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Biochemical mechanism of irreversible cell injury caused by free radical-initiated reactions

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Abstract

Effects of oxidative stress on isolated rat ventricular myocytes were studied. Myocyte viability was determined by the ability of these cells to retain rod-shaped morphology and to exclude trypan blue. The mean life time of myocytes was quantitated using the Weibull distribution function. Superfusion with 200 μM tert-butyl hydroperoxide (t-BHP) led to a time-dependent loss of cell viability, generation of the products of lipid peroxidation, oxidation of protein and non-protein thiols, a decrease in $[ATP]_i$ and in the cellular energy charge. Dithiothreitol (DTT, 5 mM) prolonged survival of myocytes exposed to t-BHP, attenuated oxidation of protein and non-protein thiols, and preserved the energy charge. Exposure to DTT did not affect the concentration of t-BHP-generated lipid peroxidation products. Promethazine (1 μM) prevented t-BHP-induced increase in the concentration of lipid peroxidation products, but did not prevent either loss of thiols or loss of cell viability. Superfusion with N-ethylmaleimide (NEM, 5 μM) also led to loss of cell viability, with accompanying decreases in protein and non-protein thiols, ATP and energy charge without the accumulation of the products of lipid peroxidation. Superfusion with $FeSO_4$ (400 μM) and ascorbate (1 mM), (Fe-Asc) did not result in loss of cell viability or a decrease protein thiols or the energy charge. Superfusion with Fe-Asc, did, however, lead to a slight decrease in the concentration of non-protein thiols and ATP and a large increase in the concentration of lipid peroxidation products. Accumulation of lipid peroxidation products induced by Fe-Asc was prevented by promethazine. These results indicate that free radical-induced irreversible cell injury results from a loss of protein thiols. Changes in the cellular energy charge and lipid peroxidation do not bear a simple relationship to the survival of cardiac myocytes under oxidative stress. (Mol Cell Biochem **137**: 9–16, 1994)

Key words: free radicals, rat cardiac myocytes, N-ethylmaleimide, tert-butyl hydroperoxide, promethazine, lipid peroxidation, energy charge, and oxidative stress

Introduction

Aerobic cells maintain a highly reduced intracellular environment [1, 2]. Cellular antioxidants such as glutathione, vitamins C and E and antioxidant enzymes such as, superoxide dismutase, glutathione peroxidase and catalase rapidly detoxify oxidizing species generated by cellular metabolism [2]. However, pathological conditions that result in excess generation of free radicals or weaken antioxidative defenses, lead to irreversible alterations in cellular metabolism and structure. Such 'oxidative' damage has been implicated to be the underlying cause of tissue injury associated with a number of disease processes, including ischemia-reperfusion, atherosclerosis, alcohol and drug toxicity, etc. [3, 4]. In addition, recent studies suggest that free radicals contribute to tissue dysfunction associated with aging [5].

Since reactions involving free radicals are very rapid, it is difficult to study the chemical evolution of specific oxidative insults. Moreover, free radicals initiate chain reactions that generate a number of different reaction products. These

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reaction products participate in other free radical reactions and have varying toxicities of their own. Identification and characterization of cellular free radical reactions and reaction products is a formidable task, which is currently being attempted by several laboratories. Even though several cellular processes sensitive to oxidative stress and free radicals have been identified, it is not clear which of these changes directly contribute to irreversible cell injury.

Previous investigations suggest that acute free radical injury results from oxidation of protein thiols, and membrane lipids, which leads to a decrease in the intracellular concentrations of ATP and an influx of calcium (6–10). However, the relative contribution of thiol oxidation, lipid peroxidation and depletion of ATP to irreversible cell injury has not been determined. In this communication, studies on isolated rat ventricular myocytes are reported. Oxidative stress was induced by agents that cause primarily lipid peroxidation, or sulfhydryl oxidation, or both. Results obtained suggest the cellular energy charge bears no simple relationship to oxidative hypercontracure and demonstrate a limited contribution of lipid peroxidation to the pathogenesis of oxidative stress. Oxidation of protein thiols appears to be the critical determinant of irreversible free radical injury.

