A Novel Peroxide-induced Calcium Transient Regulates Interleukin-6 Expression in Cardiac-derived Fibroblasts*

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James T. Colston, Bysani Chandrasekar, and Gregory L. Freeman‡

From the Division of Cardiology, University of Texas Health Science Center, South Texas Veterans Health Care System, Audie L. Murphy Division, San Antonio, Texas 78284-7872

Reperfusion of ischemic myocardium leads to a local burst of free radicals, increased \([\text{Ca}^{2+}]\)i, and the release of proinflammatory cytokines. The purpose of this study was to determine whether brief exposure of cardiac fibroblasts to \(H_2O_2\) is associated with transient changes in \([\text{Ca}^{2+}]\)i and whether this stimulus is sufficient to induce interleukin-6 (IL-6) expression. Cardiac derived fibroblasts were isolated from adult male rats and cultured under standard conditions. Individual coverslip-attached fibroblasts were loaded with the calcium probe Fura-2/AM and exposed to a single 3-min pulse of 100 \(\mu M\) \(H_2O_2\). In addition, low passage cultures were exposed to a pulse of \(H_2O_2\) and assayed for IL-6 expression. A brief exposure of \(H_2O_2\) led to a large intracellular \([\text{Ca}^{2+}]\)i transient with a mean magnitude of 318 ± 28 nM (mean ± S.D., \(n = 12\)). Stimulation in the absence of \([\text{Ca}^{2+}]\)i resulted in a 59% reduction in mean transient magnitude (129 ± 23 nM, \(n = 10\), \(p < 0.001\)), whereas pretreatment with the inositol 1,4,5-trisphosphate receptor blocker xestospongin C resulted in a 37% reduction (199 ± 25 nM, \(n = 10\), \(p < 0.01\)). Cells treated with xestospongin C and stimulated in the absence of \([\text{Ca}^{2+}]\)i did not exhibit a \([\text{Ca}^{2+}]\)i transient. Time-dependent IL-6 release was significantly elevated by 4 h (368 ± 64 pg/mg protein, \(p < 0.01\)) and increased further by 24 h (1030 ± 76 pg/mg protein). The depletion of cellular \([\text{Ca}^{2+}]\)i by pretreatment with thapsigargin in the absence of \([\text{Ca}^{2+}]\)i, attenuated \(H_2O_2\)-induced IL-6 mRNA expression while blocking protein release. These data show that the exposure of cardiac fibroblasts to a brief pulse of physiological levels of \(H_2O_2\) resulted in a large \([\text{Ca}^{2+}]\)i transient with intracellular and extracellular \([\text{Ca}^{2+}]\)i contributions. Furthermore, brief \(H_2O_2\) exposure led to calcium-dependent IL-6 expression.

For the past several years, it has been known that when viable myocardium is reperfused following ischemia, there is a brief and substantial spike in reactive oxygen species (ROS) levels in the tissue. Through the use of electron paramagnetic resonance spectroscopy and spin-trapping techniques, the bulk of ROS production was shown to occur during the first 5 min of reperfusion, being maximal at 2 min (1). Initial studies on this event focused on the process of myocardial stunning, the reversible reduction in contractile performance that follows non-lethal durations of ischemia (2). Studies by the laboratories of Bolli et al. (3) and Gross et al. (4) show that this impairment of contractile performance could be mitigated by treatment with ROS scavengers, and studies by McDonough et al. (5) have suggested that stunning is attributed at least in part to oxidative damage targeted to troponin species (5). ROS release also causes lipid peroxidation, and 4-hydroxynonenal has been shown to impair mitochondrial function at the level of cytochrome c oxidase (6). In addition, we have previously shown that reperfusion is followed by the activation of redox-sensitive transcription factors with the generation of proinflammatory cytokines and chemokines (7–9). Thus, a broad range of biological effects results from the post-ischemic ROS transient.

