Abstract—Apoptosis of cardiomyocytes is increased in heart failure and has been implicated in disease progression. The activation of “proapoptotic” caspases represents a key step in cardiomyocyte apoptosis. In contrast, the role of “proinflammatory” caspases (caspases 1, 4, 5, 11, 12) is unclear. Here, we study the cardiac function of caspase-1. Gene array analysis in a murine heart failure model showed upregulation of myocardial caspase-1. In addition, we found increased expression of caspase-1 protein in murine and human heart failure. Mice with cardiomyocyte-specific overexpression of caspase-1 developed heart failure in the absence of detectable formation of interleukin (IL)-1β or IL-18 and inflammation. Transgenic caspase-1 induced primary cardiomyocyte apoptosis before structural and molecular signs of myocardial remodeling occurred. In contrast, deletion of endogenous caspase-1 was beneficial in the setting of myocardial infarction–induced heart failure. Furthermore, caspase-1–deficient mice were protected from ischemia/reperfusion-induced cardiomyocyte apoptosis. Studies in primary rat cardiomyocytes indicated that caspase-1 induces cardiomyocyte apoptosis primarily through activation of caspases-3 and -9. In contrast to previous findings, which imply a proinflammatory role of caspase-1, these data suggest a primary proapoptotic role for caspase-1 in cardiomyocytes. Our findings support a functional role for caspase-1–mediated myocardial apoptosis contributing to the progression of heart failure. (Circ Res. 2007;100:645-653.)

Key Words: caspase-1 • transgenic mouse • heart failure

Heart failure is among the most frequent causes of death and the leading cause of hospitalization in industrialized countries. Despite significant progress in the pharmacological treatment of heart failure, the clinical course of the disease is still nearly always progressive. Apoptosis of cardiomyocytes is increased in human heart failure1-2 and several studies have subsequently suggested that cardiomyocyte apoptosis is a central mechanism in heart failure.3,4 Both the intrinsic (mitochondrial) and the extrinsic (death receptor mediated) pathway have been shown to activate caspase-3. Activation of caspase-3 is followed by cardiomyocyte apoptosis, which is sufficient per se to induce cardiac failure.5 Inhibition of apoptosis through low-molecular-weight caspase inhibitors has been shown to slow the progression of experimental heart failure,6,7 and this correlated with a reduction of caspase-3 activity.

Caspases are cysteine proteases that cleave their specific substrates after an aspartate residue. Caspases have been divided into proapoptotic (Caspases 2, 3, 6, 7, 8, 9, 10) and proinflammatory (Caspases 1, 4, 5, 11, 12) respectively.8,9 Whereas proapoptotic caspases have been implicated in the pathogenesis of various cardiovascular disorders including heart failure and cardiac ischemia,10-12 the role of the proinflammatory caspases in cardiovascular diseases is less understood.

Caspase-1 (also termed interleukin-converting enzyme), a member of the proinflammatory family of caspases, was discovered through its proteolytic activation of pro-IL-1β and -18 into their active form.13,14 Caspase-1 is synthesized as an weakly active 45-kDa precursor that is autocatalytically processed via an intermediate into p10 and p20 subunits, forming the enzymatically full active heterodimeric or tetrameric enzyme.15-18

Controversial data regarding the functional role of caspase-1 have been obtained in different species and assay systems. Whereas few studies have implicated caspase-1 in programmed cell death in certain cell types,19,20 other studies argue against a general role for caspase-1 in the regulation of apoptosis.21,22 Recent studies indicate a important role for caspase-1 in cardiac ischemia.23-25 These studies implicate both the formation of interleukins25 and the induction of apoptosis23,24 in the detrimental effects of caspase-1 activation during ischemia. In contrast, the role of caspase-1 in the development of heart failure has not been studied to date.

Here we provide evidence that caspase-1 expression is increased in murine and human heart failure and that
caspase-1 constitutes a critical regulator of cardiomyocyte programmed cell death in the mammalian heart.

Materials and Methods

Generation of Transgenic Mice
Caspase-1 transgenic mice were generated by pronuclear injection of fertilized oocytes from FVB/N mice with a transgene construct containing the coding sequence of the human caspase-1α isoform under the control of the murine α-myosin heavy chain (α-MHC) promoter. Experiments were performed with mice of line TG10, unless indicated otherwise. All mice were housed in a specified pathogen free facility. All animal experiments were approved by the responsible authorities (protocol no. 6212531.01-10/98, 28/01, and 31/03).

Adenovirus Generation
Recombinant adenoviral vectors were generated according to standard procedures. Caspase-1 (human caspase-1α isoform) was cloned by PCR and introduced into a adenoviral vector (pAd/CMV/5-DEST, Invitrogen, Carlsbad, Calif) by homologous recombination. A corresponding LacZ-expressing adenoviral vector (pAd/CMV/LacZ) was used as control. Adenovirus titers were determined by plaque assays in HEK293 monolayer cultures embedded in agarose.

