

Effect of Ischemia and Reperfusion on Protein Oxidation in Isolated Rabbit Hearts

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Summary

Reactive oxygen species and other oxidants are involved in the mechanism of postischemic contractile dysfunction, known as myocardial stunning. The present study investigated the oxidative modification of cardiac proteins in isolated Langendorff-perfused rabbit hearts subjected to 15 min normothermic ischemia followed by 10 min reperfusion. Reperfusion under these conditions resulted in only 61.8±2.7 % recovery of developed pressure relative to preischemic values and this mechanical dysfunction was accompanied by oxidative damage to cardiac proteins. The total sulfhydryl group content was significantly reduced in both ventricle homogenates and mitochondria isolated from stunned hearts. Fluorescence measurements revealed enhanced formation of nitrotyrosines and conjugates of lipid peroxidation-end products with proteins in cardiac homogenates, whereas these parameters were unchanged in the mitochondrial fraction. Reperfusion did not alter protein surface hydrophobicity, as detected by a fluorescent probe 1-anilino-8-naphthalenesulfonate. Our results indicate that oxidation of proteins in mitochondria and possibly in other intracellular structures occurs during cardiac reperfusion and might contribute to ischemia-reperfusion injury.

Key words

Ischemia • Reperfusion • Protein oxidation • Free radicals • Myocardial stunning

Introduction

It is well established that oxidative stress is involved in the development of myocardial stunning – cardiac contractile dysfunction occurring during reperfusion after brief ischemia. A burst of free radical generation in postischemic myocardium was demonstrated in several studies using electron spin resonance (ESR) spectroscopy technique (Bolli *et al.*

1988, Vavřínková *et al.* 2001). It has also been demonstrated that free radical scavengers can attenuate cardiac reperfusion injury (Kim and Akera 1987, Ambrosio *et al.* 1991, Nediani *et al.* 1997). However, relatively less is known about cellular targets and oxidative damage to various cellular components in ischemic-reperfused myocardium. Oxygen free radicals are potentially damaging lipids, proteins and nucleic acids. Whether oxidation of membrane lipids plays an

important role in postischemic injury is still uncertain. Several studies have demonstrated increased lipid peroxidation (LPO) after reperfusion of isolated hearts or after global ischemia in intact animals (Kim and Akera 1987, Ambrosio *et al.* 1991, Nediani *et al.* 1997, Paradies *et al.* 1999, Venditti *et al.* 2001), while some studies have failed to show evidence for accumulation of lipid peroxidation products (Brasch *et al.* 1989, Ceconi *et al.* 1991, Coudray *et al.* 1992, Marchant *et al.* 1993, Dobsak *et al.* 1999).

On the other hand, growing evidence has suggested that proteins are the major targets for free radicals in postischemic myocardium (Packer *et al.* 1991, Eaton *et al.* 1999, Ceconi *et al.* 2000, Powell *et al.* 2001, Schwalb *et al.* 2001, Eaton *et al.* 2002). These studies have shown that ischemia and reperfusion results in carbonyl formation, thiol group modification or LPO-mediated modification of protein amino acids. However, little is known about other oxidative lesions on proteins in ischemic-reperfused hearts, although studies on protein oxidation by exogenously generated radicals have demonstrated that all amino acids in proteins can be oxidatively damaged (for review see Davies *et al.* 1999).

The aim of the present study was to investigate the potential oxidative modifications of protein amino acids and membranes in isolated Langenorff perfused rabbit hearts subjected to 15 min ischemia and 10 min after reperfusion. The thiol group content, tyrosine formation and lysine conjugates with LPO-end products were determined as indices of oxidative modification of proteins in heart homogenates and in isolated mitochondria, which are considered to be both a major source of free radicals and a target for their damaging effects (Škárka and Ošťádal 2002, Lehotský *et al.* 2003). Fluorescence spectra of anionic probe 1-anilino-8-naphthalenesulfonate were measured to assess structural alterations in cardiac proteins.