Materials and methods

Tertiary-butyl hydroperoxide (t-BHP), N-ethylmaleimide (NEM), 5,5′dithiobis (2-nitrobenzoic acid) (DTNB), and promethazine HCl were purchased from Sigma Chemical Company. Collagenase was purchased from Boheringer, Indianapolis, IN (lot BJA119). All other chemicals were of the highest purity available.

Isolation of adult rat ventricular cells

Rat ventricular myocytes were isolated from male Sprague-Dawley rats weighing 200–260 g. Rats were injected intraperitoneally with heparin (300 units) 30 min or more prior to pentobarbital anesthesia (20 mg). The hearts were rapidly removed and perfused in a rectrograde fashion through the aorta with Tyrode containing 1.0 mM $CaCl_2$ at 37°C until all signs of blood were removed with gentle squeezing of the heart. The hearts were then perfused with a nominally Ca^{2+}-free Tyrode (free $[Ca^{2+}] = 3$ μM) for 5 min, followed by perfusion with collagenase solution containing 60–75 mg of collagenase B (Boehringer, Indianapolis, IN, lot BJA119) in 50 ml of low Ca^{2+} Tyrode (free $[Ca^{2+}] = 10$ μM) to which 1 mg aprotinin had been added. All solutions were oxygenated. After 5 min of enzyme perfusion, the hearts were removed from the cannula, the atria and right ventricles removed, and the left ventricular wall and septum cut vertically into 4–6 pieces and allowed to incubate longer in the enzyme solution.

After variable periods of time (2.5–15 min) of additional digestion at 37°C, the pieces were dipped in a 'KB'-like solution [11], 85 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4$, 1 mM K_2EGTA, 2 mM $Tris_2$ATP, 5 mM pyruvate, 5 mM creatine, 20 mM taurine, 20 mM glucose, pH 7.2) or an ATP-free variant of it (106 mM KCH_3SO_3, 3.9 mM KCl, 2.4 mM $MgSO_4$, 22 mM glucose, 22 mM taurine, 6 mM creatine, 5 mM pyruvate, 8 mM K-phosphate, pH 7.3, osmolarity adjusted to 300 mosm/kg), gently swirled in 3–3.5 ml of the same solution until the other pieces had been similarly treated, then triturated, filtered through a 200 μm nylon mesh, and centrifuged at 22 g for 3–5 min. The supernatant was drained and cells re-suspended in 2 ml of the same solution and centrifuged again. Finally cells were re-suspended in 4 ml of the same solution for storage until use in a refrigerator. At this time, 10 μl of cells were transferred to 1.0 ml of 1 mM $CaCl_2$-containing Tyrode solution for counting purposes. After 15–30 min of room temperature incubation in this Tyrode solution, fractions generally exhibited 50–60% rod-shaped quiescent cells. Experiments were performed on the fractions yielding the highest proportion of rod-shaped cells. Cell obtained by this method retained rod-shaped morphology on exposure to 2 mM calcium and remained viable for 20–25 h at 4°C as tested by trypan blue exclusion.

Solutions

The cells were perfused with or without additives in normal Ringers (n-Ringers) solution containing (in mM): NaCl 150, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 2 and HEPES 10. The pH was adjusted to 7.4 with 0.1 M NaOH.

Cell survival experiments

Isolated myocardial cells were layered on a cover slip at the bottom of a water jacketed chamber, maintained at 35°C. The Ringer solution containing 0.1% trypan blue was stored in glass jacketed funnels maintained at 35°C and bubbled with 100% oxygen and was superfused through this cell chamber using a peristaltic pump (Pharmacia Inc., model P1) at a rate of 2–3 ml per min. The chamber was placed on an inverted microscope (C. Reichert, Austria), the eye-piece of which was connected to video camera (Cohu CCD Camera), connected to a data acquisition system (Image 1, version 4.0, Universal Imaging Corporation) and a VHS video recorder (DX-900, Toshiba). Images were displayed on a NEC Multisync 3D monitor and were digitized and analyzed using the Image 1 software. Cells which maintained rod-shaped morphology and excluded trypan blue were counted at the indi-

cated times during the experiment. Results are expressed as the fraction of total cell population that was rod-shaped and excluded trypan blue. Cells which underwent ≥ 50% reduction in the resting cell length were considered to be in hypercontracture. Cells that were hypercontracted, but excluded trypan blue were not counted as viable or rod-shaped. Transition from rod shaped morphology to hypercontracture was considered irreversible injury. Fraction of rod-shaped cells at the beginning of the experiment; therefore, the fraction of rod-shaped cells in the beginning of the experiment is always 1.

