Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 316 (2004) 256–262
BBRC
www.elsevier.com/locate/ybbrc

Impact of brief oxidant stress on primary adult cardiac fibroblasts

James T. Colston,[a,*] Sam D. de la Rosa,[a] and Gregory L. Freeman[a,b]

[a] *Janey Briscoe Center of Excellence in Cardiovascular Disease, Department of Medicine, Division of Cardiology, University of Texas, Health Science Center at San Antonio, San Antonio, TX 78284, USA*
[b] *South Texas, Veterans Health Care System-Audie L. Murphy Division, San Antonio, TX 78284, USA*

Received 9 February 2004

Abstract

Reperfusion of ischemic myocardium (I/R) is associated with local release of a brief pulse of reactive oxygen species. The purpose of this study was to determine the effects of brief H_2O_2 stimulation on primary adult cardiac fibroblast phenotype. We demonstrate that brief H_2O_2 exposure results in transient phosphorylations of p38 and ERK which peaked by 15 min. Proliferation was minimally affected by either H_2O_2 or MAPK inhibition. Pretreatment with SB203580 or U0126 revealed that p38 enhances or maintains migration rates while ERK retarded migration. Peroxide exposure increased necrosis from 4% at baseline to >12% while reducing apoptosis by 3.5-fold. p38 inhibition resulted in increased necrosis and apoptosis while ERK inhibition had minimal effects. In conclusion, primary adult cardiac fibroblasts exposed to brief H_2O_2 exhibit an altered phenotype characterized by reduced migration and apoptosis and increased necrosis resulting, in part, from the differential effects of p38 and ERK signaling.

Keywords: Cardiac fibroblast; Hydrogen peroxide; p38 MAPK; Migration; Apoptosis

Cardiac fibroblasts represent the most numerous cell type in the mammalian heart [1–3]. These cells reside in the interstitium between contracting cardiomyocytes where they provide structural support, regulate extracellular matrix deposition and turnover, and play a central role in post-ischemic cardiac remodeling and fibrosis [1,4–6]. In addition, we [7] and others [8–10] have shown that cardiac fibroblasts provide a rich source of locally produced paracrine factors including cytokines, chemokines, and growth factors. Given the relative abundance of cardiac fibroblasts in the heart and their close proximity to working myocytes and vascular cells, it is likely that fibroblasts play an important role in sensing changes in the physical and biochemical milieu. However, elucidating the mechanisms by which fibroblasts sense local changes and transduce this information intracellularly to produce appropriate responses will be essential for a complete understanding of their role in the normal and stressed heart.

Coronary bypass surgery, coronary angioplasty, and heart transplant surgery are three clinical scenarios that subject the heart to potentially significant harm associated with reperfusion of ischemic myocardium. Spin trap studies have revealed that reperfusion of ischemic myocardium leads to a brief and substantial spike in reactive oxygen species (ROS) levels, the bulk of which occurs during the first 5 min of reperfusion [11,12]. In an effort to model this phenomenon in vitro, we recently exposed primary adult cardiac fibroblasts (CF) to a 3-min pulse of physiologically elevated levels of H_2O_2, and we found that this results in a large, delayed calcium transient [7]. In that setting the ROS-mediated calcium transient led to a transitory upregulation of IL-6 expression that required both ROS and Ca^{2+} for full induction. This suggests that when fibroblasts sense environmental oxidative stress they respond in ways that can signal other cells in the biological locale. How episodes of brief oxidative stress impact the fibroblasts themselves has not been evaluated.

The purpose of the present study was to define how an exposure to a brief burst of ROS, as occurs with ischemia/reperfusion, alters the cellular phenotype of primary CF. Our results demonstrate that this perturbation alters fibroblast migration, necrosis, and apoptosis, while exerting minimal effects on proliferation. These responses

* Corresponding author. Fax: 1-210-567-6960.
E-mail address: colstonj@uthscsa.edu (J.T. Colston).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.02.042

follow activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signaling pathways, suggesting that oxidative stress engages an intracellular signaling mechanism that leads to specific cellular responses. These results contribute to a growing body of evidence that cardiac fibroblasts, in addition to their traditionally recognized roles in cardiac remodeling, fibrosis, and structural support, function as sensors capable of responding to dramatic as well as subtle changes in the local environment and act, in concert with cardiomyocytes, to provide appropriate biological responses in the heart.

Materials and methods

Fibroblast isolation and culture. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health [NIH; 13]. Cardiac-derived fibroblasts (CF) were isolated from the hearts of adult male WKY rats (200–250 g) using a method developed in our laboratory [7]. Briefly, after induction of deep anesthesia with an i.m. injection (0.2–0.3 ml) containing a cocktail of ketamine–acepromazine–xylazine (9:3:1), hearts were rapidly removed, rinsed, and mounted via the aorta onto an 18-gauge cannula attached to a Langendorff-type apparatus allowing retrograde perfusion of the coronary arteries. Hearts were perfused for 5 min with 37 °C sterile-filtered calcium-free Krebs–Ringers–bicarbonate buffer (KRB; NaCl 110 mM, KCl 2.6 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, and glucose 11 mM) at 80 mm Hg. Hearts were perfused for a further 20–25 min with KRB–enzyme solution containing 0.5 mg/ml type II collagenase (Worthington Biochemical, Freehold, NJ), 25 μM $CaCl_2$, and 1 mg/ml fatty-acid-free albumin. After digestion, the ventricles were trimmed free and minced in KRB–enzyme solution containing 10 mg/ml albumin, filtered through sterile nylon mesh, and centrifuged at 25*g* for 5 min to remove cardiomyocytes, RBCs, and debris. The resultant supernatant was then centrifuged at 1000*g* for 8 min. The cell pellet was resuspended in 20 ml CF medium (CF; Hepes 15 mM, $NaHCO_3$ 16.7 mM, BME-vitamins, and MEM-amino acids each 1× [Gibco-BRL, Grand Island, NY], glutamine 2 mM, heat-inactivated FBS 10%, and antibiotics, pH 7.3) and plated into T175 tissue-culture flasks (Falcon, Becton–Dickinson Labware, Franklin Lakes, NJ). Non-adherent cells were removed by aspiration after 2 h and discarded. Cells were fed with fresh medium three times per week using CF medium and split 1:2 when confluent.

