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Differential Vulnerability to Oxidative Stress in Rat Cardiac Myocytes Versus Fibroblasts

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OBJECTIVES This study was designed to test the hypothesis that cardiac myocytes have greater vulnerability to oxidative stress compared with cardiac fibroblasts.

BACKGROUND The function of cardiac myocytes differs from that of fibroblasts in the heart, but differences in their response to oxidative stress have not been extensively studied.

METHODS Cardiomyocytes and fibroblasts from F344 neonatal rat hearts were cultured and exposed to different concentrations of hydrogen peroxide (H_2O_2) and menadione (superoxide generator). The mitogen-activated protein kinase (MAPK) proteins were assayed after oxidative stress; cell death was determined by trypan blue staining and deoxyribonucleic acid (DNA) ladder electrophoresis.

RESULTS The cardiac myocytes were significantly more vulnerable than the fibroblasts to oxidative damage, showing substantial DNA fragmentation and consistently poor cell survival after exposure to H_2O_2 (100 to 800 μM), while the cardiac fibroblasts demonstrated little or no DNA fragmentation, and superior cell survival rates both over time (from 1 to 72 h after 100 μM) and across increasing doses of H_2O_2 (100 to 800 μM). The p42/44 extracellular signal-regulated kinases were phosphorylated in both cell types after exposure to H_2O_2, but significantly more in cardiac fibroblasts. However, p38 MAPK and c-jun NH_2-terminal kinase were phosphorylated more in the cardiac myocytes compared to cardiac fibroblasts. This was also the case after exposure to menadione.

CONCLUSION Taken together, these results suggest that oxidative stress causes greater injury and cell death in cardiac myocytes compared with cardiac fibroblasts. It is possible that the signaling differences via the MAPK family may partly mediate the observed differences in vulnerability and functional outcomes of the respective cell types. (J Am Coll Cardiol 2001;38:2055–62)

Oxidative stress occurs in myocardial ischemic syndromes as well as in heart failure (1–10). The reactive oxygen species (OH- and 0-) can cause widespread cellular damage, and also by oxidation of key cysteine residues or S-H groups in membrane proteins, can trigger one or more cascades of signal transduction events, culminating in altered transcription factor binding and gene expression regulation (2–8,11–23).

As far as we know, ours is the first study in which the responses of cardiac myocytes and fibroblasts are compared simultaneously under similar experimental conditions, using the same intensity of oxidative stress. We studied components of the mitogen-activated protein kinase (MAPK) pathway after exposure to hydrogen peroxide (H_2O_2) and menadione. Our results suggest that following oxidative stress, p42/44 extracellular signal-regulated kinases (ERKs) were activated more in cardiac fibroblasts than in cardiac myocytes. However, p38 and c-jun NH_2 terminal kinase (JNK) were activated more in cardiac myocytes. We also found that the cardiac myocytes were significantly more sensitive to damage by H_2O_2 and showed far lower cell survival rates across a wide range of concentration of H_2O_2.

The role of bcl2 and bax as antiapoptotic and proapoptotic, respectively, has been well established in various cell types (24–28). Our study results suggest that differential expression of bcl2 and bax in cardiac myocytes and fibroblasts might contribute in part to the differences in susceptibility to stress.

The main objective of this study was to enable us to better understand the in vivo situation in which cardiac myocytes and fibroblasts are exposed to similar degrees of oxidative stress during ischemia/reperfusion. Secondly, we wanted to delineate the different responses of the cardiac myocytes and fibroblasts under identical experimental conditions of oxidative stress. This information will hopefully advance the knowledge for design of future cell-type specific interventional therapies in myocardial ischemia and heart failure.

MATERIALS AND METHODS

Study materials. All cell culture reagents were purchased from Gibco-BRL. All sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) reagents were obtained from Biorad. Phospho-specific antibodies for phospho-ERK-1/2 (Tyr-204), phospho-p38 MAPK (Tyr-182), phospho-JNK (Thr-183/Tyr-185) and the appropriate expression antibodies were obtained from New England Biolabs. Menadione, H_2O_2 and other chemical reagents were obtained from Sigma Chemical Co. PD-98059 was

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Abbreviations and Acronyms

ANOVA	= analysis of variance
DNA	= deoxyribonucleic acid
ERK	= extracellular signal-regulated kinases
H_2O_2	= hydrogen peroxide
JNK	= c-jun NH_2 terminal kinase
MAPK	= mitogen-activated protein kinase
PBS	= phosphate-buffered saline solution
PD-98059	= Parke-Davis compound 98059
SB-202190, 203580	= Smith Kline and Beecham 202190, 203580
SDS-PAGE	= sodium dodecyl sulfate-polyacrylamide gel electrophoresis

purchased from New England Biolabs. SB-202190 and SB-203580 were purchased from Calbiochem.

Study methods. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1996).