Determination of thiobarbituric acid reactive substances (TBARS)

Isolated myocytes were superfused as described above. After indicated times, myocytes (0.5 ml) were withdrawn from the cell chamber and suspended in 1 ml of 10% trichloroacetic acid. The protein precipitate was removed by centrifugation and 1 ml of the supernatant was mixed with 1 ml of 0.67% thiobarbituric acid containing 0.1% butylated hydroxy toluene (BHT). The resultant mixture was boiled for 10 min and the color developed was read at 532 nm. The concentration of thiobarbituric acid reactive substances (TBARS) was calculated as malondialdehyde (MDA) equivalents using an extinction coefficient of 156,000 $mole^{-1}$ cm^{-1} [12]. Protein concentration was determined by the dye-binding method of Bradford [13].

Determination of protein and non-protein thiols

An aliquot of 0.5 ml cells, superfused as described above were precipitated with 0.5 ml of 10% perchloric acid. The mixture was spun at 5000 rpm for 5 min to settle the precipitate. The pellet was dissolved in Tris (0.5 M, pH 8.8)-EDTA (5 mM)-SDS (1.0%) and 0.5 ml aliquots was treated with 0.5 ml of either 50 mM NEM or 500 μM DTNB. A 25 μl aliquot was used to determine protein by the method of Lowry [14]. The supernatant (0.9 ml) was neutralized with 0.2 ml of 2 M KOH + 2.5 M $KHCO_3$. To 0.8 ml of the neutralized extract 0.1 ml of 0.1 M Tris-HCl (pH 8.0) and 0.1 ml of DTNB (in 1% Na-citrate) were added. Thiol concentrations were determined by the absorbance of the mixture at 412 nm, using an extinction coefficient of 13,600 $mole^{-1}$ cm^{-1} [15].

Nucleotide assay

Superfused myocytes were collected and centrifuged at 22 g for 3 min. The cells were then re-suspended in n-Ringer and an equal volume of cold 10% perchloric acid was added to the mixture (final volume = 500 μl). The protein precipitate was removed by centrifugation and the supernatant was neutralized by the addition of 50 μl of 2 M KOH + 2.5 M $KHCO_3$ to a pH of 7.0. The precipitated salts were removed by centrifugation, and the neutralized extract was filtered through a 0.22 μ filter.

Nucleotides in the extract were assayed by using reverse phase high-pressure liquid chromatography (HPLC). Solvent A was 0.1 M K-phosphate and 8 mM tetrabutylammonium hydrogen sulfate, and solvent B was made by the addition of 30% methanol to solvent A. The column (C-18, Whatman, EQC 5 μm, S-80, 4.6 mm X 25 cm) was equilibrated with solvent A and the gradient was started at the time of injection (t = 0) to 100% B programmed linearly, in 12 min, using Beckman pumps 110 A. This was followed by 6 min of an isocratic period, after which the concentration of solvent A was increased in 4 min, from 0 to 100% using a linear gradient. Pump control, peak integration and data analysis were performed by System Gold software (Beckman). The absorbancne of the eluate was monitored at 254 nm by a Beckman 406 detector. Peak identity was confirmed by the retention times. Standard curves were constructed with solutions of known concentrations of ATP, ADP and AMP. Concentrations of the nucleotides were determined by the peak area. Protein was determined by Bradford's dye-binding method [13].