CF and non-fibroblast contaminants were identified by immunofluorescence (IF) using routine methods [7,14] with the following antibodies; FITC-conjugated monoclonal anti-β-actin (Sigma Chemical, St. Louis, MO), anti-PECAm-1 (CD-31; Research Diagnostics, Flanders, NJ), anti-vimentin (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-smooth muscle actin (Sigma). After 2 serial passages >99.7% of cells in these cultures exhibited vimentin and β-actin immunoreactivity, were CD-31 and smooth muscle actin negative, and displayed typical fibroblast-like morphology. Non-fibroblast cells typically account for less than 0.1% of total cells as determined by IF. Cardiac-derived fibroblasts were used in these experiments between the second and third passages.

Cell proliferation assay. Low passage CF were plated into tissue culture treated 96-well clear bottom, black-sided plates (VWR Scientific Products, West Chester, PA) at 500, 1000, and 2000 cells/well. Cells were fed with serum-free CF medium containing 0.5% BSA 24 h prior to study. CF were pretreated for 20 min with the p38 MAPK inhibitor SB203580 (10 μM; Biomol, Plymouth Meeting, PA) [15–18], or MEK inhibitor U0126 (10 μM, Biomol) [19–21], or DMSO vehicle (1:1000). CF were then exposed to log doses of H_2O_2 (0.1–1000 μM; prepared from 30% stock; Sigma) or vehicle (CF medium) for 3 min. Following H_2O_2 exposure, cells were rinsed and re-fed with CF medium and cultured overnight. After 24 h the medium was removed and the plates were frozen at −80 °C for 2 h prior to assay. Plates were then thawed, stained with CyQUANT GR dye according to manufacturer's protocol (Molecular Probes, Eugene, OR), and read on a FL_X800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT) using 485/20 excitation and 528/20 emission filters, and analyzed using KC^4 software (Bio-Tek).

Cell migration assay. The effects of H_2O_2 and MAPK inhibitors on CF cell migration (haptotaxis) were determined by Boyden chamber assay (QCM-FN; Chemicon International, Temecula, CA). CF were plated into Boyden chambers (2.5×10^5 cells/chamber) and pretreated with either SB203580 (10 μM) or U0126 (10 μM) or vehicle (CF medium with 1:1000 DMSO) for 20 min prior to 3 min stimulation with 100 μM H_2O_2. The cells were then rinsed with serum-free CF medium (0.5% BSA) and allowed to migrate towards the fibronectin gradient or BSA (control) for 24 h. The Boyden chambers were then removed, washed, and stained according to manufacturer's protocol. Samples were collected and read at 570 nm on a SpectraMAX 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

p38 and ERK 1/2 MAPK assay. Total and phosphorylated p38 and ERK 1/2 were determined by ELISA (Biosource, Camarillo, CA). CF were plated into 100 × 20 mm polystyrene tissue culture dishes and grown to 85% confluence. Cells were fed with serum-free CF medium (0.5% BSA) 24 h prior to stimulation. CF were stimulated with 100 μM H_2O_2 for 3 min or vehicle (medium), rinsed, and re-fed with serum-free medium ($n = 4$ cultures/time point). Cells were harvested at 0, 5, 15, 30, 45, 60, and 120 min and analyzed for total and *phospho*-p38 [pTpY 180/182] and *phospho*-ERK 1/2 [pTpY 185/187] and total protein (DC Protein assay; Bio-Rad, Hercules, CA) using a SpectraMAX 190 microplate spectrophotometer.

Viability assay. CF viability was measured using the Annexin-V-FLUOS staining kit (Roche, Indianapolis, IN). CF were plated into 100 × 20 mm tissue culture plates and grown to 85% confluency. Cells were fed with serum-free medium (0.5% BSA) 24 h prior to assay. Cultures were stimulated with 100 μM H_2O_2 for 3 min or vehicle (HBSS) with or without 20 min pretreatment with either 10 μM SB203580 or 10 μM U0126. After peroxide stimulation, cells were fed with serum-free medium (0.5% BSA) and harvested by mild trypsinization after 3, 6, 12, or 24 h. CF were washed and stained with Annexin V and propidium iodide (PI) and subjected to FACS analysis (25,000 cells/plate read). Fluorescence analysis was performed using a FACS Calibur flow cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA). Cells were illuminated with 15 mW of 488 nm laser light from an argon-ion laser. FITC fluorescence was read through a 530/30 nm bandpass filter and PI fluorescence was read through a 625/35 nm bandpass filter. Data were collected, stored, and analyzed using a Macintosh G3 computer running Cell Quest V3.1 software (Becton–Dickinson). Viable cells stained negative for Annexin V and PI, apoptotic cells were Annexin V-positive and PI-negative, and necrotic cells were Annexin V- and PI-positive.

Statistical analysis. Data were subjected to analysis of variance (ANOVA) followed by, when appropriate, Bonferroni corrected *t* tests.

