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RESEARCH PAPER

Dose-dependent protective effect of propofol against mitochondrial dysfunction in ischaemic/reperfused rat heart: role of cardiolipin

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Background and purpose: Ischaemia damages to the cardiac mitochondria by increasing generation of reactive oxygen species (ROS) and peroxidation of cardiolipin. The inhibited mitochondrial function leads to the cardiac injury during reperfusion. Propofol (2, 6-diisopropylphenol), an intravenous anaesthetic, has been shown to decrease cardiac ischaemia and reperfusion injury. In the present study, we propose that propofol protects mitochondrial function and decreases cardiac injury by prevention of cardiolipin peroxidation during ischaemia and reperfusion.
Experimental approach: After isolation of mitochondria from isolated rat heart perfused on a Langendorff model, various mitochondrial bioenergetic parameters were evaluated such as rates of mitochondrial oxygen consumption, H_2O_2 production, complex I and III activity as well as the degree of lipid peroxidation and cardiolipin content. The action of propofol was also explored in isolated mitochondria. And the effect of cardiolipin was evaluated by fusing cardiolipin liposome with mitochondria.
Key results: Propofol treatment had strong dose-dependent protection attenuating these parameters alterations in reperfused rat heart and isolated mitochondria. Additionally, cardiolipin treatment had the same protective effect, compared with propofol treatment at high concentration.
Conclusions and implications: The protective effect of propofol appears to be due, at least in part, as a chemical uncoupler, to the interruption of the vicious circle of ROS–cardiolipin–complexes of the respiratory chain–ROS through preserving the content and integrity of cardiolipin molecules by ROS attack. These findings may provide an explanation for some of the factors responsible for cardioprotection and one approach exploring an available antioxidant.
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Keywords: propofol; cardiolipin; reactive oxygen species; mitochondria; ischaemia/reperfusion

Abbreviation: ROS, reactive oxygen species

Introduction

Reactive oxygen species (ROS) are involved in cardiac ischaemia and reperfusion injury (Hess and Manson, 1984; Zweier *et al*., 1987; Ambrosio *et al*., 1991; Das, 1994; Vanden Hoek *et al*., 1996). The source of ROS generation and cellular and subcellular target of ROS attack are still under investigating. In addition to xanthine/xanthine oxidase (Chambers *et al*., 1985; Xia *et al*., 1996) and NADPH oxidase (Shandelya *et al*., 1993), complex I and complex III at the mitochondrial electron transport chain are suggested to be the main source of ROS during ischaemia and reperfusion (Boveris *et al*., 1976; Ambrosio *et al*., 1993; Paradies *et al*., 2001, 2002; Chen *et al*., 2003). Mitochondria-mediated ROS generation only leads to primary reaction and damage in the immediate area surrounding where these ROS are produced due to their highly reactive and short-lived life span. Therefore, as major sources of ROS production, mitochondria could also be major targets of ROS attack. The effects of ROS should be greatest at the level of mitochondrial membrane constituents, including phospholipids constituents particularly rich in unsaturated fatty acids, such as cardiolipin.

Cardiolipin, a phospholipid located almost uniquely within the inner mitochondrial membrane, is sensitive to oxidative stress because of the rich in unsaturated fatty acids. Thus, mitochondrial cardiolipin molecules are a possible early target of ROS attack, either because of their high content of unsaturated fatty acids or because of their location in inner mitochondrial membrane near to the site

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of ROS production, mainly at the level of complexes of the respiratory chain (Paradies *et al*., 2001, 2002). Recent studies showed that cytochrome *c* tightly bound on cardiolipin formed cytochrome *c* and cardiolipin peroxidase. When H_2O_2 is availed as substrate, this peroxidase will oxidize H_2O_2 to H_2O, and simultaneously leads to cardiolipin peroxidation (Kagan *et al*., 2005, 2006; Gorbenko *et al*., 2006; Tyurina *et al*., 2006). Cardiolipin peroxidation leads to decreased activities of complexes at the electron transport chain (Petrosillo *et al*., 2003; Paradies *et al*., 2004; Lesnefsky *et al*., 2004a). The impairment of mitochondrial complex activity may increase electron leak from the electron transport chain, generating more superoxide anion radical and perpetuating a cycle of oxygen-radical-induced damage.

Propofol (2,6-diisopropylphenol), an intravenous sedative–hypnotic agent popular for sedation, is chemically similar to phenol-based free-radical scavengers such as the endogenous antioxidant vitamin E, while its lipophilic nature allows its rapid access to cellular and subcellular membranes compartments. Propofol has been found to be effective in protecting against pathological states characterized by an increase in basal rate of ROS production in brain (Lee *et al*., 2005), lung (Yumoto *et al*., 2005), liver (Tsao *et al*., 2003), testicle (Unsal *et al*., 2004) and hearts (Xia *et al*., 1996), but the mechanism by which it exerts the cardioprotective effect is not well established. In the current study, we propose that propofol treatment will decrease cardiac ischaemia and reperfusion injury by preservation of mitochondrial function via prevention of cardiolipin peroxidation at the mitochondrial level.

Materials and methods

Animal experiment and heart preparation

Male Sprague–Dawley rats weighing 250–300 g were supplied by the Animal Center of Xuzhou Medical College, P R China. All animal protocols were approved by the Animal Care and Use Committee at the College. After intraperitoneal injection of heparin ($1000\,UI\,kg^{-1}$) and administration of thiopental (50 mg), hearts were rapidly excised and mounted for retrograde perfusion of the coronary circulation according to Langendorff constant flow model ($10\,ml\,min^{-1}$) using Krebs–Henseleit buffer. The hearts were placed in a water-jacketed chamber 310.15 K (37 °C). Fifty-six hearts were used ($n=8$, each); hearts in the control group were only perfused for 70 min. Propofol-treated control hearts were perfused for 70 min in the presence of 100 μM propofol (control + P). In the untreated ischaemia and reperfusion group, hearts were first perfused for 20 min, and then subjected to global ischaemia for 30 min, followed by 20 min of reperfusion. In the cardiolipin-treated group, hearts were treated with liposome during ischaemia and reperfusion (reperfused + CL group). In the propofol-treated group, hearts were treated with propofol (25, 50 and 100 μM) during ischaemia and reperfusion (Lo-P, Mi-P and Hi-P, respectively). Left ventricle end-diastolic pressure and developed pressure left ventricle developed pressure were measured with a balloon inserted into ventricular and recorded by the MacLab/4S system (AD Instruments, Castle Hill, New South Wales, Australia). Left ventricle end-diastolic pressure was adjusted to 0.53–0.80 kPa at the beginning of perfusion.

