Study of the Mechanisms of Hydrogen Peroxide and Hydroxyl Free Radical-induced Cellular Injury and Calcium Overload in Cardiac Myocytes*

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There is evidence that myocardial injury, as would occur on post-ischemic reperfusion, may be caused by the generation of oxygen radicals, as well as by the induction of intracellular calcium overload; however, the relationship between these two mechanisms of injury is not known. To test the hypothesis that oxidants and oxygen radicals can cause cardiac myocyte injury and intracellular calcium overload, isolated adult rat ventricular myocytes were exposed to H2O2 (1–10 mM) and Fe3+-nitrilotriacetate. EPR measurements confirmed the production of the highly reactive *OH radical by this system. The oxygen radical generating system initially caused a transient augmentation of twitch amplitude in single field stimulated myocytes. This was followed by contractile oscillations occurring during the twitch prior to full cell relaxation, and spontaneous mechanical oscillations occurring between electrically stimulated contractions. Eventually, cells became inexcitable and abruptly underwent contracture. In the presence of lower bathing calcium concentrations, these oxidant-induced alterations were prevented or delayed. However, cells exposed to the radical generating system in the absence of extracellular calcium still eventually underwent contracture but stimulated contractions or mechanical oscillations were not seen. Measurements in single myocytes loaded with the fluorescent probe of intracellular calcium, Indo-1, demonstrated a rise in both systolic and diastolic fluorescence ratio, as well as oscillations and widening of the fluorescence transient, suggestive of cellular calcium loading, following exposure to the radical generating system. Injured myocytes did not take up trypan blue dye. Contractile dysfunction and calcium overload were prevented by the calcium channel blocker, nifedipine. NMR measurements of cellular ATP demonstrated that these alterations in cellular calcium preceded the depletion of ATP. Subsequent depletion of ATP was accompanied by the appearance of increased concentrations of sugar phosphates indicative of a block in glycolysis and ATP depletion correlated with cellular rigor. Thus, oxygen free radicals can cause cardiac myocyte injury with contractile abnormalities which occur due to myocyte calcium loading. The mechanism of oxidant-induced calcium loading is not due to nonspecific membrane damage, or energy depletion, but rather due to increased calcium influx through voltage gated calcium channels. This early calcium overload state as well as oxidant induced block of glycolysis result in cellular energy depletion and cell death with the induction of contracture.

There is evidence that reperfusion of ischemic myocardium while limiting ischemic damage may induce a form of injury due to reperfusion itself (1–5). The pathophysiology of this reperfusion injury is incompletely understood, but recent evidence suggests that it is associated with a marked increase in the generation of oxidants and oxygen free radicals (6–10) as well as the development of myocardial calcium overload (11–14). The cellular mechanism of oxygen radical induced injury and the relationship to intracellular calcium overload is not well established.

A number of mechanisms have been identified as sources of oxidants in post-ischemic tissues including the conversion of the enzyme xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase generates the oxidants hydrogen peroxide, superoxide anion, O2·−, and hydroxyl radical, *OH (15). It has been suggested that the highly reactive *OH which is generated from O2·− and H2O2 via the iron mediated Fenton reaction is the oxidant species which causes cellular injury (1). EPR studies and biochemical studies have demonstrated that *OH and H2O2 are generated in reperfused myocardium (6–9, 16).

Techniques have been developed in the isolated myocyte model which enable the simultaneous, real-time measurement of cellular contractile function and cytoplasmic calcium transients during individual twitch contractions (17). NMR and EPR spectroscopy can be applied to measure cellular ATP and pH as well as free radical generation, respectively, in cell suspensions. In this study we have applied these techniques to perform studies in isolated rat ventricular myocytes and myocyte suspensions in order to determine the mechanisms by which hydrogen peroxide and hydroxyl free radicals generated via the Fenton reaction induce cellular injury and myocardial calcium overload.

MATERIALS AND METHODS

Myocyte Isolation—Myocytes were prepared by a modification of the technique of Silver et al. (18). In brief, 2–3-month-old male Wistar rats from the aging colony of the Gerontology Research Center were killed, the hearts quickly excised, the ascending aorta cannulated, and the heart retrogradely perfused with a collagenase containing

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buffer. When the heart became soft, the left ventricle was dissected free, gently minced, filtered through 200-μ nylon gauze, and myocytes harvested by gravity sedimentation. Greater than 70% of the myocytes were calcium-tolerant, free of membrane blebs, possessed clear sarcomere striations, and demonstrated no spontaneous twitch contractions. They exhibited brisk contractions to electrical field stimulation. A xenon arc powered by a modified stroboscope was used consisting of hydrogen peroxide and the ferric iron chelate \( \text{Fe}^{3+} \)-nitritolactate (1:2) (20 μM). The \( \text{Fe}^{3+} \)-NTA was prepared as described previously (19). A final concentration of either 1 mM or 10 mM hydrogen peroxide was used in all experiments.