CARDIAC CELL CULTURE. *Primary neonatal cardiac myocyte cultures.* The ventricles of hearts that were removed from etherized Fisher 344 neonatal rats were dissociated by mechanical mincing and enzymatic digestion with 0.1% collagenase (Worthington Biochem. Corp., Freehold, New Jersey) and 0.1% trypsin (Sigma Chem.; St. Louis, Missouri). The ventricular myocytes were separated from nonmyocyte cells that were recovered from the digestion by density gradient sedimentation through 6% bovine serum albumin, followed by differential attachment to preplated laminin-coated flasks for 1 h, to remove the remaining attached noncardiomyocytes. The resultant suspension of >95% cardiomyocytes was collected and seeded at a density of 2×10^6 cells per 100 mm^2 in gelatin-coated culture plates. The cardiac myocytes were cultured in DMEM medium with 10% heat-inactivated newborn bovine serum, 10 mM HEPES, 1% penicillin and streptomycin. Arabinoside (10 nM) was added to inhibit fibroblast growth. After 1 to 2 days in culture, the myocytes began to beat spontaneously. Over 90% of the cells were spontaneously beating, and stained positively with monoclonal antisarcomeric actin antibody. Cardiomyocytes were subjected to oxidative stress 2 days after culture. Results of cardiomyocytes cultured on collagen-coated and noncoated plates were similar.

Primary neonatal cardiac fibroblast cultures. Hearts were removed from other etherized F344 neonatal rats. The hearts were placed in ice-cold phosphate-buffered saline solution (PBS). Blood vessels and fibrous tissues were trimmed from the hearts. The hearts were washed five times with 20 ml of PBS, and were minced with scissors until very small pieces were produced. The pellet of minced tissue was then resuspended in 1% collagenase (Worthington CLS-2) and incubated at 37°C for 2 h. Next, 40 ml of PBS was then added and the mixture was spun down at 1,100 rpm for 5 min, after which the supernatant was aspirated and the minced tissue was resuspended in 0.25% trypsin and allowed to incubate for 1 h at 37°C. The digested tissue was again washed with PBS and spun down, and the pellets were then resuspended in DMEM (Gibco BRL) supplemented with 10% fetal calf serum, 10 mM HEPES, 1% penicillin and streptomycin. The fibroblast pellet from each heart was plated onto 1 × 100-mm tissue culture plates; 24 h later, the media were aspirated and the fresh media were added to the plates. Cells were grown in an air/Co_2 (49:1) humidified incubator at 37°C. The cells were passaged at a 1:5 ratio and subcultured in 100-mm tissue culture plates. After three passages, the cells were subjected to oxidative stress (described in the following text).

OXIDATIVE STRESS. The cells were treated at 37°C with H_2O_2 (at various concentrations ranging from 100 μM to 800 μM) or menadione (at various concentrations ranging from 50 to 200 μM) and were studied at various times as indicated, ranging from 30 min to 72 h after exposure.

After the treatment with either H_2O_2 or menadione, the cells were washed three times in ice-cold PBS, then lysed with addition of cold lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM ethylene diamine tetra-acetic acid, 1 mM ethelene glycol-bis (beta-aminoethyl ether) tetra-acetic acid, 0.2 mM sodium vanadate, 0.5% NP-40) containing the fresh cocktail of protease inhibitors (Boehringer Mannheim Co). Homogenates were centrifuged for 15 min at 12,000 × *g* at 4°C, protein concentration was determined and the samples were stored at −80°C.

A subset of the neonatal cardiac myocyte and fibroblast cultures was pretreated with an MEK 1 and 2 inhibitor, PD-98059, and the p38MAPK inhibitors, SB-202190 and SB-203580. The concentrations of PD-98059, SB-202180 and SB-203590 used were 0, 1, 5, 10, 20, 30 and 50 μM, and the pretreatment period was 30 min. After 30 min of pretreatment with a specific inhibitor, H_2O_2 was added at different concentrations of 0, 100, 300, 500 and 800 μM. Cell death was then assessed by configuration (cell detachment and flotation) and trypan blue exclusion after 30 min, 1 h and 4 h.

TIME COURSE OF CELL DEATH ANALYSIS. After treatment with H_2O_2 at 37°C for 4 h, cardiac myocytes and fibroblasts were harvested with 0.25% trypsin-EDTA and then stained with 0.1% trypan blue solution. The unstained (live) cells and stained (dead) cells were counted, and the percentage of cell survival was determined as the ratio of the unstained cells to the stained plus unstained cells. A time course of cell survival was done, immediately after treatment with H_2O_2 and then at 30 and 60 min and 4, 8, 12, 24, 48 and 72 h.