In nonexcitable cells, exposure to oxidative stress has been shown to cause a calcium transient. Qin et al. (10) have shown that exposure of B cells to peroxide leads to an increase of intracellular calcium with a very rapid onset. In a series of elegant experiments, these workers showed that phospholipase Cγ mediated the hydrolysis of phosphatidylinositol 4,5-biphosphate to IP3, which led to the calcium mobilization. Given the fact that the ultrastructural work by Nag et al. (11) and Eghbali et al. (12) has shown that 65–70% of the cells in the myocardium are non-myocytes with fibroblasts widely scattered throughout the tissue, these findings suggest that these cells may manifest a calcium transient following ischemia reperfusion. Given the known role of calcium as a regulator of gene transcription, it is possible that this could be a regulatory mechanism in the post-ischemic myocardium. Fibroblasts are a rich source of cytokines, chemokines, and growth factors and play a vital role in remodeling, making this possibility one that is of considerable interest.

The purpose of this study was to determine whether the exposure of cardiac derived fibroblasts (CDFs) to \(H_2O_2\) for a brief time would cause a rise in intracellular calcium. We hypothesized that this would be the case and that the calcium signal would modulate gene expression in these cells. Our results demonstrate the occurrence of this phenomenon and that abrogation of the calcium signal following \(H_2O_2\) exposure blunted the expression of IL-6, indicating that the ROS-induced calcium transient has a role in gene regulation that is distinct from the direct effects of the oxidative stress.

EXPERIMENTAL PROCEDURES

Fibroblast Isolation and Culture—The investigation conforms with a report published previously (for review see Ref. 13). CDFs were isolated from the hearts of adult male Wistar Kyoto rats (200–250 g) using a method developed in our laboratory. After induction of deep anesthesia with an intramuscular injection (0.2–0.3 ml) containing a mixture of...
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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-Ringer bicarbonate buffer (110 mm NaCl, 2.6 mm KCl, 1.2 mm CaCl₂, 1.2 mm MgSO₄, 25 mm NaHCO₃, 11 mm glucose) at 80 mm Hg. Hearts were then perfused for 25 min with Krebs-Ringer bicarbonate buffer enzyme solution containing 0.5 mg/ml type II collagenase (Worthington), 25 μM CaCl₂, 1 mg/ml fatty acid-free albumin. After digestion, the ventricles were trimmed free and minced in Krebs-Ringer bicarbonate buffer enzyme solution containing 10 mg/ml collagenase, filtered through sterile nylon mesh, and centrifuged at 25 × g for 5 min to remove cardiomyocytes, red blood cells, and debris. The resultant supernatant was then centrifuged at 1000 × g for 8 min. The cell pellet was resuspended in 20 ml of CDF medium (15 mm HEPES, 16.7 mm NaHCO₃, 1× each of basal medium Eagle vitamins and minimum Eagle's medium-amino acids (Invitrogen), 2 mm glutamine, 10% heat-inactivated fetal bovine serum, antibiotics, pH 7.3) and plated into T75 tissue culture flasks (Falcon, BD PharMingen). Non-adherent cells were removed after 2 h and discarded. Cells were fed with fresh medium three times per week using CDF medium and split 1:3 when confluent.

CDF and non-fibroblast contaminants were identified by immunofluorescence using routine methods (14) with the following antibodies: fluorescent-conjugated anti-β-actin (Sigma), anti-platelet-endothelial cell adhesion molecule-1 (CD-31, Research Diagnostics, Flanders, NJ), anti-vimentin (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-smooth muscle actin (Sigma). After two serial passages, >99.7% of cells in these cultures exhibit vimentin and β-actin immunoreactivity, are CD-31- and smooth muscle actin-negative, and display typical fibroblast-like morphology. Non-fibroblast cells typically account for <0.1% of total cells as determined by immunofluorescence. Cardiac-derived fibroblasts were used in these experiments between the third and sixth passage.

Rat heart-derived vascular endothelial cells (EC) were kindly provided by Dr. C. A. Digio (Wayne State University, Detroit, MI) (15). Cultured EC showed positive vimentin and CD31 immunoreactivity as determined by immunofluorescence and exhibited the cobblestone growth pattern typical of EC in culture. Smooth muscle actin immunoreactivity was not detected in these cultures. Embryonic mouse fibroblasts (NIH-3T3) were obtained from the American Type Culture Collection (CRL-1658). Both EC and NIH-3T3 cells were cultured under conditions identical to those described for CDFs and split 1:3 when confluent.

