Free Rad. Res. Comms., Vol. 18, No. 3, pp. 127-137
Reprints available directly from the publisher
Photocopying permitted by license only

ISCHEMIA/REPERFUSION IMPAIRS MITOCHONDRIAL ENERGY CONSERVATION AND TRIGGERS $O_2^{\cdot -}$ RELEASE AS A BYPRODUCT OF RESPIRATION

H. NOHL, V. KOLTOVER and K. STOLZE

Institute of Pharmacology and Toxicology, Veterinary University of Vienna, A-1030 Vienna, Linke Bahngasse 11, Austria

(Received October 2nd 1992; in final form December 4th 1992)

The aim of the present study was to elucidate the role of mitochondria in the development of heart failure following ischemia/reperfusion. Although mitochondria were increasingly assumed to be responsible for the establishment of an oxidative stress situation the lack of suitable methods to prove it required new concepts for an evaluation of the validity of this hypothesis. The principal idea was to expose isolated mitochondria to metabolic conditions which are developed during ischemia/reperfusion in the cell (anoxia, lactogenesis) and study how they respond. Heart mitochondria treated in that way responded with an incomplete collaps of the transmembraneous proton gradient, thereby impairing respiration-linked ATP generation. The membrane effect affected also the proper control of e^- transfer through redox-cycling ubisemiquinone. Electrons were found to leak at this site from its normal pathway to O_2 suggesting that ubisemiquinone becomes an active $O_2^{\cdot -}$ generator. It was concluded from these observations that mitochondria are likely to play a pathogenetic role in the reperfusion injury of the heart both, by an impairment of energy conservation and their transition to a potent $O_2^{\cdot -}$-radical generator. Furthermore, there is considerable evidence that the exogenous NADH-dehydrogenase of heart mitochondria is mainly responsible for functional changes of these organelles during ischemia/reperfusion.

INTRODUCTION

It is still unclear whether reperfusion of temporarily ischemic organs with oxygenated perfusate simply unmasks functional and structural disorders which occurred in the absence of oxygen or whether oxygen salvage following ischemia triggers the damage. The great majority of publications on ischemia-reperfusion related tissue damage is concerned with the theory that alterations of oxygen metabolism leading to the production of oxygen radicals is the ultimate pathogenetic event. A number of potential radical generating systems have been described which include catecholamines (1) xanthine-oxidase (2) polymorphonuclear leucocytes (PMN) (3) and mitochondria (4-6). Reperfusion injury is mainly studied at isolated organs perfused with a cell-free isoosmotic buffer. The fact that these organs also show the damaging effect of ischemia-reperfusion exclude catecholamines and PMN as critical factors in tissue damage. Reoxygenation following ischemia was repeatedly shown to convert xanthine-dehydrogenase to xanthine-oxidase (7) and allopurinol, a potent inhibitor of xanthine-oxidase reduces tissue injury (8). These findings were taken as evidence of the involvement of oxygen radicals since xanthine-oxidase catalyses the generation of $O_2^{\cdot -}$-radicals. The beneficial effect of allopurinol must not necessarily indicate a role of xanthine-oxidase in tissue damage

since this drug can also directly scavenge OH· radicals and sequestrate iron, thereby preventing its catalytic function in the generation of OH· radicals via a Fenton reaction. Furthermore, xanthine-oxidase activities do not exist in the hearts of many animal species including man while ischemia-reperfusion in these individuals causes the same damage. Corresponding considerations which more or less exclude the operation of the above systems as radical generators under conditions of ischemia-reperfusion are not valid for mitochondria. One reason is that these bioactive organelles are present in all tissues and are equipped with various redox systems directly or indirectly involved in the stepwise reduction of oxygen. Mainly in myocytes mitochondria are packed in high concentrations where they consume nearly hundred percent of total cellular oxygen. It has also been demonstrated by many investigators that mitochondrial respiration when inhibited (9,10) or hindered from energy conservation (11,12) generate relatively high amounts of $O_2^{\cdot-}$, H_2O_2 and OH· (9–13). It was mainly based on these facts and concluded from some indirect evidences that mitochondria are increasingly considered as the major source of free radicals in the ischemic-reperfused heart.

Until now there is no direct evidence which supports this assumption; even the existence of free oxygen radicals in the tissue after ischemia-reperfusion could not yet be demonstrated by direct methods. The main reason for these uncertainties are due to the lack of suitable methods, e.g. the availability of membrane-permeable spin traps which do not exert additional damaging effects at concentrations required to trap the expected radicals at their sites of generation. Another unresolved problem is the identification of these sites in the intact organ.

