Oxidative Modification of Rat Cardiac Mitochondrial Membranes and Myofibrils by Hydroxyl Radicals

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Abstract. The effect of hydroxyl radicals generated by the FeSO₄/H₂O₂ system on structural properties of proteins and membranes was studied in rat cardiac mitochondria and myofibrils. Exposure of mitochondria to 0.1 mmol/l FeSO₄/EDTA plus 1 mmol/l H₂O₂ at 37°C for 30 or 60 min caused conjugated diene formation, but it was not accompanied by accumulation of fluorescent lipid-protein conjugates. On the other hand, fluorescence measurements revealed radical-induced and time-dependent loss of tryptophans and production of bityrosines. Under the same conditions, the gradual decrease in tryptophan fluorescence and increase in bityrosine formation was also observed in radical-treated myofibrils. These results suggest that 'OH radicals can alter the mitochondrial and myofibrillar function via oxidation of amino acid residues and might be implicated in the pathogenesis of myocardial injury.

Key words: Free radicals — Oxidative stress — Protein oxidation — Fluorescence

Abbreviations: 1,8-ANS, 1-anilino-8-naphthalenesulfonate; EC, excitation-contraction; EDTA, ethylenediamine tetraacetic acid; HEPES, N-2-hydroxyethylpiperezine-N′-2-ethanesulfonic acid; ROS, reactive oxygen species, LPO, lipid peroxidation; OS, oxidative stress; SR, sarcoplasmic reticulum.

Introduction

Reactive oxygen species (ROS) are implicated in the mechanisms of biological ageing and myocardial injury in various clinical conditions. It has been proposed that mitochondrial oxidative metabolism is one of the major sources of oxygen free radicals (Zhang et al. 1998; Ide et al. 1999). ROS are extremely reactive and cause extensive molecular, cellular and tissue damage. It has been suggested that mitochondrion is also an important target of free radical attack and mitochondrial lipid, protein and DNA oxidation contribute to ageing and degenerative diseases.

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ROS-induced mitochondrial lipid peroxidation (LPO) has been documented in studies using exogenous radical-generating systems (Iwase et al. 1998; Paradis et al. 1998), during aging (Lucas and Szweda 1998) or postischemic reperfusion (Ambrosio et al. 1991). Several studies have documented that LPO results in the inactivation of mitochondrial proteins such as cytochrome c oxidase (Paradies et al. 1998) or creatine kinase (Miura et al. 1994), possibly due to oxidation of protein sulphydryl (SH) groups. However, the role of LPO in the enzyme inhibition is not yet clear, since it has been shown that lipid-soluble antioxidants could prevent LPO, but did not prevent loss in enzymatic activities (Zhang et al. 1990). Thus, LPO independent modifications of proteins may also be involved in radical-induced enzyme inhibitions, but their mechanisms are not well understood.

Although mitochondria appear to be the major target organelles of oxidative injury, modification of other cellular structures mediated by ROS may also contribute to cardiac dysfunction. ROS have been implicated in disorders of cardiac excitation-contraction (EC) coupling. Large number of studies has documented alterations in sarcoplasmic reticulum (SR), the intracellular Ca\(^{2+}\) store that plays a dominant role in EC coupling (Castilho et al. 1996; Xu et al. 1997; Kaplan et al. 2003). Radical treatment of cardiac myofibrils, another component of EC coupling, was found to inhibit myofibrillar creatine kinase and Ca\(^{2+}\), Mg\(^{2+}\)-ATPase activities (Robert et al. 1991; Suzuki et al. 1991; Kaneko et al. 1993; Mekhfi et al. 1996). It has been proposed that the oxidation of SH groups is the possible mechanism responsible for changes in enzyme activities (Suzuki et al. 1991; Kaneko et al. 1993; Mekhfi et al. 1996), but little is known about other radical-induced modifications of contractile proteins.

To clarify the involvement of different oxidative modifications of proteins, we investigated the in vitro effects of free radical-generating system Fe\(^{2+}\)/H\(_2\)O\(_2\) on mitochondrial membranes and myofibrils. Iron is generally believed to play a significant role in free radical producing reactions during aging and diseases (Stadtman 1993; Horáková et al. 2002). The hydroxyl radical formed from iron and H\(_2\)O\(_2\) in the Fenton or Haber–Weiss reactions is well known factor of myocardial oxidative damage (Dhalla et al. 2000).