Isolation of cardiac mitochondria

Cardiac mitochondria were isolated according to the method of Palmer *et al*. (1977), except that a modified Chappell–Perry buffer (buffer A (in mM): 100 KCl, 50 MOPS, 1 ethylenediamine tetraacetic acid, 5 $MgSO_4 \cdot 7H_2O$ and 1 ATP, pH 7.4) was used for mitochondrial isolation. All equipment and solutions were kept at 277.15 K (4 °C) during the isolation process. Briefly, cardiac tissue was finely minced, placed in buffer A containing 0.2% bovine serum albumin and homogenized with a Polytron tissue processor for 2.5 s at a rheostat setting of 6.0. The Polytron homogenate was centrifuged at 500 *g*. The combined supernatants were centrifuged at 3000 *g* to sediment mitochondria, washed twice and suspended in KME (in mM: 100 KCl, 50 MOPS and 0.5 ethylene glycol tetraacetic acid). Mitochondrial protein concentration was determined by modified Lowry's method, using bovine serum albumin as standard.

Preparation of liposome and fusion of liposome with mitochondrial membranes

Liposome (small unilamellar vesicle) was prepared by sonicating 1.7 mg cardiolipin in 1 ml of incubation medium containing 25 mM phosphate buffer (pH 6.7) with the microtip probe of a Branson sonifier at 40 W for six cycles of 2.5 min in an ice bath under N_2 stream. Then, 1 ml of freshly sonicated liposome was added to 1 mg of mitochondrial proteins at 303.15 K (30 °C) with constant stirring. After 40 min of incubation, mitochondria were centrifuged to remove excess cardiolipin. The mitochondrial pellet was then washed and resuspended in 250 mM sucrose and 10 mM Tris, pH 7.4.

Lipid peroxidation

Lipid peroxidation was estimated by the appearance of conjugated dienes as follows. Lipids were extracted from mitochondria by the Bligh and Dyer procedure (Folch *et al*., 1957). Lipid extracts from 4 mg of mitochondrial membrane were dissolved in 2.5 ml of chloroform: methanol (1:1) and absorption spectra were measured between 210 and 310 nm (Buege and Aust, 1978) with a UV-VIS recording spectrophotometer (UV-2401PC; Shimadzu, Tokyo, Japan).

Determination of mitochondrial H_2O_2 production

The rate of mitochondrial H_2O_2 production was obtained by measuring linear fluorescence increase induced by H_2O_2 oxidation of dichlorofluorescein to the fluorescent dichlorofluorescein in the presence of horseradish peroxidase (Black and Brandt, 1974). Rat heart mitochondria (0.5 mg protein) were suspended in 3 ml of a medium containing 100 mM sucrose, 100 mM KCl, 5 mM Tris, pH 7.4, supplemented with 8.0 μg horseradish peroxidase and 1 μM dichlorofluorescein. Production of H_2O_2 was induced by addition of 5 mM

malate + 2 mM pyruvate or 5 mM succinate as substrates (state 4). The amount of H_2O_2 produced was calculated by measuring fluorescence changes upon addition of known amounts of H_2O_2.

Mitochondrial oxygen consumption

Mitochondrial ADP-dependent state 3 respiration was measured polarographically with an oxygen electrode at 298.15 K. (25 °C). Respiration was initiated by addition of 2 mM pyruvate + 5 mM malate or 5 mM succinate. After 2 min, state 3 respiration was induced by adding 1 mM ADP.

Analysis of cardiolipin in mitochondrial membranes

Cardiolipin was analysed by high-pressure liquid chromatography (HPLC) using a Waters liquid chromatograph device. HPLC-grade solvents were obtained form TEDIA company, and cardiolipin standard were obtained form Avanti Polar Lipids (Alabaster, AL). Lipids from heart mitochondria were extracted with chloroform/methanol by the procedure of Bligh and Dyer (Folch *et al*., 1957). Lipid extraction was carried out on ice immediately after preparation of mitochondria in the presence of butylated hydroxytoluene (BHT) (50 μM) and under a nitrogen atmosphere. Phospholipids were separated by the HPLC method described previously (Ruggiero *et al*., 1984) using an Lichrosorb@Si60 (5 μm) column (4 × 250 mm). The chromatographic system was programmed for gradient elution using two mobile phases, solvent A: hexane/2-propanol (6:8, v/v), and solvent B: hexane/2-propanol/water (6:8:1.4, v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0 to 100%. Flow rate was 1 ml min^{-1} and detection was at 206 nm. The peak of cardiolipin was identified by comparison with the retention time of standard cardiolipin.

Complex I activity

Complex I (NADH–CoQ reductase) activity was measured in mitochondrial particles prepared by sonicating, under nitrogen atmosphere, 1 mg of rat heart mitochondria dissolved in 1 ml of 50 mM phosphate buffer pH 7.2. The assay mixture contained 3 mM sodium azide, 1.2 μM antimycin A, 50 μM decylubiquinone and 50 mM phosphate buffer, pH 7.2. The mitochondrial sample (50 μg) was added to 3 ml of the assay mixture and the reaction was started by the addition of 60 μM NADH. The reaction was measured by measuring rotenone-sensitive decrease in the absorbance of NADH at 340 nm with a diode array spectrophotometer. Activity was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH. Specific activity of the enzyme was expressed as the amount of NADH oxidized (nmol min^{-1} mg^{-1}).

Complex III activity

Complex III activity (decylubiquinol/ferricytochrome *c* oxidoreductase) was measured in mitochondrial particles prepared by sonicating 1 mg of rat heart mitochondria dissolved in 1 ml of 50 mM phosphate buffer, pH 7.2. The assay mixture contained 3 mM sodium azide, 1.5 μM rotenone, 50 μM ferricytochrome *c* and 50 mM phosphate buffer, pH 7.2. The sample (10 μg) was added to 3 ml of assay mixture and reaction was started by addition of 30 μM decylubiquinol. The reaction was measured with a diode array spectrophotometer by measuring the increase in reduced cytochrome *c* absorbance at 550–540 nm. Activity was calculated using an extinction coefficient of 19.1 mM^{-1} cm^{-1}. Specific activity of the enzyme was expressed as the amount of cytochrome *c* reduced (nmol min^{-1} mg^{-1}).

Decylubiquinol was synthesized by reduction of decylubiquinone (10 μM) with $NaBH_4$ in 2 ml of 1:1 ethano/H_2O mixture (v/v, pH 2). Ubiquinol so formed was extracted twice with 1 ml of diethylether/isooctane 2:1 (v/v). The combined organic phases were washed with 2 ml of 2 M NaCl and evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in ethanol and the resulting light-yellow solution was acidified with 10 μl of 0.1 M HCl and stored at 253.15 K (–20 °C).