Human Cardiac Tissue
Failing hearts were obtained from patients undergoing heart transplantation resulting from terminal heart failure. Nonfailing donor hearts that could not be transplanted for technical reasons were used as controls. Donor patient histories and echocardiography revealed no signs of heart disease. Immediately after removal of the hearts, samples were taken, snap-frozen in liquid nitrogen and stored at −80°C. In accordance with the Declaration of Helsinki, written informed consent was obtained from all patients or the families of prospective heart donors before cardiectomy. The experiments were approved by the Ethics Committee of the Würzburg Medical Faculty.

Cardiomyocyte Isolation
Neonatal and adult cardiomyocytes were isolated as described previously.26,27 Cardiomyocyte length and width were determined from digitally recorded images using the MetaVue software package (Visitron, Puchheim, Germany).

Determination of IL-1β and -18
IL-1β ELISA (mouse IL-1β ELISA Kit, EMIL1B, Pierce Endogen, Ill) and IL-18 ELISA (mouse IL-18 ELISA, BMS618, Bender Med Systems, Vienna, Austria) were performed with lysates from whole left ventricular myocardium and sera of 2-month-old caspase-1 transgenic and corresponding wild-type animals. For details, see the expanded Materials and Methods section in the online data supplement.

Determination of Cardiomyocyte Apoptosis and Cell Viability
Nuclear DNA fragmentation was detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining using a commercial kit (Roche Diagnostics, Indianapolis, Ind). For details, see the expanded Materials and Methods section in the online data supplement.

Myocardial Infarction, Ischemia/Reperfusion, Hemodynamic and Echocardiographic Analysis
See the expanded Materials and Methods section in the online data supplement.

Histochemical and Immunohistochemical Analyses
Interstitial fibrosis, cardiomyocyte hypertrophy, and determination of necrosis and reactive oxygen species formation were determined as described previously.28–31 For further details and immunohistochemical analysis, see the expanded Materials and Methods section in the online data supplement.

RNA Isolation and Real-Time RT-PCR
See the expanded Materials and Methods section in the online data supplement.

Immunoblotting
See the expanded Materials and Methods section in the online data supplement.

Statistical Analysis
Average data are presented as mean±SEM. Statistical analysis was performed using the Prism software package (GraphPad, San Diego, Calif). ANOVA followed by Bonferroni test and Student’s t test were used as appropriate. Differences were considered significant when P<0.05 and are indicated by an asterisk in the figures.

Results

Upregulation of Caspase-1 in Heart Failure
To identify genes differentially expressed early during the development of heart failure, we used a well-characterized mouse model of β,1-adrenergically induced heart failure.32 Using cDNA arrays, we screened the expression of 1176 mouse genes and observed consistent upregulation of caspase-1 mRNA (data not shown). We then determined caspase-1 expression in murine and human heart failure. We found a significant upregulation of caspase-1 mRNA and protein in the left ventricular myocardium of β,1-adrenergic receptor transgenic mice at 6 months of age, ie, before the clinical onset of heart failure (Figure 1A and 1B). Analysis of left ventricular lysates from 4 nonfailing and 9 failing human hearts revealed a significant upregulation of caspase-1 also in human heart failure (2.2-fold versus nonfailing, P<0.05) (Figure 1C).

Caspase-1 Induces Cardiomyocyte Hypertrophy Despite Unchanged Ventricular Weights
To test the hypothesis that increased expression of caspase-1 contributes to the development of heart failure, transgenic mice with overexpression of caspase-1 in the heart were generated. We used the murine α-MHC promoter to target expression of the caspase-1 cDNA to cardiac myocytes (Figure 2A, top). We identified 4 positive founder mice, 2 of which carried the transgene in their germline and gave rise to 2 independent transgenic lines (termed TG1 and TG10; Figure 1A in the online data supplement). Western blotting demonstrated significant overexpression of transgenic caspase-1 protein, which was confined to the ventricular myocardium (endogenous caspase-1 is expressed in the spleen and, to a lesser extent, in the lung33,34), with slightly higher levels in TG10 as compared with TG1 (supplemental Figure IB and IC). Figure 2A, bottom, shows expression of caspase-1 (45 kDa) and its processing.

Despite the marked ventricular dilatation observed in caspase-1 transgenic mice (Figure 3A and 3B, bottom left), the ventricular weights of caspase-1 transgenic hearts were

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not increased at any age studied (Figure 2B, top). However, we detected intense remodeling of the ventricular myocardium from 4 months onward. Whereas no significant cardiomyocyte hypertrophy was present at 1 month of age, cardiomyocyte size progressively increased starting at 4 months of age (Figure 2B, bottom). In addition, we analyzed the cell dimensions of isolated adult cardiomyocytes from 5-month-old caspase-1 transgenic and wild-type mice. The average cardiomyocyte length increased from 117 to 130 μm (P<0.01), and the width increased from 26 to 37 μm (P<0.0001) through overexpression of caspase-1 (n=60 cells per genotype).

