Short-term impairment of energy production in isolated rat liver mitochondria by hypoxia/reoxygenation: involvement of oxidative protein modification

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The aim of the present study was to elucidate the role of mitochondria in liver impairment after ischaemia/reperfusion. It is commonly assumed that mitochondria are in part responsible for tissue damage by impaired oxidative phosphorylation as a consequence of the attack of radicals generated within the mitochondria. The principal support for this hypothesis was found by exposing isolated mitochondria to temporary hypoxia in combination with alterations of substrate supply. Rat liver mitochondria treated in this way responded with impaired ADP-stimulated respiration after reoxygenation, which decreased with time of hypoxia and reoxygenation. The decline of the activity of the NADH-cytochrome *c*-oxidoreductase complex found under these conditions is likely to cause the drop in active respiration. No changes in the content of respiratory chain complexes,

determined by Blue Native PAGE, could be demonstrated. However, oxidative modifications of mitochondrial proteins, indicated by carbonyl formation, were found. Likewise, products of lipid peroxidation, such as lipid peroxides and malondialdehyde, were formed. Mitochondria were still able to build up a transmembrane potential and did not show drastic changes in membrane conductivity after hypoxia/reoxygenation stress. The presence of water-soluble antioxidants exhibited a beneficial effect, diminishing the decline of active respiration after 5 min of hypoxia and 10 min of reoxygenation. These observations strongly suggest that mitochondria play a pathogenic role in ischaemia/reperfusion injury, which is at least in part mediated by an oxygen-derived free-radical-linked mechanism.

INTRODUCTION

There is a body of evidence that an excess of free radicals derived from oxygen are formed during ischaemia/reperfusion of various tissues, which causes the impairment of cellular components [1–6]. Furthermore, the ability of isolated hepatocytes to produce large quantities of oxygen free radicals during post-anoxic reoxygenation has recently been demonstrated [7,8]. Besides other cellular sources, mitochondria generate superoxide within the respiratory chain [9–14]. Specifically, in the coenzyme Q cycle, ubiquinone is oxidized to quinone with concomitant production of superoxide. A second mitochondrial source of superoxide formation is NADH dehydrogenase [15]. Recently, mitochondria during post-anoxic reoxygenation were found to be the main generators of superoxide in hepatocytes [16]. Investigations with isolated heart mitochondria, exposed to metabolic conditions which mimic the situation of ischaemia/reperfusion, provide evidence for superoxide production at complex 3 of the respiratory chain [17].

The aim of the present investigation was to study the effect of reactive oxygen species (ROS), generated within the mitochondria by hypoxia/reoxygenation, on mitochondrial integrity and function. Therefore we exposed isolated rat liver mitochondria, incubated in a calcium-free medium, to hypoxia/reoxygenation, and determined malondialdehyde and lipid peroxides as indicators of lipid peroxidation, the protein-bound carbonyls representing protein modifications, the activity of the NADH-cytochrome *c*-oxidoreductase complex, respiration and membrane potential.

MATERIALS AND METHODS

Chemicals

Rabbit IgG directed against the 2,4-dinitrophenyl moiety was obtained from Sigma (St. Louis, MO, U.S.A.). Sheep anti-rabbit IgG conjugated with peroxidase was from Boehringer Mannheim (Germany). The protein assay reagent was from Bio-Rad Laboratories GmbH, Munich, Germany. All other reagents were of the purest grade available.

Preparation of mitochondria

Liver mitochondria were prepared from the livers of 220–240 g male Wistar rats in ice-cold medium containing 250 mM sucrose, 20 mM Tris/HCl (pH 7.4), 2 mM EGTA and 1% (w/v) BSA using a standard procedure [18]. After the initial isolation, Percoll was used for purification of mitochondria from a fraction containing some endoplasmatic reticulum, Golgi apparatus and plasma membranes [19]. The mitochondria were well coupled, as indicated by a respiratory control index greater than 5 with glutamate plus malate as substrates.