Materials and Methods

Animals

Male adult Wistar rats (weighing 250–350 g) were used for study (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobrá Voda, Slovakia). The animals were allowed free access to food and water, and were maintained in air-conditioned room.

Preparation of myofibrils and mitochondria

Myofibrils were prepared from rat myocardium according to the method described by Solaro et al. (1971) with modification. Briefly, the tissue was washed, minced
and homogenized in 10 vol of 30 mmol/l KH$_2$PO$_4$, 5 mmol/l EDTA, 0.3 mol/l sucrose, 0.5 mmol/l dithiothreitol, 0.3 mmol/l phenylmethylsulfonyl fluoride, 1 µmol/l leupeptine, 1 µmol/l pepstatine (pH 7.0) with a Ultra-Turrax T25 homogenizer (three times for 10 sec, 20,500 rpm). The homogenate was centrifuged at 1000 × g for 10 min. The pellet was resuspended in 100 mmol/l KH$_2$PO$_4$, 5 mmol/l EDTA (pH 7.0) and centrifuged at 3500 × g for 10 min. This procedure was repeated twice. The resulting pellet was then suspended in 100 mmol/l KH$_2$PO$_4$, 5 mmol/l EDTA (pH 7.0) with 1% (vol/vol) Triton X-100, incubated at 0°C for 30 min and centrifuged at 3500 × g for 10 min. The pellet was washed three times as described about in 30 mmol/l imidazole, 60 mmol/l KCl, 2 mmol/l MgCl$_2$ (pH 7.0). The final pellet was suspended in 30 mmol/l imidazole, 60 mmol/l KCl, 2 mmol/l MgCl$_2$ (pH 7.0) with 50% glycerol, stored in −70°C and then used as cardiac myofibril preparation.

Mitochondria were prepared from tissue homogenate (see above) by differential centrifugation. The supernatant from homogenate centrifugation (1000 × g for 10 min) was centrifuged at 18,000 × g for 35 min. The pellet was suspended in 30 mmol/l imidazole, 60 mmol/l KCl, 2 mmol/l MgCl$_2$ (pH 7.0) and stored at −70°C. All isolation steps were performed at 4°C. Protein assays were performed by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Oxidation of myofibrillar proteins and mitochondria

Oxidative stress (OS) was induced by incubation myofibrils and mitochondria (3 mg/ml) with 1 mmol/l H$_2$O$_2$, 0.1 mmol/l FeSO$_4$/EDTA at 37°C for 30 or 60 min. Controls were incubated in the same manner without the treatment with free radical generating system. After the appropriate time intervals, the aliquots of myofibrils and mitochondria were taken for conjugated diene determination and fluorescence measurements.

Measurement of conjugated dienes and fluorescence studies

Conjugated diene formation was determined from the absorbance ratio $A_{233}/A_{215}$ of mitochondria dispersed in concentration 20 µg/ml protein in solution with 10 mmol/l phosphate buffer containing 1% Lubrol (Braughler et al. 1986).

Fluorescence measurements were performed in solution containing 50 µg protein per ml, 10 mmol/l HEPES, 100 mmol/l KCl (pH 7.0) at 25°C using Shimadzu RF 540 spectrofouimeter.

The fluorescence emission spectra (from 300 to 450 nm, 5 nm slit width) of tryptophan were measured by excitation at 295 nm (2 nm slit width) (Douisset et al. 1994).

Emission spectra of dityrosine, a product of tyrosine oxidation, were recorded in range 380 to 440 nm (5 nm slit width) at excitation wavelength 325 nm (5 nm slit width) (Giulivi and Davies 1994).

Emission spectra (from 425 to 480 nm, 5 nm slit width) of lysine conjugates with LPO products were recovered at excitation of 365 nm (5 nm slit width). Excitation spectra (from 325 to 380 nm, 5 nm slit width) were measured at 440
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nm (5 nm slit width) (Dousset et al. 1994). 1,8-ANS fluorescence was measured following 15-min incubation of the probe with mitochondria. The excitation and emission wavelengths were 365 and 480 nm, respectively (5 nm slit widths).