EPR Measurements of Free Radical Generation—EPR spectra were recorded at room temperature using an IBM-Bruker ER 300 spectrometer operating at X-band with a TM 110 cavity and TM flat cell. The spectrometer settings were: modulation frequency, 100 kHz; modulation amplitude, 0.5 G; scan time, 1.0 min; microwave power, 20 mW; microwave frequency, 9.722 GHz; and magnetic field were precisely measured, respectively, with an EIP 575 Source Locking Microwave Counter and a Bruker ER 035M NMR Gausus.

Repetitive 1-min acquisitions were performed and the digitized Bruker spectral data transferred to an AST 286 or AST 386 personal computer (PC) for analysis. Spectral simulations were done on the PC and directly matched with the experimental data to extract the spectral parameters. A multipurpose computer software capable of simulating and handing as many as 24 simulated and/or experimental spectra at a time was developed and used in the present study for analyzing experimental spectra as described previously (7, 15).

Contraction Measurements—Isolated myocytes were placed in a chamber on the stage of an inverted microscope (Modified Zeiss IM-35) and superfused with the HEPES buffer. Cells were field stimulated by 5-ms pulses at 0.2-0.5 Hz, via platinum wire electrodes with a Grass S90 stimulator. The image of the cell was projected onto a Reticon 1024G/RC100R photodiode array, and the instantaneous length of the cell calculated from the number of photodiodes darkened by that image (Fig. 1). The photodiode array was scanned every 5 ms, and thereby the length of the cell as a function of time was measured. On-line calculation of rest length, maximal velocity of shortening, and maximal extent of shortening was performed by a VAX 11/730 (Digital Equipment Corporation). Cell shortening from slack length was measured before and during exposure to the oxidants under varying extracellular calcium concentrations, in the presence of the calcium channel blocker nitrendipine (1 μM), and in the presence of the membrane cholesterol EGTA (5 mM, Sigma). The effect of the oxidants on resting cell length, twitch amplitude, and the development of mechanical oscillations was measured.

Intracellular Calcium Measurements—In some experiments intracellular calcium was measured with the fluorescent indicator Indo-1. The acetoxymethyl ester form of this dye was loaded into suspensions of cells according to a previously described method (20). Briefly, a solution containing 50 μg of Indo-1 acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR) in 50 μl of dimethyl sulfoxide (Sigma), 2.5 μl of 25% w/w Pleuronic F-127 (BASF Wyandotte Corp., Wyandotte, MI), and 75 μl of fetal bovine serum (Sigma) was added to a 2 ml myocyte suspension. The myocytes were gently agitated at room temperature for 10 min and then centrifuged at 30 g for 30 s and resuspended in HEPES-buffered solution. Intracellular esterases then cleaved the acetoxymethyl ester groups trapping the membrane impermeant calcium sensitive form of the dye. These cells were then studied in a chamber on the stage of the modified Zeiss inverted microscope as described previously (17). A xenon arc powered by a modified strobescope was used as the source of illumination for epifluorescence, producing a 3.8-μs light pulse with a repetition rate of 200 Hz (Fig. 1). An interference filter selected a wavelength of 550 ± 5 nm to excite Indo-1 and a 395-nm longpass diroch blue filter directed the exciting light to a 100 × objective. Following excitation the calcium bound and free forms of Indo-1 emitted fluorescence peaking at approximately 410 and 490 nm, respectively. Fluorescent light was collected from the cell and passed through a series of dichroic mirrors and interference filters to two photomultiplier tubes, one selecting for light with a wavelength of 391-434 nm ("419-nm channel") and the other selecting for light with a wavelength of 457-507 nm ("490-nm channel"). The signals evoked by each flash were individually digitized and stored after each flash. The ratio of the fluorescence intensities in the two channels was computed off-line by a VAX 11/730 computer, providing a calcium estimate for each flash.

Red light (650-750 nm) was used for the brightfield image of the cell which was directed to the photodiode array and television camera to avoid interference with the epifluorescent light (391-507 nm) resulting from excitation of Indo-1 which was directed to the photomultiplier tubes.

Effect of Hydrogen Peroxide on Indo-1—Emission spectra of the pentapotassium salt (calcium sensitive) of Indo-1 (Molecular Probes, Inc.) were measured during exposure to 10 mM \( \text{H}_2\text{O}_2 \) + \( \text{Fe}^{3+} \)-NTA in a buffer solution that was similar to cytoplasm: HEPES (20 mM), KCl (120 mM), NaCl (10 mM), MgCl₂ (5 mM); \( \text{pH} = 7.2 \), to evaluate the direct effect of the oxidant system on Indo-1 fluorescence. These measurements were performed on a dual emission spectrophotometer (Delta Scan 1, Photon Technology International, Inc., Princeton, NJ).

\[ \text{pK} = \log \left( \frac{[\text{Indo-1}]}{[\text{Indo-1}^\text{calc}} \right) \]

where \( \text{pK} = 6.90 \), \( \text{d} = 3.290 \) ppm, and \( \text{d} = 5.805 \) ppm, as reported previously (21, 22). All chemical shift values are defined relative to the PCr resonance.