Results

We first determined the effects of brief H_2O_2 exposure on phosphorylated p38 and ERK levels using ELISA. As shown in Fig. 1A, a 3 min exposure of CF to 100 μM H_2O_2 resulted in transient phosphorylation of p38; increased levels were detected by 5 min, were maximal by

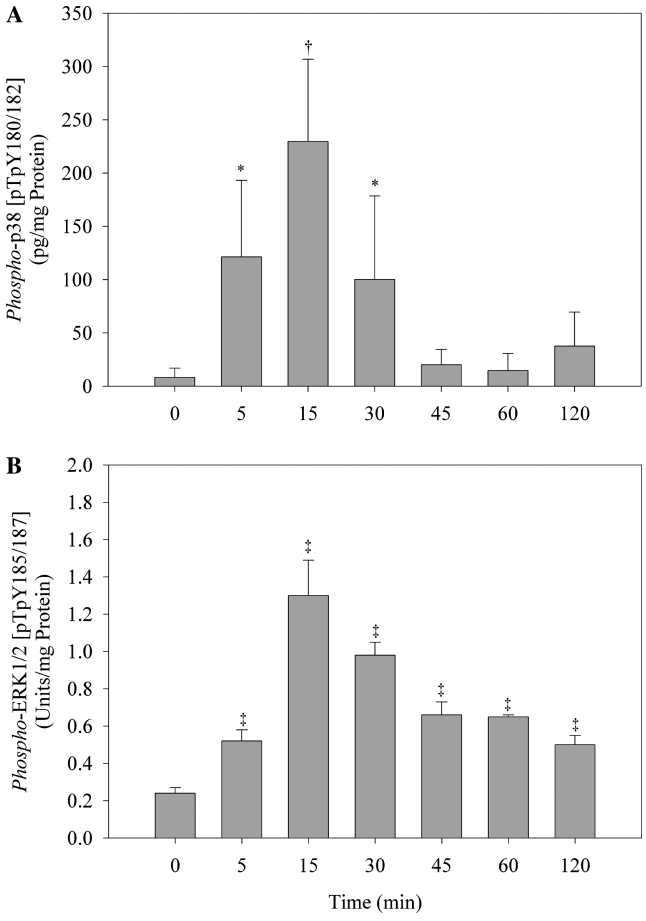

Fig. 1. ERK 1/2 and p38 MAP kinase are phosphorylated in response to brief oxidant stress. Cardiac fibroblasts were stimulated with 100 μM H_2O_2 for 3 min and harvested after the indicated time periods. Total and phosphorylated kinase levels were determined by ELISA assay and normalized to total protein. (A) p38 MAPK is transiently phosphorylated (*phospho*-p38 pTpY [180/182]) following brief oxidant stress. (B) ERK 1/2 is activated similarly (*phospho*-ERK 1/2 [pTpY 185/187]) though exhibiting slower deactivation kinetics. Bars represent means ± SD from 4 cultures/time (*$p < 0.05$, †$p < 0.01$, and ‡$p < 0.001$ vs. vehicle-treated controls).

15 min (27-fold over baseline; $p < 0.01$), and declined thereafter returning to baseline by 45 min. Fig. 1B demonstrates that ERK phosphorylation exhibited similar activation kinetics to p38, becoming maximal by 15 min (5.5-fold; $p < 0.001$) and declining thereafter. However, unlike the peroxide-induced activation profile of p38, ERK phosphorylation returned towards baseline more slowly, remaining marginally elevated at 2 h. These results demonstrate that while brief peroxide exposure leads to similar rapid and transient peak phosphorylations of p38 and ERK 1/2 MAPKs, differences in inactivation (dephosphorylation) kinetics were manifested in the slower return of ERK 1/2 to baseline levels of phosphorylation.

Since activation of various intracellular signaling pathways, including p38 and ERK, is known to influence cellular proliferation and migration, we investigated whether H_2O_2 led to changes in these parameters of CF phenotype, and whether specific inhibition of either signaling pathway modulated that response. As shown in Fig. 2A, brief exposure of CF to log doses of H_2O_2 from 0.1 to 100 μM had no significant effect on cell proliferation—each sample showed an increase from 2000 to approximately 3500 cells after 24 h. Exposure of CF to more severe oxidative stress, 3 min of 1 mM H_2O_2, did result in the presence of 22.7% fewer cells as compared to untreated controls ($p < 0.001$), though there remained an overall increase in cell number. Fig. 2B shows that the proliferation occurring in the presence of 100 μM H_2O_2