INTERNUCLEOSOMAL DEOXYRIBONUCLEIC ACID (DNA) PATTERNS (DNA LADDER). After the cells were treated with H_2O_2 (range, 25 to 800 μM) for 60 min, internucleosomal

DNA fragmentation assay was performed. Briefly, tissues were homogenized in 5 ml lysis buffer containing TE (10 mM Tris-HCl at a pH of 8.0 and 100 mM EDTA), SDS (0.5 ml) and ribonuclease (RNase; Promega; Madison, Wisconsin) and incubated at 37°C for 60 min. A second incubation was performed at 50°C for 3 h after the addition of proteinase K (100 μg/ml; Promega). The final incubation was completed in NaCl 1 mol/l overnight at 4°C. The solution was then spun at 12,000 rpm for 20 min and the supernatant was extracted twice with phenol and chloroform/isopropanol (49:1). DNA was precipitated in cold ethanol at −20°C. Twenty micrograms of DNA was then loaded onto 1.6% agarose gel containing 0.5 μg/ml ethidium bromide, electrophoresed in 1 × TBE running buffer and visualized under UV illumination.

WESTERN BLOTTING. For Western blot analysis, 50 μg of protein was subjected to SDS-PAGE in a 10% gel, and proteins were then transferred to nitrocellulose (Bio-Rad). The membranes were incubated in blocking solution (5% nonfat milk, 0.01% Tween-20 in Tris-buffered saline solution) for 1 h before being exposed to primary antibodies for 2 h at room temperature. After washing, the membranes were incubated with peroxidase-conjugated secondary antibody for 1 h before ECL (Amersham Life Science) detection.

Densitometric analysis of ECL autoradiographs was performed using a Personal Densitometer and ImageQuant software (Molecular Dynamics).

Statistical methods. Each experiment was performed at least three times and the mean value ± SD was used in the results and calculations. Densitometric quantification of all blots was performed with beta actin as control. For the calculation of ratios of proteins, the densitometric values for each blot were standardized against a control of 1.0. Two-way analysis of variance (ANOVA) testing was performed on all the densitometric results. The myocyte and fibroblast interaction across different dosages as well as the myocyte fibroblast interaction across time with a fixed dose were tested. If a significant difference of <0.05 was detected between myocytes and fibroblasts with the two-way ANOVA test, the *t* test with Bonferroni correction was then applied for comparisons between groups.

RESULTS

Cell survival analysis after exposure to H_2O_2. The cultured cardiac myocytes and cardiac fibroblasts were exposed to H_2O_2 at concentrations of 300, 500 and 800 μM, for 4 h. The cells were then stained with trypan blue and analyzed for viability. At every concentration of H_2O_2, the cardiac myocytes showed greater injury and cell death, and had significantly lower rates of cell survival compared to the cardiac fibroblasts (Fig. 1A, two-way ANOVA, $p < 0.01$). At 300 μM of H_2O_2, there was a cell survival of 52% in the cardiac myocytes while 80% of the cardiac fibroblasts survived ($p < 0.01$). At 500 μM of H_2O_2, the cell survival was 18% for cardiac myocytes and 62% for cardiac fibroblasts ($p < 0.01$); and at 800 μM, it was 4% for myocytes and 43% for fibroblasts ($p < 0.01$).

In a separate series of experiments, the time course of the effect of H_2O_2 at a concentration of (100 μM) on cell survival was determined for up through 72 h (Fig. 1B). At all times tested, the cardiomyocytes showed greater cell death compared with cardiac fibroblasts (two-way ANOVA, $p < 0.01$). At 8 h, the cell survival was 42% in cardiomyocytes and 74% in cardiac fibroblasts ($p < 0.05$). At 24 h after H_2O_2 exposure, 12% of cardiomyocytes compared with 54% of cardiac fibroblasts survived ($p < 0.01$); at 72 h, only 9% of cardiomyocytes versus 46% of cardiac fibroblasts survived ($p < 0.05$). The interactions of myocytes and fibroblasts across the doses and the time course were significant (two-way ANOVA, $p < 0.01$).

In the cardiomyocytes, DNA fragmentation by gel electrophoresis was clearly visible after 1 h of exposure to H_2O_2 at concentrations of 100 and 300 μM, but not at the lower (25 to 50 μM) or higher (500 to 800 μM) concentrations (Fig. 1C). In contrast, no DNA fragmentation was seen in the cardiac fibroblasts after similar treatment at any of the concentrations of H_2O_2 (25 to 800 μM).

PD-98059, a mitogen activated protein kinase 1/2 inhibitor by itself, at ranges between 1 to 30 μM, resulted in no significant change in cell survival in cardiomyocytes. At concentrations between 30 and 50 μM, PD-98059 caused a slight (4 ± 2%, p = NS) increase in cardiomyocyte cell death as assessed by trypan blue staining compared with vehicle. SB-202190, a p38 MAPK inhibitor, at ranges from 1 to 50 μM, resulted in no significant change in myocyte survival. For pretreatment with inhibitors, 10 μM SB-202190 was employed because at higher concentrations, SB-202190 can inhibit both p38 and JNK pathways. Pretreatment of the cardiac myocytes with PD-98059 (20 μM) or SB-202190 (10 μM) for 30 min prior to the H_2O_2 treatment (300, 500, 800 μM) resulted in slightly increased cell death in the cardiomyocytes. Cell survival was mildly reduced with PD-98059 pretreatment (8 ± 3%, NS) and also with SB-202190 pretreatment (6 ± 4%, NS). Fibroblast pretreatment with PD-98059 (20 μM) or SB-202190 (10 μM), for 30 min prior to the H_2O_2 treatment (at concentrations of 300, 500, 800 μM), also resulted in slightly increased cell death but less than that in cardiomyocytes. The fibroblast survival with PD-98059 pretreatment was 3 ± 1% (NS) and for SB-202190 pretreatment it was $<1\%$.