Methods

Preparation of isolated hearts and perfusion

Male New Zealand white rabbits (2–2.5 kg) were intravenously injected with heparin (1000 IU/kg) for anticoagulation and were killed by injection of sodium pentobarbital (100 mg/kg). The hearts were excised, placed in ice-cold Krebs-Henseleit (K-H) solution and cannulated through the aorta for perfusion with K-H solution at a constant pressure of 65 mm Hg. The K-H solution (pH 7.4) consisted of 135 mmol/l NaCl,

5.4 mmol/l KCl, 0.9 mmol/l MgCl₂, 24 mmol/l NaHCO₃, 1.2 mmol/l NaH₂PO₄, 1.8 mmol/l CaCl₂ and 10 mmol/l glucose. The solution was saturated with 95 % O₂ and 5 % CO₂ and maintained at 37 °C. To measure isovolumetric left ventricular pressure (LVP) a fluid-filled latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium. Coronary flow was measured by a timed collection of coronary effluent.

After an initial stabilization period of 10 min the ischemic hearts underwent 15 min ischemia followed by 10 min reperfusion. Subsequent to a stabilization period the control hearts were perfused with K-H solution for 25 min without ischemia.

At the end of the perfusion protocol the hearts were removed from the perfusion system, the left ventricles were freeze-clamped in liquid nitrogen and then stored at –80 °C until use.

Preparation of tissue homogenates and mitochondria

Frozen powdered tissue (about 0.5 g) was thawed in 10 volumes of ice-cold homogenization buffer containing 30 mM imidazol, 60 mM KCl and 2 mM MgCl₂, pH 7.0 and homogenized for three times for 10 s with Ultra-Turrax T25 homogenizer at 13 500 rpm.

Mitochondria were isolated from homogenate by differential centrifugation according to the method of Lass *et al.* (1997). The homogenate was centrifuged at 1000 x g for 10 min, and the supernatant was centrifuged at 18 000 x g for 35 min. The resulting mitochondrial pellet was washed and resuspended in the homogenization buffer. The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Measurement of total sulfhydryl groups

The total –SH group content was determined following the 2,2-dithiobisnitrobenzoic acid (DTNB) assay (Jocelyn 1987) as described by (Kaplan *et al.* 2003). Briefly, homogenates or mitochondria were transferred to a buffer containing 0.4 mM DTNB, 30 mM imidazole (pH 7.4) and 5 mM EDTA and the absorbance was measured at 412 nm after 10 min incubation at room temperature. The sulfhydryl group content was calculated using molar absorption coefficient of 13 600 M⁻¹.cm⁻¹.

Fluorescence studies

The fluorescence measurements were performed on Shimadzu RF-540 spectrofluorometer at 25 °C.

Binding of 1-anilino-8-naphthalenesulfonate (ANS) was measured at 480 nm (slit width 5 nm) with excitation at 365 nm (slit width 5 nm).

The formation of bityrosines was evaluated by measuring the emission spectra (380-440 nm) at excitation wavelength 325 nm (slit width 5 nm).

Fluorescence emission spectra in range 420-480 nm were measured in order to assess the production of conjugates of LPO-end products with free amino groups

of proteins. The excitation wavelength was 365 nm (slit width 5 nm).

Statistical analysis

The results are presented as mean \pm S.E.M. Unpaired Student's t-test was used when the control and ischemic group were compared. A value of $p < 0.05$ was considered as statistically significant.

Table 1. Effect of ischemia and reperfusion on cardiac contractile function

	HR (bpm)	CF (ml/min)	sLVP (mm Hg)	LVEDP (mm Hg)	devLVP (mm Hg)	+LVdP/dt (mm Hg/s)	-LV dP/dt (mm Hg/s)
Control							
0 min	176 \pm 13	76.5 \pm 9.6	134 \pm 6	9.0 \pm 1.0	125 \pm 5	1900 \pm 108	1275 \pm 83
25 min	177 \pm 13	70.8 \pm 13.1	127 \pm 11	10.5 \pm 1.5	116 \pm 12	1763 \pm 206	1200 \pm 134
Stunned							
Before I	166 \pm 12	55.5 \pm 12.3	120 \pm 12	10.0 \pm 0.8	110 \pm 11	1588 \pm 268	1125 \pm 132
After I	153 \pm 16	63.0 \pm 16.3	92 \pm 4 \dagger	23.5 \pm 5.0* \dagger	68 \pm 3* \ddagger	900 \pm 91* \ddagger	750 \pm 46* \dagger