Since mitochondria are assumed to play a key role in reperfusion related oxygen activation we designed an experimental procedure which avoids methodological restraints discussed above to study these questions. The principal idea of our concept was to study isolated mitochondria from normal hearts under metabolic conditions established during ischemia and reoxigenation. The aim of this study was to find out whether or not this treatment would affect the capacity of mitochondria to generate reactive oxygen species.

MATERIAL AND METHODS

Triethanolamine, KCl, phosphate, glutamate, succinate, $K_3Fe(CN)_6$, EGTA (Titriplex VI) and acetonitrile were obtained from Merck (Darmstadt-Germany); $NADH_2$, ADP, catalase and antimycin A (AA) were from Boehringer (Mannheim, Germany); EDTA and sucrose were obtained from Loba Feinchemie (Fischamend, Austria); BSA (bovine serum albumin, fraction V), L-malic acid, succinate, fumarate, L-epinephrine, superoxide dismutase (SOD) and reduced glutathione (GSH) were purchased from Sigma Chemical (Deisenhofen, Germany); α-tocopherol was obtained from ICN Nutritional Biochemicals (Cleveland, Ohio-USA). All chemicals were of the highest grade of purity.

Mitochondria from livers (RLM) and hearts (RHM) of male Sprague-Dawley rats (250–300 g weight) were isolated according to (14). The isolation buffer contained 20 mM of triethanolamine (pH 7.4), 2 mM EDTA and 0.25 or 0.30 M sucrose for RLM and RHM, respectively. The pellets of RLM or RHM were finally resuspended in 20 mM triethanolamine (pH 7.4) and 125 mM KCl (reaction buffer). ATP/oxygen ratios and respiratory control values were determined in the reaction buffer at 25°C with a micro Clark type electrode of own design. The reaction buffer

was in equilibrium with ambient oxygen tension. Respiratory control values were calculated from the ratio of O_2 consumption in the presence and absence of ADP plus inorganic phosphate. Generation of ATP per atoms of oxygen (P/O) was calculated from the amount of O_2 consumed during state 3 respiration (in the presence of ADP and P_i). $O_2^{\cdot-}$ generation was calculated according to ref. 10 from the SOD-sensitive adrenochrome formation from epinephrine (0.5 mM), measured in a Shimadzu model 3000 UV-VIS spectrometer in the dual wavelength mode (450 nm/575 nm).

Ubisemiquinone populations (SQ·) in mitochondria established under conditions of "ischemia and reperfusion" were evaluated from the ESR spectra of these paramagnetic compounds, using a Bruker ER-200 D-SRC-spectrometer. Since the generation of steady state concentrations of SQ· in mitochondria require oxygen to run redox cycling of ubiquinone (UQ) (see ref. 15) we have replaced oxygen in the case of "ischemia" by $K_3Fe(CN)_6$. It has been recently shown that steady state concentrations of mitochondrial SQ· exhibited the same level when $K_3Fe(CN)_6$ was used as oxidant instead of O_2 to keep redox cycling UQ running (15). This technique allows to study whether O_2 can directly interact with SQ· in mitochondria. SQ· related ESR signals resulting from "ischemia" (= anaerobia) or aerobic respiration during "reperfusion" of mitochondria were determined following rapid freezing in a standard quartz tube down to 205°K.

RESULTS

Insufficient oxygen supply to an organ or total anoxemia shifts aerobic ATP-synthesis via oxidative phosphorylation to anaerobic side chain phosphorylation. As a consequence cellular ATP levels decrease and lactate/pyruvate ratios increase. This metabolic change does not only cause lactacidosis with pH-shifts down to 6.5 it also affects cellular $NADH_2/NAD^+$ ratios which are in equilibrium with lactate/pyruvate ratios via lactate-dehydrogenase. Mitochondria were therefore supposed to operate in a $NADH_2$-rich acid environment during ischemia. To approach these conditions experimentally we incubated isolated heart-mitochondria in an acid buffer (pH 6.5) with $NADH_2$ concentrations assumed to exist during total global ischemia. Final $NADH_2$ concentrations were evaluated on the basis of cardiac lactate/pyruvate ratios measured at the end of 30 min of total global ischemia (experiments not shown). Anoxia was mimicked by removing oxygen of the suspension by bubbling with N_2 at 25°C. After 30 min of anaerobia the suspension was equilibrated with pure oxygen for 10 min to mimic the reestablishment of oxygen supply following ischemia. Different types of controls were performed to differentiate between the effect of 30 min plus 10 min storing at 25°C' and the imitation of ischemia/reoxygenation. Control 1 was obtained from untreated mitochondria immediately after isolation. For control 2 mitochondria were stored under conditions of ischemia/reperfusion but without $NADH_2$ at pH 7.4.