MAPK pathway signaling following oxidative stress. EXPOSURE TO H_2O_2. After the cells were exposed to different concentrations of H_2O_2 for a period of 30 min, protein extraction and analysis were performed.

Protein expression of ERK1 and ERK 2 was slightly greater in cardiac fibroblasts than in cardiac myocytes, both at baseline and after H_2O_2 treatment. However, phosphorylation of ERK1 and ERK2 after H_2O_2 was significantly

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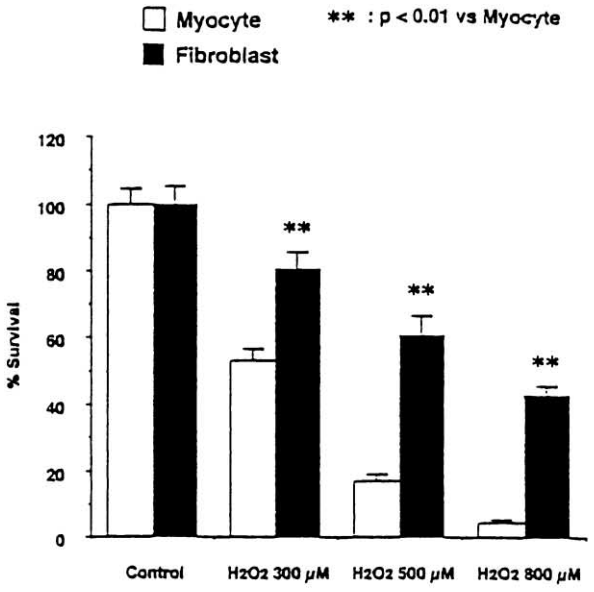

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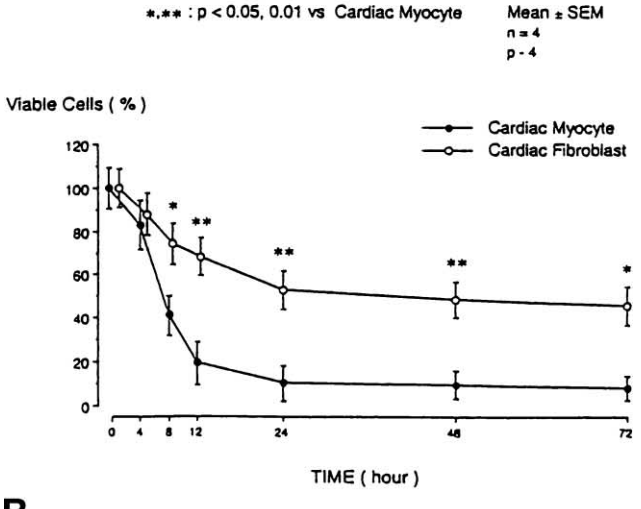

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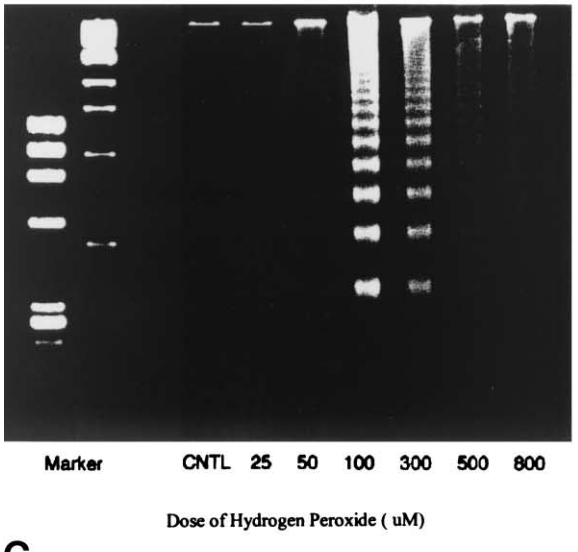