Morphology of mitochondria

Morphological analysis of the isolated mitochondria was performed as previously described (Palmer *et al*., 1998). Isolated mitochondria were fixed in suspension by addition of an equal volume of phosphate-buffered, full-strength Karnovsky's fixative (Lesnefsky *et al*., 1997).

Statistical analysis

All data are presented as mean ± s.e. and their statistical significance was determined by one-way analysis of variance, with Dunnett (compare with reperfused group) and Student–Newman–Keuls (multiple comparisons among cardiolipin-treated and propofol-treated groups) and Student's test (comparison between control and reperfused groups, as well as between control and control + P group). $P<0.05$ was considered statistically significant.

Results

Lipid peroxidation by ROS is accompanied by rearrangement of the polyunsaturated fatty acid double bonds, leading to the formation of conjugated dienes, which absorb at 233 nm. Figure 1 shows absorbance values obtained from all these mitochondrial lipid extracts at 233 nm. Mitochondria from reperfused heart showed a large increase in the level of lipid peroxidation compared with the control. Mi-P and Hi-P and cardiolipin treatment markedly decreased the mitochondrial lipid peroxidation. However, 25 uM propofol did not prevent cardiolipin peroxidation.

ADP-stimulated respiration (state 3) and respiratory control ratio were markedly decreased in mitochondria isolated from reperfusion heart with pyruvate and malate as complex I and complex II substrates. Propofol treatment dose dependently attenuated the decrease of state 3 in mitochondria following reperfusion. Cardiolipin given before ischaemia also protects mitochondria during ischaemia and reperfusion. With succinate as complex-II substrate, ischaemia and reperfusion also decreases the rate of state 3, and

both propofol and cardiolipin protect respiration of mitochondria after ischaemia and reperfusion (Table 1). State 4 rates of respiration were slightly decreased in all these preparations of mitochondria.

H_2O_2 can be generated by addition of respiratory substrates pyruvate + malate or succinate to aerobic mitochondria. H_2O_2 production was significantly enhanced in mitochondria isolated from ischaemia and reperfusion heart with pyruvate + malate as complex I substrate or succinate as complex II substrate. This H_2O_2 production was decreased in mitochondria treated by cardiolipin and propofol (50 and 100 uM) (Figure 2).

As shown in Figure 3, reperfusion decreased the content of cardiolipin compared with the control. Propofol treatment prevents cardiolipin loss in a concentration-dependent manner. Mitochondria from reperfused rat heart

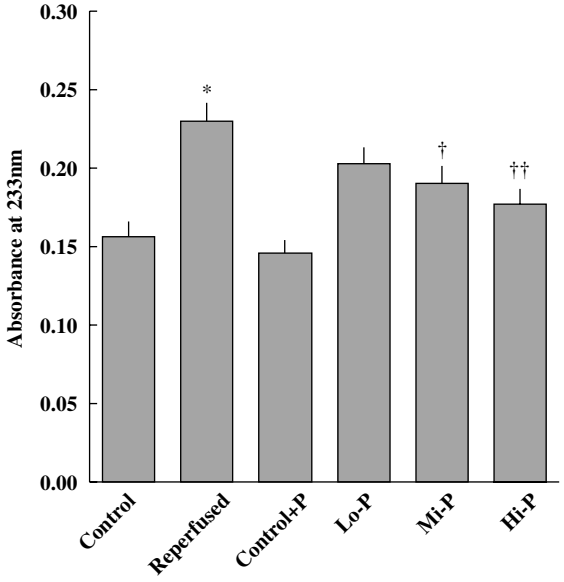

Figure 1 Absorbance values of the conjugated dienes of mitochondrial lipids extracted from control, reperfused, control + P, Lo-P, Mi-P and Hi-P groups. The conjugated dienes spectra were recorded as described under Materials and methods. Each group includes eight rats. Data were expressed as mean ± s.e. $^{*}P<0.05$ vs control; $^{\dagger}P<0.05$ vs reperfused; $^{\dagger\dagger}P<0.01$ vs reperfused; P, propofol.

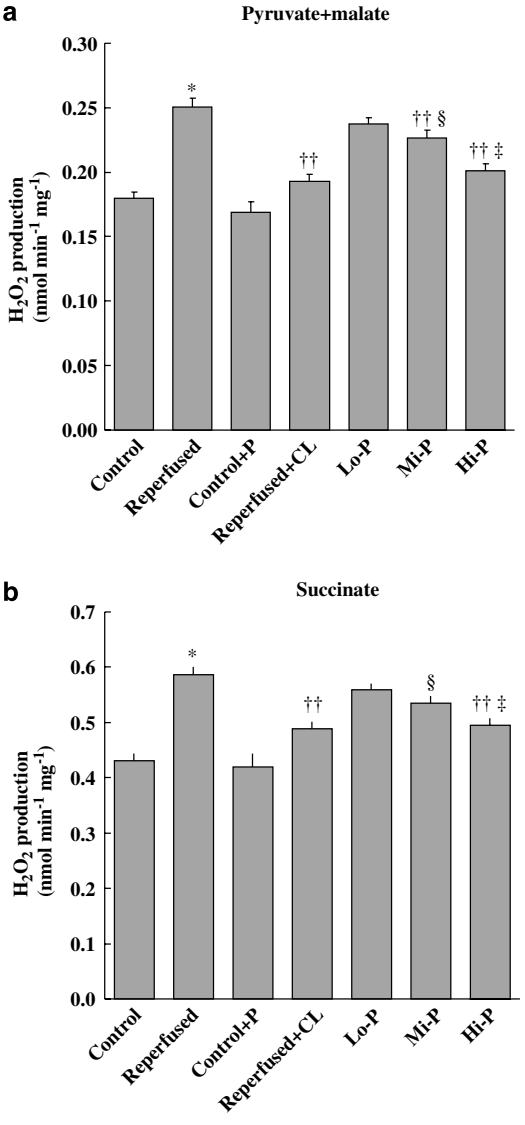

Figure 2 H_2O_2 production in mitochondria isolated from control, reperfused, control + P, reperfused + CL, Lo-P, Mi-P and Hi-P groups. Pyruvate and malate (**a**) are used as complex I substrate and succinate (**b**) is used as complex II substrate. Each group includes eight rats. Data were expressed as mean ± s.e. $^{*}P<0.05$ vs control; $^{\dagger\dagger}P<0.01$ vs reperfused; $^{\ddagger}P<0.05$ vs Lo-P; $^{\S}P<0.05$ vs Hi-P. CL, cardiolipin; P, propofol.