Incubation of mitochondria

Mitochondria (1–2 mg of protein/ml) were incubated in a medium containing 10 mM sucrose, 120 mM KCl, 20 mM Tris, 15 mM NaCl, 5 mM potassium phosphate, 0.5 mM EGTA and 1 mM free Mg²⁺ at pH 7.4.

Hypoxia was produced by bubbling 2 ml of the incubation medium with N₂ until oxygen was not detectable by means of a

Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline-n-oxide; ROS, reactive oxygen species; TCA, trichloroacetic acid; TPP⁺, tetraphenyl-phosphonium ion.

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Clark electrode. Afterwards, the mitochondria added to this medium further decreased the oxygen concentration via the respiratory chain. The final oxygen concentration was less than 2 nmol/ml. As the K_m value of the mitochondrial cytochrome oxidase is 0.3 μM , the limiting effect of oxygen concentration is independently illustrated by the collapse of the membrane potential measured by means of a tetraphenylphosphonium ion (TPP^+)-sensitive electrode in separate experiments (results not shown). A 2 ml volume of the air-saturated incubation medium was added to achieve reoxygenation.

Determination of mitochondrial respiration

The oxygen consumption of mitochondria was measured with a Clark-type electrode. The oxygen content of the air-saturated medium was taken to be 435 ng-atoms/ml at 30 °C.

Determination of the membrane potential

The membrane potential was determined from the distribution of TPP^+ by means of a TPP^+ -sensitive electrode. In accordance with [20], the binding of TPP^+ to lipophilic components was taken into account. The matrix volume was assumed to be 1 μl /mg of mitochondrial protein.

Activity of the NADH-cytochrome *c*-oxidoreductase complex

Aliquots of 500 μl of the mitochondrial suspension (1–2 mg/ml) were withdrawn from the incubations. The mitochondria were disintegrated by sonication. A 15 μl volume was used to run the assay at 30 °C as described in [21]. The reduction of cytochrome *c* was followed by measurement of the absorption at 550 nm with a Varian spectrophotometer (Cary 1E). The rotenone-sensitive absorption was used for quantification.

Electrophoretic separation of the respiratory chain complexes using 'Blue Native PAGE'

The procedure described by Schaeffer and von Jagow [22] and Reinheckel et al. [23] was used to separate the respiratory chain complexes. Aliquots of 500 μl of mitochondrial suspensions from the incubation experiments were immediately centrifuged at 20000 *g* at 4 °C for 10 min. Pellets were dissolved by the addition of 250 μl of detergent containing 2.5 % n-dodecyl- β -D-maltoside/0.75 M ϵ -amino-n-capronic acids, 50 mM Bistris and 2 mM PMSF at pH 7.0. After centrifugation at 100000 *g* for 30 min, supernatants were supplemented with 20 μl of 5 % Coomassie Blue G 250 and 75 μl were applied to a 6–13 % *T*, 3 % *C* gradient gel, in which *T* represents the total concentration of monomer (acrylamide + bisacrylamide) and *C* is the percentage (by weight) of total monomer *T* which is due to the cross-linking agent (bisacrylamide).

Spectrometric quantification of protein-bound carbonyls

For assessment of carbonyls the reaction with dinitrophenylhydrazine was employed [24]. For each determination, three trichloroacetic acid (TCA)-precipitated samples containing 1 mg of mitochondrial protein were redissolved in 300 μl of 0.1 M NaOH for 5 min. Two of the samples were treated with 3 ml of 10 mM dinitrophenylhydrazine in 2.5 M HCl for 1 h at room temperature. The third tube, used as the blank, was incubated with 2.5 M HCl. The reaction was stopped by addition of 3.3 ml of 20 % TCA. The pellets were washed twice with 3 ml of absolute ethanol/ethylacetate (1:1) and once using 3 ml of 10 % TCA. The protein pellets were finally dissolved in 6 M guanidine hydrochloride and the absorption at 370 nm (dinitrophenylhydrazine

minus sample blank) was determined. Protein recovery was estimated for each sample. Carbonyl content was calculated using the molar absorption coefficient of aliphatic hydrazones of 22000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ and expressed as nmol carbonyl/mg of protein.