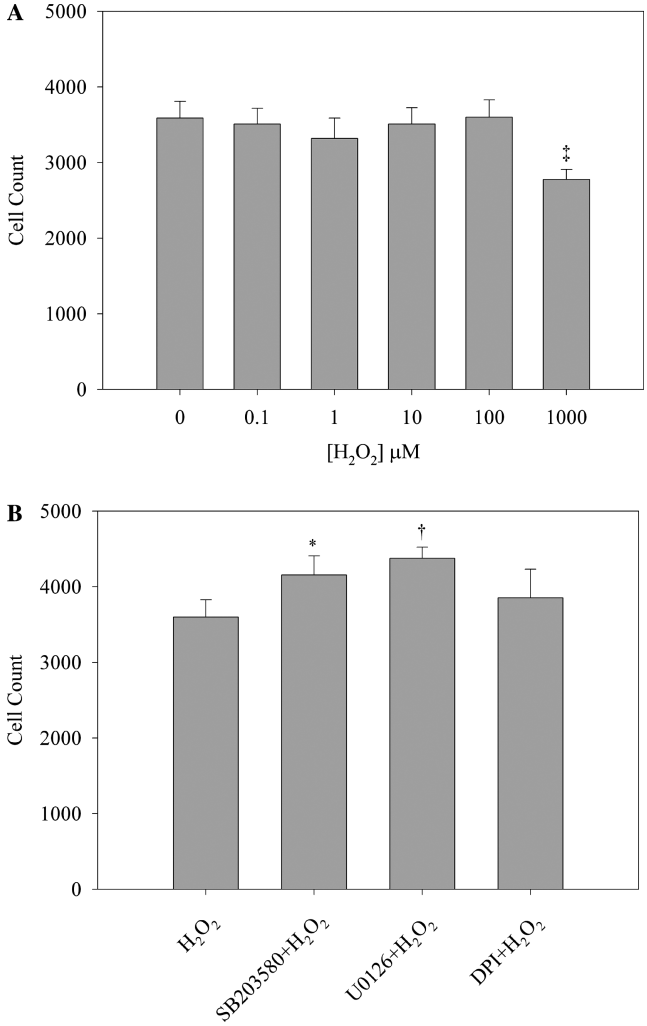

Fig. 2. Cardiac fibroblast proliferation rate is insensitive to brief H_2O_2 stimulation. CF were plated into 96-well microtiter plates at 2000 cells/well and cultured overnight in serum-free medium (0.5% BSA). (A) Three minute stimulation of CF with log doses of H_2O_2 ⩽ 100 μM had no effect on proliferation after 24 h. At higher doses (1000 μM) proliferation was suppressed. (B) Pretreatment with the p38 MAPK inhibitor SB203580 (10 μM; 20 min) or ERK 1/2 inhibitor U0126 (10 μM; 20 min) resulted in modest increases in proliferation. Pretreatment with the flavoprotein inhibitor diphenylene iodonium (DPI) was without effect. Bars represent means ± SD ($n = 4$/treatment). *$p < 0.05$, †$p < 0.01$, and ‡$p < 0.001$ vs. vehicle treatment (A) or H_2O_2 (B).

was enhanced by inhibition of either p38 with SB203580 or MEK (activator of ERK 1/2) with U0126 ($p < 0.05$ and $p < 0.01$, respectively). These results suggest roles for p38 and ERK 1/2 MAP kinases in the regulation of cell cycle following H_2O_2 exposure, such that elevated kinase levels favored quiescence. We have previously observed that exposure of CF to H_2O_2 activates endogenous production of superoxide/H_2O_2 and nitric oxide in a dose-dependent manner (unpublished observation). Of note, the influence of peroxide-stimulated endogenous superoxide and/or nitric oxide on CF proliferation was minimal since H_2O_2 exposure after treatment with the flavoprotein inhibitor diphenylene iodonium (DPI) was without effect. The fact that treatment of CF with either SB203580, U0126, or DPI in the absence of exogenously applied H_2O_2 had no significant effect on proliferation (data not shown) suggests that the effects of these molecules are minimal at baseline, and that they serve to blunt the cellular response to H_2O_2.

Exposure of CF to a 3 min pulse of H_2O_2 resulted in a dose-dependent decrease in the number of cells migrating towards fibronectin (haptotaxis) after 24 h, as defined by Boyden Chamber assay. Fig. 3 shows that in the absence of the chemotactic stimulus of a fibronectin gradient few cells migrated, indicating the cells are intrinsically quiescent. Baseline migration rate towards a fibronectin gradient over 24 h was decreased following oxidative stress: 10 and 100 μM H_2O_2 led to 34% and 45% reductions in the numbers of cells migrating (both $p < 0.001$). Treatments with lower doses (0.1 and 1 μM H_2O_2) were without effect (data not shown), suggesting a threshold response pattern. Since p38 has been shown to mediate cell migration in other cell types, we assessed its role in CF. Treatment of CF with SB203580 led to a 33% reduction in migration, confirming a positive effect of p38 on migration in this cell type. Of note, pretreatment with this inhibitor followed by 100 μM H_2O_2 exposure resulted in a further reduction in migration. The data suggest the negative impact of oxidative stress is independent of p38, and of sufficient magnitude to outweigh the positive effect of p38 upregulation induced by H_2O_2. Generally opposite effects were seen with ERK 1/2 and cell migration. Treatment with U0126 alone

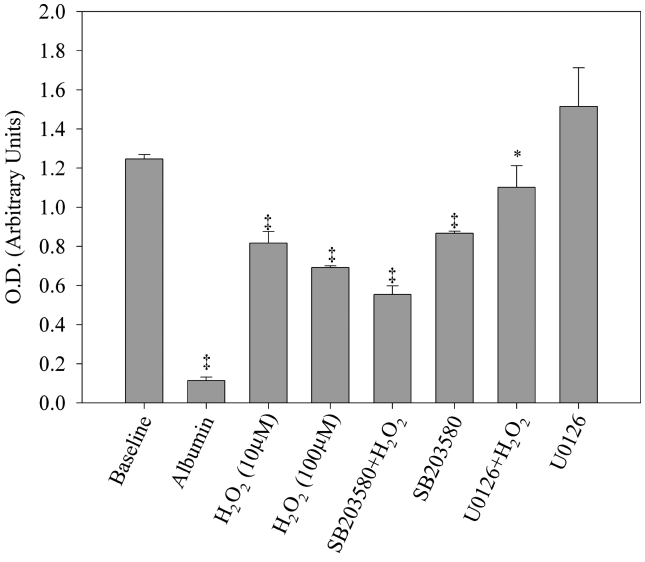

Fig. 3. Brief oxidant stress retards migration. Cardiac fibroblasts were plated into Boyden chambers (2.5×10^5 cells/chamber), pretreated with either SB203580 (10 μM; 20 min) or U0126 (10 μM; 20 min) or DMSO vehicle (1:1000), and stimulated with H_2O_2 or HBSS vehicle. Extracellular matrix-stimulated (baseline) and unstimulated (BSA) migration rates were established by allowing CF to migrate towards fibronectin and albumin for 24 h, respectively. Bars represent means ± SD ($n = 4$/treatment). *$p < 0.05$, ‡$p < 0.001$ vs. baseline.