Intracellular Calcium and ROS Measurements—Intracellular calcium measurements were made in individual coverslip-attached fibroblasts using the calcium-sensitive probe Fura-2/AM (Molecular Probes, Eugene, OR) (16). CDFs were plated onto sterilized round coverglass (25-mm thickness 1, Warner Instrument Corp., Camden, CT) and cultured overnight in CDF medium. Prior to study, coverslip-attached CDFs were loaded with 5 μM Fura-2/AM in perfusion buffer for 45 min at 37 °C. Following incubation, the coverslip-attached fibroblasts were washed three times with perfusion buffer and mounted in a perfusion chamber for study. Intracellular ROS levels were measured in individual CDFs loaded with dichlorodihydrofluorescein diacetate (Molecular Probes). Dichlorodihydrofluorescein diacetate is converted to the highly fluorescent 2',7'-dichlorofluorescein in the presence of ROS. Coverslip-attached CDF cells were incubated with 5 μM dichlorodihydrofluorescein diacetate for 20 min in perfusion buffer and then washed three times prior to study (17).

Fluorescence Measurement System—Coverslip-attached CDF cells were mounted in a closed perfusion chamber (RC-21BRF, Warner) and maintained at 37 °C constant temperature (Temperature Controller (TC-324B, Warner) throughout the experiment. The cells were continually superfused at a rate of 2 ml/min with oxygen-saturated perfusion buffer (137 mm NaCl, 1.2 mm MgSO₄, 7 H₂O, 4.9 mm KCl, 1.2 mm NaHPO₄, 20 mm HEPES, 15 mm t-glucose, 1.8 mm CaCl₂, pH 7.4). The perfusion chamber was mounted on the stage of an inverted fluorescent microscope (Nikon Eclipse TE200, Nikon Inc., Melville, NY) mated to an imaging and photon counting system (Ion Optix, Milton, MA). Individual CDFs were studied using a ×20 objective after being optically isolated from the control chamber. Images were collected in the region that ranged from 0.01 to 0.04 mm². Photon counts, image storage, and rapid filter changes were controlled by microcomputer. Data storage and analysis were performed using IonWizard analysis software (Ion Optix).

Intracellular calcium levels were calculated automatically using the equation described by Grynkiewicz et al. (18) after routine calibration. Rₘₐₓ and Rₘᵢₙ were determined routinely using two methods, Fura-2/AM pentapotassium salt (5 μM) with known [Ca²⁺] and in situ calibration using ionomycin with nominal and high [Ca²⁺].

Interleukin-6—Interleukin-6 protein release into CDF culture supernatants was determined by enzyme-linked immunosorbent assay following protocols by the manufacturer (BIOSOURCE, Camarillo, CA). Interleukin-6 standards were run in parallel with CDF culture supernatants and analyzed with a computer-controlled enzyme-linked immunosorbent assay plate reader (SpectraMax 190, Molecular Devices). Triplicate cultures for each treatment and time point were used for IL-6 analyses with individual samples assayed in duplicate. IL-6 levels were determined from standard curves performed for each assay and normalized to total protein content. IL-6 mRNA levels were determined in treated and control CDFs using Northern blotting as described previously (8). Statistical Analyses—For each study, ANOVA was performed to determine whether statistically significant differences were present across treatments or times within treatments. If the F-test from ANOVA was statistically significant (p < 0.05), Bonferroni-adjusted Student’s t-tests were performed for the pairwise treatment or time comparisons of interest with p < 0.05 considered as significant. The F-test for peak Ca²⁺ across treatments was significant (p < 0.001); therefore, pairwise comparisons between control and each of the five other treatments were performed. The F-test for IL-6 across time within H₂O₂ was significant (p < 0.001); therefore a profile analysis was performed to identify time intervals at which significant increases of IL-6 were observed. The experiment was repeated with expanded time intervals for H₂O₂, TG, and TG + H₂O₂ treatments. The F-tests for H₂O₂ (p < 0.001) and TG + H₂O₂ (p < 0.04) were significant, so profile analyses were performed for these treatments; however, the F-test for TG (p = 0.06) was not significant. The F-test for optical density across time within H₂O₂ was significant (p < 0.001), so a profile analysis was performed. The F-test for optical density across treatments at 4 h was significant (p < 0.001), and for this experiment, all possible pairwise comparisons among treatments were performed.