Immunoblotting for detection of protein-bound dinitrophenylhydrazones

Protein carbonyls were derived by using dinitrophenylhydrazine and prepared for reductive SDS/electrophoresis as recommended by Levine et al. [25]. The samples were applied to a 10 % *T*, 3 % *C* polyacrylamide gel using the separating system of Schaeffer and von Jagow [26]. After semi-dry blotting [27], immunostaining was performed with a rabbit IgG directed against the 2,4-dinitrophenyl moiety at a dilution of 1:1500. Secondary antibody was a sheep anti-rabbit IgG conjugated with peroxidase at a 1:1000 dilution.

HPLC analysis of uric acid

Reverse-phase chromatography, as described in [28], was applied. For the determination, 300 μl aliquots of mitochondrial suspension were added to ice-cold perchloric acid (final concentration 1.04 M) and centrifuged at 20000 *g* for 1 min.

The supernatant was neutralized by KOH/Hepes (2 M/0.3 M) and subjected to HPLC analysis with an L-6200 pump and an L-4250 UV/VIS detector (Merck-Hitachi, Darmstadt, Germany) at 254 nm using a 250 $\times$ 4 mm RP18 column packed with 5 μm Si particles.

Uric acid was eluted with 0.2 M KH_2PO_4 , pH 5.95, by a step gradient of methanol: 5 min, 0 % CH_3OH ; 6 min, 4 % CH_3OH ; 5 min, 12 % CH_3OH ; and 1 min, 40 % CH_3OH , at a flow rate of 1.3 ml/min.

Determination of the malondialdehyde-thiobarbituric acid complex

HPLC analysis as described previously [29] was applied. Aliquots of 500 μl of mitochondrial suspension were added to 1 ml of a mixture containing 5 % TCA and 1 M acetic acid to precipitate protein, then 0.5 ml of H_2O and 1 ml 0.71 % thiobarbituric acid were added. The reaction was supported by 20 min of boiling. After centrifugation at 10000 *g* the supernatant was subjected to HPLC analysis with a L6200 pump and an L-4250 UV/VIS detector (Merck-Hitachi) at 535 nm using an RP18 column packed with 5 μm Si particles. The thiobarbituric acid-malondialdehyde complex was isocratically eluted with 50 mM KH_2PO_4 , pH 7.0/35 % CH_3OH , at a flow rate of 0.65 ml/min. Tetramethoxypropane was used as the standard.

Determination of lipid peroxides

Lipid peroxides were determined by using the assay kit produced by the Kamiya Biomedical Company (Seattle, WA, U.S.A.). In the presence of haemoglobin the lipid hydroperoxides formed were reduced to hydroxyl derivatives (lipid alcohols), whereby a special chromogen (10-*N*-methylcarbamoyl-3,7-dimethylamino-OH-phenothiazine) is oxidatively cleaved to Methylene Blue, which is measured spectrophotometrically at 675 nm. Before running the assay, the lipids were extracted as described [30].

Determination of protein

Protein content was measured according to the method of Bradford [31] using BSA as a standard.

RESULTS

Respiration after hypoxia/reoxygenation

When freshly isolated rat liver mitochondria were exposed for different times to hypoxia followed by 1 min of reoxygenation, the active respiration was diminished (Figure 1). The degree of the decline increased with the duration of hypoxia up to 15 min. When additional hypoxia was combined with a deficiency of available substrates the respiration declined to a lower value. Furthermore, we observed a clearly higher sensitivity of NADH-linked respiration to hypoxia/reoxygenation stress in comparison with FADH-linked respiration.

Figure 2 demonstrates that the impairment of oxidative phosphorylation proceeded with time of reoxygenation after 5 min of hypoxia when substrates were not available during

Figure 1 Dependence of respiration in rat liver on hypoxia time

Rat liver mitochondria (1–2 mg/ml) were incubated in N_2 -saturated incubation medium either with (+) or without (–) substrates. At the times indicated mitochondria were reoxygenated by addition of air-saturated incubation medium. After 1 min active respiration was stimulated by 200 μ M ADP. Substrates were added simultaneously with ADP when hypoxia was induced in the absence of substrates. The concentrations of the substrates were: 5 mM glutamate (glut) plus 5 mM malate (mal), 10 mM succinate (succ) plus 10 μ M rotenone (rot). All values are means $\pm$ SD from five independent experiments on different preparations of mitochondria.