The spectra were manually digitized. The digitised spectra were then smoothed, baseline corrected, and integrated. This processing of the spectra allowed integration of each individual peak and enabled the calculation of percent change in phosphorous metabolite concentrations.
RESULTS

EPR Spectroscopy—EPR measurements were performed in order to confirm that the radical generating system actually generated the reactive OH radical in both the presence and absence of cardiac myocytes and to determine the kinetics of this radical generation. In the presence of iron chelates which facilitate the Fenton reaction, H2O2 is reduced to OH as shown in the following reactions.

\[
\text{H}_2\text{O}_2 + 2\text{Fe}^{3+} \rightarrow 2\text{Fe}^{2+} + \text{O}_2 + 2\text{H}^+
\]

(1)

\[2(\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{Fe}^{3+} + \text{OH}^-)\]

(2)

This reaction is a well known source of OH and there is evidence that the iron mediated Fenton reaction of H2O2 to OH is an important source of the oxidative injury which occurs on reperfusion of the ischemic heart (23, 24).

On mixing the ferric iron chelate Fe3+-NTA (20 μM) and H2O2 (10 mM) in the presence of the EPR spin trap 5,5'-dimethyl-1-pyrroline-N-oxide, DMPO, a prominent 1:2:2:1 quartet signal was observed with hyperfine coupling constants \(a_H = 14.71\), \(a_N = 14.99\) G characteristic of DMPO-OH (25) (Fig. 2A). With H2O2 alone or Fe3+-NTA in the presence of DMPO no significant radical signal was seen (Fig. 2, B and C). These experiments suggest that OH is generated on mixing Fe3+-NTA and H2O2 and reacts with DMPO to form DMPO-OH. In addition, it appears that H2O2 alone or the iron chelate alone give rise to little if any radical generation. Over a 5-min period, the intensity of the observed radical signal continues to increase suggesting continued free radical generation. In order to further confirm that OH is generated, additional experiments were performed in the presence of ethanol. The hydroxyl radical will extract a hydrogen from ethanol to form an ethyl radical which would give rise to a DMPO-ethyl adduct signal. In the presence of 5% ethanol, a prominent 1:1:1:1:1 six-peak spectrum with \(a_H = 23.29\), \(a_N = 15.90\) was observed, indicative of the DMPO ethyl adduct with a decrease in the magnitude of the DMPO-OH spectrum (Fig. 3). These spectra demonstrate that an ethyl radical was generated and thus confirm that OH was formed.

Additional experiments were performed in the presence of cardiac myocytes in order to determine that measurable OH radical generation occurs and that the myocytes did not quench the OH generating system. In the presence of cardiac myocytes, a prominent DMPO-OH signal was again observed, with only partial cell-induced quenching of radical generation. In most preparations a smaller DMPO-R signal, \(a_H = 22.0\), \(a_N = 16.0\), was also observed and the magnitude of this signal increased with increasing cell concentration. The DMPO-OH radical signal observed in the presence of the cells was similar to that without cells except that the observed linewidth was broader due to partial immobilization of the radical adduct, probably secondary to partitioning into the cell membrane. Even with cell suspensions 10-fold more concentrated than those used in the subsequent studies, radical generation was still measurable. Upon addition of H2O2 to myocytes in the absence of Fe3+-NTA, a small DMPO-OH signal was observed. This signal was approximately \(\frac{1}{3}\) the magnitude of that observed in the presence of the iron chelate suggesting that a slow reaction of H2O2 may occur with cellular iron to generate OH, even in the absence of added exogenous iron.

These experiments were repeated with varying buffer calcium concentrations ranging from 0 to 4.0 mM, and there were no alterations observed in the magnitude of radical generation. Similarly, neither 1 mM EGTA nor 1 μM nitrendipine had any measurable effect on radical generation.

Contractile Measurements—When bathed in an extracellular calcium of 1.0 mM and stimulated according to the protocol outlined above (0.5 Hz), the myocytes demonstrated a steady state twitch amplitude (rest length change/rest length) of 8.26 ± 1.05% (X ± S.E., n = 15). The addition of Fe3+-NTA to the superfusate did not alter any of the measured parameters, and therefore all subsequent experiments were performed with this catalyst present. Addition of hydrogen peroxide, which initiates the rapid generation of hydroxyl radicals (see above), resulted in a transient augmentation of twitch amplitude of 46 ± 8.1% versus the base line (Fig. 4). This rise in twitch amplitude, which occurred 2.7 ± 0.4 min after the addition of hydrogen peroxide, was followed (at 3.3 ± 0.5 min after the addition of hydrogen peroxide) by the presence of frequent contractile oscillations occurring during the twitch prior to full cell relaxation and occasional spontaneous mechanical oscillations between the stimulated beats.