HR, heart rate; bpm, beats per minute; CF, coronary flow; sLVP, systolic left ventricular pressure; LVEDP, left ventricular end diastolic pressure; devLVP, developed LVP; +LV dP/dt, maximum rate of pressure development; -LV dP, maximum rate of relaxation. Values are expressed as means \pm SEM of five hearts. * $p < 0.05$; significantly different when compared to the preischemic value in the same group. \dagger $p < 0.05$, \ddagger $p < 0.01$; significantly different when compared to control hearts.

Results

Contractile function

Myocardial contractile parameters for control and ischemic/reperfused hearts are shown in Table 1. The initial values of the contractile parameters of both groups were not significantly different, indicating similar starting myocardial function of all hearts. Ischemia, initiated by clamping the perfusion line, resulted in rapid decline of systolic LVP, while end-diastolic LVP increased from 10.0 \pm 0.8 to 17.2 \pm 3.2 mm Hg. Reperfusion, achieved by opening the perfusion line, recovered the systolic pressure to 76.7 \pm 3.3 % of the preischemic value. During reperfusion, end-diastolic pressure further increased and reached 235 \pm 50 % by the end of the reperfusion period ($p < 0.05$). A fall of systolic pressure and an increase in diastolic pressure resulted in the decrease of developed pressure (devLVP) to 61.8 \pm 2.7 % of the preischemic value. During reperfusion the rate-pressure product (product of heart rate and devLVP) was significantly reduced (10462 \pm 1369 mm Hg/min) when compared to the preischemic value (18464 \pm 2925 mm Hg/min, $p < 0.05$) or control group (20417 \pm 2219 mm Hg/min, $p < 0.01$).

Both, maximum rate of pressure development (+LV dP/dt) and maximum rate of relaxation (-LV dP/dt) were also significantly depressed during reperfusion (56.7 \pm 5.7 and 66.7 \pm 4.1 % of preischemic value, respectively). Heart rate at the end of reperfusion was the same in control and ischemic/reperfused group. Similarly, coronary flow in stunned hearts did not significantly differ from the control value.

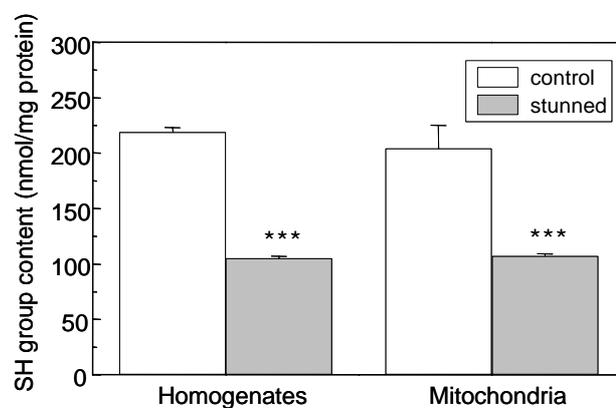


Fig. 1. Total sulfhydryl group content in homogenates and mitochondria isolated from control and stunned hearts. Values are given as means \pm S.E.M. of 5 experiments. *** $P < 0.001$; significantly different as compared to the control value.

Sulfhydryl group content

To test the hypothesis that ischemia and reperfusion leads to oxidative modification of proteins, the sulfhydryl group content was measured in homogenates and mitochondria isolated from control and stunned hearts. Ischemia and reperfusion resulted in a significant decrease in the SH group content (Fig. 1). A 52 ± 1 % loss in $-SH$ groups was observed in ventricle homogenates from stunned hearts when compared to control values. For mitochondria from stunned hearts a 48 ± 1 % loss was found in the sulfhydryl groups.