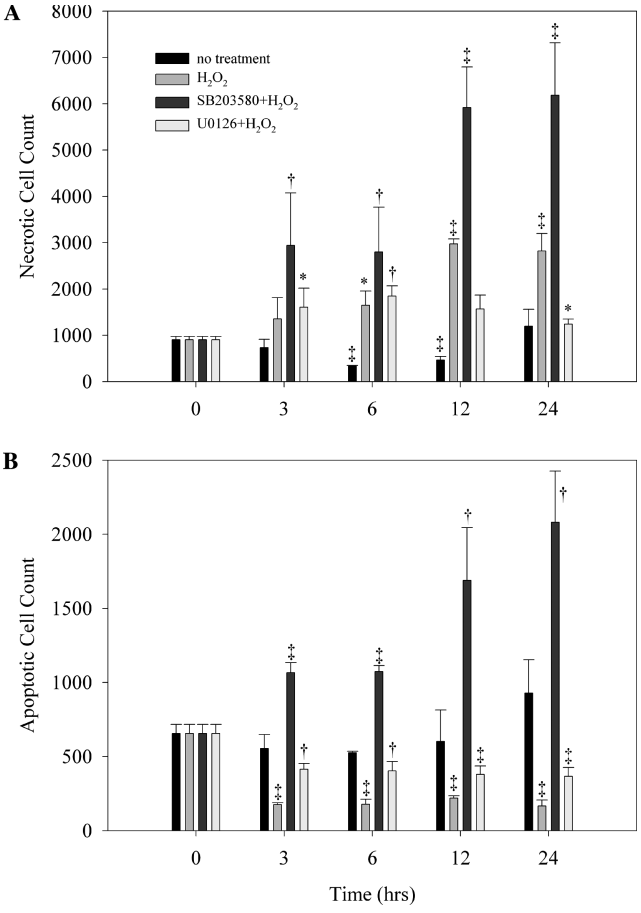

Fig. 4. Impact of brief oxidant stress on cardiac fibroblast viability. CF were plated into 100 × 20 mm tissue culture plates and grown to 85% confluency. Cultures were stimulated for 3 min with 100 μM H_2O_2 or vehicle (HBSS) with or without 20 min pretreatments with either SB203580 (10 μM) or U0126 (10 μM). After peroxide stimulation, cells were re-fed with serum-free medium (0.5% BSA) and harvested by mild trypsinization after 3, 6, 12, or 24 h. CF were washed and stained with Annexin V and propidium iodide (PI) and subjected to FACS analysis (25,000 cells/plate read) using a FACS Calibur flow cytometer as described under "Materials and methods." Viable cells stained negative for Annexin V and PI, necrotic cells (A) were Annexin V- and PI-positive, and apoptotic cells (B) were Annexin V-positive and PI-negative. Bars represent means ± SD ($n = 4$/treatment). *$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$ vs. Time 0.

tended to cause increased migration, suggesting ERK 1/2 retards migration. Exposure of the cells to H_2O_2 following pretreatment with U0126 caused migration to be reduced by only 12% compared to baseline rates ($p < 0.05$), showing that ERK 1/2 may participate in oxidation-induced decreases in migration since it is upregulated under these conditions, and when ERK 1/2 is blocked H_2O_2 has a blunted impact on cell migration.

Hydrogen peroxide has been shown to affect cell viability by inducing necrosis and/or apoptosis in some contexts while providing protection from cell death in others. We determined the consequences of brief peroxide exposure on CF viability by stimulating cells for 3 min with 100 μM H_2O_2 and measuring necrosis and apoptosis by FACS analysis. Fig. 4A shows that baseline incidence of necrosis in untreated CF cultures was approximately 4%, and that brief exposure to H_2O_2 led to a necrotic rate of >12% by 12 and 24 h (both $p < 0.001$). Treatment with SB203580 prior to H_2O_2 exposure resulted in further increases in necrosis compared to peroxide-treated CF (2-fold by 24 h; $p < 0.01$), suggesting p38 plays a role in preventing necrosis following oxidant stress. By contrast, treatment with U0126 prior to H_2O_2 led to reductions in H_2O_2-induced necrosis to levels approaching baseline, indicating ERK 1/2 participates in necrosis following peroxide exposure.

As shown in Fig. 4B, CF exhibit a baseline apoptotic rate of nearly 3%, while cells exposed to H_2O_2 showed a 3.5-fold reduction in apoptosis over the 24 h study period ($p < 0.001$). Inhibition of p38 with SB203580 led to a marked increase in apoptosis, demonstrating that p38 plays a significant anti-apoptotic role in this context. Inhibition of ERK 1/2 activation had little effect on the impact of H_2O_2 on rates of apoptosis.