Figure 2 Time-course of mitochondrial respiration upon reoxygenation after 5 min of hypoxia

Mitochondria were exposed to 5 min of hypoxia in the absence of substrates. The times indicated represent the times of reoxygenation at which 5 mM glutamate plus 5 mM malate or 10 mM succinate plus 10 μ M rotenone were added simultaneously with 200 μ M ADP to stimulate active respiration. All values are means $\pm$ SD from six independent experiments on different preparations of mitochondria.

Figure 3 'Blue Native PAGE' patterns of respiratory chain complexes during hypoxia/reoxygenation of rat liver mitochondria

A total of 130 μ g of mitochondrial protein was applied to each lane. Complexes were identified according to their subunit patterns in two-dimensional SDS/PAGE, exactly as shown for rat liver mitochondria previously [23]. Lane 1, freshly prepared mitochondria; lane 2, 15 min control incubation (30 $^{\circ}$ C); lane 3, 5 min hypoxia + 10 min reoxygenation (30 $^{\circ}$ C). Complex I (I); complex III (III); complex IV (IV); ATP-synthetase (V); unidentified peak (X).

hypoxia and reoxygenation. Active respiration with glutamate/malate as substrates dramatically declined to about $32.5 \pm 6\%$ of the initial value after 15 min of reoxygenation. In contrast, FADH-dependent respiration was less affected ($72.88 \pm 5.41\%$ of the initial value).

Respiratory chain complexes after hypoxia/reoxygenation

Since the impaired respiration with glutamate/malate and succinate/rotenone after hypoxia/reoxygenation may be caused by functionally altered complexes of the respiratory chain, the activity of the NADH-cytochrome *c*-oxidoreductase complex was determined after 5 min of hypoxia and 10 min of reoxygenation for comparison with a normoxic control incubation of 15 min in the presence of glutamate plus malate. Complexes I and III of the respiratory chain are very likely to be favoured targets of an oxidative attack because they are known as generators of oxygen-derived free radicals [9–15]. The activity of the NADPH-cytochrome *c*-oxidoreductase decreased drastically from 84 ± 7 to 30.2 ± 8.7 nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ after 5 min of hypoxia and 10 min of reoxygenation in the absence of substrates, whereas a normoxic control incubation of 15 min with glutamate and malate exhibited a slightly diminished activity of 71.8 ± 3.8 nmol mg $^{-1}$ min $^{-1}$ (results are means $\pm$ SD from five different preparations of mitochondria). Surprisingly, the content of respiratory chain complexes determined by 'Blue Native PAGE' remained unchanged under these conditions (Figure 3).

Oxidative protein modification

Apparently, mitochondrial proteins underwent modifications by hypoxia/reoxygenation which cannot be detected by means of electrophoresis, but are reflected in changes of their function. To confirm the postulated oxidative modification of proteins during

Figure 4 Formation of protein-derived carbonyls during hypoxia/reoxygenation

Carbonyls were derived with dinitrophenylhydrazine, washed and quantified spectrometrically at 370 nm as described in the Materials and methods section. The absorption ratio 260 nm/276 nm was less than 0.75 in all samples, indicating that nucleic acids are not likely to interfere with the assay under the conditions used. The control incubation was performed under normoxic conditions in the presence of 5 mM glutamate plus 5 mM malate with an additional preincubation time of 5 min. Reoxygenation for up to 15 min was performed after 5 min of hypoxia without substrates. The initial content of carbonyls was 3.59 ± 0.27 nmol/ml. All values are means $\pm$ SD from five independent experiments on different preparations of mitochondria.

reoxygenation, we determined the overall formation of protein-bound carbonyls in rat liver mitochondria. In Figure 4 results are presented which document the massive carbonyl production after 5 min of hypoxia and different times of reoxygenation. It should be noted that after 20 min the carbonyl content of the control incubation was also elevated. We therefore chose 5 min hypoxia and times of reoxygenation up to 10 min as relevant incubation conditions for further investigations.