Table 1 Respiratory activities in mitochondria isolated from control, reperfused, control + P, reperfused + CL, Lo-P, Mi-P and Hi-P groups[a]

	Control	*Reperfused*	*Control + P*	*Reperfused + CL*	*Lo-P*	*Mi-P*	*Hi-P*
Pyruvate + malate							
State 3	193 ± 9.3	84 ± 4.6*	197 ± 5.6	165 ± 7.9††	103 ± 4.4	137 ± 7.1†,‡,§	162 ± 7.9††,‡
State 4	45 ± 1.7	31 ± 1.8*	46 ± 1.9	41 ± 2.3††	35 ± 1.7	38 ± 2.0†	40 ± 2.2††
RCR	4.4 ± 0.3	2.8 ± 0.3*	4.3 ± 0.1	4.1 ± 0.2††	3 ± 0.2	3.7 ± 0.3	4.2 ± 0.3††,‡
Succinate							
State 3	192 ± 12.4	88 ± 5.1*	197 ± 10.2	159 ± 6.7††	115 ± 4.9†	139 ± 7.6††,‡	151 ± 7.3††,‡
State 4	55 ± 2.7	38 ± 1.9*	56 ± 2.7	47 ± 2.4††	40 ± 2.3	44 ± 2.7	46 ± 2.2†
RCR	3.6 ± 0.4	2.3 ± 0.1*	3.6 ± 0.2	3.4 ± 0.2††	2.9 ± 0.2	3.2 ± 0.2††	3.3 ± 0.2††

Abbreviations: CL, cardiolipin; P, propofol; RCR, respiratory control ratio.
[a]Mitochondrial respiratory activities were measured as described under Materials and methods. Each value represents the mean ± s.e. of eight separate experiments.
$^{*}P<0.05$ vs control; $^{\dagger}P<0.05$ vs reperfused; $^{\dagger\dagger}P<0.01$ vs reperfused; $^{\ddagger}P<0.05$ vs Lo-P; $^{\S}P<0.05$ vs Hi-P.

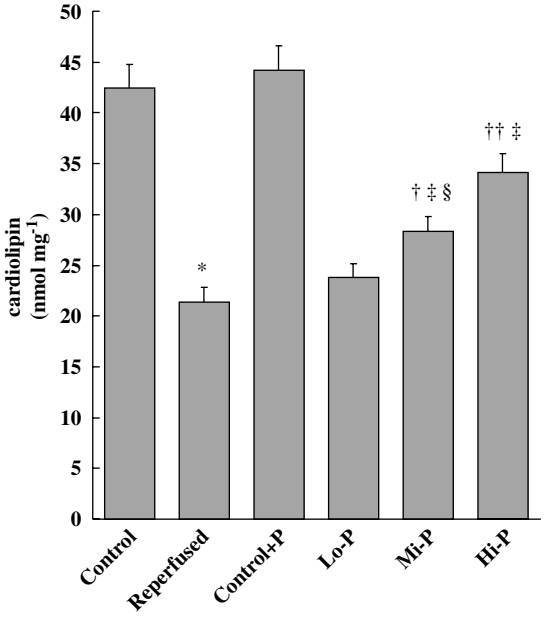

Figure 3 Cardiolipin content in mitochondria isolated from control, reperfused, control + P, Lo-P, Mi-P and Hi-P groups. Mitochondrial cardiolipin content was determined by HPLC technique. Each group includes eight rats. Data were expressed as mean ± s.e. *$P<0.05$ vs control; $^{\dagger}P<0.05$ vs reperfused; $^{\dagger\dagger}P<0.01$ vs reperfused; $^{\ddagger}P<0.05$ vs Lo-P; $^{\S}P<0.05$ vs Hi-P. HPLC, high-pressure liquid chromatography; P, propofol.

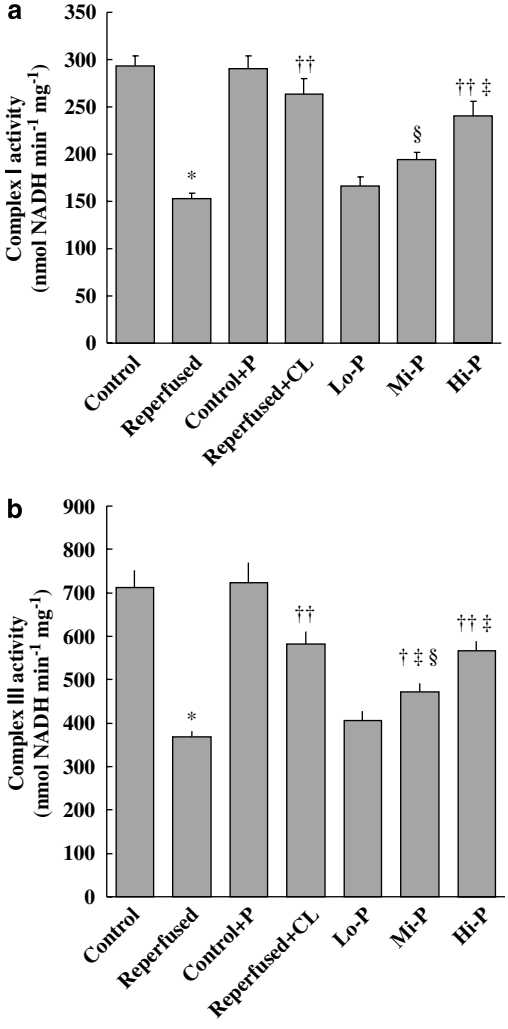

Figure 4 Complex I and complex III activities in mitochondria isolated from control, reperfused, control + P, reperfused + CL, Lo-P, Mi-P and Hi-P groups. The activities of complex I (**a**) and complex III (**b**) were measured as described under Materials and methods. Each group includes eight rats. Data were expressed as mean ± s.e. *$P<0.05$ vs control; $^{\dagger}P<0.05$ vs reperfused; $^{\dagger\dagger}P<0.01$ vs reperfused; $^{\ddagger}P<0.05$ vs Lo-P; $^{\S}P<0.05$ vs Hi-P. P, propofol.

exhibited a remarkable decrease in the activity of the complex I and complex III activity, compared with the control. Propofol treatment dose dependently attenuated the decline in the amount of both these enzyme complexes. Cardiolipin treatment can completely reverse the decrease in the activity of the two enzyme complexes (Figure 4). To prevent the possibility of a morphological gradient of mitochondria being produced artificially in unfixed pellets, we fixed all isolated mitochondrial preparations in suspension. Thus, any given view of the resultant pellets is truly representative of the pellet as a whole. Using this approach, which is sensitive to relatively minor changes in mitochondria, mitochondria from reperfused group showed decreases of integrity compared with control, whereas integrity of mitochondria from Hi-P-treated reperfused heart appears to be, at least in part, restored (Figure 5).