Figure 1 demonstrates the effects of this treatment on bioenergetic parameters of mitochondria reflecting coupling of energy conservation to respiratory activities. Respiratory control values (RC) and ATP/oxygen ratios (P/O) were found to decrease significantly in mitochondria exposed to conditions of ischemia/reperfusion. This indicates incomplete uncoupling from oxidative phosphorylation. Uncoupling effects were also observed in control 2. This was not unexpected since

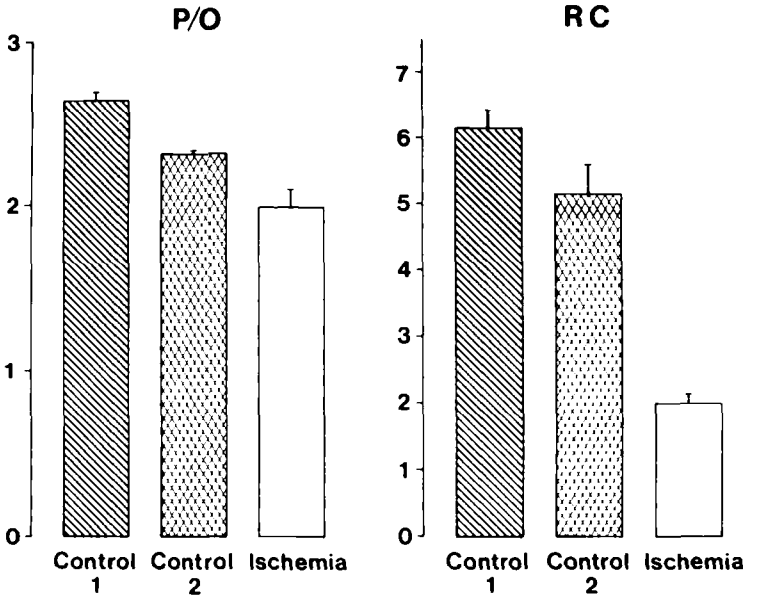

FIGURE 1 The effects of "ischemia/reoxygenation" on bioenergetic indicators of the integrity of the inner mitochondrial membrane. Conditions: 20 mg/ml RHM, 0.05% BSA, and 5 mM glutamate/malate to start state 4 respiration. Transition to state 3 respiration was initiated with 4.16 mM potassium phosphate and 4.16 μM ADP. Control 1: untreated RHM (n = 14); control 2: RHM kept 30 min at 25°C under N_2 followed by 10 min of reoxygenation (n = 5); Ischemia: as control 2 but in the presence of 10 mM $NADH_2$ at pH 6.5 (n = 5). The bars represent mean values ± standard deviations. The statistical significance between values of control 2 and ischemia was p = 0.05.

it is long known that isolated mitochondria increasingly develop these "aging effects" with time when not being stored on ice during the experimentation period. However, the presence of $NADH_2$ together with unusual acid pH-conditions caused a much stronger uncoupling effect on mitochondrial oxidative phosphorylation. The decrease of the physiological pH to pH 6.5 was not critical, because we observed no uncoupling effect as long as $NADH_2$ was absent (exp. not shown). Heart mitochondria exposed to the above conditions of ischemia/reperfusion were also found to release $O_2^{\cdot-}$ in the reaction medium as side products of normal respiration (Figure 2). This was in contrast to control 1 where $O_2^{\cdot-}$ radicals could only be observed when the mitochondrial electron transfer to cytochrome oxidase was inhibited by antimycin A ("classical conditions"). Storing of RHM (control 2) caused the generation of small amounts of $O_2^{\cdot-}$ while the presence of $NADH_2$ in an acid incubation buffer (conditions of "ischemia") exerted an almost 80% increase.