Data analysis

The results are presented as mean ± S.E.M. One-way analysis of variance was first carried out to test for differences among groups. Between individual groups comparisons were made using an unpaired Student’s t-test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Formation of conjugated dienes was used as an index of LPO in mitochondrial membranes. The 30- and 60-min incubation periods were chosen because recently we have shown that these exposure durations are required to induce significant LPO in SR membranes. The degree of peroxidation increased gradually with increasing time of incubation in the medium containing 0.1 mmol/l FeSO$_4$/EDTA plus 1 mmol/l H$_2$O$_2$ (Fig. 1). Comparing to corresponding control, 123.2 ± 6.4% and 137.3 ± 6.9 increase in LPO index was reached at 30 and 60 min, respectively.

The effect of the free radical-generating system on protein structure was examined by measuring tryptophan and bityroline fluorescences. Fig. 2 shows that incubation of mitochondria with an oxidant system significantly decreased the intensity of intrinsic tryptophane fluorescence. After 30-min incubation the fluorescence was decreased to 78.9 ± 1.8% and 60-min treatment led to further decrease to 60 ± 1.5% of the control value. A 30-min incubation of mitochondria with radical-generating system caused also an increase in bityroline fluorescence 125.7 ± 4.2% (Fig. 3), however, prolonged treatment did not lead to further accumulation of bityrolines (126.7 ± 2.4% of the control value).

Table 1. Effect of H$_2$O$_2$ + FeSO$_4$/EDTA oxidative system on fluorescence in mitochondrial membranes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fluorescence intensity of</th>
<th>Fluorescence intensity of</th>
<th>1,8-ANS probe</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lys$_\text{em}$-LPO</td>
<td>Lys$_\text{ex}$-LPO</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>71.62 ± 2.6</td>
<td>72.92 ± 1.5</td>
<td>87.98 ± 1.5</td>
</tr>
<tr>
<td>OS 30 min</td>
<td>74.92 ± 1.3</td>
<td>74.10 ± 1.5</td>
<td>83.44 ± 2.7</td>
</tr>
<tr>
<td>Control</td>
<td>72.80 ± 0.9</td>
<td>75.72 ± 1.5</td>
<td>85.76 ± 2.5</td>
</tr>
<tr>
<td>OS 60 min</td>
<td>76.12 ± 2.4</td>
<td>77.26 ± 2.6</td>
<td>78.84 ± 3.2</td>
</tr>
</tbody>
</table>

OS, oxidative stress; Lys$_\text{em}$-LPO, emission of conjugates of lysine with lipid peroxidation products; Lys$_\text{ex}$-LPO, excitation of conjugates of lysine with lipid peroxidation products; 1,8-ANS, 1-anilino-8-napthalenesulfonate. Values are expressed as means ± S.E.M. of five experiments.
Figure 1. Effect of 30- and 60-min oxidative stress (OS) on formation of conjugated dienes in rat cardiac mitochondria. The results are expressed as mean ± S.E.M. of five animals in each group. * $p < 0.01$; significantly different as compared to control.

Figure 2. Effect of 30- and 60-min oxidative stress (OS) on tryptophan fluorescence in rat cardiac mitochondria. The results are expressed as mean ± S.E.M. of five animals in each group. ** $p < 0.001$; significantly different as compared to control.

Figure 3. Effect of 30- and 60-min oxidative stress (OS) on bitryrosine fluorescence in rat cardiac mitochondria. The results are expressed as mean ± S.E.M. of five animals in each group. ** $p < 0.001$; significantly different as compared to control.

Figure 4. Effect of $\text{H}_2\text{O}_2 + \text{FeSO}_4/\text{EDTA}$ induced oxidative stress (OS) on tryptophan fluorescence in rat cardiac myofibrils. The results are expressed as mean ± S.E.M. of five animals in each group. *$p < 0.01$; ** $p < 0.001$; significantly different as compared to control.
In order to assess modification of mitochondrial proteins by LPO end products, the fluorescence excitation (350–360 nm) and emission (440–450 nm) spectra were measured. As shown in Table 1, incubation of mitochondria with FeSO₄/EDTA plus H₂O₂ resulted only in small non-significant rise of fluorescent products. Similarly, no significant differences between control and oxidized mitochondria were observed in 1,8-ANS fluorescence. A similar pattern of changes in tryptophan fluorescence was observed when isolated myofibrils were exposed to 0.1 mmol/l FeSO₄/EDTA plus 1 mmol/l H₂O₂ (Figs. 4 and 5). Analysis of data revealed a loss of tryptophan fluorescence to 84.3 ± 3% and 74.4 ± 2.9% after 30-min and 60-min incubation, respectively. In contrast to mitochondria, we observed a gradual increase in bityrosine formation in radical-treated myofibrils; the values were 114.2 ± 2.8% and 123.5 ± 4% for 30- and 60-min treated myofibrils, respectively.

