζ and PP2A are sensitive to oxidative stress and alterations in this pathway affect phosphorylation and hence the regulation of the contractile proteins cTnI and cTnT in the heart.

**P120**

**Induction of Cytosolic Phospholipase A2 by Atorvastatin is eNOS- and iNOS-Dependent Whereas Pioglitazone Upregulates cPLA2 by eNOS- and iNOS-Independent Mechanism**

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**Background:** Three-day treatment with oral atorvastatin (ATV) or pioglitazone (PIO) increases the expression of cytosolic phospholipase A2 (cPLA2) in the rat heart. As the upregulation of Cyclooxygenase-2 (COX2) by ATV is eNOS- and iNOS-dependent, we sought to determine whether ATV and PIO upregulates cPLA2 expression and activity in eNOS and iNOS−/− mice. **Methods:** In 2 separate experiments WT, eNOS−/− and iNOS−/− mice received PIO (10 mg/kg) or water alone or ATV (10 mg/kg) or water alone by oral gavage for 3 days. On the fourth day hearts were explanted and assayed for cPLA2 activity (immunoblotting) and activity (ELISA). **Results:** ATV increased cPLA2 expression in the WT (227±3%; p<0.0001), eNOS−/− (237±4%; p<0.0001) and iNOS−/− (211±3%; p<0.0001) mice; however, cPLA2 activity increased only in the WT mice (Figure 1a). PIO increased cPLA2 expression in the WT (189±2%; p<0.0001) and iNOS−/− (141±7%; p<0.0002) mice. In the eNOS−/− mice the PIO effect was much smaller, yet significant (110±2%; p<0.0007). However, in contrast to ATV, PIO augmented cPLA2 activity in all three strains of mice (Figure 1a), suggesting a role for post-translation modification. **Conclusions:** In contrast to ATV, PIO activates cPLA2 in an eNOS- and iNOS-independent mechanism. As eNOS activity decreases with age, diabetes and advanced atherosclerosis, this particular effect may be relevant in the clinical setting. Further studies are needed to elucidate the mechanism of cPLA2 activation by the two classes of drugs.

**P121**

**Both β1- and β2-Adrenergic Receptors (ARs) Are Required for Pressure Overload Cardiac Hypertrophy**

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In isolated myocytes, hypertrophy induced by noradrenaline is mediated via α-ARs, however, in vivo, mice with deletions of both major cardiac β-ARs still develop hypertrophy with pressure overload. The mechanism by which the heart adapts to pressure overload, producing either adaptive or maladaptive remodeling is still not completely understood. To study the role of β-ARs in pressure overload hypertrophy, we performed transverse aortic constriction (TAC) in congenic mice with targeted deletions of β1, β2 and both β1 and β2-ARs and in sham controls. After 3 weeks, β1−/− mice showed a 21% increase in heart weight to body weight ratio (HW/BW) vs. sham, similar to WT (HW/BW 5.02±0.72 for β1−/− vs. 5.20±0.92 for WT). β2−/− mice showed an exaggerated (49%) hypertrophic response (HW/BW 5.81±0.53; p<0.001 vs. WT). Only when both β1 and β2-ARs were ablated was hypertrophy fully attenuated: in β1/2−/− mice HW/BW was not different from sham (β1/2−/− 4.88% vs. sham 4.69% ±0.31; p=1 vs. WT). Echocardiography showed that peak band gradient was not different between groups (WT 45.3±4.1; β1−/−: 47.2±10.2, β2−/−: 49.0±9.7, β1/2−/−: 53.2±11.3 mmHg), and all groups maintained normal LV function. Morphometric analysis confirmed the absence of hypertrophy in the β1/2−/−: mean cross-sectional area for WT was 254±7.49 μm2 vs. β1/2−/− 115.6±16.7 μm2, which was not different from sham. Gene microarray analysis detected a set of genes which were differentially expressed in β1−/−, β2−/− or β1/2−/− (β1−/− and β2−/−): (1) 113 calcium binding protein A8/calgranulin B (S100a5, 4.5-fold up); (2) Cyclin-dependent kinase inhibitor 1A/2B (Cdkn1a, 3.8-fold up); (3) Metallothioneins Mt1 (3-fold up) and Mt2 (2.7-fold up); (4) FK506 binding protein 5, a B (S100a9, 4.5-fold up); (2) Cyclin-dependent kinase inhibitor 1A/P21 (Cdkn1a, 3.8-fold up); (3) DNA tumor suppressor BRCA1-associated protein 1, a (Btrca1, 2.3-fold up); (4) Ring finger protein 56, tumor suppressor 2 (Rfp56, 2.3-fold up). To explain the proteolysis induced by H2O2 in wild-type PKCε, Thr-phosphorylated cTnT. To explain the proteolysis induced by the two classes of drugs. As eNOS activity decreases with age, diabetes and advanced atherosclerosis, this particular effect may be relevant in the clinical setting. Further studies are needed to elucidate the mechanism of cPLA2 activation by the two classes of drugs.