We have recently shown that ubisemiquinone pools of those mitochondria exhibiting $O_2^{\cdot-}$ release without the requirement of AA are susceptible to molecular oxygen (15). Figure 3 demonstrates that the ESR spectra of redox-cycling ubisemiquinones from "ischemia/reperfusion" treated mitochondria respond similarly. Redox-cycling ubisemiquinones of untreated mitochondria (control 1) were not affected by the presence of oxygen. When mitochondria were exposed to conditions of ischemia/reperfusion oxygen exerted however a 33% decrease of

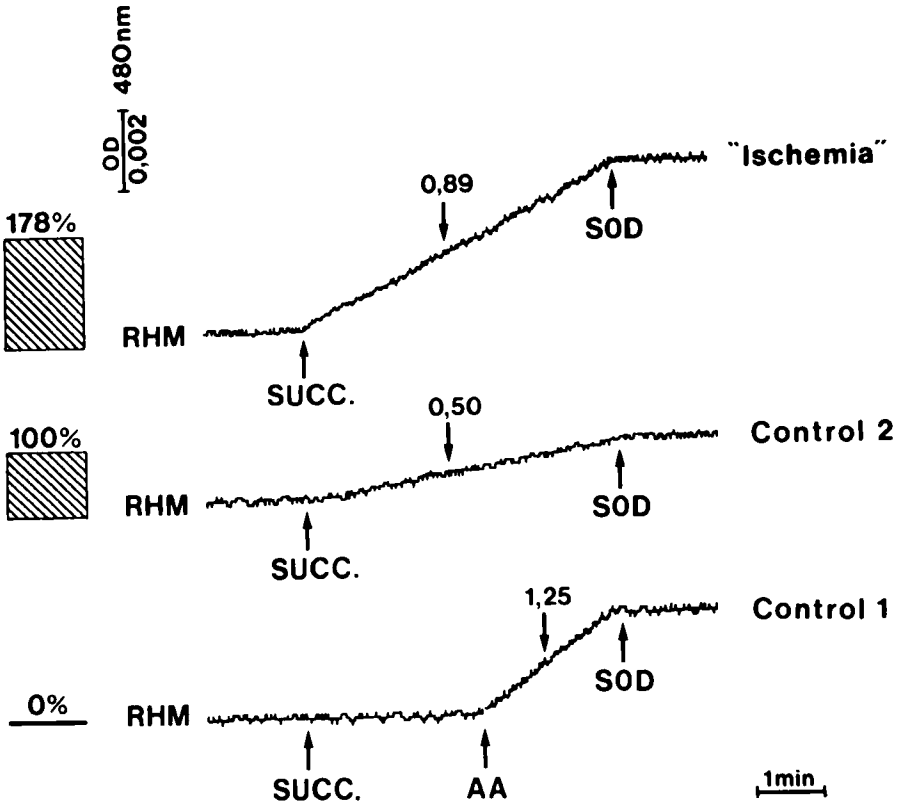

FIGURE 2 The effect of "ischemia/reoxygenation" on the capacity of isolated RHM to release $O_2^{\cdot -}$ radicals into the reaction medium. Conditions: 0.3–0.4 mg of RHM were suspended in 1 ml of the reaction buffer saturated with pure oxygen. Succinate (12 mM final concentration) was added to start the reaction. Other additives were catalase (725 U/ml), and 1 μg of AA per ml of acetonitrile (0.5% v/v). 71.4 U/ml of SOD was added to prove the involvement of free $O_2^{\cdot -}$ radicals in adrenochrome formation. $O_2^{\cdot -}$ formation rate of control 2 following equilibration with succ (0.50 nmol × min^{-1} × mg^{-1}) was set 100% (mean values ± SE from 5 experiments). The statistical significance between values of control 2 and "ischemia" was $p = 0.001$.

ubisemiquinone-related ESR signal intensities. In the absence of NADH at physiological pH-values (control 2) the oxygen effect was less pronounced. We concluded from these observations that ischemia/reperfusion conditions affected mitochondria in a way that oxygen will directly interact with ubisemiquinones. Since oxygen is a one electron acceptor and ubisemiquinone a one electron donor the occurence of the one electron reduction product of dioxygen in our reaction system as side product of normal respiration was in agreement with this concept. Accordingly also storage of non-incubated mitochondria (control 2) made SQ· slightly susceptible to oxygen. In a recent paper we reported that redox-cycling ubisemiquinones do not directly interact with molecular oxygen unless the integrity of the inner mitochondrial membrane is altered such that protons can penetrate (15). Energy coupling is a sensitive indicator of this type of membrane alteration.