Messenger RNA expression of atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), and sarcoplasmic reticulum ATPase (SERCA) in the myocardium of caspase-1 transgenic mice was unchanged in 1-month-old mice (Figure 2C). ANF and β-MHC expression increased, whereas SERCA mRNA decreased at 4 months of age.

In addition, we stained ventricular sections from wild-type and caspase-1 transgenic hearts from different age groups with Sirius red to assess the extent of interstitial collagen deposition. We detected interstitial fibrosis throughout the ventricular myocardium in caspase-1 transgenic mice from 4 months of age onward but not in 1-month-old animals (Figure 2D). Likewise, line TG1 displayed significant cardiomyocyte hypertrophy and increased interstitial collagen content as compared with wild-type control mice (data not shown).

Taking the marked cardiomyocyte hypertrophy and the prominent interstitial fibrosis into account, the absence of an increase in the left ventricular weight of these animals suggested a marked loss of cardiomyocytes. To prove loss of cardiomyocytes in this setting, one would ideally like to determine precisely the total number of myocytes as well as other cell types for an adult mouse heart, a technically very difficult undertaking. Rather, we aimed to test a direct apoptotic effect of caspase-1 in cardiomyocytes in a defined in vitro setting (see Figure 5).

Expression of Caspase-1 Is Sufficient to Induce Cardiac Failure, Whereas Deletion of Endogenous Caspase-1 Ameliorates Postinfarction Heart Failure

Although caspase-1 transgenic mice displayed normal cardiac morphology at young age (2 months), we observed thinning of the left ventricular wall and cardiac dilatation with increasing age. In addition, left atrial dilatation and thrombus formation were frequently observed in caspase-1 transgenic mice, consistent with the development of cardiac failure (Figure 3A). When the left ventricular function of these mice was assessed in vivo by left ventricular catheterization using a 1.4 F micromanometer tip catheter, a significant impairment of left ventricular contractility in caspase-1 transgenic mice (1 year of age) became apparent (P<0.05) (Figure 3B, top left). This was not caused by alterations in heart rate, as the beating frequency was the same in wild-type and caspase-1 transgenic hearts (471±18 bpm, wild-type, versus 468±22 bpm, caspase-1 transgenic) (data not shown). In addition, there was a modest but significant decline of the maximal left ventricular pressure (105±2 mm Hg, wild-type, versus 88±2 mm Hg, caspase-1 transgenic, P<0.0001) and a marked increase of left ventricular end-diastolic pressure, indicating impaired cardiac function (Figure 3B, top right). Furthermore, echocardiographic analysis demonstrated cardiac dilatation and a decrease in fractional shortening in caspase-1 transgenic mice (Figure 3B, bottom left and right).

We then assessed the functional role of endogenous caspase-1 in a model of postinfarction cardiac failure. Homozygous caspase-1–deficient mice were subjected to myocardial infarction through permanent left anterior descending ligation and followed for 8 weeks thereafter. Although we did not observe a significant influence of endogenous caspase-1 on infarct size (Figure 3C, top left), caspase-1–deficient mice displayed reduced cardiomyocyte hypertrophy in comparison with wild-type control mice (Figure 3C, top middle and right). Survival was doubled in caspase-1–deficient mice compared with wild-type mice (Figure 3C, bottom left). Furthermore, deletion of caspase-1 rescued in part the decline of left ventricular function observed in wild-type animals after myocardial infarction (Figure 3C, bottom right). Interestingly, this occurs independent of infarct size. These data provide evidence that endogenous caspase-1 activity indeed contributes to the deterioration of left ventricular function after myocardial infarction.
Caspase-1 Induces Cardiomyocyte Apoptosis Independent of Interleukin Formation and Inflammation

Given that caspase-1 and its downstream regulated cytokines have been implicated in the proinflammatory response to a variety of stimuli in various cell types, we determined sera and cardiac IL-1β and IL-18 levels of caspase-1 transgenic and wild-type mice. Despite robust overexpression of caspase-1, IL-1β, and IL-18 levels in the myocardium and sera of caspase-1 transgenic mice were essentially indistinguishable from those of control hearts (Figure 4A). The absence of IL-1β formation was confirmed by immunohistochemistry (Figure 4B, left). Furthermore, we studied several proinflammatory mediators that have been implicated in the downstream response of IL-1β formation. IL-1α, IL-4, IL-6, tumor necrosis factor-α, interferon-γ, and granulocyte/macrophage colony-stimulating factor were essentially unchanged within the myocardium of caspase-1 transgenic compared with wild-type mice (data not shown). In parallel, we assessed the presence of inflammatory cells such as neutrophilic granulocytes and lymphocyte subsets, which represent the prototypical inflammatory response to necrotic cell death. All of these markers yielded identical results for wild-type and caspase-1 transgenic mice. However, staining for macrophages (CD18 positive and CAE negative) as typical postapoptotic response showed increased numbers of macrophages in the myocardium of caspase-1 transgenic mice already at 2 months of age (supplemental Figure II).