The degree of carbonyl formation was different for individual proteins. In particular, small proteins with low molecular mass seem to be more susceptible to oxidative attack and show higher increases in the amount of carbonyls as compared with high-molecular-mass proteins (Figure 5).

Effect of antioxidants on active respiration after hypoxia/reoxygenation

We provided further evidence for an oxidative-linked mechanism for the impairment of oxidative phosphorylation by hypoxia/reoxygenation by demonstrating beneficial effects on respiration when 5,5-dimethyl-L-pyrroline-n-oxide (DMPO), ascorbic acid and uric acid were present during 5 min of hypoxia and 10 min of reoxygenation in the absence of substrates. The ratios of active respiration in the presence of antioxidants and of a control incubation in which the mitochondria were exposed to 5 min of anoxia and 10 min of reoxygenation alone were 1.11 ± 0.03 with 10 mM DMPO, 1.11 ± 0.03 with 10 mM ascorbic acid and 1.13 ± 0.04 with 3 mM uric acid respectively. Interestingly, the OH-radical scavenger DMSO did not affect the hypoxia/reoxygenation-induced decline of oxidative phosphorylation. The data are means $\pm$ SD from four preparations of mitochondria.

To support the suggestion of the antioxidative effect of uric acid, which may be oxidized by ROS forming the uric acid radical, we followed the dependence of uric acid concentration on reoxygenation time. In fact, we found a stronger consumption of externally added uric acid in comparison with a control incubation within the phase of reoxygenation (Figure 6). The

Figure 5 Western blot showing dinitrophenylhydrazine-derived protein-bound carbonyls

(A) Anti-dinitrophenyl immunostain of 5 min control incubation (lane 1), 5 min hypoxia (lane 2), 15 min control incubation (lane 3) and 5 min hypoxia followed by 10 min reoxygenation (lane 4). The numbers represent the ratios of the peak areas from lanes 3 and 4 $\times 100\%$. Peak areas were determined in reflection mode employing a DESAGA-densitometer. (B) Coomassie Blue G 250 stain of 5 min control incubation (lane 1), 5 min hypoxia (lane 2), 15 min control incubation (lane 3) and 5 min hypoxia followed by 10 min reoxygenation (lane 4). One example of three reproducible experiments from different preparations of mitochondria is shown.

lower concentration of uric acid in the control incubation found after 6 min is probably due to scavenging of ROS, which were usually formed in the presence of oxygen.

Conductivity of the membrane after hypoxia/reoxygenation

Apart from the functional response of rat liver mitochondria to hypoxia/reoxygenation, observed as a decrease in active respiration, we found an increase in the conductivity of the mitochondrial membrane, demonstrated by the slightly increased leak respiration (respiration in the absence of adenine nucleotides), with glutamate plus malate, as well as with succinate plus rotenone, as substrates (Table 1). Since the respiration with both substrate couples was elevated, we rule out a considerable breakage of mitochondria as an explanation for this observation.

Lipid peroxidation

The increased conductivity of the membrane may be caused by modifications of membrane proteins and/or by the modification of membrane lipids. We therefore determined, additionally to the carbonyls as a parameter of protein modification, the con-

Figure 6 Utilization of uric acid during hypoxia/reoxygenation

Mitochondria were exposed to 5 min of hypoxia and different times of reoxygenation in the absence of substrates, or were incubated under normoxic conditions with 5 mM glutamate plus 5 mM malate (control). The times indicated represent the duration of reoxygenation. The corresponding controls were incubated over a period of time which is equal to the sum of the times of hypoxia and reoxygenation. Samples of 1 ml were withdrawn for HPLC analysis as described in the Materials and methods section. The initial concentration of uric acid in these experiments was 850 nmol/ml. All values represent means $\pm$ SD from four independent experiments on different preparations of mitochondria.