Interestingly $O_2^{\cdot -}$ release during respiration was only dependent upon supplementation of mitochondria with $NADH_2$. Changing acid pH values used during

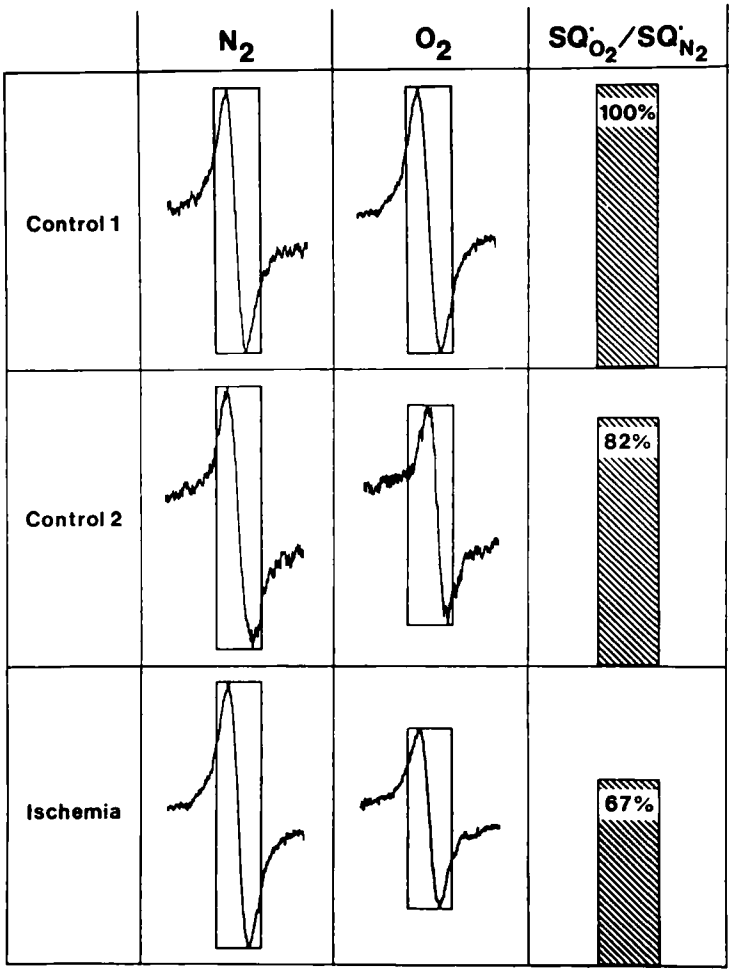

FIGURE 3 Response of mitochondrial ubisemiquinone populations (SQ·) to ischemia (N_2) and reoxygenation (O_2). The effect of oxygen is expressed as the percentage of the ESR signal height of mitochondrial SQ· under nitrogen (4 sets of experiments). Statistical deviations between the controls and "ischemia" were significant ($P = 0.05$). Experimental details: 20 mg of RHM were suspended in 1 ml of the reaction buffer which was equilibrated with pure N_2 or O_2, respectively. Immediately after the addition of $K_3Fe(CN)_6$ (20 mM) and succinate/fumarate (20/4 mM) the reaction mixture which was placed in a quartz tube (3 mm inner diameter) was frozen by liquid nitrogen and subjected to ESR measurements. ESR settings: microwave power −9,46 GHZ, frequency −1 mW; magnetic field modulation frequency −100 KHz; amplitude 5 G; receiver gain -2.10^4.

"ischemia", to the physiological level had no significant effect on any parameter measured (see Table I "ischemia"/control 3). On the other hand, incubation of freshly isolated RHM with $NADH_2$ alone affected energy coupling parameters as well as redox-cycling SQ· in the same way as shown for "ischemia" (control 4/ "ischemia"). Furthermore these RHM also released $O_2^{\cdot-}$ as side products of respi-

TABLE I
Experimental evidences on the major role of NADH in "ischemia"-induced alterations of mitochondrial respiratory activities. Details are given in the legends of figures 1–3