As shown in Figure 6, ischaemia/reperfusion resulted in compromised cardiac function. Treatment of hearts with propofol significantly improved functional recovery of the heart during reperfusion, as shown by the less increased left ventricle end-diastolic pressure and better left ventricle developed pressure, in a concentration-dependent manner.

To test if propofol directly affected cardiac mitochondrial function, we performed *in vitro* experiments for the effects of propofol at concentrations of 5, 10 and 20 μM on isolated mitochondria, respectively. Cardiolipin liposome was also used as positive control. When exposed to free-radical-generating system tert-butylhydroperoxide (t-BuOOH)/Cu^{2+}, mitochondria isolated from rat heart undergo lipid peroxidation (Paradies *et al*., 1998). As shown in Figure 7, the activity of complex I and complex III decreased in t-BuOOH-treated mitochondria, whereas addition of propofol dose dependently prevented this change, and propofol at 20 μM and exogenous cardiolipin totally attenuated the decrease. Similarly, treatment of heart mitochondria with t-BuOOH resulted in marked reduction in the content of cardiolipin, which was completely prevented by propofol in a concentration-dependent manner, and propofol at more than 20 μM, and exogenous cardiolipin, completely prevented the loss (Figure 8).

Discussion and conclusions

In present study, we demonstrated that propofol exerts dose-dependent cardiac protection by decreasing the generation of ROS and preventing cardiolipin peroxidation during

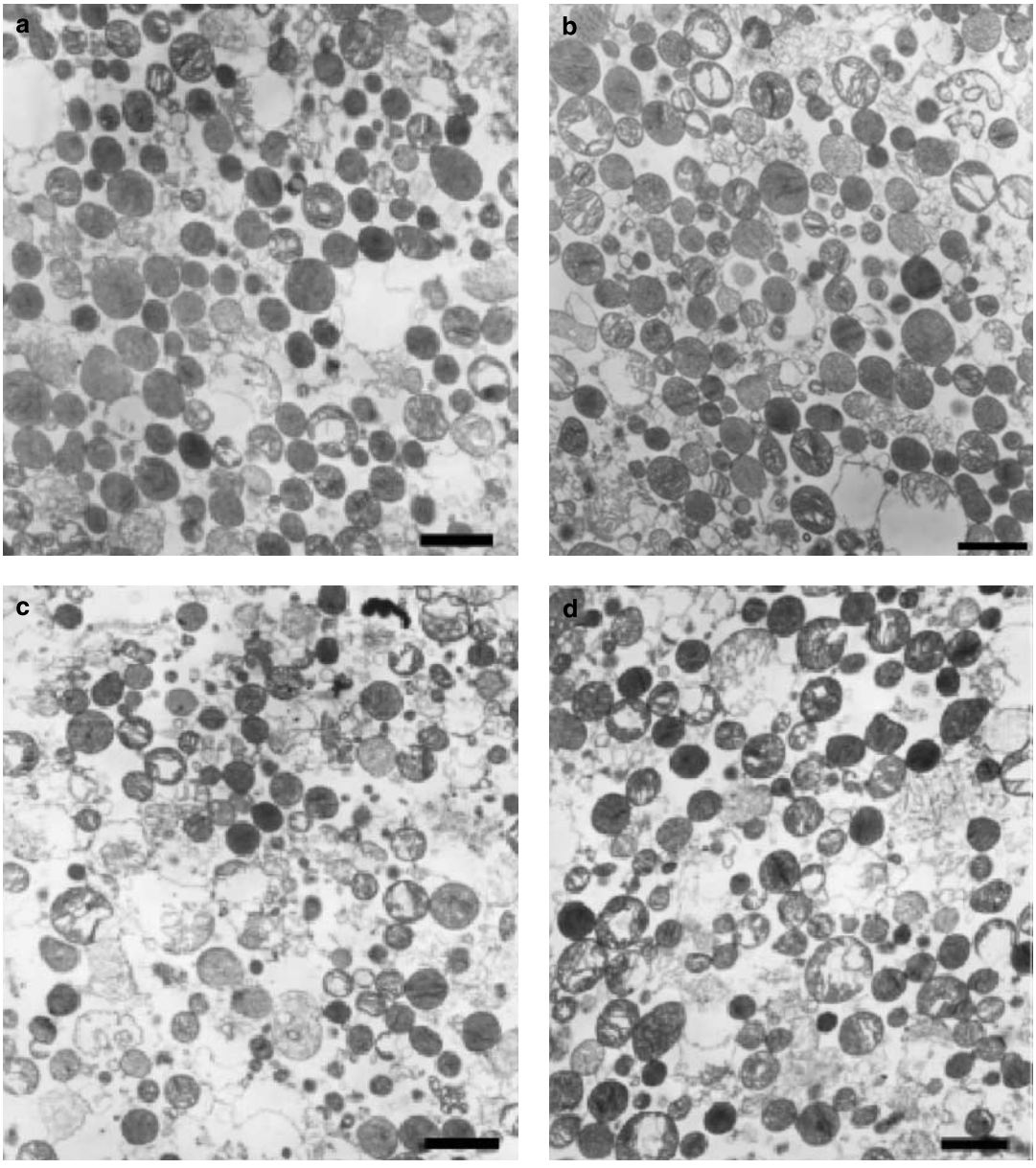

Figure 5 Morphology of mitochondria isolated from control (**a**), control + P (**b**), reperfused (**c**) and Hi-P (**d**) groups. Electron micrographs of all preparations of mitochondria were obtained as described under Materials and methods. Each group includes eight rats. Magnification, × 5000. Bars represent 2 μm. P, propofol.

global myocardial ischaemia/reperfusion. With the correlation of ROS production, the content of cardiolipin and the activities of respiratory chain complexes (Petrosillo *et al*., 2003; Paradies *et al*., 2004), our study also demonstrated that propofol can limit the source of ROS and reduce its generation by restoring the activity of complex I and complex III, whose optimal activity requires a cardiolipin-rich environment in the inner mitochondrial membrane. In brief, propofol can exert its action at mitochondria level through interrupting the vicious circle of ROS–cardiolipin–complexes of the respiratory chain–ROS.