Data analysis

In order to estimate survival of myocytes in t-BHP containing solutions the Weibull distribution was used. The Weibull distribution is a generalization of the exponential distribution and has been used to analyze survival in carcinogenesis experiments [16]. In this distribution the probability density function of time t is:

$$f(t) = \lambda\gamma(\lambda t)^{\gamma-1} \exp[-(\lambda t)^{\gamma}] \quad (1)$$

where γ is the scaling factor, λ determines the shape of the distribution curve and are called the scale and shape factor respectively. The normalized survival function (at t = 0, S = 1) is:

$$S(t) = \exp[(-\lambda t)] \quad (2)$$

The mean life time of a myocyte was estimated using Eq. (3):

$$\mu = \frac{\Gamma\left(1 + \frac{1}{\gamma}\right)}{\lambda} \quad (3)$$

where $\Gamma(\gamma)$ is the gamma function defined as

$$\Gamma(\gamma) = \int_0^\infty x^{\gamma-1} e^{-x} dx \qquad (4)$$
$$= (\gamma - 1)!$$

Comparison of Weibull distributions were made using the property of the maximum likelihood estimator (MLE). Differences were considered significant at 95% ($P < 0.05$).

All non-linear curve fitting were performed using NFIT (Island Products, Galveston TX). In all cases best fit to the data was chosen on the basis of the standard error of the fitted parameter and the lowest value of σ, which is defined as the sum of the squares of the residuals divided by the degrees of freedom (number of observations minus the number of parameters calculated). Data are expressed as means ± SE. For comparison of means, analysis of variance (ANOVA) was performed, followed by corrected student's t test for multiple comparisons [17]. Data were considered statistically significant when the p value was <0.05.

Results

Cell survival

Isolated rat ventricular myocytes, superfused with n-Ringer, maintained rod-shaped morphology for more than 3 hours (maximum observation period) at 35°C. Under these conditions, less than 10% cells underwent hypercontracture. When the myocytes were superfused with n-Ringer containing 200 μM t-BHP, no change in the rod shaped morphology of most cells was observed for the first 30 min of superfusion. Sustained exposure, however, induced mechanical oscillations in myocytes (indicative of calcium overload), following which the myocytes underwent hypercontracture. The time course of loss of rod-shaped morphology of cardiac myocytes is shown in Fig. 1A. The mean life time of myocytes (μ), determined by their ability to maintain rod-shaped morphology and exclude trypan blue, was 39 min (Table 1). When the myocytes were superfused with n-Ringer containing 200 μM t-BHP and the antioxidant promethazine, the mean life time of the myocytes was 42 min, which is not statistically different ($p > 0.05$) from the mean life time of myocytes exposed only to t-BHP (Fig. 1A). However, if the myocytes were superfused with n-Ringer containing t-BHP and a thiol reducing agent, DTT [18], the mean life time of the myocytes was significantly ($p < 0.05$) prolonged (= 82 min) as compared to the life time of the myocytes superfused with t-BHP alone (Fig. 1A).

Superfusion of myocytes with n-Ringer containing 5 μM NEM, a membrane permeable thiol alkylating reagent [19], also resulted in time-dependent loss of cell viability and