To directly determine whether cardiomyocyte necrosis occurs to a significant extent, we performed immunohistochemistry and stained for complement component C9, a marker for necrotic cell death. We did not observe a significant increase of complement 9 staining in caspase-1 transgenic mice as compared with wild-type mice (Figure 4B, right). In accordance with these findings, we found reactive oxygen species formation as assessed by 8-hydroxy-2′-deoxyguanosine and dihydroethidium staining to be essentially unaltered between wild-type and caspase-1 transgenic mice (data not shown).

Given that an alternative mechanism of action of caspase-1 is the induction of programmed cell death, we determined the extent of apoptosis in the myocardium of caspase-1 transgenic mice by TUNEL. In contrast to the structural alterations observed during the remodeling process, which appeared only with increasing age of the animals (see Figure 2), apoptosis was markedly increased already at the youngest age group studied (Figure 4C, top and bottom left). In addition, we found a massive increase of apoptosis in adult cardiomyocytes isolated from caspase-1 transgenic mice (Figure 4C, right), confirming that caspase-1 is sufficient to induce cardiomyocyte apoptosis.

In a series of complementary experiments, we demonstrate the contribution of endogenous caspase-1 to the induction of cardiomyocyte apoptosis in vivo. For the rapid induction of a high extent of cardiomyocyte apoptosis, we used a 30-minute
Caspase-1 Induces Cardiomyocyte Apoptosis via Activation of Caspases-3 and -9

To assess the proapoptotic effect of caspase-1 in a defined experimental setting in vitro, we generated recombinant adenoviral vectors (Adv-Casp-1) to express caspase-1 in neonatal rat cardiomyocytes. Western blot analysis confirmed activation of adenoviral-expressed caspase-1 (Figure 5A). Adv-Casp-1 led to a dose-dependent induction of cardiomyocyte apoptosis 48 hours after infection. Already at low multiplicity of infection (MOI) (MOL), cardiomyocyte apoptosis increased 4.6±0.6-fold relative to neonatal rat cardiomyocytes infected with a adenovirus expressing LacZ (Adv-lacZ) (Figure 5B, left and top right). Analysis of cell viability confirmed the dose-dependent cardiomyocyte death induced by expression of caspase-1 (Figure 5B, bottom right).

We further assessed the activation of caspase-3 as a hallmark of cardiomyocyte apoptosis. Expression of
caspase-1 in neonatal rat cardiomyocytes led to a significant increase of caspase-3 activation (Figure 5C, left). To investigate whether the intrinsic and/or extrinsic pathways are activated through caspase-1 in neonatal rat cardiomyocytes, we determined the activation of caspase-9 (intrinsic pathway) and caspase-8 (extrinsic pathway). Whereas we found caspase-8 activity essentially unchanged (data not shown), cleaved caspase-9 was significantly increased after incubation of neonatal rat cardiomyocytes with Adv-Casp-1 (P<0.0001; Figure 5C, right). The apoptotic response induced by caspase-1 was blocked both by the broad-spectrum caspase inhibitor zVAD-fmk and the caspase-1–specific inhibitor RU 36384 but not by the caspase-8–specific inhibitor zIETD-fmk (Figure 5D).

Discussion

This work indicates a important role for caspase-1 in the development of heart failure. Caspase-1 is upregulated in experimental and in human heart failure and acts as a potent proapoptotic caspase both in isolated cardiomyocytes and in vivo.

We confirmed that caspase-1 protein is upregulated in murine heart failure, which corroborates earlier data obtained on the mRNA level.37-39 The early and parallel occurrence of
Figure 5. Caspase-1 induces cardiomyocyte apoptosis via activation of caspases-3 and -9. A, Western blot analysis demonstrating activation of adenovirally expressed caspase-1 (10 mosi, 48 hours after adenoviral infection). B, left, Overlay of TUNEL staining (green cardiomyocyte nuclei) and immunofluorescence for α-actinin (red) of isolated rat cardiomyocytes 48 hours after adenoviral infection with Adv-Casp-1 or Adv-LacZ (30 mosi). Nuclei are stained with Hoe33258 (blue). B, top right, Detection of apoptosis in neonatal rat cardiomyocytes after adenoviral expression of caspase-1. Quantification of TUNEL staining 48 hours after adenoviral transfection with MOI ranging from 1 to 30 (2 or Adv-LacZ (30 mosi). Nuclei are stained with Hoe33258 (blue). B, top right, Detection of apoptosis in neonatal rat cardiomyocytes after adenoviral infection) using the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) viability assay. Each bar represents 3 individual experiments with n = 6 each. B, bottom right, Quantitative determination of cell viability after adenoviral transfection (MOI from 1 to 30, 34 hours of incubation after adenoviral infection) using the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) viability assay. Each bar represents 3 individual experiments with n = 2 to 6. C, Western blot analysis to determine activation of caspases-3 and -9 after adenoviral expression of caspase-1 (10 mosi; 24 hours of incubation). Membranes were probed with antibodies directed against the cleaved isoforms of caspases-3 and -9. Coomassie staining served as protein loading control. Each bar represents the mean of 7 individual determinations from 2 independent sets of experiments. D, Determination of cell viability (MTT viability assay) after incubation with a broad-spectrum caspase inhibitor (zVAD-fmk) (left), a caspase-8–specific inhibitor (zIETD-fmk) (middle), and a caspase-1–specific inhibitor (RU 36384) (right). Each bar represents 3 to 5 individual experiments with n = 3 to 4.