C

Figure 1. (A) Cell survival of rat cardiac myocytes versus cardiac fibroblasts after hydrogen peroxide (H_2O_2) (300 to 800 μM). Neonatal rat cardiac myocytes and fibroblasts were cultured for 5 days before exposure to H_2O_2 for 4 h. n = 6 experiments at each time point (two-way analysis of variance [ANOVA], $p < 0.01$). Results are expressed as mean ± SEM. *t* test was used for individual comparisons. $^{**}p < 0.01$. **(B)** Graph showing time course of the effect of H_2O_2 (100 μM) on survival of neonatal cardiac myocytes and fibroblasts. Cells were exposed to H_2O_2 (100 μM) for a period of up to 72 h before cell survival analysis was performed. n = 4 experiments for both cardiac myocytes and fibroblasts at each time point (two-way ANOVA, $p < 0.01$). Results are expressed as mean ± SEM. *t* test was used for individual comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$. **(C)** Deoxyribonucleic acid (DNA) ladder electrophoresis of neonatal cardiac myocytes treated with H_2O_2 (in concentrations from 25 to 800 μM) for 60 min. The DNA fragmentation ladder is most intense at 100 and 300 μM H_2O_2, and becomes much less visible at lower concentrations (25 and 50 μM) as well as higher (500 and 800 μM) concentrations.

higher in cardiac fibroblasts compared with myocytes (Fig. 2A, 2B and 2C; two-way ANOVA, $p < 0.01$).

The p38 MAPK protein appeared to be expressed similarly in both cardiac myocytes and cardiac fibroblasts, with minimal change after treatment. With 300 μM H_2O_2, phosphorylation of p38 MAPK was seen in both cell types, but tended to be higher in cardiac myocytes (Fig. 2A and 2D).

There was an appreciable level of JNK protein expression in both cardiac myocytes and fibroblasts at baseline that did not change after H_2O_2 treatment. A heightened phosphorylation of JNK was observed in both cell types with H_2O_2 treatment, but was significantly greater in cardiac myocytes than in fibroblasts (Fig. 2A and 2E; two-way ANOVA, $p < 0.05$).

The ratios of phosphorylated p38 to phosphorylated Erk2 and phosphorylated JNK to phosphorylated Erk2 were significantly different in myocytes compared with fibroblasts (Fig. 2F; two way-ANOVA, $p < 0.05$).

EXPOSURE TO MENADIONE. Cells were exposed to different concentrations of menadione (50, 100 and 200 μM) for a period of 30 min before they underwent protein extraction and analysis.

Exposure to menadione caused a similar type of response in the cardiac myocytes and fibroblasts as compared with H_2O_2 (Fig. 3). There was somewhat greater activation of ERK1 and ERK2 in cardiac fibroblasts than in cardiac myocytes. Also similarly, the p38 and JNK activation was greater in cardiac myocytes compared with cardiac fibroblasts.

bcl2 and bax protein expression after exposure to H_2O_2. The bcl2 protein expression after exposure to H_2O_2 was induced more in cardiac fibroblasts than in cardiac myocytes (Fig. 4A and 4B). The bax protein level was higher at baseline in cardiac myocytes compared to fibroblasts and it did not appear to be further induced with oxidative stress. In cardiac fibroblasts, bax was slightly induced between 300 to 500 μM H_2O_2 (Fig. 4A and 4B).

The densitometric analysis of bcl2 and bax in cardiac myocytes and fibroblasts showed that bcl2 or bax seemed to be less induced with oxidative stress in cardiac myocytes,