Fluorescence measurements

To determine the effect of stunning on membrane surface and proteins a binding of anionic membrane probe ANS was studied. ANS fluorescence in homogenates from stunned hearts was higher than in the controls, but this change did not reach statistical significance (124.1 ± 13.2 % of control value, $p > 0.05$). Similarly, there were no significant changes in ANS binding to mitochondrial membranes (105.2 ± 2.2 %, $p > 0.05$).

In an attempt to determine whether ischemia and reperfusion result in protein oxidation other than cysteine $-SH$ group oxidation, the bityrosine fluorescence was measured. Changes in fluorescences after ischemia and reperfusion in homogenates and mitochondria are shown in Figure 2. The bityrosine fluorescence was significantly increased in homogenates (126 ± 8 % of the control, $p < 0.05$), but the increase in mitochondria did not reach statistical significance (113 ± 3 %, $p > 0.05$).

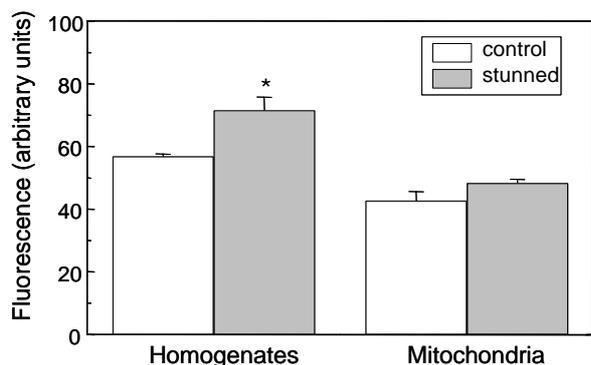


Fig. 2. Effect of ischemia and reperfusion on bityrosine formation in ventricle homogenates and mitochondria. Values are given as means \pm S.E.M. of five experiments. * $P < 0.05$: significantly different as compared to the control value.

In order to assess lipid-mediated modifications of membrane proteins, we measured the fluorescence

emission spectra resulting from derivatization of lysine residues of proteins by LPO-end products. Ischemia and reperfusion resulted in significant accumulation of fluorescent complexes in ventricle homogenates (135 ± 9 %, $p < 0.05$), but fluorescence intensity of isolated mitochondria was unchanged (Fig. 3).

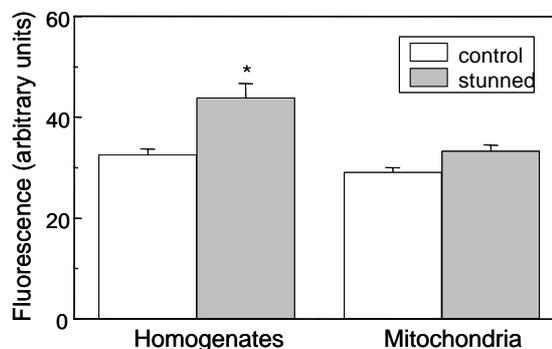


Fig. 3. Effect of ischemia and reperfusion on the formation of conjugates of LPO-end products with free amino groups of proteins. Values are given as means \pm S.E.M. of five experiments. * $P < 0.05$; significantly different as compared to the control value.

Discussion

In the present study, we attempted to explore oxidative modifications of proteins in homogenates and mitochondria isolated from stunned rabbit hearts. We have found that 15 min ischemia followed by 10 min reperfusion resulted in contractile dysfunction, accompanied by a loss of sulfhydryl groups in both ventricle homogenates and mitochondria. The formation of bityrosines and conjugates of LPO-end products with free amino groups of proteins were increased in homogenates, but remained unaltered in mitochondria. The results are consistent with the hypothesis that oxidative injury during reperfusion plays a key role in the pathogenesis of myocardial stunning.