caspase-1 expression and cardiomyocyte apoptosis supports the notion that these 2 events are causally related. This is followed (but not paralleled) by myocardial remodeling, the induction of a fetal gene expression program, and, finally, the development of overt heart failure. Deletion of endogenous caspase-1 was without effect under basal conditions but ameliorated the development of heart failure after myocardial infarction. In the setting of ischemia/reperfusion (known to be a very strong stimulus for cardiomyocyte apoptosis), deletion of caspase-1 effectively protected against cardiomyocyte apoptosis. Comparably modest expression of caspase-1 in isolated cardiomyocytes at low multiplicities of infection revealed the potent proapoptotic activity of caspase-1. Thus increased caspase-1 expression appears to primarily induce cardiomyocyte apoptosis which, consecutively, leads to the development and progression of heart failure.

In contrast to heart failure, several studies have investigated the role of caspase-1 in the pathogenesis of ischemic heart disease. Most importantly, mice deficient in caspase-1 have been subjected to myocardial infarction and followed for up to 9 days.24 These mice displayed a significant reduction in mortality within 24 hours after myocardial infarction, correlating with reduced levels of cardiomyocyte apoptosis. We also saw a marked improvement of survival resulting from deletion of endogenous caspase-1. Syed et al reported that cardiomyocyte caspase-1 induces apoptosis during cardiac ischemia,25 whereas a study on isolated human atrial tissue found ischemic myocardial dysfunction to be a result of caspase-1–dependent IL-1 formation.25

Our data argue for activation of caspase-1 under basal conditions in the heart in vivo and thus are in line with several other studies that have defined an autocatalytic mechanism of caspase-1 activation.17,18 However, there is 1 recent study in which caspase-1 activation was detected only under conditions of cardiac ischemia.23 At present, we cannot identify the relevant factors, which might contribute to the observed differences to what Syed et al have reported. Potential differences are the gender of the animals studied (whereas we have studied exclusively male mice, Syed et al did not indicate the sex of the mice used for their study) and factors such as the animal chow and the level of transgene expression.

Our data suggest that the marked increase of caspase-1 expression in human failing myocardium is pathophysiological relevant, however, not through the formation of proin-
flammatory cytokines, as might be assumed. Increased IL-1β serum levels have been described in patients with heart failure and have been suggested to contribute to the progression of the disease.\textsuperscript{41,42} Although we initially speculated that this might be caused, at least partly, by the marked increase of caspase-1 expression in the failing heart, our data do not support a role for cardiac caspase-1 in this regard. We have determined serum and tissue levels of IL-1β in caspase-1 transgenic mice and found that IL-1β levels were low and unaltered. This is unlikely attributable to the expression of the human caspase-1 isoform in our transgenic model, as human caspase-1 has been found to be highly similar to murine caspase-1 with respect to its substrate requirements and its inhibition by specific inhibitors.\textsuperscript{43} Specifically, the cleavage of the murine IL-1β precursor is nearly identical to that of the human IL-1β precursor.\textsuperscript{44}

As an additional test for potential subthreshold quantities of IL-1β formation, we assessed typical IL-1β-mediated effects on cardiomyocytes that have been previously reported. In this respect, several studies have shown cardiomyocyte hypertrophy and activation of the fetal gene expression program through IL-1β.\textsuperscript{45–48} In sharp contrast, we found unchanged cardiomyocyte dimensions and a completely normal fetal gene expression profile in 1-month-old animals. Furthermore, IL-1β, as well as various proinflammatory mediators known to be upregulated through IL-1β were not detectably elevated in caspase-1 transgenic mice. However, even at this early age, cardiomyocyte apoptosis was very prominent throughout the left ventricular myocardium, thus providing evidence for caspase-1 exerting a proapoptotic function specifically in cardiomyocytes.