Discussion

The present study shows that hydroxyl radicals generated by Fenton reaction can induce LPO and oxidize several amino acid residues of mitochondrial and myofibrillar proteins. Free radical-induced structural and functional changes of myofibrillar proteins have been reported in several studies (Zhang et al. 1990; Miura et al. 1994; Iwase et al. 1998; Paradies et al. 1998). Paradies et al. (1998) found a close correlation between LPO and inhibition of the cytochrome c oxidase activity in cardiac mitochondria exposed to t-butylhydroperoxide/Cu²⁺, suggesting that lipid-mediated damage plays an important role in protein modification. To evaluate the role of lipid-mediated protein modification we measured the fluorescence of conjugates formed by LPO end-products, such as molondialdehyde, and free amino-groups of proteins. Although our study shows that FeSO₄ plus H₂O₂ induces LPO, it was not accompanied by accumulation of fluorescent lipid-protein conjugates. The lack of lipid-mediated protein modification, which we observed, is in agreement with results of Zhang et al. (1990). These authors reported that inactivation of electron transport chain proteins by various ROS is not prevented by lipid-soluble antioxidants. Recently, we have shown that LPO in cardiac
SR induced by FeSO$_4$ without H$_2$O$_2$ is accompanied by production of fluorescent lipid-protein conjugates (Kaplan et al. 2003). The differences might be related to the different mechanism of Fe$^{2+}$ and Fe$^{2+}$/H$_2$O$_2$-induced damage or to the different vulnerability of SR and mitochondrial membranes to the OS. Several studies have indicated that ROS-induced changes in mitochondrial proteins are more likely related to oxidation of SH groups, because the inhibition of mitochondrial creatine kinase activity was reversed by SH group reductants (Yuan et al. 1992; Miura et al. 1994). Although SH group oxidation in mitochondrial proteins is well documented, little is known about the ROS-induced oxidation of other amino acid residues and conformational changes of proteins. To further investigation whether protein modification occurs during the exposure of mitochondria to radical-generating system, we used 1,8-ANS probe, a sensitive indicator of protein conformation (Slavik 1982). Our results show that radicals cause only a slight decrease in 1,8-ANS fluorescence, suggesting that an OS is unrelated to substantial changes in protein conformation and mitochondrial membrane surface. To test whether 'OH radicals modify specific amino acids despite the lack of gross conformational changes, the intrinsic fluorescence of tryptophan and dityrosine was measured. Recently, we have shown that tryptophan fluorescence is a sensitive marker of oxidative damage during ischemia and reperfusion (Murin et al. 2001). The time-dependent loss in tryptophan fluorescence observed in the present study suggests a radical induced degradation of this amino acid residue or perturbations in its surroundings. Increased fluorescence of dityrosine provides further evidence for the direct oxidation of mitochondrial proteins. Our observation that the exposure of myofibrils to 'OH radicals also results in changes of tryptophan and dityrosine fluorences support the possibility that oxidation of tryptophan and tyrosine is involved in modification of contractile proteins, besides the alterations related to oxidation of SH groups (Suzuki et al. 1991; Kaneko et al. 1993). Present results showing that 'OH radicals modify tryptophan and tyrosine residues in mitochondria and myofibrils are consistent with those previously obtained by others (Davies et al. 1987; Welch et al. 2001). These studies with different hydroxyl radical-generating systems indicate that tryptophan and tyrosine belong to the most vulnerable amino acid residues in proteins and are good markers of 'OH radical mediated protein modification and damage. Our findings indicate that OS could lead to oxidation of amino acid residues without gross structural alterations and might be related to functional inhibition of mitochondrial and myofibrillar proteins. As shown previously (Dean et al. 1997), limited modifications, which occur without pronounced structural changes in proteins, are sufficient for enzyme inactivation.

Taken together, our results suggest that treatment of mitochondria and myofibrils with 'OH radicals results in modification of tryptophan and tyrosine residues, but is unrelated to LPO-dependent modification of proteins. Although it has not been examined which specific protein or proteins were modified during an OS, the present results support the possibility that 'OH radicals can alter the mitochondrial and myofibrillar function via direct oxidation of several amino acid residues. These
mechanisms may result in cardiac contractile dysfunction and might be implicated in the pathophysiology of ischemic damage or aging.

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References


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