Results

The exposure of cardiac-derived fibroblasts to a brief 3-min pulse of 100 μM H₂O₂ led to a large transient increase in [Ca²⁺]. Fig. 1A shows a typical [Ca²⁺]Transient tracing from one treated CDF along with a representative tracing from a non-H₂O₂-stimulated cell monitored for the same time period. As shown in this figure, [Ca²⁺] was observed to rise several minutes after initial H₂O₂ exposure peaking at ~25 min (23.5 ± 6.2 min) after which levels returned to below base line with an average transient duration of 44 ± 7.6 min. Basal [Ca²⁺] was 81 ± 21 nM (mean ± S.D., n = 12) prior to H₂O₂ exposure and 19 ± 7 nM post-transient. Non-H₂O₂-exposed CDFs did not exhibit any large fluxes in [Ca²⁺]. Similarly, neither heart-derived endothelial cells nor NIH-3T3 fibroblasts exhibited detectable Ca²⁺ fluxes following brief peroxide exposure.

A protocol was developed enabling the production of a calcium transient in CDFs in the absence of exogenously applied H₂O₂. As shown in Fig. 1B, this method produced a transient of similar magnitude and duration to those observed in peroxide-treated cells. Conversely, cells were stimulated with H₂O₂ following pretreatment with thapsigargin in the absence of extracellular Ca²⁺. This treatment allowed peroxide exposure in the absence of detectable Ca²⁺ fluxes following brief peroxide exposure.

The source(s) of Ca²⁺ that gave rise to the observed transients in CDF, whether it was from intracellular or external pools, and the mechanisms responsible for these fluxes were investigated. Fig. 2A shows that the absolute Ca²⁺ transient magnitude in cells exposed briefly to H₂O₂ was 318 ± 28 nM (Con). When cells were stimulated with H₂O₂ in the absence of extracellular Ca²⁺ (no Ca), the transient magnitude was significantly reduced by 59% to 129 ± 23 nM (p < 0.001). In contrast, when cells were pretreated with the IP₃ receptor blocker xestospongin C (XeC), which then was exposed to H₂O₂, the transient magnitude was reduced by 37% to 195 ± 25 nM (p < 0.01). These results suggest that approximately two-thirds of the H₂O₂-induced Ca²⁺ flux is transplasmalemmal, whereas one-third was from internal IP₃-sensitive stores. Furthermore, pretreatment with XeC followed by H₂O₂ stimula-
tion in the absence of extracellular Ca^{2+} (XeC no Ca) completely inhibited the development of an intracellular Ca^{2+} transient. These results indicate that the H_{2}O_{2}-induced Ca^{2+} transient arises from extracellular and IP_{3}-sensitive stores without significant contributions from mitochondrial, nuclear, or other intracellular pools.

Because the major source of Ca^{2+} was extracellular, we investigated whether this was attributed to the opening of L-type calcium channels in the plasma membrane. L-type calcium channels are ubiquitous large conductance voltage-activated channels and have been demonstrated in fibroblasts (19). As shown in Fig. 2B, pretreatment with the calcium channel blocker verapamil (Ver) had no significant effect on peak transient magnitude. These results suggest this route of entry is unlikely to contribute significantly to the observed transient.