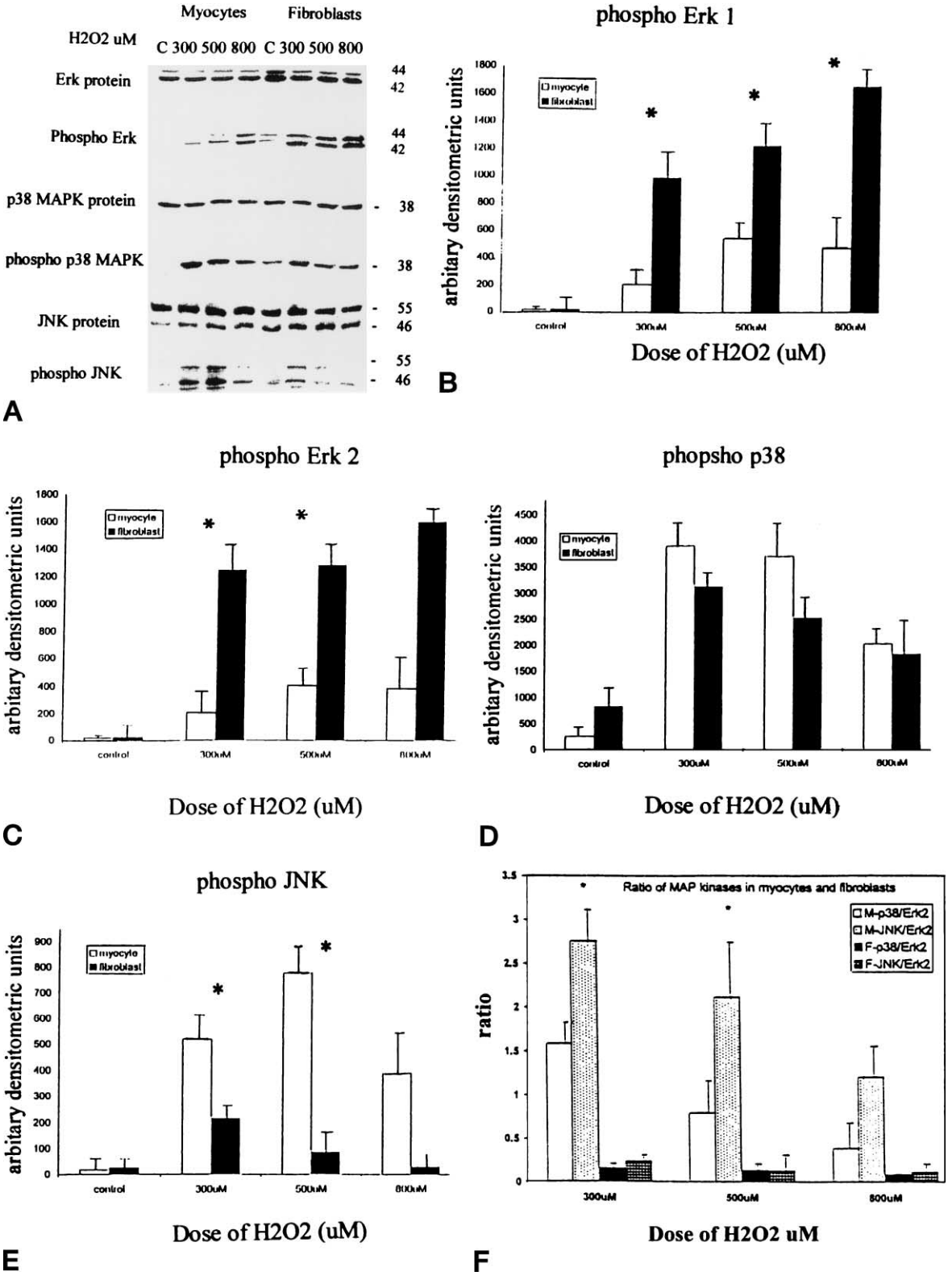

Figure 2. **(A)** Representative Western blot showing effect of (H_2O_2) (300 to 800 μM) on the activation and phosphorylation of the mitogen-activated protein kinases (MAPK) in neonatal rat cardiac myocytes and fibroblasts. Cells were exposed to different concentrations of H_2O_2 for a period of 30 min before protein extraction and analysis. **(B–E)** Densitometric evaluation of phosphoextracellular signal-regulated kinases (Erk) 1 **(B)**, phospho Erk 2 **(C)**, phospho p38 MAPK **(D)** and phospho JNK **(E)** activation after H_2O_2. n = 3 at each dose (two-way analysis of variance (ANOVA), $p < 0.01$). Results are expressed as mean ± SD. *t* test was used for individual comparisons. *$p < 0.05$, myocyte versus fibroblast. **(F)** Ratios of phosphorylated p38 to phosphorylated ERK 2 and phosphorylated c-jun NH_2 terminal kinase (JNK) to phosphorylated ERK 2 in cardiac myocytes and cardiac fibroblasts after treatment with H_2O_2 (300, 500, 800 μM) for 30 min. Ratios are based on means of n = 3, at each dose and after standardization of the controls to a value of 1 (two-way ANOVA, $p < 0.02$). M = myocyte, F = fibroblast. *t* test was used for individual comparisons. *$p < 0.05$, myocyte versus fibroblast.

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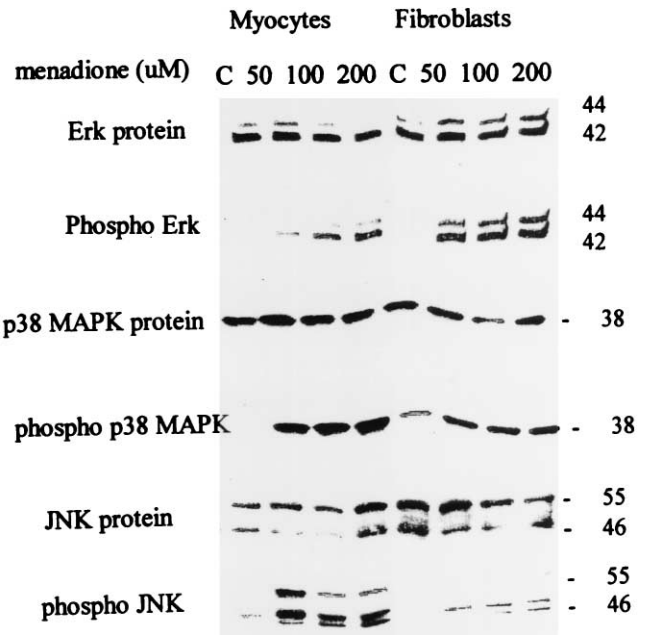

Figure 3. Representative Western blot showing the effect of menadione (vitamin K) on the activation and phosphorylation of the mitogen-activated protein kinases (MAPK) in neonatal rat cardiac myocytes and fibroblasts. Cells were exposed to different concentrations of menadione (50, 100, 200 μM) for a period of 30 min before protein extraction and analysis. Erk = extracellular signal-regulated kinases; JNK = c-jun NH_2 terminal kinase.

while in cardiac fibroblasts, there was an earlier induction of bcl2 at 300 μM and a later induction of bax at 500 μM of H_2O_2. Also in cardiac myocytes, bax was significantly higher than in cardiac fibroblasts, both at baseline and after H_2O_2 (300, 500 and 800 μM (Fig. 4B; two-way ANOVA, $p < 0.05$)).