In contrast to previous findings obtained in other cell types implying a proinflammatory role of caspase-1, our data suggest a primary proapoptotic role for caspase-1 in the heart. Caspase-1 expression results in cleavage of caspases-9 and -3, but not caspase-8, suggesting activation of the intrinsic pathway of apoptosis. Taken together, our data reveal a novel functional role for caspase-1 in the pathogenesis of experimental and human heart failure. Contrary to previous assumptions, cardiac caspase-1 appears to primarily induce apoptotic cell death, as opposed to the formation of inflammatory cytokines. Given the availability of highly specific caspase-1 inhibitors,\textsuperscript{49} caspase-1 may represent a therapeutic target molecule to prevent the progression of cardiac remodeling during the course of heart failure.

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Disclosures

None.

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A Role for Caspase-1 in Heart Failure
Sabine Merkle, Stefan Frantz, Michael P. Schön, Johann Bauersachs, Monika Buitrago, Robert J.A. Frost, Eva M. Schmitteckert, Martin J. Lohse and Stefan Engelhardt

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Figure Legends to Supplementary Figures

Figure S1. Generation and Characterization of Caspase-1-Transgenic Mice

(A) Identification of the F₀-generation of caspase-1-transgenic mice by PCR from genomic DNA. Two out of four transgenic animals gave rise to independent transgenic lines (TG1 and TG10).

(B) Western blot analysis comparing the protein-expression levels of the two transgenic mouse lines TG1 and TG10 to wild-type animals. To visualize the expression of transgenic caspase-1 in direct comparison to endogenous caspase-1 within a single experiment, protein lysates from caspase-1-transgenic mice were diluted 1:5.

(C) Organ-panel of caspase-1-transgenic and wild-type mice to demonstrate cardiac-specificity of the α-myosin heavy chain promoter based transgenesis. Endogenous caspase-1 is expressed in the spleen and (to a lesser extent) in the lung of both wild-type and caspase-1-transgenic animals (n=3).

Figure S2. Macrophage Infiltration but Absence of Leukocyte Infiltration in Caspase-1-Transgenic Hearts

The presence of inflammatory cells was analyzed using cryosections (7 µm) of left ventricular myocardium from wild-type and caspase-1-transgenic mice at 2 months of age. Primary antibodies directed against CD4, CD8, CD18, and CAE-stain were used to determine the cellular infiltration of immune cells. Staining against CD31 served as positive control. Significant infiltration of macrophages (CD18 positive and CAE negative), but not of neutrophilic granulocytes (CD 18 positive and CEA positive) and lymphocytes (CD4, 8, 19) was detected. Examples shown are representative for 4 animals studied per genotype.
**Supplementary Methods**

**Determination of Interleukin-1β and -18**

Probes and biotinylated antibody reagents were applied to 96-well plates precoated with anti-mouse IL-1β antibody or anti-mouse IL-18 respectively, and incubated for 2 hours. After washing and addition of streptavidin-HRP plus tetramethylbenzidine (TMB) as substrate, the absorbance was measured on an ELISA plate reader set at 450 nm. IL-1β and IL-18 values were calculated using FLUOstar OPTIMA Software (BMG Labtechnologies, Offenburg, Germany).

**Determination of Cardiomyocyte Apoptosis and Cell Viability**

Briefly, deparaffinized tissue sections or formalin-fixed neonatal rat cardiomyocytes were permeabilized with proteinase K and counterstained with propidium iodide to visualize cell nuclei. Tissue sections were incubated with the TUNEL reaction mixture in the dark for 60 min at 37°C. For a positive control, pre-incubation with DNase was carried out, reactions performed without TdT served as negative controls. For the quantification of apoptosis in tissue sections, the number of TUNEL-positive cardiomyocytes (that displayed propidium iodide staining and that were located intracellularly in cardiomyocytes) was normalized to the total number of cardiomyocytes.

For TUNEL-staining of neonatal rat cardiomyocytes, isolated cells were seeded on glass cover slips in 24-well plates (2 x 10⁵ cardiomyocytes per well), TUNEL-staining and propidium iodide counterstain were performed 48 hours after adenoviral transfection as described above. The number of TUNEL-positive cells was normalized to the number of all cell nuclei. For Fig. 6a, immunofluorescence was performed after TUNEL-staining employing an α-actinin-specific antibody (clone EA-53, Sigma) diluted 1:1000. Cell nuclei were stained with Hoe33258 (Molecular Probes).

For the MTT viability assay, isolated cardiomyocytes were plated on 24-well plates (2 x 10⁵ cardiomyocytes per well) with the assay carried out 34 hours after adenoviral transfection. The medium was replaced by 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide in PBS) and incubated for 1 hour at 37°C. Cells were lysed and absorbance was measured at 570 and 650 nm. The broad-spectrum
caspase inhibitor (zVAD-fmk, final concentration 20 µM, R&D, Wiesbaden, Germany), or the caspase-8 specific inhibitor (zIETD-fmk, final concentration 20 µM, Calbiochem, CA) or the caspase-1 specific inhibitor RU 36384 (final concentration 5 µM, Aventis, Frankfurt, Germany) were added at the time of adenoviral infection and incubated for 34 hours.