As shown earlier, the minor component of the H_{2}O_{2}-induced Ca^{2+} transient resulted from intracellular IP_{3}-sensitive stores. IP_{3} may be formed as a result of phospholipase C activation following ligand binding to specific G-protein-coupled receptors in the plasma membrane. Alternately, IP_{3} may be formed by PI3K through a non-receptor-mediated mechanism. Pretreatment with the PI3K inhibitor wortmannin (WM) resulted in a similar reduction in H_{2}O_{2}-induced Ca^{2+} transient magnitude as compared with pretreatment with the IP_{3} receptor blocker XeC (229 ± 23 and 199 ± 25 nM, p < 0.05 and p < 0.01 versus Con, respectively). These results indicate that de novo IP_{3} formation through the activity of PI3K is a probable mechanism leading to H_{2}O_{2}-induced intracellular Ca^{2+} release.

Previous work from our laboratory has shown that reperfusion of previously ischemic myocardium is associated with the up-regulation of various proinflammatory mediators including interleukin-6. Thus, we investigated whether the H_{2}O_{2}-induced transient is linked to the expression of IL-6. Cells grown in culture were stimulated with a brief pulse of H_{2}O_{2} and assayed at various times for IL-6 production. As shown in this figure, significant amounts of IL-6 were detected by 4 h (p < 0.01) and increased thereafter until 24 h. By comparison, nonstimulated cells accumulated significantly lower levels of IL-6 by 24 h as compared with H_{2}O_{2}-treated cells (p < 0.001).

Because the exposure of cells to a brief pulse of H_{2}O_{2} resulted in a large Ca^{2+} transient and IL-6 release in CDFs, we investigated whether the transient influx of Ca^{2+} was a necessary component of the H_{2}O_{2}-induced signaling pathway, leading to IL-6 expression. To address this possibility, we depleted the cells of [Ca^{2+}], by pretreating CDFs with thapsigargin in the absence of extracellular Ca^{2+} (see Fig. 1B, dashed trace). Im-

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**FIG. 1.** Brief peroxide exposure induces Ca^{2+} transients in cardiac fibroblasts. Coverslip-attached adult cardiac derived fibroblasts were loaded with 5 μM Fura-2/AM, washed, and treated as indicated. Intracellular Ca^{2+} levels were monitored using a computer-controlled microscope-based fluorescence measurement system as described under “Experimental Procedures.” A, this figure shows a representative Ca^{2+} transient from one cell superfused with physiological buffer containing 1.8 mM Ca^{2+} and exposed to 100 μM H_{2}O_{2} for 3 min (solid trace). Also shown is a representative trace from a non-H_{2}O_{2}-exposed cell monitored for a similar time period (dashed trace). B, a method was developed to induce calcium transients exhibiting similar characteristics to peroxide-induced transients in CDFs without exogenous H_{2}O_{2} exposure. Cells were pretreated with thapsigargin in Ca^{2+}-free buffer, superfused with Ca^{2+}-free buffer containing 10 μM ionomycin for 1 min, and then exposed to Ca^{2+}-containing buffer after 30 min. The solid trace shows a typical transient from one fibroblast treated with ionomycin using this protocol. Alternately, cells were pretreated with thapsigargin and exposed to H_{2}O_{2} in the absence of extracellular Ca^{2+} (dashed trace). This treatment allowed peroxide exposure without the production of a Ca^{2+} transient. Physiological Ca^{2+} was reintroduced to the superfusate 30 min after peroxide exposure as indicated. The re-introduction of extracellular Ca^{2+} did not induce a detectable influx of Ca^{2+} in CDFs over a 2-h monitoring period.
These results taken together suggest that it is Ca\(^{2+}\) influx and not the H\(_2\)O\(_2\) exposure per se that leads to IL-6 protein release by CDF.

Fig. 4A shows the time-dependent increase in IL-6 mRNA in peroxide-stimulated CDFs. Because IL-6 induction was significant by 4 h following H\(_2\)O\(_2\) exposure (p < 0.001 versus 2 h), we compared mRNA levels at that time in peroxide-stimulated and untreated control CDFs to those stimulated with H\(_2\)O\(_2\) in which calcium fluxes were not allowed and to cells that were not exposed to peroxide but in which calcium transients were induced. This approach allowed individual assessment of the impact of peroxide and calcium in IL-6 regulation (Fig. 4B). The data indicate that either peroxide exposure or calcium influx alone is sufficient to significantly induce IL-6 mRNA expression (p < 0.01 and p < 0.001, respectively, versus untreated), although neither was as potent a stimulus as peroxide exposure and calcium influx together (p < 0.001 versus untreated).