Table 1 Leak respiration of rat liver mitochondria after hypoxia/reoxygenation

Mitochondria were incubated as described in Figure 2. At the reoxygenation times indicated 5 mM glutamate and 5 mM malate or 10 mM succinate plus 10 μ M rotenone were added. The initial values of respiration were 17.59 ± 0.35 ng-atoms $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ with glutamate plus malate and 24.27 ± 1.21 ng-atoms $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ with succinate plus rotenone. All values are means $\pm$ SD from five independent experiments on different preparations of mitochondria.

Time of reoxygenation (min)	Leak respiration (% of initial)	
	Glutamate/malate	Succinate/rotenone
1	100 $\pm$ 8	127 $\pm$ 2
5	120 $\pm$ 11	118 $\pm$ 5
10	120 $\pm$ 6	129 $\pm$ 4
15	130 $\pm$ 7	121 $\pm$ 7

centration of lipid peroxides and malondialdehyde as parameters of lipid peroxidation during reoxygenation. After 1 min of reoxygenation following 5 min of hypoxia, the concentrations of lipid peroxides and malondialdehyde remained practically unchanged at the level of untreated mitochondria (2.05 ± 0.41 nmol/mg malondialdehyde; 3.58 ± 1.49 nmol/mg lipid peroxides). However, within the first 5 min of reoxygenation, the relatively small concentration of malondialdehyde slightly increased to 2.73 ± 0.35 nmol/mg, whereas lipid peroxides increased to 15.52 ± 2.31 nmol/mg, indicating strong lipid peroxidation during this phase. The concentration of malondialdehyde was not further changed in up to 15 min of reoxygenation, whereas the concentration of lipid peroxides fell to 5.00 ± 1.97 nmol/mg after 15 min of reoxygenation. One can speculate that lipid peroxides were removed by different mechanisms. These data are means $\pm$ SD from five different preparations of mitochondria. In comparison with iron-ascorbate-induced lipid peroxidation in rat liver mitochondria, resulting in the formation of about 30 nmol/mg thiobarbituric reactive substances [32], the

degree of lipid peroxidation after hypoxia/reoxygenation was rather low (about 3 nmol/mg malondialdehyde).

DISCUSSION

From a large number of investigations addressing ischaemia/reperfusion-induced injury of tissues, it became clear that mitochondria are involved in the mechanism by which the damage occurs. Central to this consideration is the property of mitochondria to be generators of oxygen-derived free radicals such as superoxide. As was found by Caraceni et al. [16], mitochondria are the main sources of superoxide in hepatocytes during reoxygenation. Under conditions of ischaemia, the mitochondria become anoxic, accompanied by a diminished supply of substrates. The aim of the present investigation was to study the effect of these components of ischaemia, in combination with reoxygenation and modification of substrate supply, on the function of isolated mitochondria.

Being aware of the necessity of the balance between production and scavenging of ROS in intact biological systems, we interpret the beneficial effect of substrates against hypoxia/reoxygenation stress as a result of the elevated redox energy over the whole period of incubation, which may be used to reduce oxidized glutathione, thus regenerating this antioxidant within the mitochondria [33].

It is very likely that the observed decline in NADH-cytochrome *c*-oxidoreductase activity is responsible for the diminished active respiration. The cytochromes containing iron are highly susceptible to ROS, which may induce metal-catalysed oxidation within the active centres of respiratory chain complexes. Subsequently, a chain reaction involving protein peroxides may occur within the enzyme complexes, finally leading to their inactivation [34,35]. An alternative explanation for the observed functional impairment would be to consider changes in phospholipid-protein interactions to be essential, as has been shown for cardiolipin and complex III of the respiratory chain [36].

The observation that NADH-dependent respiration was more affected than FADH-dependent respiration underlines the high susceptibility of complex I of the respiratory chain to hypoxia/reoxygenation stress. This is not surprising because of the complex structure of NADH:ubiquinone oxidoreductase which is assembled from about 40 subunits. Therefore, superoxide, known to be generated by complex I [15], has a good chance of attacking this macromolecule in a way that impairs the function of the enzyme.