CONDITIONS	P/O	RC	$SQ^{\cdot}_{O_2}/SQ^{\cdot}_{N_2}$ ESR-signal hight	$O_2^{\cdot}$ nmol/min/mg	REMARKS
Control 1 - no anoxia - no addition - pH: 7,4	2,63 ±0,06	6,17 ±0,29	1,00 ±0,00	0,00	"freshly isolated RHM untreated"
Control 2 - 30'anoxia 25°C - 10' reoxygenation - no addition - pH:7,4	2,32 ±0,01	5,16 ±0,47	0,82 ±0,02	0,50 ±0,09	The effect of in vitro aging at 25°C
"Ischemia" - 30'anoxia 25°C - 10' reoxygenation - +NADH - pH:6,5	1,99 ±0,13	2,00 ±0,13	0,67 ±0,02	0,89 ±0,07	"Ischemia" at pH:6,5
Control 3 - 30'anoxia 25°C - 10' reoxygenation - +NADH - pH:7,4	1,87 ±0,28	2,20 ±0,27	0,62 ±0,07	0,92 ±0,08	"Ischemia" at pH:7,4 (pH effect)
Control 4 - no anoxia - +NADH - pH:6,5	1,96 ±0,18	2,26 ±0,25	0,77 ±0,08	0,50 ±0,02	"freshly isolated RHM +NADH" (NADH effect)

ration from any substrate for respiration (also after $NADH_2$ was completely exhausted).

Considering the prompt establishment of these effects (without the need of storage under conditions of anoxia and reoxygenation) it appears that $NADH_2$ was mainly responsible for alterations of mitochondrial bioactivities. The more pronounced response of redox-cycling UQ and the higher amounts of $O_2^{\cdot}$ release in RHM exposed to conditions of ischemia seem to be due to the superimposed effects of *in vitro* aging (compare "ischemia/control 4/control 2 of Table I).

Since ischemia/reperfusion conditions caused a drastic impairment of energy coupling we became interested in factors affecting the integrity of the mitochondrial membrane. Enzymatic antioxidants (SOD: 71,4 U/ml; catalase: 125 U/ml) and non-enzymatic antioxidants (GSH: 0.1 mM; α-tocopherol: 0.1 mM), inhibition of mitochondrial phospholipase A_2 activation by adsorbing free fatty acids to BSA (0.5%) and chelating of free Ca^{++} (EGTA: 0.1 mM) could not prevent the response to "ischemia" or $NADH_2$ pretreatment alone as described above (exp. not shown). Thus both, oxidative stress and Ca^{++} mediated membrane damage seem unlikely to account for oxygen susceptibility of ubisemiquinones and the decrease of energy coupling.

When liver mitochondria were exposed to conditions of ischemia/reperfusion or high $NADH_2$-levels alone no functional alterations with respect to the controls could be detected (experiments not shown).

DISCUSSION

Ischemia Induces the Generation of $O_2^{\cdot-}$ Radicals as Byproduct of Mitochondrial Respiration

The exposure of intact mitochondria to metabolic conditions of ischemia led to unexpected clear results which may be indicative of mitochondrial *in vivo* alterations during ischemia/reperfusion. Our experimental concept allows to circumvene possible artifacts which may arise from isolation of mitochondria from preinjured organs following ischemia/reperfusion; e.g. preparative damaging effects may mask real mitochondrial alterations or may cause effects which do not occur *in situ*. The most remarkable effect observed in this study was the transition of originally non-radical generating mitochondria to mitochondria releasing $O_2^{\cdot-}$ while respiring without the need of any further additive. Instead exposure of RHM to a $NADH_2$-rich acid pH buffer was sufficient to bring about this change. Control mitochondria could not be made to release $O_2^{\cdot-}$ radicals unless respiration was inhibited by AA or when $NADH_2$ was used as substrate. The latter effect was earlier demonstrated to depend on the existence of the exogenous $NADH_2$-dehydrogenase (16,17). This direct pathway of $O_2^{\cdot-}$ generation could be excluded in mitochondria subjected to conditions of ischemia. $NADH_2$ added to these mitochondria was completely consumed during the reoxygenation period while $O_2^{\cdot-}$ release was observed following the addition of substrates for complex I (glutamate/malate) or for complex II (succinate).

Identity of the $O_2^{\cdot-}$ Generator in Mitochondria

We have recently reported that mitochondria start releasing $O_2^{\cdot-}$ in the reaction medium when the resistence of the inner membrane to the penetration of protons was decreased (15).

RC values together with P/O ratios sensitively indicate to what extent the transmembraneous proton gradient of mitochondria is dissipated by non-specific proton leakage and reconductance through the proton translocating ATP synthase respectively. A significant decrease of these parameters was observed in mitochondria following exposure to conditions of ischemia-reperfusion (see Figure 1). This finding was therefore taken as a strong indicator that the hydrophobic membrane has developed polar properties during this treatment allowing rediffusion of H^+ into the matrix.