**P122**

**Activation of Phosphoinositide 3-Kinases/Akt Pathway Is Responsible for Cardioprotection Induced by Modulation of TR2**

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Activation of the phosphoinositide 3-kinase/Akt (PI3K/Akt) signaling pathway protects cells from ischemic injury. We have reported that the immune modulator glucan, an immune-activating lectin, induces cardioprotection. Dectin-1 is a specific receptor for glucan and Toll-like receptor 2 (TLR2) is required for transmitting the signal from Dectin-1 into cells. However, the role of modulation of TLR2 in cardioprotection has not been investigated. We hypothesized that modulation of TLR2 will induce cardioprotection through a PI3K/Akt-dependent mechanism. To evaluate our hypothesis, we examined the effect of modulation of TLR2 on myocardial ischemic injury. TLR2 knockout (KO) (n=8) and wild type mice (n=8) were treated with glucan (1 mg/kg) or pdotebodycon (PDG, 75 μg/25g), a specific TLR2 ligand, one hr before the hearts were subjected to ischemia (1 hr)/reperfusion (4 hrs). Untreated controls (n=8) were treated with PBS. Myocardial infarction was determined by TTC staining. Infarct size was significantly reduced in glucan (11.6 ± 2.38% vs 36.1 ± 3.48%, p<0.01) and PDG (10.5 ± 3.03% vs 30.1 ± 7.59%, p<0.01) treated mice vs untreated mice. The levels of phospho-Akt (0.10 ± 0.15 vs 0.45 ± 0.09) and phospho-GSK3β (0.66 ± 0.14 vs 0.33 ± 0.10) were significantly increased in the myocardium of glucan treated mice compared with untreated mice. However, both glucan and PDG-induced cardioprotection were completely abolished in TLR2 KO mice. To investigate whether PI3K/Akt signaling is involved in cardioprotection induced by modulation of TLR2, we administered glucan (1 mg/kg) or PDG (75 μg/25g) to kdstat (kinase deficient) transgenic mice (n=8) one hr before myocardial I/R. Both glucan and PDG-induced cardioprotection were completely abolished in kdStat mice. The results suggest that modulation of TLR2, either direct or indirect, will induce cardioprotection through a PI3K/Akt dependent mechanism and that there is a critical link between TLR2 and PI3K/Akt signaling during myocardial I/R.
or empty plasmid. The transient transfection of C2C12 cells with pHL indicates an increased activity (by 2.3fold) of the reporter gene implying a regulatory role for the pHL sequence. In conclusion, our data indicate that FAK activation regulates phospholamban gene expression possibly by an interaction with MEF2 and that the intronic of pHL sequence plays a role in the expression of PLN in response to mechanical stress in cardiac myocytes.

**P125**

A Novel Role for Mitochondrial-Localized Stat3 in Cardiac Mitochondria

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Regulation of immune responses and cell growth by pro and anti-inflammatory cytokines is mediated through activation of the Stat3 transcription factor. The current model dictates that binding of cytokines including IL-6 and IL-10 to cell surface receptors induces tyrosine and serine phosphorylation of Stat3 resulting in nuclear translocation, binding to a GAS enhancer in Stat3-responsive genes, and downstream transcriptional responses. We provide the first evidence for the presence of Stat3 in mitochondria (MITO) of cultured cells and primary tissues including liver and heart. In Stat3-/- primary pro B cells the activities of complexes I and II of the electron transport chain (ETC) are decreased 50-90%. MITO-targeted Stat3 expressed in Stat3-/- cells restores the function of complexes I and II. Floxed Stat3 mice were crossed with transgenic mice that express Cre recombinase under the control of the α-myosin heavy chain promoter whose expression is restricted to cardiomyocytes. MITO from the hearts of 8 week old Stat3 flox/flox mice (Stat3-/-) and Stat3 +/+ mice (WT) were isolated. Hearts are normal as assessed by histology and physiological parameters. Stat3 content in heart tissue and MITO from Stat3 flox/flox mice varied between 5 and 20% of Stat3 +/+ mice. Stat3-/- mice exhibit decreased rates of oxidative phosphorylation with complex I (72% decrease, n=5 Stat 3 -/- vs. n=5 WT, p<0.01) and complex II (64% decrease, n=7 Stat 3 -/- vs. n=8 WT, p<0.01) substrates. These defects are due to decreased complex I and complex II enzyme activities. Thus, MITO-localized Stat3 contributes novel functions which may orchestrate responses to cellular stress and innate immunity through modulating the activity of the ETC. Since partial inhibition of the ETC during ischemia or early reperfusion can attenuate cardiomyocyte injury, inactivation of stat3 during these periods opens a new approach to address mitochondrial-driven cardiomyocyte injury during ischemia and early reperfusion.
4th Annual Symposium of the American Heart Association Council on Basic Cardiovascular Sciences: Cardiovascular Repair and Regeneration: Structural and Molecular Approaches in the Cellular Era July 30–August 2, 2007 Keystone Conference Center Keystone, CO

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