The suggestion of a mechanism of injury linked to oxidative protein modification is strongly supported by the observed formation of protein-bound carbonyls. It has recently been demonstrated that protein-bound carbonyls, as early parameters of oxidative damage, were formed within the initial phase of lipid peroxidation during iron-ascorbate-induced oxidative stress in rat liver mitochondria [37]. The observed base level of protein-derived carbonyls is in the range known for other biological materials (0.5–3.5 nmol/mg) [24], and may be at least partially due to stress during mitochondrial preparation. This level is, in terms of mitochondrial function, tolerated well, but the high rate of carbonyl formation during reoxygenation (5 nmol of carbonyls/mg of protein over 9 min) is likely to contribute to the observed decline in function.

The specific amount of carbonyls in individual proteins and the distinct susceptibility of proteins to oxidative modification (Figure 5) might be determined by the amino acid composition, the conformation and the surface-volume relationship of these proteins. It remains speculative whether the observed phenomenon is primary, i.e. the result of direct oxidation within the

amino acid side-chains in proteins by ROS, or secondary, i.e. the result of modifications by lipid-derived aldehydes such as 4-hydroxynonenal and/or glycoxidation. However, any of these processes is likely to result in the impairment of protein function. It is a task for the future to identify those proteins which are highly sensitive to oxidative modification.

Besides the oxidative modification of proteins, we were able to detect remarkable amounts of lipid peroxides formed during reoxygenation. It should be noted that, in contrast, the late product of lipid peroxidation, malondialdehyde, was only 1.5-fold increased in comparison with controls. Therefore, direct oxidation products of unsaturated fatty acids (e.g. arachidonic acid), such as hydroperoxy fatty acids or isoprostanes, should be determined in order to demonstrate sensitively the degree of lipid peroxidation.

Although products of lipid peroxidation and oxidative modifications of proteins were observed, the mitochondrial membrane seems to be little affected in its function with respect to oxidative phosphorylation. This becomes evident by the slightly increased leak respiration with both NADH- and FADH-dependent substrates, as well as by the ability to maintain a high membrane potential of about 140 mV after reoxygenation in the resting state, determined by means of a TPP⁺-sensitive electrode (results not shown).

It should be borne in mind that the multiprotein complexes of the respiratory chain, although anchored in the mitochondrial inner membrane, contain large, functionally relevant domains outside of the membrane, thus providing targets for radical attack. Other parts of the protein may be protected by the membrane lipid bilayer and antioxidative defences, such as ubiquinol and α -tocopherol. We provided evidence for a ROS-linked mechanism of injury by demonstrating the beneficial effect of the water-soluble antioxidants, such as ascorbate and uric acid, which may protect the domains outside of the lipophilic environment by scavenging protein peroxides [35]. The dominant role of superoxide in damaging mitochondrial function may be concluded from the observation that the spin-trap DMPO, which forms adducts with superoxide and the OH[•] radical, but not DMSO, which is an OH[•] radical scavenger, decreased the decline in active respiration caused by hypoxia/reoxygenation. It is likely that the presence of 0.5 mM EGTA in the incubation medium prevents the formation of OH[•] radicals via the Fenton reaction by complexing free iron.

Because of the calcium-free incubations, our results are only related to calcium-independent effects of anoxia/reoxygenation in isolated mitochondria. The high membrane potential after reoxygenation, the low leak respiration and the lack of an effect of cyclosporin A applied at a final concentration of 1 μ M (results not shown), rule out a permanent permeability transition of the mitochondrial inner membrane under the conditions used here, which is discussed as playing an important role in the pathology of ischaemia/reperfusion injury [38].

We clearly demonstrate the pathological role of ROS produced by hypoxia/reoxygenation even before permeability transition occurs. Our data support the suggestion that functional impairment of mitochondria by self-generated ROS may be at least in part responsible for ischaemia/reperfusion injury of the liver.

Apart from the time of ischaemia, the substrate supply and the

availability of antioxidants can be considered as factors determining the degree of reperfusion injury in the liver.

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