Discussion

Our findings show for the first time that stimulation of primary cardiac fibroblasts with brief oxidant stress, similar to the in vivo condition of ischemia/reperfusion, leads to upregulation of p38 and ERK 1/2 and to the acquisition of a phenotype characterized by reduced apoptosis and migration. In the normal heart, cardiac fibroblasts are numerous, long-lived, and generally quiescent. Prior studies have shown the importance of this cell type for maintenance of structural integrity of the heart, and for their responses to a variety of stressful stimuli. They play a key role in the increased cardiac fibrosis present in states of cardiac hypertrophy, and are critically important for healing of myocardium damaged, either in diffuse inflammatory states such as myocarditis, or following regional damage in myocardial infarction. In this situation CF re-enter the cell cycle, proliferate, and migrate to the infarcted region where they affect extracellular matrix deposition leading to fibrosis and healing [1,22]. How fibroblasts respond to less severe, but clinically common, episodes of oxidative stress, such as occurs with reperfusion of ischemic myocardium, has not been studied.

Using electron paramagnetic resonance spectroscopy and spin trapping techniques Garlick et al. [11] and others [12] have demonstrated that reperfusion of ischemic myocardium leads to a brief and substantial spike in ROS production. We recently reported that in vitro stimulation of CF with brief oxidant stress, mimicking ischemia/reperfusion, leads to large Ca^{2+} fluxes and IL-6 induction [7]. Thus, CF are capable of sensing extracellular oxidants that activate intracellular signaling mechanisms, leading to specific cellular responses such as cytokine expression. In the current study we show that brief oxidant stress also leads to rapid activations of ERK 1/2 and p38 signaling pathways. Activation of these MAPK pathways has been demonstrated in myriad cell types and may be receptor-mediated or may be activated in response to physical or biochemical stress. The fact that activation of these pathways is ubiquitous, yet can impact cell survival, proliferation, migration or growth, indicates that responses are determined by the context, amplitude, and duration of the stimulus. In the specific context of ischemia/reperfusion it is clear that the CF responds to oxidative stress in a coordinated pattern mediated at least in part by these signaling pathways.

Hydrogen peroxide and superoxide anions have been shown to stimulate proliferation in a number of cell types (see [23] for review). On the other hand, studies have demonstrated that H_2O_2 exposure leads to reduced proliferation in keratinocytes and epithelial cells [24,25], and acquisition of a senescence-like state in human diploid fibroblasts [26], F65 primary human fibroblasts [27], and NIH 3T3 cells [28]. Activation of ERK 1/2 is known to participate in the stimulation of CF proliferation and migration [3,29,30]. We observed that brief exposure of CF to oxidant stress (0.1–100 μM H_2O_2) had little effect on CF proliferation despite transient activations of both ERK 1/2 and p38 MAPK. Surprisingly, inhibition of either ERK 1/2 or p38 prior to H_2O_2 resulted in modest but significant increases in cell proliferation as compared to either H_2O_2 treatment alone or to baseline proliferation rates (see Fig. 2A). These data indicate that p38 and ERK 1/2 counteract a primary proliferative effect of H_2O_2, such that the cell replication is not enhanced following oxidant stress in this model. Our results may reflect differences in the duration and amplitude of the oxidant stress compared to other studies where higher concentrations and/or longer exposures are common; conditions that we have observed to be profoundly cytotoxic to primary adult rat CF (unpublished observation). In the context of the intact heart this would suggest that brief ischemic episodes may not induce fibroblast proliferation.

In the heart, cell migration is an essential process during embryonic development, angiogenesis, wound healing, and inflammation. Cardiac fibroblasts, once activated by extracellular cues, undergo phenotypic transition to myofibroblasts and migrate as individual cells in a complex process requiring dynamic changes in extracellular matrix, cytoskeletal architecture, cell–cell, and cell–substrate adhesions [31]. Reperfusion of myocardium subjected to sublethal durations of ischemia results in temporary and reversible depression of function (stunning) but does not exhibit the histological changes typically seen following longer durations of ischemia that are associated with areas of infarction. As such, fibroblast migration, as a precursor to wound repair and cardiac remodeling, should not be a feature of ischemia/reperfusion. We found that following oxidative stress CF exposed to a fibronectin gradient exhibited reduced migration rates (Fig. 3). A ramification of this finding is that in an intact heart episodes of ischemia/reperfusion may retard cell migration of fibroblasts, such that healing of infarcted myocardium may be impaired if ongoing episodes of ischemia occur in border-zone areas from which fibroblasts are recruited for the healing process.

Allen et al. [32] recently showed that treatment with the free radical scavenger *N*-acetyl cysteine suppressed the stimulatory effects of fibronectin on myoblast migration. Our data show that inhibition of p38 MAPK led to significant reductions in CF migration, and that peroxide-induced reductions in CF migration rates were more pronounced following p38 MAPK inhibition. Thus, p38 MAPK contributes to ECM-stimulated migration and maintains migration rates during oxidant stress. On the other hand, ERK inhibition with the MEK inhibitor U0126 had little effect on fibronectin stimulated migration and resulted in increased migration rates following brief oxidant stress. These results demonstrate opposing effects of different MAPK pathways, with p38 promoting migration while ERK acts to retard migration following brief oxidant stress.

Oxidant stress is well known to lead to necrosis, and our results confirm this: even a 3 min exposure to100 μM H_2O_2 caused a 3-fold increase in necrosis at 24 h. Inhibition of p38 led to substantial further increase in necrosis, demonstrating a beneficial effect of p38 in prevention of cell death following ROS exposure. ERK 1/2 had an opposing effect, such that CF subjected to inhibition of ERK 1/2 prior to oxidant stress had necrotic cell counts very close to those of untreated cells. Thus, p38 and ERK 1/2 activation have opposing effects on H_2O_2-induced necrosis in CF; p38 MAPK activity appears necessary for limiting necrosis while ERK 1/2 activation was responsible, at least in part, for peroxide-induced necrosis.