A recognized important pathway for protein oxidation in ischemic-reperfused hearts is $-SH$ group oxidation. Several studies, including our previous study, (Lesnefsky *et al.* 1991, Ceconi *et al.* 2000, Kaplan *et al.* 2002) have shown a decreased $-SH$ group content in heart homogenates after ischemia and reperfusion. Moreover, the sulfhydryl group containing agents have been shown to attenuate myocardial reperfusion injury by preventing $-SH$ group oxidation in functional proteins (Pauly *et al.* 1987, Yanagishita *et al.* 1997). Our findings that $-SH$ content was equally reduced in heart mitochondria indicate that sulfhydryl oxidation may be

associated with alterations in mitochondrial function. As has previously been reported (Korge *et al.* 2001) the –SH reducing agents were fully protective against mitochondrial permeability transition, which is also implicated in cardiac reperfusion injury. Derangements in other mitochondrial functions, observed in postischemic hearts, such as release of cytochrome c, defects in electron transport and reduction in oxidative phosphorylation activity (for review see Škárka and Ošťádal 2002) may also be related to the loss of –SH groups or other oxidative modifications.

The production of bityrosine, which arises from direct oxidation of tyrosine residues by free radicals, is a sensitive marker of protein oxidation (Davies *et al.* 1999). In a similar model of ischemia (20 min) on rat hearts, Yasmin *et al.* (1997) and Cheung *et al.* (2000) showed an enhanced bityrosine level in the coronary effluent, which was caused by peroxynitrite generated during reperfusion. These findings are consistent with our results showing that bityrosine levels are significantly elevated in tissue homogenates from postischemic hearts.

Changes in membrane proteins are often associated with changes in the lipid bilayer. Using Western blot analysis, Eaton *et al.* (1999) have shown that 30 min ischemia led to formation of protein conjugates with 4-hydroxy-2-nonenal (HNE), an end product of LPO. Lucas and Szveda (1998) reported that mitochondrial dysfunction, which occurred during reperfusion of ischemic (30 min) aged rat hearts, is related to accumulation of HNE-protein adducts. In our study the fluorescence of modified proteins was measured as a marker of protein oxidation by lipid-derived species. Decomposition products of LPO such as HNE and other aldehydes react with lysine residues of proteins to produce conjugates with characteristic fluorescence spectrum (Steinbrecher 1987). The increase of conjugate fluorescence in ventricle homogenates, but not in mitochondria, suggests that lipid-derived protein oxidation occurs in other cardiac membranes, yet unidentified. With regard to our previous works on the same model of isolated stunned heart, which failed to show alterations in sarcoplasmic reticulum function (Kaplan *et al.* 1992, 1997), but there was the inhibition of

Na⁺,K⁺-ATPase activity (unpublished results), we hypothesized that lipid mediated oxidation may be attributed to plasma membrane. However, further studies are required to identify the site of membrane modification. On the other hand, we cannot exclude the possibility that the lack of major changes in fluorescence parameters in the mitochondrial fraction is due to delayed oxidation of mitochondrial proteins.

To study the possible structural changes induced by protein oxidation, we measured the interactions of hydrophobic probe, ANS, with cardiac homogenates and mitochondria. ANS is noncovalently incorporated into proteins or membranes and its fluorescence is sensitive to changes in conformation and surface hydrophobicity (Slavík 1982). Addition of ANS to cardiac homogenates and mitochondria resulted in an increase of fluorescence, but the changes were not statistically significant. This indicates that ischemia and reperfusion do not cause substantial conformational changes or modification of hydrophobicity in cardiac membranes. Such findings are in agreement with data from *in vitro* studies, which show that conformational and hydrophobicity changes of proteins occur only to a limited extent during the oxidative insult (Dean *et al.* 1997).

In summary, this study provides evidence that myocardial stunning is associated with oxidative modifications of cardiac proteins, both in the whole ventricle and in the mitochondria. Because the observed changes in whole ventricle are different from those found in isolated mitochondria, it is possible that there are also other targets of free radicals in reperfused myocardium and they may differ in their susceptibility to oxidative stress. Additional investigations of the specific intracellular targets will provide the insight into the mechanism of oxidative injury in myocardial ischemia and reperfusion.

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