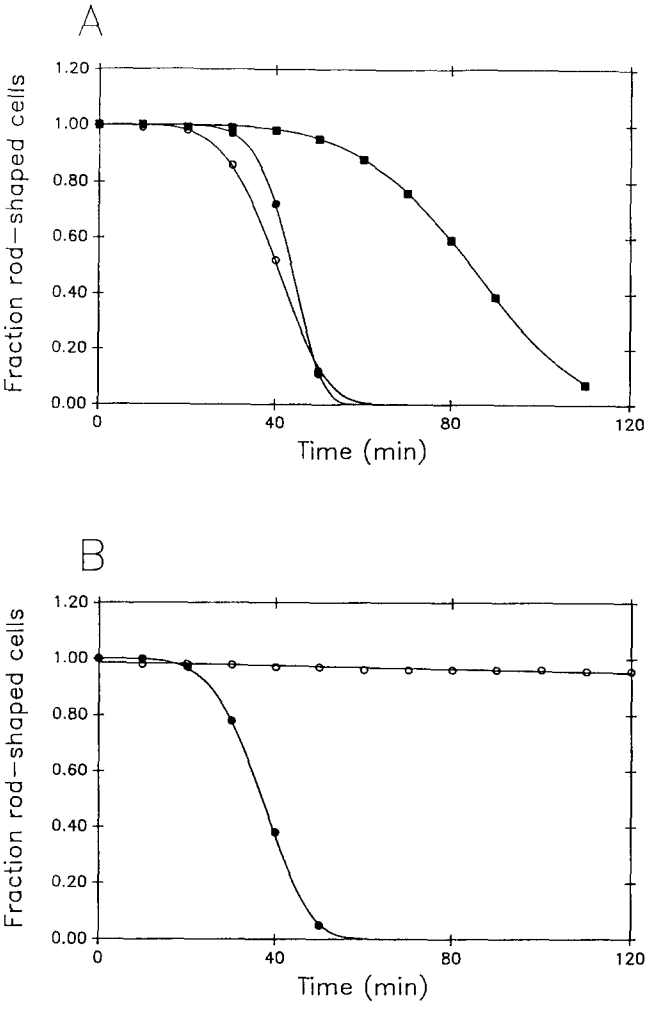

Fig. 1. Survival of isolated rat ventricular myocytes in the presence of oxidant and antioxidants. Plots show the loss of rod-shaped morphology of isolated myocytes as a function of time of superfusion with n-Ringer containing 200 μM t-BHP (A, open circles), 200 μM t-BHP + 1 μM promethazine (A, filled circles), 200 μM t-BHP + 5 mM DTT (A, filled squares), 5 μM NEM (B, filled circles) and 1 mM Ascorbate + 400 μM $FeSO_4$ (B, open circles). Data are presented as discrete points and the curves are a fit to Eq. (2), except in case of the Fe-Asc group, in which case the decrease in the population of rod shaped cells followed a linear time course. The calculated life times of myocytes under various experimental conditions are shown in Table 1.

myocytes underwent hypercontrature with a mean life time of 36 min (Fig. 1B). When, however, the myocytes were superfused with n-Ringer containing 400 μM $FeSO_4$ + 1 mM ascorbate (Fe-Asc), no significant los of viability was observed (Fig. 1B). After 120 min of superfusion with Fe-Asc in n-Ringer more than 90% myocytes excluded trypan blue and retained their rod-shaped morphology. When myocytes were superfused with n-Ringer containing promethazine alone or that containing promethazine and Fe-Asc, no significant loss of viability was observed, and more than 90%

Table 1. Effect of oxidants on survival of isolated cardiac myocytes and generation of thiobarbituric acid reactive substances

Superfusate	Mean life time (min)	MDA equivalents nmoles/mg protein
n-Ringer	> 200	0.16 ± 0.06
+ 200 μM t-BHP	39.0 ± 9.5*	1.29 ± 0.62*
+ 200 μM t-BHP + 1 μM Promethazine	42.4 ± 4.3*	0.14 ± 0.07†
+ 200 μM t-BHP + 5 mM DTT	82.2 ± 17.8†*	1.35 ± 0.20*
+ 5 μM NEM	35.8 ± 9.4*	0.11 ± 0.12
+ $FeSO_4$ (400 μM)-Ascorbate (1 mM)	> 120	1.26 ± 0.40*
+ 1 μM Promethazine	> 120	0.13 ± 0.08

Isolated cardiac myocytes were superfused with n-Ringers containing the indicated concentration of the additives. Cell survival was determined by monitoring transitions of the cells from rod shaped morphology to hypercontracture. Thiobarbituric acid reactive substances were determined as described under Materials and methods and are expressed as MDA equivalents per mg protein. Data are given as mean ± S.E. (n = 5–12 hearts). Mean life time was calculated using Eq. (3). *$p < 0.05$ compared to the value in n-Ringer and † $p < 0.05$ compared to the value in 200 μM t-BHP.

myocytes retained rod-shaped morphology and excluded trypan blue for an observation time of 120 min. The calculated mean life time of myocytes under various experimental conditions are shown in Table 1.

Thiobarbituric-acid reactive substances

Table 2 lists the amounts of thiobarbituric acid reactive substances (TBARS) generated upon superfusion of isolated myocytes under various experimental conditions. Superfusion of isolated cardiac myocytes with n-Ringer containing 200 μM t-BHP led to a large increase in the concentration of TBARS. No increase in TBARS was observed when the myocytes were superfused with n-Ringer containing 200 μM t-BHP and 1 μM promethazine. In contrast, addition of 5 mM DTT did not prevent the increase in TBARS generated by t-BHP. Moreover, superfusion with n-Ringer containing 5 μM NEM did not lead to a significant increase in the concentration of TBARS in the myocyte extracts. When the myocytes were superfused with n-Ringer containing Fe-Asc, a large increase in the TBARS was evident. The increase in the concentration of TBARS generated by Fe-Asc was similar in magnitude to the concentration of TBARS generated by 200 μM t-BHP. Inclusion of 1 μM promethazine to the n-Ringer containing Fe-Asc, prevented the increase in the concentration of TBARS substances (Table 1).