ROS exposure had opposite effects on apoptosis. We found that stimulation of CF with brief oxidant stress results in greater than a 3-fold decrease in apoptosis compared to baseline rates. Here again, p38 appeared important, since inhibition of p38 abolished protection leading to a significant (3-fold) increase in apoptosis. These observations are similar to those of Han et al. [33] who showed that protection against H_2O_2-induced apoptosis was seen following oxidative preconditioning characterized by transient p38 activation and sustained protein kinase B (Akt) activation. These authors and others showed that inhibition of p38 with SB203580 eliminated protection against apoptosis providing evidence of a protective role for p38 in H_2O_2-induced apoptosis. We also observed that ERK inhibition was somewhat protective although apoptosis levels in CF were intermediate between unstimulated and H_2O_2 stimulated cells.

Our results indicate brief oxidant stress leads to transient activations of p38 and ERK resulting in protection from H_2O_2-induced apoptosis in CF. The mechanism for this protection is unclear although we have previously reported H_2O_2-induced Ca^{2+} fluxes and previous reports demonstrate modest increases in $[Ca^{2+}]_i$ protect cells through Ca^{2+}/calmodulin-dependent phosphorylation of Akt initiating a known cell survival signaling pathway [34]. It is of interest that in a situation where oxidant stress augments CF fibrosis there is a reduction in apoptotic cell death. Since CF are capable of proliferation it is reasonable to speculate that under ordinary conditions there is some degree of steady-state cellular turnover. Down-modulation of apoptosis in circumstances of increased necrosis may be a mechanism to preserve overall cell numbers in times of stress, allowing sufficient cells to survive to allow myocardial scar formation.

In conclusion, the present study defined the cellular phenotype resulting from the exposure of primary cardiac fibroblasts to brief respiratory bursts as occurs in ischemia/reperfusion. Our results demonstrate that brief stimulation of cardiac fibroblasts with H_2O_2 leads to activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signaling pathways. We demonstrate that brief H_2O_2 alters fibroblast migration, apoptosis, and necrosis while exerting minimal effects on proliferation. These results provide evidence that cardiac fibroblasts, in addition to their traditionally recognized roles in cardiac remodeling, fibrosis, and providing structural support, are capable of responding to dramatic as well as subtle changes in the local environment enabling them to act, in concert with cardiomyocytes, to provide appropriate biological responses in the stressed heart.

Acknowledgments

This work was supported by the Research Service of the Department of Veterans Affairs and the Biomedical Research Foundation of South Texas.

References

[1] M. Eghbali, M.J. Czaja, M. Zeydel, F.R. Weiner, M.A. Zern, S. Seifter, O.O. Blumenfeld, Collagen chain mRNAs in isolated heart cells from young and adult rats, J. Mol. Cell. Cardiol. 20 (1988) 267–276.

[2] A.C. Nag, Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution, Cytobios 28 (1980) 41–61.

[3] W. Schorb, K.M. Conrad, H.A. Singer, D.E. Dostal, K.M. Baker, Angiotensin II is a potent stimulator of MAP-kinase activation in neonatal rat cardiac fibroblasts, J. Mol. Cell. Cardiol. 27 (1995) 1151–1160.

[4] B. Maisch, Extracellular matrix and cardiac interstitium: restriction is not a restricted phenomenon, Herz 20 (1995) 75–80.

[5] D.W. Powell, R.C. Mifflin, J.D. Valentich, S.E. Crowe, J.I. Saada, A.B. West, Myofibroblasts. I. Paracrine cells important in health and disease, Am. J. Physiol. 277 (1999) C1–C19.

[6] J.J. Tomasek, G. Gabbiani, B. Hinz, C. Chaponnier, R.A. Brown, Myofibroblasts and mechano-regulation of connective tissue remodeling, Nat. Rev. Mol. Cell. Biol. 3 (2002) 348–363.

[7] J.T. Colston, B. Chandrasekar, G.L. Freeman, A novel peroxide-induced calcium transient regulates interleukin-6 expression in cardiac-derived fibroblasts, J. Biol. Chem. 277 (2002) 23477–23483.

[8] C. Ancey, P. Corbi, J. Froger, A. Delwail, J. Wijdenes, H. Gascan, D. Potreau, J.C. Lecron, Secretion of IL-6, IL-11 and LIF by human cardiomyocytes in primary culture, Cytokine 18 (2002) 199–205.

[9] M.O. Gray, C.S. Long, J.E. Kalinyak, H.T. Li, J.S. Karliner, Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF-beta 1 and endothelin-1 from fibroblasts, Cardiovasc. Res. 40 (1998) 352–363.

[10] C.S. Long, K. Kariya, L. Karns, P.C. Simpson, Trophic factors for cardiac myocytes, J. Hypertens. Suppl. 8 (1990) S219–S224.

[11] P.B. Garlick, M.J. Davies, D.J. Hearse, T.F. Slater, Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy, Circ. Res. 61 (1987) 757–760.

[12] R. Bolli, B.S. Patel, M.O. Jeroudi, E.K. Lai, P.B. McCay, Demonstration of free radical generation in "stunned" myocardium on intact dogs with the use of the spin trap α-phenyl-*N*-*tert*-butyl nitrone, J. Clin. Invest. 82 (1988) 476–485.

[13] National Institutes of Health (1985) Guide to the Care and Use of Animals, Office of Science and Health Reports (DHEW Publication Number 85-23), DRR/National Institutes of Health, Bethesda, MD.

[14] J.T. Colston, B. Chandrasekar, G.L. Freeman, Expression of apoptosis-related proteins in experimental coxsackievirus myocarditis, Cardiovasc. Res. 38 (1998) 158–168.