Myocardial Infarction, Ischemia-Reperfusion, Hemodynamic and Echocardiographic Analysis

Caspase-1-deficient (NOD.Casp-1) and wild-type mice (NOD/L+S) were purchased from Jackson Laboratories (Boston, MA). Mice (2-3 months old) underwent coronary artery ligation for the production of myocardial infarction as described before 1,2. After echographic measurements, hearts were excised and the right and left ventricle were separated. The left ventricle was cut into transverse sections as previously reported 1. From the middle ring, 4 µm-sections were cut and stained with sirius red. Infarct size was determined by planimetric measurement using a digital image analyzer and calculated by dividing the sum of endocardial and epicardial circumferences of infarct areas by the sum of the total endocardial and epicardial circumferences.

Coronary ligation was carried out as described previously3. Briefly, mice (four months old) were anesthetized, placed on a heating pad, intubated and ventilated with a mixture of oxygen and isoflurane. After left lateral thoracotomy and exposure of the heart by retractors, the left anterior descending coronary artery (LAD) was ligated with a slipknot, which was removed 30 minutes later to allow reperfusion for 24 hours. After reperfusion, mice were anesthetized again and the left anterior descending artery was religated. To stain nonischemic tissue, 1 ml of 1% Evan’s blue solution was injected through the apex into the left cavity. Hearts were excised, quickly rinsed with PBS, cut into 5 transverse slices and incubated for 10 minutes with 1.5% 2,3,5-triphenyltetrazolium chloride (TTC, red) to stain for viable myocardium, the infarcted areas appear pale. After taking the weight of each slice, formalin-fixation was carried out overnight and each side was photographed. Area at risk (AAR) and infarct area (IA) were determined as described previously3.

Left ventricular catheterization was performed via the right carotid artery under anesthesia with tribromoethanol using a miniaturized pressure sensing catheter (1.4F Micro-tip catheter, Millar Instruments, Houston, TX). Increasing doses of dobutamine
were infused via the left jugular vein. Chart software (Chart 4.2, ADInstruments, Castle Hill, Australia) was employed for data recording (2000Hz) and determination of parameters of cardiovascular function.

Echocardiographic studies were performed under light anesthesia with spontaneous respiration using isoflurane as recently described. An ultrasonographer experienced in rodent imaging and blinded to the mouse genotype performed the echocardiography, operating a Toshiba PowerVision 6000 and a 15 MHz transducer. Short-axis two-dimensional echocardiographic images were obtained at the mid-papillary and apical levels of the left ventricle and stored as digital loops. Frame acquisition rates using the loop mode reached 100 MHz, allowing excellent temporal resolution for two-dimensional analysis. At the same anatomic levels, short-axis M-mode images were obtained with a sweep speed of 100 mm/s. Echocardiographic studies were performed after the surgical procedure at weeks four and eight.

Endocardial borders were traced at end-systole and end-diastole utilizing a prototype off-line analysis system (NICE, Toshiba Medical Systems, The Netherlands) as recently described. Using the end-systolic and end-diastolic areas, fractional area changes were calculated at both levels as \[
\frac{\text{end-diastolic area} - \text{end-systolic area}}{\text{end-diastolic area}}.
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**Histochemical and Immunohistochemical Analyses**

For analysis of cardiomyocyte hypertrophy, tissue sections (4 µm) of left ventricular myocardium were stained with hematoxylin/eosin for determination of myocyte cross-sectional areas. By digitizing the images and computerized pixel counting individual cells were analyzed. Only nucleated cardiac myocytes from areas of transversely cut muscle fibres were included in the analysis (see reference 28 of the manuscript).

For the analysis of interstitial collagen deposition, sirius red stained tissue sections of left ventricular myocardium were analyzed as described previously (see reference 28).

The following antibodies were used in immunohistochemistry to detect murine antigens: mAb 30311.11 (IgG1 anti IL-1β, R&D Systems, Wiesbaden, Germany), mAb 500A2 (anti-CD3, BD Pharmingen, Heidelberg, Germany), RM4-5 (anti-CD4, BD Pharmingen), mAb 53-6.7 (anti-CD8, BD Pharmingen), mAb C71/16 (anti-CD18, BD Pharmingen), mAb 1D3 (anti-CD19, BD Pharmingen), mAb MEC 13.3 (anti CD31, BD Pharmingen). Immunohistochemical studies of murine hearts (n=4 from
each genotype) were performed on acetone-fixed serial cryostat-cut sections (7 µm) by the ABC immunoperoxidase method (Vector, Burlingame, CA) as described\(^5\). Briefly, sections were incubated with 10% goat serum in PBS for 10 min followed by the primary mAb at 10 µg/ml for 30 min. After three washes in PBS, endogenous peroxidase was blocked by 0.3% H\(_2\)O\(_2\), and slides were washed three times. Sections were then incubated with the secondary biotin-conjugated antibody (Vector) at 20 µg/ml for 30 min, washed three times again, and incubated with streptavidin-horseradish-peroxidase (HRP)-complex (DAKO, Hamburg, Germany). Bound mAb was visualized using 3-amino-9-ethyl-carbazole (AEC, Sigma) as chromagen. Sections were counterstained with Gill's hematoxylin (Sigma) and LiCO\(_3\). Analysis of chloroacetate esterase (CAE) reactivity using naphthol-AS-D-chloroacetate (Sigma, Deisenhofen, Germany) as substrate and methyl green as counterstain was performed as described previously\(^6\).