Changes of the physical property of the inner mitochondrial membrane had also consequences on the regular e^--flow through redox-cycling ubiquinones. The most striking effect was the sensitivity of mitochondrial ubisemiquinones to oxygen (see Figure 3). A direct interaction of O_2 with $SQ\cdot$ results in a one e^- transfer to O_2 thereby generating $O_2^{\cdot-}$ radicals and decreasing steady state concentrations of $SQ\cdot$ by autoxidation. Both effects were observed (see Figures 3,2). It is known from the literature that autoxidation of $SQ\cdot$ requires the presence of H^+ on thermodynamic grounds (18–20). Due to the observed uncoupling effect of ischemia-reperfusion we can assume that protons have become access to redox cycling $SQ\cdot$ in these mitochondria. We have recently shown that pretreatment of isolated mitochondria with toluene causes a collapse of the transmembraneous proton gradient via a non-specific proton-leak pathway (15). According to the observed effect of ischemia-reperfusion in this study redox-cycling $SQ\cdot$ became susceptible to oxygen following

toluene pretreatment. The simultaneous detection of $O_2^{\cdot -}$ radicals in both cases can be assumed to result from thermodynamic changes of the redox couple $SQ\cdot/SQ - O_2^{\cdot -}/O_2$ which favours autoxidation of redox-cycling $SQ\cdot$ while protons rediffuse accross the inner mitochondrial membrane.

The Role of the Exogenous NADH-dehydrogenase in the Development of Ischemia-induced $O_2^{\cdot -}$ Release During Respiration

All changes observed as a consequence of "ischemia-reperfusion" were found to depend exclusively on the presence of $NADH_2$ in the incubation system. The time period of anoxia and imitation of acidosis were not critical. Thus raising cytosolic $NADH_2$ levels during ischemia had to be considered as the essential factor which triggers hazardous alterations of mitochondrial activities. Furthermore, the deleterious $NADH_2$-effect required the presence of the exogenous $NADH_2$-dehydrogenase since liver mitochondria which do not have this particular enzyme remained unaffected by exposure to $NADH_2$. Although this enzyme was shown to be an active $O_2^{\cdot -}$ generator enzymatic and non-enzymatic antioxidants failed to protect heart-mitochondria from the damaging effect of $NADH_2$. Also chelation of Ca^{++} (EGTA) and removal of fatty acids (by binding to BSA) in order to inhibit activation of mitochondrial phospholipase A_2 had no protecting effect. Thus, oxidative degradation of membrane lipids seems not to be responsible for functional membrane changes observed. It is remarkable that the "aging effect" of stored mitochondria (control 2) established identical patterns of functional changes in mitochondria although less intensively pronounced as in ischemia/reperfusion treated mitochondria. *In vitro* aging is well known to increase the permeability of the inner membrane to protons (due to phospholipase A_2-mediated accumulation of lysophosphatides). This observation therefore strongly supports the existence of a correlation between the physical condition of the membrane and the loss of control of e^- cycling through $SQ\cdot$.

The Role of Mitochondria in the Development of Ischemia/Reperfusion Induced Heart Injury

Although the mechanism of ischemia-reperfusion induced activity changes of mitochondria is not fully understood our findings allow the following conclusions:

The increase of cytosolic $NADH_2$ levels in heart cells which occurs as a result of ischemia-induced lactogenesis decreases the resistence of mitochondria to transmembraneous proton conductance. This transition is tightly related to an impairment of mitochondrial ATP generation and a loss of control of e^- flow through redox cycling ubiquinones. Thermodynamic restraints which normally prevent a direct interaction of $SQ\cdot$ with oxygen (15) become less effective during ischemia-reperfusion allowing the establishment of a one-e^- shuttle to oxygen out of sequence. These functional changes of mitochondria may contribute to organ injury both by the decrease of energy supply and the development of oxidative stress. Mitochondria are therefore likely to play a central ontogenetic role in the reperfusion injury of the heart.

Furthermore, deleterious functional changes in heart-mitochondria are likely to depend on the existence of the exogenous $NADH_2$-dehydrogenase (16). This can be concluded from the lack of response of liver mitochondria to incubation with corresponding $NADH_2$ levels. In contrast to the classical matrix-directed

$NADH_2$-ubiquinone-oxidoreductase which is present in all types of mitochondria the exogenous $NADH_2$-dehydrogenase is a constituent of heart mitochondria only and may oxidize cytosolic $NADH_2$ directly. Thus, metabolic changes of cytosolic $NADH_2$-levels is assumed to modulate the activity of this enzyme and its interactions with other compounds involved in energy-linked respiration. The way by which this may affect functional changes observed is still unclear. We have work in progress to study this correlation.