FIG. 2. EPR spectra recorded in the presence of 50 mM DMPO of A: 10 mM H2O2 + 20 μM Fe3+-NTA in HEPES buffer; B: as in A but Fe3+-NTA alone; C: H2O2 alone; and D: H2O2 + Fe3+-NTA in the presence of cardiac myocytes 104 cells/ml. A prominent 1:2:2:1 quartet signal of DMPO-OH is observed with H2O2 + Fe3+-NTA but not H2O2 or Fe3+-NTA alone.

FIG. 3. A, EPR spectrum of H2O2 + Fe3+-NTA, OH generating system in the presence of 50 mM DMPO. B, a simulation of spectrum in A with \(a_H = 14.99\) G, \(a_N = 14.71\) G, and line width = 1.45 G. C, a simulation of spectrum in B consisting of a linear combination of DMPO-OH, \(a_H = 14.99\) G, \(a_N = 14.71\) G, line width = 1.45 G with a weight of 0.58, and DMPO-ethyl \(a_H = 23.29\), \(a_N = 15.9\). line width = 1.45 G with a weight of 0.42.
Mechanisms of Oxidant Injury in Cardiac Myocytes

FIG. 4. Cell length is displayed for a single cell electrically field stimulated at 0.2 Hz prior to and during exposure to H$_2$O$_2$ and Fe$^{3+}$-NTA. Left panel, twitch amplitude initially increases as diastolic cell length decreases. Contractile oscillations occurring prior to full cell relaxation then develop. This is followed by failure of the twitch and then an abrupt shortening to contracture. Right panel, figure showing cell twitches under control conditions (A) and following the onset of mechanical oscillations (B), plotted with an expanded time base.

FIG. 5. Photograph of a cardiac myocyte before (A) and after (B) exposure to the radical-generating system. The photograph in B was taken after the cell underwent contracture.

Spontaneous sarcoplasmic reticulum calcium release is known to cause localized activation of sarcomeres which then propagates through the cell as spontaneous mechanical waves which occur between electrically stimulated beats (28). This phenomenon is dependent upon calcium loading, and is markedly increased in calcium overload states. The cells also demonstrated a gradual decrease in their rest length, and later a decline in twitch amplitude (Fig. 4). The cells then became inexcitable and abruptly shortened further to a square contracture state, which occurred 7.5 ± 0.9 min after the addition of hydrogen peroxide. The cells remained in this contracture form for the duration of the experiment (30 min) and failed to demonstrate relengthening following H$_2$O$_2$ washout (Fig. 5). When the [Ca$^{2+}$] in the extracellular buffer was decreased, much longer periods of exposure to the radical generating system were required before these alterations in mechanical function were observed. As shown in Fig. 6, a 4-fold reduction in buffer calcium concentration from 2.0 to 0.5 mM resulted in a 5-fold delay in the time to peak contractility, as well as marked delays in the onset of contractile oscillations and contracture. Thus, these observations suggest that external calcium mediates the development of free radical induced myocyte injury. To further explore this relationship, myocytes were superfused with buffer containing 1 µM nitrendipine, a sarcolemmal calcium channel blocker. The base-line twitch was reduced in amplitude, in accordance with prior studies in bulk preparations. Oxidant mediated injury was significantly altered by the presence of nitrendipine with peak twitch amplitude occurring at 13.5 ± 1.00 versus 7.5 ± 0.90 minutes (p < 0.003). No contractile oscillations or spontaneous waves developed. Three cells were exposed to H$_2$O$_2$ + Fe$^{3+}$-NTA in the presence of 5 mM EGTA and 0 mM calcium. These cells demonstrated no spontaneous or stimulated contractions, but eventually did progress to contracture.

Indo-1 Fluorescence Measurements—Addition of hydrogen peroxide and Fe$^{3+}$-NTA to Indo-1-loaded myocytes resulted in changes in cellular length, function, and morphology analogous to cells not exposed to the fluorescent dye. Under basal conditions the 410/490 nm fluorescence ratio demonstrated a rapid transient following each electrical stimulation. Addition of hydrogen peroxide and Fe$^{3+}$-NTA to Indo-1 loaded myocytes resulted in an increase in the systolic and diastolic 410/490 nm fluorescence ratio, consistent with a rise in intracellular systolic and diastolic calcium (Fig. 7). The amplitude of each fluorescence transient increased as well. This was seen to occur with a time course similar to the transient increase in cell twitch amplitude described previously. During the next phase of oxidant mediated injury, that of contractile oscillations and spontaneous waves, oscillations in the fluorescence signal and widening of the fluorescence transient occurred. When twitch amplitude declined, diastolic 410/490 ratio increased and the systolic transient declined. Finally the cell
underwent contracture and the 410/490 ratio continued to increase. Following the onset of augmented contractility and the rise in Indo-1 fluorescence ratio which occurs during \( \text{H}_2\text{O}_2 \) and \( \text{Fe}^{3+}-\text{NTA} \) exposure, two Indo-1-loaded cells were switched to superfusion with 0 mM Ca\(^{2+}\) and the calcium chelator EGTA, 5 mM. The contraction immediately failed and the diastolic fluorescence ratio quickly fell toward control diastolic levels. This confirms that the early rise in the diastolic and systolic fluorescence ratios reflect a true rise in intracellular Ca\(^{2+}\).