Anticomplement 9 staining was performed using an anti-NCL-CCC9 antibody (1:50, Novocastra, Newcastle, UK). Counterstain for identification of myocytes was done with Alexa Fluor 488 phalloidin (A-12379), cell nuclei were stained with DAPI (see reference 29,30 of the manuscript ). Superoxide anion generation was performed on 5 µm cryosections using dihydroethidium (DHE, Invitrogen) fluorescence. Upon oxidation to fluorescent ethidium, it intercalates into DNA and can be analyzed at 585 nm as a red fluorescence signal. The staining was quantified by determination of pixel intensity (see reference 29,31 of the manuscript). For detection of oxidative stress-related DNA damage, paraformaldehyde-fixed paraffin-embedded heart tissue sections were incubated with an anti-8-OHdG antibody (7,5µg/ml, #24328, Oxis,OR). Envision+kit (DAKO) was used as a secondary reagent. Stainings were developed using DAB (brown precipitate) and slides were counterstained with hematoxylin.

**RNA Isolation and Real-Time RT-PCR**

For extraction of total RNA from frozen tissues RNeasy Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer`s instructions. The integrity of the isolated RNA was verified with denaturing agarose gel electrophoresis. First-strand cDNA was synthesized using oligo-dT primers. For real-time PCR we employed an ABI PRISM Sequence Detection System 7700 (Applied Biosystems, Darmstadt, Germany) with Sybr Green (Cambrex Bioscience, Rockland, ME) as fluorescent and 6-carboxy-X-rhodamine (ROX, Molecular Probes, Eugene, OR) as reference dye. We
applied the Sequence Detector 1.7 software to determine the threshold cycle (Ct) values. To control the specificity of the amplification product, dissociation curves were analyzed for each experiment. Corresponding GAPDH experiments were used as a reference. The \( \Delta\Delta CT \) method. The following primer sequences were used for real-time PCR analysis: Murine caspase-1 (forward 5´-TGGTCTTGTGACTTGGAGGA-3´, reverse 5´-GGTCTACCATCAGCAGTGG-3´), murine GAPDH (forward 5´-TGCAAGTGGAGATTGTTG-3´, reverse 5´-CATTATGGCCTTGACTGTG-3´), murine ANF (forward 5´-TTCAAGAAGCTGCTGACC-3´, reverse 5´-CCCTGCTTCTCAGTGTGCT-3´), murine \( \beta \)-MHC (forward 5´-CAATGCAGAGTCGGTGAAGG-3´, reverse 5´-GCCGATTAAGTTCTTCTGT-3´), murine SERCA (forward 5´-TGACAATGGGACTTTCTTCGTCACGAGACTG-3´).

PCR conditions using heat-activatable Taq polymerase (Hot Master Taq, Eppendorf, Germany) were as follows: 40 cycles of 94°C for 20 sec (2 min initial cycle), 60°C for 20 sec and 65°C for 35 sec.

**Immunoblotting**

For Western blot analysis from tissue, 30-40 \( \mu g \) (mouse lysates) or 70 \( \mu g \) (human lysates) of protein were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA) which were subsequently blocked with 2% BSA for 2 hours. The membranes were incubated overnight at 4°C with the primary antibody (anti-human caspase-1 p10 antibody, sc-515, diluted 1:850, Santa Cruz, CA) followed by chemiluminescent detection (ECL-Plus, Amersham Pharmacia, Freiburg, Germany).

For Western blot analysis from neonatal rat cardiomyocytes, cells were seeded on 6 cm cell culture plates (3 x 10^6 cardiomyocytes per plate). Lysis buffer was added 24 hours after adenoviral transfection. 25 \( \mu g \) of protein were seperated by SDS-PAGE and transferred to Immobilon-P membranes. Membranes were blocked with 5% dry milk for 2 hours followed by incubation with primary antibody (anti cleaved caspase-3 (Asp175), #9661, diluted 1:1000, Cell Signaling; anti cleaved caspase-9 (Asp353), #9507, diluted 1:1000, Cell Signaling; anti Caspase-8 p20, sc-7890, diluted 1:1000, Santa Cruz, CA) overnight. Chemiluminescent detection was performed with ECL-Plus (Amersham Pharmacia).
References for Supplementary Methods


Fig. S1
Fig. S2

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