DISCUSSION

There are four major findings in this study. First, the cardiac myocytes were more vulnerable to injury by oxidative stress, and showed significantly lower cell survival rates compared with cardiac fibroblasts after exposure to H_2O_2. Second, in response to oxidative stress, p42 and p44 ERK kinase activation was similar in cardiac fibroblasts and cardiac myocytes, but there was a tendency toward somewhat higher p38 MAPK activation in cardiac myocytes. Third, JNK activation was substantially greater in cardiac myocytes than in cardiac fibroblasts.

Cell survival. Our results showed significantly poorer survival of cardiomyocytes compared with cardiac fibroblasts after all concentrations of H_2O_2. Since high concentrations of free radicals are generated in the heart during ischemia or ischemia-reperfusion and also in severe heart failure, it is important that the mechanisms of oxidative damage to cardiomyocytes be better understood (1–4,6–9). The much greater resistance of fibroblasts than cardiac myocytes to oxidative stress is of interest, given the fact that fibroblasts are instrumental in implementing myocardial wound repair.

The MAPK pathway. ERK 1 AND ERK 2. We observed that the ERKs, which usually mediate the growth and survival response in different cell types (10–14), were activated in both cardiac fibroblasts and cardiac myocytes on exposure to oxidative stress. The activation of ERKs occurred to a significantly greater extent in cardiac fibroblasts than in myocytes. This correlated with the much higher survival rate of cardiac fibroblasts. Also pretreatment with PD-98059, a MEK inhibitor (of ERK1 and ERK2), before exposure to oxidative stress, produced slightly higher cardiomyocyte death, further suggesting a possible protective effect of the ERKs in cardiomyocytes. These findings were in accordance with studies reported by Aikawa et al. (3) which also showed increased cardiomyocyte death with MEK inhibitors.

P38 MAPK. The activation of p38 MAPK was higher in cardiac myocytes compared to cardiac fibroblasts, given the same degree and duration of oxidative stress. Although studies have previously demonstrated p38 MAPK activation during ischemic stress in the heart (15–20), the p38 MAPK response in cardiac myocytes compared to cardiac fibroblasts has not been clearly defined. Opposing roles of the ERKs (p42/44) and of p38 MAPK may exist in cells, with ERKs being antiapoptotic and p38 MAPK being apoptotic (21). Activation of p38 MAPK might also be associated with the development of ischemic tolerance and preconditioning (15,17).

Four different subtypes of p38 MAPK have now been identified, with p38 beta MAPK being most highly expressed in the heart and skeletal muscle (14,18,19). The functional role of each of the different p38 subtypes has not been completely determined. In the present study, we used a commercially available antibody that detected all four subtypes of the p38 MAPK. In this study, the protective role of p38 MAPK was somewhat supported by the fact that pretreatment with p38 MAPK inhibitors (SB-202190) resulted in slightly increased cardiomyocyte cell death in response to H_2O_2 in the present studies.

JNK ACTIVATION. Our experiments in the present study showed a substantially higher level of JNK activation in cardiac myocytes versus cardiac fibroblasts. We have previously found that a dominant negative form of JNK prevented DNA fragmentation and also reduced caspase activation in H9c2 cells (8). In other studies, with the various cell types that have been studied using different stressors, JNK seems to be more of an indicator of cellular stress and damage than the other markers of the MAPK family (7–21).

The higher levels of JNK activation in cardiac myocytes in the present study may indicate the presence of a lower injury/death threshold in the cardiomyocytes compared to the more resilient cardiac fibroblasts.

Significance of the phosphorylated p38/ERK, phosphorylated JNK/ERK ratios. It is probably not the effect of any particular single protein that decides the fate of the cell, but