Reactive oxygen species generated from mitochondria contribute to ischaemia/reperfusion injury (Hess and Manson, 1984; Zweier *et al*., 1987; Ambrosio *et al*., 1991). Decreasing ROS production or increasing ROS dissipation will protect mitochondria and decrease cardiac injury during ischaemia and reperfusion. Antioxidants work effectively when they are administrated before and immediately after reperfusion, as previous studies have shown that production of ROS mainly occurs during ischaemia and during the first minutes after cardiac reperfusion (Zweier *et al*., 1987; Ambrosio *et al*., 1991; Lesnefsky *et al*., 2004b). Thus, new antioxidants or free-radical scavengers of high potency, low toxicity and easy permeability to cellular and subcellular compartments need to be identified. Propofol has significant protective activity against cardiac damage and altered physiology, which occur during ischaemia–reperfusion injury (Xia *et al*., 1996; Tsao *et al*., 2003), and it appears to fulfill most of the criteria above. Our results show that propofol given before ischaemia and during reperfusion at

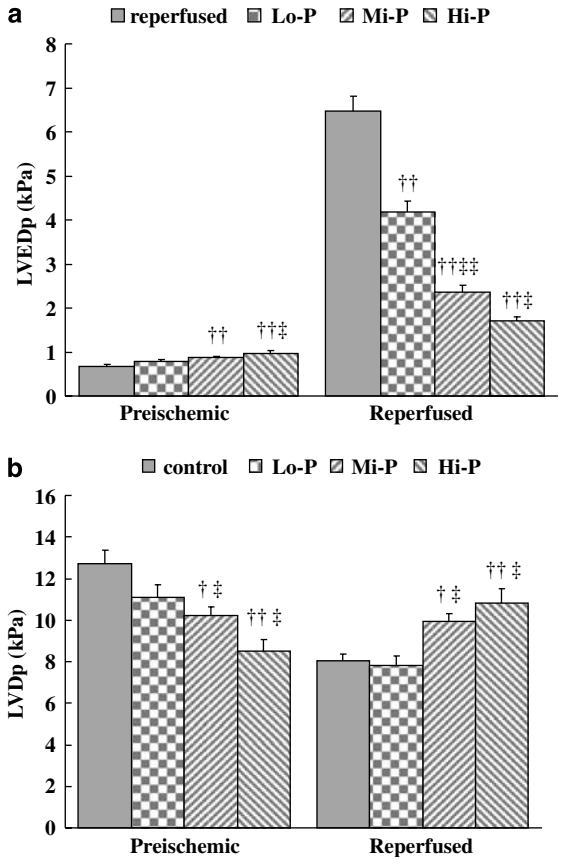

Figure 6 Changes in left ventricle end-diastolic pressure (**a**) and left ventricle developed pressure (left ventricle developed pressure = systolic−end-diastolic pressure) (**b**) in reperfused, Lo-P, Mi-P and Hi-P groups before ischaemia and at the end of reperfusion. Each group includes eight rats. Data were expressed as mean ± s.e. $^{\dagger}P<0.05$ vs reperfused; $^{\dagger\dagger}P<0.01$ vs reperfused; $^{\ddagger}P<0.05$ vs Lo-P; $^{\S}P<0.05$ vs Hi-P. P, propofol.

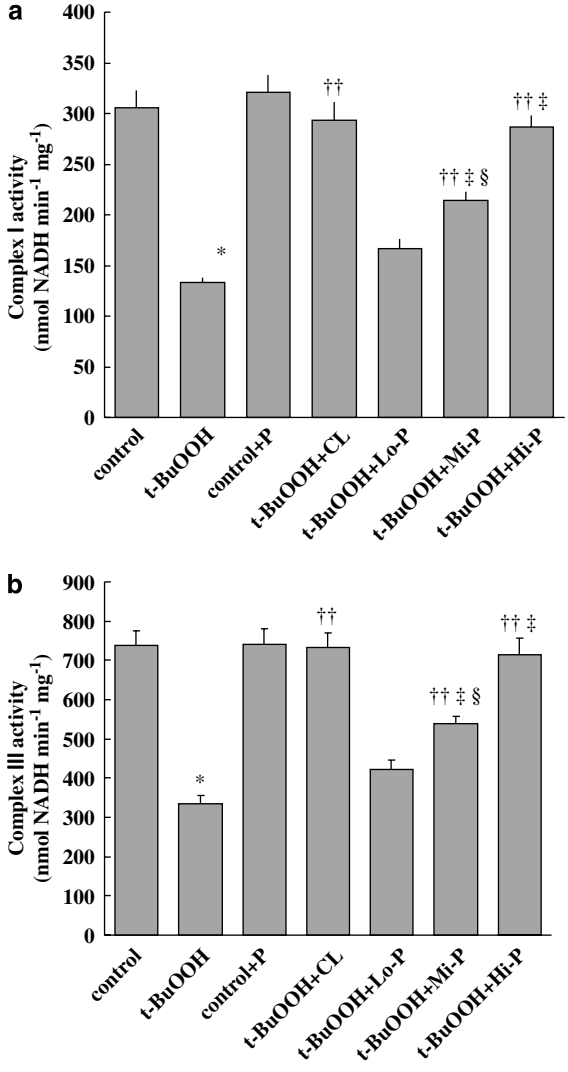

Figure 7 Lipid peroxidation-induced loss in complex I and complex III activity in mitochondria isolated form rat heart and prevention by cardiolipin liposome, propofol at 5, 10 and 20 μM (mLo-P, mMi-P and mHi-P). Lipid peroxidation in isolated mitochondria was induced by addition of 5 μM $CuCl_2$ and 100 μM t-BuOOH to the standard reaction medium at 310.15 K (37 °C). After 30 min. of incubation, the reaction was stopped by addition of 1 mM ethylenediamine tetraacetic acid. Where present, cardiolipin liposome or propofol was added at the beginning of incubation. Complex I (**a**) and complex III (**b**) activities were measured as described under Materials and methods. Each group includes eight rats. Data were expressed as mean ± s.e. $^{*}P<0.05$ vs control; $^{\dagger\dagger}P<0.01$ vs t-BuOOH; $^{\ddagger}P<0.05$ vs mLo-P; $^{\S}P<0.05$ vs mHi-P. P, propofol; t-BuOOH, tert-butylhydroperoxide.

50 or 100 μM attenuates ischaemia/reperfusion damage at the mitochondrial level, as also suggested by the protective effect of propofol against free-radical-induced peroxidative damage on isolated mitochondria (Figures 7 and 8). Propofol treatment leads to decreased ROS generation and less cardiolipin peroxidation, supporting the fact that propofol provided cardioprotection by manipulating mitochondrial function during ischaemia and reperfusion. The protection of mitochondrial function by propofol is also showed by the preserved rates of state 3, state 4 and the respiratory control ratio in mitochondria following ischaemia and reperfusion. The protection afforded by propofol treatment on all these mitochondrial bioenergetics parameters was associated with the protection observed in mitochondrial morphology and heart haemodynamics.