In addition, these data suggest that the presence of a calcium stimulus may regulate IL-6 protein release to a greater extent than mRNA levels (see Fig. 3B).

**DISCUSSION**

The results from this study demonstrate for the first time that brief exposure of cardiac fibroblasts to physiological levels of H\(_2\)O\(_2\) results in a large Ca\(^{2+}\) transient and Ca\(^{2+}\)-dependent induction of IL-6. The investigations by Bolli et al. (1) and others (20, 21) have shown that reperfusion of previously ischemic myocardium leads to a transient production and release of ROS including H\(_2\)O\(_2\). In addition, numerous studies have demonstrated that ischemia/reperfusion injury is associated with an inflammatory response characterized by significant infiltration of neutrophils into the myocardium, especially after longer ischemic episodes (22, 23). It has been shown that local concentrations of H\(_2\)O\(_2\) in the microenvironment adjacent to neutrophils may reach 100 \mu M (24, 25). Given that cardiac fibroblasts representing the most numerous cell types in the heart are in contact with working myocytes and are known to participate in post-ischemic remodeling, the response of these cells to transient physiological levels of H\(_2\)O\(_2\) probably represents an important myocardial process contributing to post-ischemic inflammatory pathobiology.

As shown in Fig. 1A, brief exposure of primary adult CDFs to H\(_2\)O\(_2\) resulted in a large nearly symmetrical Ca\(^{2+}\) transient characterized by its long duration and delayed onset. Furthermore, after [Ca\(^{2+}\)]\(_i\) returned to post-transient base-line levels, cells could be restimulated with brief H\(_2\)O\(_2\) exposure to produce an additional transient (data not shown). Roveri et al. (26) showed that exogenous peroxide exposure led to Ca\(^{2+}\) transients in smooth muscle cells characterized by their rapid onset and short duration (<5 min) and elevated post-transient base-line Ca\(^{2+}\) levels. These authors showed that the initial rise in [Ca\(^{2+}\)]\(_i\), was because of the release of IP\(_3\)-sensitive stores, whereas elevated post-transient base-line levels were because of Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels that was preventable using calcium channel blockers or antioxidants.

Our results are suggestive of a different mechanism of Ca\(^{2+}\) influx, because as shown in Fig. 2A, pretreatment with the calcium channel blocker verapamil had a minimal effect on Ca\(^{2+}\) transients in smooth muscle cells characterized by their rapid onset and short duration (<5 min) and elevated post-transient base-line Ca\(^{2+}\) levels. These differences may reflect alternate mechanisms for Ca\(^{2+}\) handling in excitable versus nonexcitable cells and/or differences in the concentration and duration of H\(_2\)O\(_2\) exposure. It is noteworthy that these authors (26) observe that at lower concentrations of H\(_2\)O\(_2\) (<150 \mu M), slower Ca\(^{2+}\) transients were seen in isolated smooth muscle cells, although the data were not presented. The fact that H\(_2\)O\(_2\) concentrations vary greatly among the many published studies on the cellular effects of exogenous H\(_2\)O\(_2\).
Brief peroxide exposure stimulates Ca^{2+}-dependent interleukin-6 production by cardiac fibroblasts. Primary adult cardiac derived fibroblasts were grown in 60 × 15-mm culture dishes and exposed to either 100 μM H_{2}O_{2} or physiological buffer for 3 min as indicated. At the specified times, cells were harvested for determination of protein content with supernatants collected and assayed for IL-6 levels by enzyme-linked immunosorbent assay. A, peroxide and time dependence of IL-6 accumulation in H_{2}O_{2}-exposed fibroblasts in vitro (H_{2}O_{2}). Significant IL-6 release was observed between 2 and 4 h in stimulated cultures. By comparison, IL-6 levels were determined in unstimulated control cultures at base line and at 24 h (no H_{2}O_{2}). Bars represent the mean ± S.D. for three individual cultures per time point with IL-6 levels normalized to protein content. B, CDFs were pretreated with 10 μM TG and exposed briefly to peroxide (TG+H_{2}O_{2}), pretreated with TG without H_{2}O_{2} exposure (TG), or exposed to H_{2}O_{2} without TG pretreatment (H_{2}O_{2}). These data show that IL-6 production by Ca^{2+}-depleted fibroblasts (TG+H_{2}O_{2}) was minimal and not significantly different from unstimulated cells (TG). By comparison, non-pretreated peroxide-exposed cells accumulated large amounts of IL-6 in vitro over the 24-h study period. Bars represent the mean ± S.D. for three individual cultures per time point with IL-6 levels normalized to protein content. Data were analyzed by ANOVA with post hoc analyses performed using profile analyses to identify time intervals at which significant increases of IL-6 protein expression were observed. In addition, when appropriate, pairwise comparisons were made using Bonferroni-adjusted Student's t tests (**, p < 0.01; ***, p < 0.001).
exposure ranging from submicromolar to millimolar concentrations contributes to the difficulty in comparing results from these studies and establishing unambiguous mechanisms that are biologically relevant. Importantly, we did not observe Ca\textsuperscript{2+} transients in either rat heart-derived vascular EC or mouse NIH-3T3 fibroblasts following brief peroxide exposure. The observed difference in calcium response among peroxide-stimulated EC, NIH-3T3, and CDF suggests that cardiac fibroblasts may possess unique signaling mechanisms essential to their role(s) in regulating cardiac growth and interstitial structural adaptations (fibrosis) in the heart.