Protein and non-protein thiols

Superfusion of myocytes with n-Ringer containing 200 μM t-BHP for 45 min led to a 75% decrease in the concentration of non-protein thiols, and a 55% decrease in the concentration of protein thiols. The t-BHP-induced decrease in protein and non-protein thiols was not prevented by 1μM promethazine. Superfusion of myocytes with 200 μM t-BHP and 5 mM DTT resulted in a slight (30%), but statistically significant decrease in the concentration of non-protein thiols with no significant loss of protein thiols. Superfusion with n-Ringer containing Fe-Asc led t a 57% decrease in the concentration of non-protein thiols with no change in the concentration of protein thiols. Furthermore, superfusion with NEM led to a severe decrease in the concentrations of both protein and non-protein thiols (Table 2).

Phosphorylated nucleotides

Isolated myocytes had high content of ATP with a high ATP/

Table 2. Protein and non-protein thiols (nmoles/mg protein) in isolated cardiac myocytes exposed to oxidants

Superfusate	Non-protein thiols	Protein thiols
n-Ringer	10.5 ± 2.0	200.0 ± 20.5
+ 200 μM t-BHP	2.5 ± 0.9*	85.5 ± 20.5*
+ 200 μM t-BHP + 5 mM DTT	7.3 ± 1.7†	206.5 ± 25.3†
+ 200 μM t-BHP + 1 μM Promethazine	1.9 ± 0.4*	90.8 ± 16.3*
+ $FeSO_4$ (400 μM)-Ascorbate (1 mM)	4.5 ± 1.3*	209.5 ± 27.7
+ 5 μM NEM	2.7 ± 1.1*	66.1 ± 13.4*

Data are given as mean ± S.E. (n = 5–12 hearts). *$p < 0.05$ compared to the value in n-Ringer and † $p < 0.05$ compared to the value in 200 μM t-BHP.

ADP ratio and an energy charge of approximately 1.0. Upon superfusion with n-Ringer containing t-BHP, a 50% decrease in the concentration of ATP, an 80% decline in the ATP/ADP ratio, and a 46% decrease in the energy charge was observed. This decrease in the ATP/ADP ratio and the energy charge, but not the decrease in ATP concentration was prevented by DTT. A similar decrease in the concentration of ATP and ATP/ADP ratio and the energy charge was observed when the myocytes were superfused with 5 μM NEM. Extract of myocytes superfused with n-Ringer containing Fe-Asc, caused a 62% decrease in ATP, with no statistically significant changes in the ATP/ADP ratio of the cellular energy charge (Table 3).

injury.

Exposure to t-BHP was used as a model for free radical injury. The plasma membrane is permeable to t-BHP, and the peroxide is reduced intracellularly to t-butyl alcohol by glutathione peroxidase [20]. Unmetabolized t-BHP, however, in the presence of trace quantities of adventitious iron generates tert-butyoxy, tert-butyl peroxy and hydroxyl radicals [21]. These radicals initiate chain reactions which result in the generation of superoxide, singlet oxygen and lipid aldehydes [21]. Exposure of cardiac myocytes to micromolar concentrations of the proxide, results in hypercontracture [22]. The hypercontracted myocytes had grossly disrupted intracellular structure and were permeable to trypan blue,

Table 3. Effect of oxidants on phosphorylated nucleotides of isolated ventricular myocytes

Superfusate	ATP/ADP	Energy charge	ATP
n-Ringer	8.28 ± 0.95	0.97 ± 0.01	17.91 ± 2.0
+ 200 μM t-BHP	1.62 ± 0.30*	0.52 ± 0.08*	8.66 ± 2.0*
+ 200 μM t-BHP + 5 mM DTT	6.88 ± 0.87†	0.84 ± 0.07†	7.40 ± 1.7*
+ 5 μM NEM	2.57 ± 0.59*	0.70 ± 0.10*	3.69 ± 2.2*
+ $FeSO_4$ (400 μM)-Ascorbate (1 mM)	6.09 ± 1.76	0.81 ± 0.09	6.72 ± 0.9*

The concentration of ATP is in nmoles/mg protein. The energy charge was calculated using the expression: energy charge = (ATP + ½ADP)/(ATP + ADP + AMP). Data are presented as mean ± S.E. (n = 3 hearts) * $p < 0.05$ compared to the value in n-Ringer and † $p < 0.05$ compared to the value in 200 μM t-BHP.