Fluorescence intensities at both emission wavelengths were monitored during exposure to the radical generating system. The intensity of fluorescence at both wavelengths was bimodal, with an initial small increase in light intensity in both wavelengths occurring over the first 3-4 min followed by a more prominent subsequent decline.

Measurement of the fluorescence spectrum of the Indo-1-free acid in the "intracellular" buffer indicated that hydrogen peroxide + \( \text{Fe}^{3+}-\text{NTA} \) did not cause an early increase in either the absolute intensity of light detected at 410 nm or the 410/490 ratio in solution. Following a prolonged exposure to \( \text{H}_2\text{O}_2 \) and \( \text{Fe}^{3+}-\text{NTA} \) (15 min), there was no change in the shape of the emission spectra of both calcium bound (Indo-1, 5 \( \mu \)M and CaCl\(_2\), 20 \( \mu \)M) and unbound forms (Indo-1, 5 \( \mu \)M and EGTA, 10 \( \mu \)M) of Indo-1-free acid. There was, however, a significant decrease in fluorescence intensity at the measured wavelengths (Fig. 8). Measurement of the fluorescence spectrum of non-Indo-1-loaded cells exposed to the oxidants showed that the 410/490 ratio remained essentially constant. Thus, the measurement of Indo-1 fluorescence in isolated myocytes is most consistent with an initial increase in myoplasmic calcium. The late decline in light intensity may be secondary to either loss of the dye from the cytoplasm (secondary to sarcoplasmal damage) or possibly destruction of the dye itself by the oxygen radicals, as was suggested by exposure of the Indo-1 directly to the radical generating system.

**Mechanisms of Cellular Injury**—There are three different types of mechanisms which could cause the oxidant induced calcium overload: 1) nonspecific membrane damage with the leakage of calcium through membrane holes; 2) ATP depletion with a rise in intracellular calcium due to the failure of ATP dependent calcium pumps; and 3) specific alterations in the function of calcium regulatory proteins resulting in impaired cellular calcium homeostasis. In an effort to determine which of these mechanisms occurs, a series of studies were performed in which the time course of contractile failure, ATP depletion, and cell membrane breakdown were measured and correlated.

Cells were exposed to the radical-generating system and measurements of the exclusion of the vital dye trypan blue were performed. The myocytes were observed to exclude the dye even after the onset of contractile abnormalities and the dye continued to be excluded up till the time of rigor. Even with the total failure of contraction and the presence of rigor most of the cells, >80%, still excluded trypan blue dye. This suggests that the cell membrane maintains its integrity and that the rise in intracellular calcium is not due to simple membrane leakage.

In order to determine the relationship between alterations in cellular ATP and \( \text{pH} \) in the induction of myocyte calcium overload, phosphorous NMR studies were performed. In these studies a lower hydrogen peroxide concentration of 1 mM was used which results in a slower time course of cellular injury and allows a complete NMR time course to be obtained. Serial
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of exposure to the oxidants prominent spectral peaks were observed due to ATP and PCr (Fig. 9). A large P1 peak was also observed due to the presence of 1 mM phosphate in the cellular buffer solution. No decrease in ATP was seen until after 1500 s. During the period 1500-2000 s after exposure, a marked decrease in ATP was observed accompanied by a rise in P1 as well as a rise in the sugar phosphate peak. No shift in the P1 resonance was seen, suggesting that no significant alteration in pH occurred. The spectra were analyzed and the time course of alterations in [ATP], pH, and sugar phosphate concentrations determined (Fig. 10). The marked rise in sugar phosphate concentration accompanied the precipitous fall in ATP consistent with the previously reported oxidant induced inactivation of glyceraldehyde-3-phosphate dehydrogenase (27).

A series of single cells were studied to assess the time course of induction of contractile abnormalities under identical conditions of oxidant exposure as the NMR studies. With the 1 mM hydrogen peroxide and 20 μM iron-NTA used, the time to peak contraction was 6.7 ± 0.8 min, the time to spontaneous oscillations was 7.3 ± 0.7 min, and the time to rigor was 27 ± 2.7 min. As shown in Fig. 7 with Indo-1-loaded cells these contractile alterations are accompanied by a rise in fluorescence ratio indicative of cellular calcium overload. Thus, contractile alterations and calcium overload occur long before the depletion of cellular ATP. The occurrence of myocyte contracture which occurs later does correlate with the observed ATP decrease.