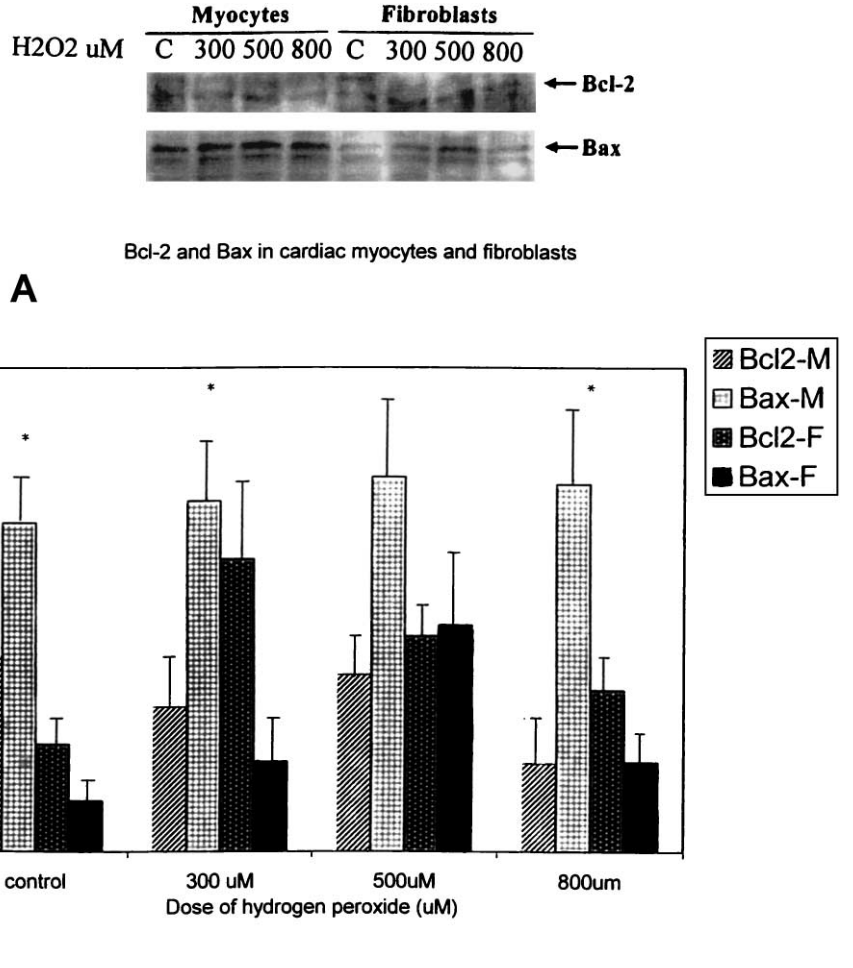

Figure 4. (A) Representative Western blot of bcl2 and bax protein expression in the cardiac myocyte and cardiac fibroblast after exposure to different dosages of hydrogen peroxide (H_2O_2) (300, 500, 800 μM) for 30 min. The experiment was repeated at least three times, with n = 3, for both cardiac myocytes and fibroblasts with similar results. C = control cells, not exposed to oxidative stress and assayed after 30 min. Coomassie blue staining was used to confirm equal loading. **(B)** Densitometric analysis of bcl2 and bax protein in the neonatal cardiomyocytes and fibroblasts (n = 3 each dose; two-way analysis of variance, $p < 0.01$). Results are expressed as mean ± SD. *t* test was used for individual comparisons. *$p < 0.05$, myocyte versus fibroblast for differences in bax protein expression (bax-M vs. bax-F). M = myocyte, F = fibroblast.

rather ratios of several proapoptotic and antiapoptotic proteins in the cell that tilt the balance either in favor or against survival. It was with this concept in mind that we sought to determine whether the ratios of the antiapoptotic (ERK1 and ERK2) and the mostly proapoptotic proteins (p38 MAPK and JNK) could correlate with the stress vulnerability of the different cell types. We have shown that in the cardiomyocyte, both the p38/ERK2 and the JNK/ERK2 ratios became quite high at all concentrations of H_2O_2. These high ratios are probably not so favorable for survival. This notion was well supported by the response in the cardiac fibroblast, which had higher ERK2 activity, and hence much lower ratios of p38/ERK2 and JNK/ERK2, and of course also much better survival than the cardiomyocyte.

bcl2 family and cell survival. Cell death, particularly apoptotic cell death in the heart, is of substantial interest because of the therapeutic potential for myocyte salvage after myocardial infarction and in heart failure (5,24–28). Overexpression of bcl2 and suppression of bax expression in the heart has been shown to be associated with reduced cardiac injury and improved cardiomyocyte survival (27,28).

Our results raise the possibility that in part, the greater resistance to injury in cardiac fibroblast might be due to a greater capacity of the cardiac fibroblasts to increase bcl2 expression in response to oxidative stress. Conversely, a higher basal expression of bax protein in the cardiomyocytes, as was seen in our experiments, could lower the threshold for injury and favor earlier cell death.

Study significance and limitations. A number of other proapoptotic and antiapoptotic factors were not measured in these experiments, such as the caspase family of proteins, p53, NFκB and other members of the bcl2 family (bclx-1, bag 1, bad). In addition, an alteration in phosphorylation status of bcl2 or bax and translocation of these proteins might have occurred and might also have contributed to differences in cell survival. It would be of interest to determine the absolute levels of particular proteins individually in cardiomyocytes and fibroblasts so that a more

accurate value of the percent of activated protein can be calculated per cell. We used neonatal cardiomyocytes and fibroblasts, which are more resistant than cells from adult hearts to oxidative stress and adult cardiomyocytes and fibroblasts might have different responses to the same stimulus. Nevertheless, the results from this study provide insight into some integral differences in the response to stress between cardiomyocytes and fibroblasts which might be employed to help enhance individual cell survival.

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