Discussion

Uncontrolled reactions involving free radical species lead to disruption of the otherwise intricately regulated cellular metabolism. Such reactions have been suggested to be responsible for tissue dysfunction and injury associated with a number of disease states, drug toxicities and aging [3–5]. Nevertheless, the mechanisms by which free radicals cause irreversible injury have not been identified. Since free radicals contain unpaired electrons, these species react avidly with most electron rich cell constituents. Membrane lipids are the most vulnerable targets [7]. It has been hypothesized that excessive production of free radicals leads to oxidation of unsaturated fatty acids which alters the structure, fluidity and the transport function of the membrane [7]. These changes lead to eventual cell lysis. In addition to unsaturated fatty acids, intracellular thiols are also sensitive to oxidative stress [6, 8], due to their low reduction potential. Oxidation of thiols could lead to changes in the structure of the cytoskeleton, and alter the functions of membrane and cytosolic enzymes, which in turn would disrupt cellular architecture and metabolism. Recent studies also suggest that free radical mediated depletion of intracellular ATP may be the critical determinant of oxidative injury [9, 10]. Experiments reported in this communication were designed to identify the contribution of each of these changes *viz* lipid peroxidation, thiol oxidation and depletion of ATP to the evolution of oxidative indicating irreversible cell injury. Several investigators have used such structural alterations in cardiac myocytes to evaluate the effects of cyto-toxic and cyto-protective agents [8, 22–24]. However, for such an assay to be quantitative, it is necessary that the distribution of the 'life-time' of myocytes be determined. Toxicological and clinical studies using a continuous insult model (such as the one used here) show that the population distribution in such instances is non-gaussian [16]. Therefore, for quantitating survival of isolated myocytes in the presence of oxidants, the Weibull distribution function was used. Previous analysis shows that the Weibull distribution function affords a better description of cell survival in this model than other survival functions [22]. However, the general applicability of this model to other models of cell injury remains untested.

An increase in the concentration of lipid proxidation products and oxidation of protein and non-protein thiols in the presence of t-BHP is in agreement with previous studies which identify lipid peroxidation and protein oxidation as the major biochemical alterations underlying oxidant-induced cell injury [6, 8, 23, 25]. The large decrease in $[ATP]_i$ is also consistent with the previous reports that alterations in the energy metabolism of myocytes are an important consequence of oxidative injury [9, 10, 26].

Experiments described above demonstrate that changes in the formation of the products of lipid peroxidation do not correlate with hypercontracture. Promethazine inhibited pro-

duction of TBARS but did not prolong survival of t-BHP exposed myocytes. Promethazine is a free radical scavenger that inhibits the breakdown of plyunsaturated fatty acids to adehydic products [27]. It reacts rapidly with the peroxy radical of carbon tetrachloride, but rather poorly with carbon-centred trichloromethyl radical [28], and therefore does not affect the primary activation of carbon tetrachloride. Cell injury caused by t-BHP in the presence of promethazine could therefore result from carbon-centred radicals or alkoxyl radicals. The ability of promethazine to inhibit t-BHP-initiated lipid peroxidation suggests a pre-dominant role of peroxyl radicals in lipid peroxidation reactions [30].