Peroxidation of lipid, which contains double bonded polyunsaturated fatty acids, is considered a major mechanism of oxygen free-radical attack and an indicator for oxidative stress and ROS production. Our results demonstrated that propofol treatment of reperfused rat heart results in a lower degree of mitochondrial lipid peroxidation, as shown in Figure 1.

Alterations of state 3 and 4 respiration determine changes in the respiratory control ratio, which is diagnostic of extensive mitochondrial damage. As reported in Table 1, ischaemia/reperfusion decreased state 3 respiration rate more significantly than that of state 4, thus leading to a decrease of respiratory control ratio in mitochondria. All alterations of the three parameters might be attenuated by propofol treatment, indicating that propofol had a protective effect on the integrity and function of the mitochondria.

Superoxide is formed at complex I and complex III at the mitochondrial electron transport chain (Boveris *et al*., 1976;

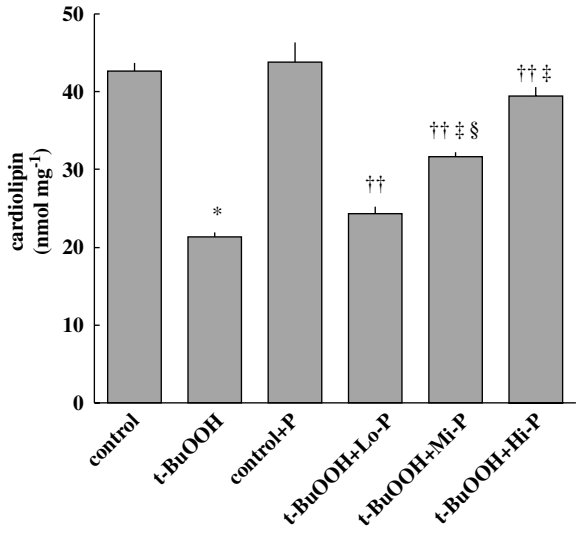

Figure 8 Lipid peroxidation-induced loss in cardiolipin content in rat heart mitochondria and prevention by propofol. Lipid peroxidation in isolated mitochondria was induced as described in the legend to Figure 7. Where present, propofol was added at the beginning of the incubation. Mitochondrial cardiolipin content was determined by HPLC technique. Each group includes eight rats. Data were expressed as mean ± s.e. *$P<0.05$ vs control; $^{\dagger\dagger}P<0.01$ vs t-BuOOH; $^{\ddagger}P<0.05$ vs mLo-P; $^{\S}P<0.05$ vs mHi-P. HPLC, high-pressure liquid chromatography; P, propofol; t-BuOOH, tert-butylhydroperoxide.

Paradies *et al*., 2001, 2002). The decreased activities of complex I and complex III increase ROS generation. Ischaemia–reperfusion decreased complex I and complex III activities (Figure 4), accompanied by enhanced H_2O_2 production (Figure 2), supporting this notion. Pyruvate can directly interact with H_2O_2 and this fact might explain the lower amount of H_2O_2 detected in the presence of pyruvate + malate with respect to succinate.

Cardiolipin participated in higher order organization of complex I and complex III of the respiratory chain in a supercomplex in the inner mitochondrial membrane (Zhang *et al*., 2002). Thus, changes of cardiolipin content, as consequence of oxidative damage by ROS attack and cytochrome *c*/cardiolipin peroxidase, as shown by changes in the level of conjugated dienes sensitively detected by HPLC, may affect the activity of complex I and complex III. The preserved cardiolipin content by propofol results in protected complex I and complex III activities, less production of oxygen radicals and oxidative damage, as well as improvement of the mitochondria (Figure 5) and heart mechanical function (Figure 6). These results suggest that propofol exerts cardiac protection by protection of mitochondria during ischaemia and reperfusion.

To explore the potential mechanism of the effect of propofol on ROS and cardiolipin, we demonstrated the effect of propofol under control conditions and the effect of cardiolipin on reperfused heart. Propofol had no remarkable action under control conditions, except for its negative effect on myocardia contractile function (data not shown). Enrichment of cardiolipin on mitochondria isolated from reperfused rat heart decreased H_2O_2 production, and increased complex I and complex III activity, with the same efficiency as antioxidant treatment with 100 μM propofol. Cardiolipin is oxidized immediately in the air, and so is very sensitive to ROS. When cardiolipin liposome was fused with mitochondria, it partly neutralized H_2O_2 production. On the basis of the above results, we concluded that propofol, chemically similar to phenol-based free-radical scavengers, may be a chemical uncoupler and thus an ROS scavenger.

Propofol is lipophilic, allowing it to target and accumulate in the inner mitochondrial membrane, thereby enabling scavenging of ROS generated during respiration at the site of production. There is evidence demonstrating that nitric oxide can act as a regulator of peroxidase activity of the cytochrome *c*/cardiolipin complex (Vlasova *et al*., 2006). Thus, the possibility that propofol may directly interact with cytochrome *c* or the cytochrome *c*/cardiolipin complex and inhibit their peroxidase, in addition to preventing cardiolipin peroxidation as an ROS scavenger, cannot be excluded.

It should be noted that antioxidant vitamins (vitamins E and C) were not able to prevent ROS production from other sources in whole blood, despite their ability to reduce ROS release from polymorphonuclear cells. Vitamins E and C impaired vascular function in the experimental diabetic rat (Palmer *et al*., 1998). Melatonin, a hormonal product of the pineal gland, showed antioxidant activity at 50 μM, but is expensive and not widely used for treatment. Propofol (49.57 mM) has been shown to effectively protect against lipid peroxidation in mouse brain homogenates, similar to trolox (60.35 mM) and melatonin (22.02 mM) (Lee *et al*., 2005). Thus, propofol, at adequate concentration, effectively preserved mitochondria antioxidant capacity and myocardial function. Propofol decreases blood pressure obviously, but it could be widely used at adequate concentration (11.2–28.1 μM in patient blood) in clinical application, without irreversible damage to heart, if the volume of blood is enough.

In conclusion, propofol dose dependently enhances mitochondrial antioxidant capability, and this leads to less cardiac injury during ischaemia and reperfusion. The mechanism of protection involves the antioxidant role of propofol to decrease ROS generation and cardiolipin peroxidation as a chemical uncoupler. Propofol given at appropriate concentration (11.2–28.1 μM in patient blood) could potentially reduce myocardial ischaemia/reperfusion injury during cardiac surgery in high-risk patients, such as those with diabetes, and provide a useful strategy for the wide range of diseases with mitochondrial oxidative damage in their aetiology in the future.

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Conflict of interest

The authors state no conflict of interest.

References

Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP *et al.* (1993). Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J Biol Chem* **268**: 18532–18541.

Ambrosio G, Zweier JL, Flaherty JT (1991). The relationship between oxygen radical generation and impairment of myocardial energy metabolism following post-ischemic reperfusion. *J Mol Cell Cardiol* **23**: 1359–1374.