In order to further determine if the observed contractile abnormalities and calcium overload were due to specific abnormalities in calcium regulatory proteins, myocytes were stimulated at a rate of 0.5 Hz and superfused with 10 mM hydrogen peroxide and 20 μM iron-NTA in the presence and absence of 1.0 μM concentrations of the sarcolemmal calcium channel blocker nitrendipine. Oxidant-mediated injury was markedly altered in the presence of nitrendipine with a delay in peak twitch amplitude from 7.5 ± 0.9 to 13.5 ± 1.0 min (p < 0.003, n = 20). No contractile oscillations or spontaneous waves occurred in the nitrendipine-treated cells, whereas all of the cells in the absence of the drug exhibited these contractile abnormalities. EPR studies in the presence of DMPO demonstrated that nitrendipine did not alter or scavenge OH (Table I). In similar experiments performed with non-stimulated cells exposed to the radical generating system, no gradual decrease in cell length or contractile oscillations or waves were seen indicating that these abnormalities are likely due to calcium entry through voltage-gated calcium channels.

**Fig. 10.** Graphs of the time course of [ATP], pH, and [sugar phosphate] as a function of time after the addition of hydrogen peroxide, obtained from analysis of the 31P NMR spectra from experiments performed as described in Fig. 9. Both [ATP] and [sugar phosphate] are plotted as the percent of control (before H2O2 addition) concentration.

**TABLE I**

<table>
<thead>
<tr>
<th>Myocyte group</th>
<th>Time to contractile oscillationsa</th>
<th>Time to rigorb</th>
<th>Radical scavengingc %</th>
</tr>
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<tbody>
<tr>
<td>1 mM H2O2 + 20 μM Fe²⁺-NTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (no added drug)</td>
<td>7.3 ± 0.7</td>
<td>27 ± 2.7</td>
<td>0</td>
</tr>
<tr>
<td>+50 mM DMTU</td>
<td>17 ± 1.2</td>
<td>92 ± 9.5</td>
<td>&gt;95</td>
</tr>
<tr>
<td>10 mM H2O2 + 20 μM Fe²⁺-NTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (no added drug)</td>
<td>3.3 ± 0.5</td>
<td>7.5 ± 0.9</td>
<td>0</td>
</tr>
<tr>
<td>+50 mM DMTU</td>
<td>6.8 ± 0.6</td>
<td>21 ± 1.2</td>
<td>&gt;95</td>
</tr>
<tr>
<td>+1 μM nitrendipine</td>
<td>None</td>
<td>13.5 ± 0.8</td>
<td>0</td>
</tr>
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*a n ≥ 10 in each group.
*b Percent scavenging of 'OH radical generation, measured from the percent decrease in the intensity of the DMPO-OH signal observed in EPR measurements.

500-s NMR spectral files were obtained before and after the addition of the radical-generating system. At the beginning of exposure to the oxidants prominent spectral peaks were observed due to ATP and PCr (Fig. 9). A large P1 peak was also observed due to the presence of 1 mM phosphate in the cellular buffer solution. No decrease in ATP was seen until after 1500 s. During the period 1500-2000 s after exposure, a marked decrease in ATP was observed accompanied by a rise in P1 as well as a rise in the sugar phosphate peak. No shift in the P1 resonance was seen, suggesting that no significant alteration in pH occurred. The spectra were analyzed and the time course of alterations in [ATP], pH, and sugar phosphate concentrations determined (Fig. 10). The marked rise in sugar phosphate concentration accompanied the precipitous fall in ATP consistent with the previously reported oxidant induced inactivation of glyceraldehyde-3-phosphate dehydrogenase (27).

A series of single cells were studied to assess the time course of induction of contractile abnormalities under identical conditions of oxidant exposure as the NMR studies. With the 1 mM hydrogen peroxide and 20 μM iron-NTA used, the time to peak contraction was 6.7 ± 0.8 min, the time to spontaneous oscillations was 7.3 ± 0.7 min, and the time to rigor was 27 ± 2.7 min. As shown in Fig. 7 with Indo-1-loaded cells these contractile alterations are accompanied by a rise in fluorescence ratio indicative of cellular calcium overload. Thus, contractile alterations and calcium overload occur long before the depletion of cellular ATP. The occurrence of myocyte contracture which occurs later does correlate with the observed ATP decrease.

In order to further determine if the observed contractile abnormalities and calcium overload were due to specific abnormalities in calcium regulatory proteins, myocytes were stimulated at a rate of 0.5 Hz and superfused with 10 mM hydrogen peroxide and 20 μM iron-NTA in the presence and absence of 1.0 μM concentrations of the sarcolemmal calcium channel blocker nitrendipine. Oxidant-mediated injury was markedly altered in the presence of nitrendipine with a delay in peak twitch amplitude from 7.5 ± 0.9 to 13.5 ± 1.0 min (p < 0.003, n = 20). No contractile oscillations or spontaneous waves occurred in the nitrendipine-treated cells, whereas all of the cells in the absence of the drug exhibited these contractile abnormalities. EPR studies in the presence of DMPO demonstrated that nitrendipine did not alter or scavenge 'OH (Table I). In similar experiments performed with non-stimulated cells exposed to the radical generating system, no gradual decrease in cell length or contractile oscillations or waves were seen indicating that these abnormalities are likely due to calcium entry through voltage-gated calcium channels.