[15] A. Clerk, P.H. Sugden, The p38-MAPK inhibitor, SB203580, inhibits cardiac stress-activated protein kinases/c-jun N-terminal kinases (SAPKs, JNKs), FEBS Lett. 426 (1998) 93–96.

[16] X.L. Ma, S. Kumar, F. Gao, C.S. Louden, B.L. Lopez, T.A. Christopher, C. Wang, J.C. Lee, G.Z. Feuerstein, T.-L. Yue, Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion, Circulation 99 (1999) 1685–1691.

[17] M.M. Mocanu, G.F. Baxter, Y. Yuankun, S.D. Critz, D.M. Yellon, The p38 MAPK inhibitor, SB203580, abrogates ischaemic preconditioning in rat heart but timing of administration is critical, Basic Res. Cardiol. 95 (2000) 472–478.

[18] P.R. Young, M.M. McLaughlin, S. Kumar, S. Kassis, M.L. Doyle, D. McNulty, T.F. Gallagher, S. Fisher, P.C. McDonnell, S.A. Carr, M.J. Huddleston, G. Seibel, T.G. Porter, G.P. Livi, J.L. Adams, J.C. Lee, Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site, J. Biol. Chem. 272 (1997) 12116–12121.

[19] C.-M. Cheng, H.-J. Hong, J.-C. Liu, N.-L. Shih, J.-H. Juan, S.-H. Loh, P. Chan, J.-J. Chen, T.-H. Cheng, Crucial role of extracellular signal-regulated kinase pathway in reactive oxygen species-mediated endothelin-1 gene expression induced by endothelin-1 in rat cardiac fibroblasts, Mol. Pharmacol. 63 (2003) 1002–1011.

[20] M.F. Favata, K.Y. Horiuchi, E.J. Manos, A.J. Daulerio, D.A. Stradley, W.S. Feeser, D.E. Van Dyk, W.J. Pitts, R.A. Earl, F. Hobbs, R.A. Copeland, R.L. Magolda, P.A. Scherle, J.M. Trzaskos, Identification of a novel inhibitor of mitogen-activated protein kinase kinase, J. Biol. Chem. 273 (1998) 18623–18632.

[21] A.C. Ranganathan, K.K. Nelson, A.M. Rodriguez, K.-H. Kim, G.B. Tower, J.L. Rutter, C.E. Brinckerhoff, T.-T. Huang, C.J. Epstein, J.J. Jeffrey, J.A. Melendez, Manganese superoxide dismutase signals matrix metalloproteinase expression via H_2O_2-dependent ERK1/2 activation, J. Biol. Chem. 276 (2001) 14264–14270.

[22] I. Manabe, T. Shindo, R. Nagai, Gene expression in fibroblasts and fibrosis: involvement in cardiac hypertrophy, Circ. Res. 91 (2002) 1103–1113.

[23] R.H. Burdon, Superoxide and hydrogen peroxide in relation to mammalian cell proliferation, Free Radic. Biol. Med. 18 (1995) 775–794.

[24] E.A. O'Toole, M. Goel, D.T. Woodley, Hydrogen peroxide inhibits keratinocyte migration, Dermatol. Surg. 22 (1996) 525–529.

[25] S. Watanabe, X.E. Wang, M. Hiros, T. Osada, H. Tanaka, N. Sato, Rebamipide prevented delay of wound repair induced by hydrogen peroxide and suppressed apoptosis of gastric epithelial cells in vitro, Dig. Dis. Sci. 43 (1998) S107–S112.

[26] C. Bladier, E.J. Wolvetang, P. Hutchinson, J.B. de Haan, I. Kola, Response of a primary human fibroblast cell line to H_2O_2: senescence-like growth arrest or apoptosis?, Cell Growth Diff. 8 (1997) 589–598.

[27] Q. Chen, B.N. Ames, Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells, Proc. Natl. Acad. Sci. USA 91 (1994) 4130–4134.

[28] J.B. de Haan, F. Cristiano, R. Iannello, C. Bladier, M. Kelner, I. Kola, Elevation in the ratio of Cu/Zn-superoxide dismutase to glutathione peroxidase activity induces features of cellular senescence and this effect is mediated by hydrogen peroxide, Hum. Mol. Genet. 5 (1995) 283–292.

[29] D.E. Dostal, G.W. Booz, K.M. Baker, Angiotensin II signaling pathways in cardiac fibroblasts: conventional versus novel mechanisms in mediating cardiac growth and function, Mol. Cell. Biochem. 157 (1996) 15–21.

[30] H. Kawano, Y.S. Do, Y. Kawano, V. Starnes, M. Barr, R.E. Law, W.A. Hsueh, Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts, Circulation 101 (2000) 1130–1137.

[31] A. Desmoulière, G. Gabbiani, The role of the myofibroblast in wound healing and fibrocontractive diseases, in: R.A.F. Clark (Ed.), The Molecular and Cellular Biology of Wound Repair, second ed., Plenum Press, New York, 1996.

[32] D.L. Allen, D.H. Teitelbaum, K. Kurachi, Growth factor stimulation of matrix metalloproteinase expression and myoblast migration and invasion in vitro, Am. J. Physiol. 284 (2003) C805–C815.

[33] H. Han, H. Wang, H. Long, S. Nattel, Z. Wang, Oxidative preconditioning and apoptosis in L-cells, J. Biol. Chem. 276 (2001) 26357–26364.

[34] S. Yano, H. Tokumitsu, T.R. Soderling, Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway, Nature 396 (1998) 584–587.