In 1986, Putney (27) first proposed a model for receptor-regulated Ca\textsuperscript{2+} entry into nonexcitable cells termed capacitative calcium entry. Noncapacitative Ca\textsuperscript{2+} entry mechanisms have also been described in cells without electrically excitable membranes (28). Our results do not entirely support the capacitative model in H\textsubscript{2}O\textsubscript{2}-induced Ca\textsuperscript{2+} transients in cardiac fibroblasts in vitro. Consistent with this model, we observed H\textsubscript{2}O\textsubscript{2}-induced Ca\textsuperscript{2+} release from internal stores involved in IP\textsubscript{3} sensitive pools, because as shown in Fig. 2A, the blockade of the IP\textsubscript{3} receptor using XeC significantly attenuated peak transient levels. Any role for ryanodine receptors was eliminated, because pretreatment with ryanodine was without effect (data not shown). Furthermore, pretreatment with XeC in the absence of Ca\textsuperscript{2+} completely blocked transient development, indicating that the Ca\textsuperscript{2+} responsible for the observed transients was largely limited to two sources, IP\textsubscript{3}-sensitive intracellular pools and extracellular medium. Similarly, as shown in Fig. 1B, the depletion of cellular calcium following pretreatment with thapsigargin in Ca\textsuperscript{2+}-free medium followed by H\textsubscript{2}O\textsubscript{2} stimulation blocked transient development. Subsequent replacement of extracellular calcium 30 min after stimulation did not result in significant Ca\textsuperscript{2+} entry or “overshoot” as predicted by the capacitative model (29). In addition, IP\textsubscript{3} receptor blockade using XeC reduced calcium transient magnitude but did not block H\textsubscript{2}O\textsubscript{2}-induced Ca\textsuperscript{2+} entry (Fig. 2A). Thus, our results indicate that the observed H\textsubscript{2}O\textsubscript{2}-induced transients in CDFs in vitro resulted from a noncapacitative entry mechanism.