**Fig. 9.** 31P NMR spectra of myocyte suspensions in the presence of 1.0 mM hydrogen peroxide and 20 μM Fe²⁺-NTA. A. spectrum obtained immediately after addition of hydrogen peroxide. B. spectrum obtained 2000 s later. Spectra consist of 200 pulse acquisitions and are identically scaled. The peaks from left to right correspond to the: SP, sugar phosphate; P1, inorganic phosphate; PCr, phosphocreatine; γ-ATP, α-ATP; β-ATP resonances.
activated by stimulation induced voltage depolarization.

A series of cells were also exposed to the radical generating system in the presence of 5 mM concentrations of the high affinity calcium chelator EGTA and 0 mM calcium. These cells demonstrated no spontaneous or stimulated contractions, but eventually did progress to a rigor state. This further supports that although the early contractile alterations occur due to calcium overload, the cellular rigor which occurs later does not require calcium overload and is presumably due to ATP depletion.

In order to determine the presence and importance of \( \cdot \)OH radical generation in the mechanisms of cell injury, additional comparative studies were performed exposing the myocytes to hydrogen peroxide in the presence and absence of iron-NTA. In the absence of iron-NTA the onset of contractile abnormalities was delayed and the time course of alterations was not reproducible from myocytes isolated from different hearts. EPR studies performed with these cell preparations in the presence of the spin trap DMPO demonstrated that the \( \cdot \)OH radical adduct, DMPO-OH, was always observed but the magnitude and rate of increase in this signal varied in different myocyte preparations presumably due to differences in the concentration of cellular iron or other transition metals available to run the Fenton reaction. Additional studies were performed in the presence of the hydroxyl radical scavenger dimethylthiourea, DMTU. As shown in Table I, 50 mM DMTU markedly delayed the onset of injury with a more than 2-fold increase in the time to contractile oscillations and the time to cell rigor. In the presence of either 1 or 10 mM hydrogen peroxide similar protection was seen. Matched EPR studies in the presence of the spin trap DMPO demonstrated a greater than 95% decrease in the concentration of the hydroxyl radical adduct, DMPO-OH, confirming that DMTU actually scavenged \( \cdot \)OH. Thus, the observed cellular injury was prevented or delayed by the hydroxyl radical scavenger DMTU suggesting that \( \cdot \)OH was indeed responsible for cellular injury.

**DISCUSSION**

There is evidence that a burst of oxygen radical generation occurs immediately following reperfusion of ischemic myocardium (6-10). Studies in the intact heart have demonstrated an increase in intracellular calcium following myocardial reperfusion (11, 14). Dramatic increases in intracellular calcium are also documented in isolated myocytes undergoing reperfusion after anoxic exposure and are associated with cellular injury (28). It may be that the oxygen radicals formed at reperfusion lead to intracellular calcium overload and myocyte injury; however, questions remain regarding the effects of oxygen radicals and oxidants on myocardial function and intracellular calcium.

We employed the isolated myocyte as a model to study radical-induced cardiac injury as it allows the study of muscle cells independent of vascular effects. Additionally it allows the simultaneous measurement of contractility and intracellular calcium in a single myocyte and assures that the measured signals are derived from the myocyte rather than other cell types as may be the case in an intact heart. Hydrogen peroxide and iron were employed to generate the highly reactive hydroxyl radical. EPR studies confirmed the formation of this radical in the presence or absence of myocytes. In the absence of myocytes, \( \text{H}_2\text{O}_2 \) alone did not generate any significant radical signal, but in the presence of myocytes, some \( \cdot \)OH radical generation still occurred suggesting that endogenous myocyte iron may catalyze this reaction. This could suggest that the cellular toxicity of \( \text{H}_2\text{O}_2 \) is at least partially due to \( \cdot \)OH generation. The cellular injury we observed in our model probably results from a combination of the direct effects of hydrogen peroxide as well as those of the more reactive hydroxyl radical generated via the Fenton reaction with the iron chelate.

Myocytes exposed to the generating system demonstrated transient increases in contractility and frequent contractile oscillations consistent with intracellular calcium loading. There was a simultaneous gradual reduction in diastolic cell length also consistent with a rise in diastolic calcium levels. Occasional spontaneous waves were observed due to enhanced spontaneous sarcoplasmic reticulum calcium release. Later contraction failed and the cells underwent contracture. These processes depended on extracellular calcium levels, since the time to development of the maximum inotropic response, the development of contractile oscillations, and the final development of contracture were inversely related to the extracellular calcium concentration.