Black MJ, Brandt RB (1974). Spectrofluorometric analysis of hydrogen peroxide. *Anal Biochem* **58**: 246–254.

Boveris A, Cadenas E, Stoppani AO (1976). Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. *Biochem J* **156**: 435–444.

Buege JA, Aust SD (1978). Microsomal lipid peroxidation. *Methods Enzymol* **52**: 302–310.

Chambers DE, Parks DA, Patterson G, Roy R, McCord JM, Yoshida S *et al.* (1985). Xanthine oxidase as a source of free radical damage in myocardial ischemia. *J Mol Cell Cardiol* **17**: 145–152.

Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ (2003). Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem* **278**: 36027–36031.

Das DK (1994). Cellular, biochemical, and molecular aspects of reperfusion injury. *Ann N Y Acad Sci* **723**: 116–127.

Folch J, Lees M, Sloane GH (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**: 497–509.

Gorbenko GP, Molotkovsky JG, Kinnunen PK (2006). Cytochrome *c* interaction with cardiolipin/phosphatidylcholine model membranes: effect of cardiolipin protonation. *Biophys J* **90**: 4093–4103.

Hess ML, Manson NH (1984). Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. *J Mol Cell Cardiol* **16**: 969–985.

Kagan VE, Tyurin VA, Jiang J, Tyurina YY, Ritov VB, Amoscato AA *et al.* (2005). Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol* **1**: 223–232.

Kagan VE, Tyurina YY, Bayir H, Chu CT, Kapralov AA, Vlasova II *et al.* (2006). The 'pro-apoptotic genies' get out of mitochondria: oxidative lipidomics and redox activity of cytochrome *c*/cardiolipin complexes. *Chem Biol Interact* **163**: 15–28.

Lee H, Jang YH, Lee SR (2005). Protective effect of propofol against kainic acid-induced lipid peroxidation in mouse brain homogenates: comparison with trolox and melatonin. *J Neurosurg Anesthesiol* **17**: 144–148.

Lesnefsky EJ, Chen Q, Moghaddas S, Hassan MO, Tandler B, Hoppel CL (2004a). Blockade of electron transport during ischemia protects cardiac mitochondria. *J Biol Chem* **279**: 47961–47967.

Lesnefsky EJ, Chen Q, Slabe TJ, Stoll MS, Minkler PE, Hassan MO *et al.* (2004b). Ischemia, rather than reperfusion, inhibits respiration through cytochrome oxidase in the isolated, perfused rabbit heart: role of cardiolipin. *Am J Physiol Heart Circ Physiol* **287**: H258–H267.

Lesnefsky EJ, Tandler B, Ye J, Slabe TJ, Turkaly J, Hoppel CL (1997). Myocardial ischemia decreases oxidative phosphorylation through cytochrome oxidase in subsarcolemmal mitochondria. *Am J Physiol* **273**: H1544–H1554.

Palmer AM, Thomas CR, Gopaul N, Dhir S, Anggard EE, Poston L *et al.* (1998). Dietary antioxidant supplementation reduces lipid peroxidation but impairs vascular function in small mesenteric arteries of the streptozotocin-diabetic rat. *Diabetologia* **41**: 148–156.

Palmer JW, Tandler B, Hoppel CL (1977). Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. *J Biol Chem* **252**: 8731–8739.

Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Federici A, Ruggiero FM (2004). Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin. *Circ Res* **94**: 53–59.

Paradies G, Petrosillo G, Pistolese M, Ruggiero FM (2001). Reactive oxygen species generated by the mitochondrial respiratory chain affect the complex III activity via cardiolipin peroxidation in beef-heart submitochondrial particles. *Mitochondrion* **1**: 151–159.

Paradies G, Petrosillo G, Pistolese M, Ruggiero FM (2002). Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage. *Gene* **286**: 135–141.

Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E (1998). Peroxidative damage to cardiac mitochondria: cytochrome oxidase and cardiolipin alterations. *FEBS Lett* **424**: 155–158.

Petrosillo G, Ruggiero FM, Di Venosa N, Paradies G (2003). Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin. *FASEB J* **17**: 714–716.

Ruggiero FM, Landriscina C, Gnoni GV, Quagliariello E (1984). Lipid composition of liver mitochondria and microsomes in hyperthyroid rats. *Lipids* **19**: 171–178.

Shandelya SM, Kuppusamy P, Weisfeldt ML, Zweier JL (1993). Evaluation of the role of polymorphonuclear leukocytes on contractile function in myocardial reperfusion injury. Evidence for plasma-mediated leukocyte activation. *Circulation* **87**: 536–546.

Tsao CM, Ho ST, Chen A, Wang JJ, Tsai SK, Wu CC (2003). Propofol ameliorates liver dysfunction and inhibits aortic superoxide level in conscious rats with endotoxic shock. *Eur J Pharmacol* **477**: 183–193.

Tyurina YY, Kini V, Tyurin VA, Vlasova II, Jiang J, Kapralov AA *et al.* (2006). Mechanisms of cardiolipin oxidation by cytochrome *c*: relevance to pro- and antiapoptotic functions of etoposide. *Mol Pharmacol* **70**: 706–717.

Unsal A, Devrim E, Guven C, Eroglu M, Durak I, Bozoklu A *et al.* (2004). Propofol attenuates reperfusion injury after testicular torsion and detorsion. *World J Urol* **22**: 461–465.

Vanden Hoek TL, Shao Z, Li C, Zak R, Schumacker PT, Becker LB (1996). Reperfusion injury on cardiac myocytes after simulated ischemia. *Am J Physiol* **270**: H1334–H1341.

Vlasova II, Tyurin VA, Kapralov AA, Kurnikov IV, Osipov AN, Potapovich MV *et al.* (2006). Nitric oxide inhibits peroxidase activity of cytochrome *c*/cardiolipin complex and blocks cardiolipin oxidation. *J Biol Chem* **281**: 14554–14562.

Xia Y, Khatchikian G, Zweier JL (1996). Adenosine deaminase inhibition prevents free radical mediated injury in the post-ischemic heart. *J Biol Chem* **271**: 10096–10102.

Yumoto M, Nishida O, Nakamura F, Katsuya H (2005). Propofol attenuates oxidant-induced acute lung injury in an isolated perfused rabbit-lung model. *J Anesth* **19**: 287–294.

Zhang M, Mileykovskaya E, Dowhan W (2002). Gluing the respiratory chain together. *J Biol Chem* **277**: 43553–43556.

Zweier JL, Flaherty JT, Weisfeldt ML (1987). Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc Natl Acad Sci USA* **84**: 1404–1407.