Exposure of the myocytes to Fe-Asc resulted in the production of TBARS, which was prevented by promethazine. However, exposure to Fe-Asc did not lead to myocyte-hypercontracture. Previous studies show that exposure to Fe-Asc results in extensive lipid peroxidation in microsomes [31–34]. Ascorbate is required to maintain iron in the ferrous form. The ferrous form of iron is oxidized by molecular oxygen, to generate superoxide, hydrogen peroxide and the hydroxy radical [3]. As shown above, Fe-Asc leads to extensive lipid peroxidation, but little oxidation of cellular thiols, and no loss of cell viability. The limited toxicity of the Fe-Asc system may be due to 'site-specific' damage. Since lipids comprise the bulk of the plasma membrane, most of the free radicals generated by the exogenous addition of iron will react with membrane lipids before they can diffuse to the cell interior to initiate damage at more critical sites. Nevertheless, these results suggest that peroxidation of membrane lipid by itself does not lead to irreversible injury. It could, however, be argued that the thiobarbituric acid test is a non-specific measure of lipid peroxidation, but although this test almost certainly measures species other than malondialdehyde it is a useful comparative index of lipid peroxidation [35]. Moreover, promethazine (in the same concentration as that used here) prevents t-BHP-induced increase in ethanol formation in hepatocytes [36] indicating that is an effective inhibitor of lipid peroxidation. The inability of this antioxidant to prolong survival of t-BHP exposed myocytes is an independent evidence against the involvement of lipid peroxidation in myocyte hypercontracture. The observed lack of effect of promethazine on the survival of t-BHP-exposed cardiac myocytes is in contrast to the results of Schnellmann [37] which show that t-BHP-induced cell death of renal proximal tubules, and the accompanying increase in TBARS, was prevented by promethazine. A similar preservation of the viability of hydrogen peroxide-exposed hepatocytes by promethazine has also been reported [38]. However, Rush *et al.* [36] reported that promethazine was unable to affect the toxicity of hydrogen peroxide to isolated hepatocytes. Reasons for such differences are not well understood and could relate to the use of different cell types used in these studies. Nevertheless, since promethazine is an antioxidant, it is not surprising that under some experimental conditions and at high concentration (50–100 μM) it may be able to prolong survival of cells exposed to oxidative stress, however, as demonstrated by the experiments reported above, promethazine did not prolong survival under conditions when it prevented lipid peroxidation, which suggests a marginal contribution of lipid peroxidation to oxidative hypercontracture of cardiac myocytes.

In contrast to the results obtained with lipid peroxidation products, inhibition of t-BHP-induced thiol oxidation, by supplemental DTT (which maintains thiols in the reduced state), resulted in a significant prolongation of cell viability. Although, addition of DTT to the superfusing solution containing t-BHP, did not prevent the formation of lipid peroxidation products (Table 2), it did prevent loss of protein and non-protein thiols (Table 3). These results, therefore, suggest that depletion of cellular thiols may be a significant contributing factor in oxidant-induced loss of cell viability. The importance of protein oxidation reactions is also underscored by the observations that depletion of thiols by NEM resulted in hypercontracture, without attendant accumulation of lipid peroxidation products, or a large decrease in the cellular energy charge.

Cellular metabolism is tightly controlled by the energy charge which regulates the balance between the consumption and synthesis of ATP, and has been suggested to be an indicator of the energy level of importance for cell survival [39]. Upon exposure to t-BHP, the energy charge decreased to 0.5. This reduction in the energy charge was accompanied by hypercontracture and cell lysis. DTT prevented the decrease in energy charge and preserved cell viability. However, hypercontracture was elicited by NEM, even though the decrease in the energy charge was marginal. Therefore, it appears unlikely that a simple correlation exists between the cellular energy charge and hypercontracture. The usefulness of the cellular energy charge and cellular damage has been assessed by Kristensen [40]. When the energy charge of quiescent fibroblasts was decreased by several metabolic inhibitors, it was found that even with an energy charge of 0.1 to 0.35, the release of lactate dehydrogenase (an index of cell damage) did not follow immediately [40]. Under some experimental conditions, a decrease in the energy charge to 0.1 could be reversed without apparent loss of cell viability [40]. These results are in agreement with those reported here. Even though the difference in the energy charge between myocytes exposed to NEM and to Fe-Asc was not statistically different, the former caused hypercontracture, whereas exposure to Fe-Asc did not affect cell viability.

In summary, the results reported in this communication are consistent with the hypothesis that free radicals-induced irreversible injury is caused by the oxidation of protein thiols. Accumulation of the products of lipid peroxidation or the loss of cellular energy charge does not correspond to loss of cell

viability. Further investigations are required to identify specific structural and functional alterations caused by protein thiol oxidation.

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