The measurement of Indo-1 fluorescence suggested that intracellular calcium changes were responsible for the observed early mechanical changes. The initial changes in intracellular calcium included a rise in diastolic Indo-1 fluorescence ratio as well as an increase in both the magnitude and duration of the fluorescence transient. Later the Indo-1 fluorescence signal demonstrated spontaneous oscillations. These early changes in the Indo-1 fluorescence signal are consistent with a rise in both diastolic and systolic calcium levels. Later the interpretation of the Indo-1 fluorescence signals are complicated by a significant loss of signal. This may be the result of a direct effect of the oxidant system on the dye as indicated by the study of solutions of Indo-1-free acid. Additionally, Indo-1 may be lost from the cytoplasm after prolonged exposure to the radical generating system. The late rise in baseline fluorescence ratio may represent a rise in cytosolic or mitochondrial calcium levels as loading of myocyte suspensions in our laboratory with Indo-1 acetoxyethyl ester has resulted in mitochondrial compartmentation of as much as 50% of the fluorescence signal (17).

Three different types of mechanisms could have caused the oxidant-induced calcium overload: 1) nonspecific membrane damage with the leakage of calcium through membrane holes; 2) ATP depletion with a rise in intracellular calcium due to the failure of ATP dependent calcium pumps; or 3) specific alterations in the function of calcium regulatory proteins resulting in impaired cellular calcium homeostasis. A series of studies were performed in which the time course of contractile failure, ATP depletion, and cell membrane breakdown were measured and correlated. These studies demonstrated that the cellular calcium overload was not due to membrane breakdown or ATP depletion but due to calcium influx from voltage gated calcium channels. The induction of cellular contracture, however, did correlate with the depletion of ATP. There are a number of specific alterations in calcium regulatory proteins which have been proposed to cause the oxidant-induced cellular calcium overload. These mechanisms include enhanced sarcoplasmic reticulum calcium release, enhanced sodium-calcium exchange, and calcium entry through voltage gated calcium channels (29-31). It has been shown that sarcoplasmic reticulum vesicles rapidly release calcium when exposed to oxygen radicals (29). Perhaps this may partially explain some of the oscillations in intracellular calcium that exist in our model. Additionally, it has been demonstrated that sodium/calcium exchange is enhanced in sarcolemmal vesicles exposed to an oxygen radical generating system (30). Other studies (31) have shown that oxygen radicals can cause early after depolarizations which most
likely represent reactivation of the sarcolemmal voltage-gated calcium channel. In this study we have demonstrated transient augmentation of contraction, the development of contractile oscillations prior to full cell relaxation and spontaneous waves, as well as changes in the Indo-1 fluorescence ratio indicative of calcium loading. These changes occurred only in field stimulated cells and were blunted or abolished by lowering extracellular calcium or by the addition of nitrendipine. Thus, our observations demonstrate that voltage-gated calcium channels are important in the process of cellular calcium loading.

The ultimate failure of contraction and the development of cell contracture which occurs much later than the induction of calcium overload probably result directly from myocardial ATP depletion induced by the radical generating system. It has been demonstrated previously that single guinea pig myocytes undergo contracture and that ATP-inhibited potassium channels are activated following exposure to hydrogen peroxide (32). This has been attributed to direct inhibitory effects on cellular glycolytic and oxidative metabolic mechanisms. In other studies (33) anoxia has been demonstrated to lead to ATP depletion, an increase in potassium conductance due to an opening of ATP-inhibited potassium channels, and the development of cell contracture; however, there was no significant increase in intracellular calcium until after the development of contracture. This suggested that myocyte contracture did not result directly from a rise in intracellular calcium, but rather because of a fall in ATP. Studies in cell suspensions (34) have confirmed that the development of contracture in single myocytes results directly from profound ATP depletion. In the current study, we observed that the time course of ATP depletion in cell suspensions paralleled the onset of contracture. In addition cells developed contracture following exposure to the oxidant system, even in the absence of extracellular calcium. The onset of contracture was, however, markedly hastened by the presence of elevated extracellular calcium, suggesting that although ATP depletion is probably the final factor leading to contracture, elevated calcium can interact to cause earlier contracture by either leading to increased cellular energy demand and, hence, more rapid cellular ATP depletion, or by sensitizing the myofilaments to reduced ATP levels, as has been suggested by others (35, 36).

We have observed that the oxidants hydrogen peroxide and the hydroxyl radical cause myocyte injury and intracellular calcium overload. These alterations occur largely due to calcium influx through voltage gated calcium channels. Additionally, we observed that oxygen radicals can cause further cellular injury by impairing glycolytic substrate metabolism which along with the cellular calcium overload would further induce intracellular ATP depletion. It is possible that the endogenous generation of hydrogen peroxide and hydroxyl radicals, which occurs on reperfusion of the ischemic heart, could similarly induce myocyte calcium overload which would, in turn, modulate the presence and magnitude of contractile dysfunction.

REFERENCES