Hydrogen peroxide is a highly reactive biologically important molecule that is released by cells in response to various stimuli. The primary effects of exogenous H\textsubscript{2}O\textsubscript{2} exposure result from the oxidation of membrane lipids, proteins, and membrane-associated molecules. Following exogenous application of H\textsubscript{2}O\textsubscript{2}, we did not observe a detectable burst of intracellular free radicals as measured by dichlorofluorescein fluorescence (data not shown) during a time when significant Ca\textsuperscript{2+} fluxes were observed. As shown in Fig. 2B, pretreatment with the PI3K inhibitor wortmannin resulted in a similar reduction in transient magnitude as compared with pretreatment with XeC, suggesting that de novo IP\textsubscript{3} formation may result from the activation of membrane-associated PI3K. This mechanism is supported by the observations of Qin et al. (10) demonstrating that H\textsubscript{2}O\textsubscript{2} is capable of activating PI3K. Our results do not exclude the possibility that other mechanisms, e.g. phospholipase C activation, may contribute to IP\textsubscript{3} production and subsequent intracellular Ca\textsuperscript{2+} release. In fact, H\textsubscript{2}O\textsubscript{2}-induced calcium fluxes in CDFs are probably a complex phenomenon resulting from internal release, external influx, and altered extravasion and re-uptake mechanisms.

Previous studies from our laboratory (7–9) have demonstrated that myocardial production and release of proinflammatory mediators including IL-6, tumor necrosis factor-\textalpha, IL-1\textbeta, and nitric oxide are associated with ischemia/reperfusion injury. Preliminary studies from our laboratory have shown that adult cardiac derived fibroblasts are capable of producing tumor necrosis factor-\textalpha and large amounts of IL-6 in vitro following lipopolysaccharide exposure (30). In addition, we have recently demonstrated a coordinated expression of IL-6, IL-6R, and the signal transducer gp130 during reperfusion of previously ischemic myocardium (31). Interleukin-6 is a proinflammatory cytokine whose expression is modulated by the redox state of the cell (32). This cytokine has been shown to depress myocardial contraction, play a role in apoptosis, and induce the expressions of adhesion and other proinflammatory molecules (for review see Ref. 33). Given the local production and release of H\textsubscript{2}O\textsubscript{2} by myocytes and neutrophils, we investigated whether such exposure would be sufficient to induce IL-6 expression. Brief peroxide exposure led to the time-dependent accumulation of IL-6 protein (Fig. 3A) and mRNA (Fig. 4A). Interleukin-6 protein levels were 5-fold higher in peroxide-exposed cells after 24 h as compared with unstimulated control cultures. We had previously established that re-exposure of Ca\textsuperscript{2+}-depleted fibroblasts to medium containing normal Ca\textsuperscript{2+} (1.8 mm) did not lead to large Ca\textsuperscript{2+} fluxes (Fig. 1B). This allowed us to stimulate CDFs with brief H\textsubscript{2}O\textsubscript{2} exposure without the production of a Ca\textsuperscript{2+} transient to investigate the importance of calcium signaling in H\textsubscript{2}O\textsubscript{2}-induced IL-6 expression. The stimulation of CDFs with H\textsubscript{2}O\textsubscript{2} in the absence of a Ca\textsuperscript{2+} transient (Fig. 3B, TG+H\textsubscript{2}O\textsubscript{2}) effectively blocked IL-6 protein release with levels similar to unstimulated Ca\textsuperscript{2+}-depleted cells...
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(7G) and unstimulated control cells (Fig. 3A, no H2O2). This treatment blunted but did not eliminate IL-6 mRNA induction (Fig. 4B). Similarly, the production of a calcium transient in the absence of exogenous peroxide also attenuated mRNA levels although to a lesser extent, indicating that neither Ca2+ nor peroxide alone exclusively regulates IL-6 gene activity. The results from these experiments demonstrate the Ca2+ dependence of H2O2-induced IL-6 release, suggesting that the peroxide-induced calcium transient may serve as a signal in the subsequent induction and release of this important proinflammatory mediator.

In conclusion, our results demonstrate that brief exposure of primary adult CDFs to physiological levels of H2O2 leads to a large symmetrical calcium transient characterized by a delayed onset and long duration. The transient arises from both a minor component resulting from intracellular release from IP3-sensitive stores and a major component represented by extracellular Ca2+ influx. In addition, our results show that brief H2O2 exposure leads to time-dependent release of IL-6, which did not occur in the absence of significant Ca2+ fluxes.

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REFERENCES

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

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