Acknowledgements

The authors are grateful to Werner Stamberg for excellent technical help. The present study was supported by the "Oesterreichische Fonds zur Förderung der wissenschaftlichen Forschung"

References

1. S.L. Jewett, L.J. Eddy and P. Hochstein (1989) Is the autoxidation of catecholamines involved in ischemia-reperfusion injury? *Free Radical Biology and Medicine*, **6**, 185–188.
2. D.E. Chambers, D.A. Parks, G. Patterson, R. Roy, J.M. McCord, S. Yoshida, L.F. Parmley and J.M. Downey (1985) Xanthine oxidase as a source of free radical damage in myocardial ischemia. *Journal Molecular Cell Cardiology*, **17**, 145–152.
3. J.L. Romson, B.G. Hook, V.H. Rijot, M.A. Schork, D.P. Swanson and B.R. Lucchesi (1982) The effect of ibuprofen on accumulation of 111-indium labelled platelets and leukocytes in experimental myocardial infarction. *Circulation*, **66**, 1002–1011.
4. J.F. Turrens, M. Beconi, J. Barilla, U.B. Chavez and J.M. McCord (1991) Mitochondrial generation of oxygen radicals during reoxygenation of ischemic tissues. *Free Radical Research Communication*, **12–13**, 681–689.
5. W. Henke and K. Jung (1991) Ischemia decreases the content of adenine nucleotide translocator in mitochondria of rat kidney. *Biochimical Biophysical Acta*, **1056**, 71–75.
6. V. Vandeplassche, C. Hermans, F. Thone and M. Borgers (1989) Mitochondrial hydrogen peroxide generation by NADH-oxidase activity following regional myocardial ischemia in the dog. *Journal Molecular Cell Cardiology*, **21**, 383–392.
7. R.S. Roy, J.M. McCord (1983) Superoxide and ischemia: Conversion of xanthine dehydrogenase to xanthine oxidase. In: Oxy Radicals and their scavenger systems. Cellular and medical aspects Vol II (R.A. Greenwald and G. Cohen eds.) N.Y *Elsevier Science*, 145–153.
8. J.M. McCord and R.S. Roy (1982) The pathophysiology of superoxide: roles in inflammation and ischemia. *Cancer Journal Physiology Pharmacology*, **60**, 1346–1352.
9. A. Boveris and E. Cadenas (1975) Mitochondrial production of superoxide anion and its relationship to the antimycin-insensitive respiration. *FEBS Letters*, **54**, 311–314.
10. H. Nohl and D. Hegner (1978) Do mitochondria produce oxygen radicals *in vivo*? *European Journal Biochemistry*, **82**, 563–567.
11. E. Cadenas and A. Boveris (1980) Entrancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochemistry Journal*, **188**, 31–37.
12. H. Nohl and W. Jordan (1980) The metabolic fate of mitochondrial hydrogen peroxide. *European Journal Biochemistry*, **111**, 203–210.
13. H. Nohl, W. Jordan and D. Hegner (1981) Identification of free hydroxyl radicals in respiring rat heart mitochondria by spin trapping with the nitrone DMPO. *FEBS Letters*, **123**, 241–244.
14. L. Szarkowska and M. Klingenberg (1963) On the role of ubiquinone in mitochondria. *Biochemische Zeitschrift*, **338**, 674–697.
15. H. Nohl and K. Stolze (1992) Ubisemiquinones of the mitochondrial respiratory chain do not interact with molecular oxygen. *Free Radical Research Communication*, accepted for publication.
16. H. Nohl (1987) Demonstration of the existence of an organo-specific NADH dehydrogenase in heart mitochondria. *European Journal Biochemistry*, **169**, 585–591.
17. H. Nohl (1987) A noval superoxide radical generator in heart mitochondria. *FEBS Letters*, **214**, 269–273.
18. Y.A. Jlan, G. Czapski and D. Meisel (1976) The one-electron transfer redox potentials of free radicals. *Biochimical Biophysica Acta*, **430**, 209–224.

19. D.L. Maride and W.G. Hodgson (1965) Reduction of oxygen to superoxide anion in aprotic solvents. *Analytical Chemistry*, **37**, 1562–1565.
20. P.M. Wood (1974) The redox potential of the system oxygen-superoxide. *FEBS Letters*, **44**, 22–24.

Accepted by Dr. H. Esterbauer