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THE MECHANISM OF TOXIC ACTION OF HYPERBARIC OXYGENATION ON THE MITOCHONDRIA OF RAT-HEART CELLS*

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Abstract—Hyperbaric oxygenation of the whole animal was examined with respect to changes in mitochondria from heart cells. Accumulation of conjugated dienes, decrease of unsaturated fatty acids and changes in phospholipid pattern of the mitochondrial membrane indicate peroxidative damage of membrane lipids during oxygen stress. Hyperoxia induces increased activities of superoxide dismutase, catalase and glutathione peroxidases, all enzymes protecting mitochondria from deleterious effects of reactive oxygen species. From the multiplicity of parameters related to pathophysiological effects of O_2 we inferred on increased formation rates of toxic oxygen species involving superoxide as the initial step in biological activation of O_2. The K_m for superoxide formation was 550 μM, ranging above physiological oxygen levels by a factor of 11. Thus, the mechanism by which hyperoxia might affect stimulation of superoxide formation is discussed on the basis of enhanced Michaelis–Menten kinetics due to elevated oxygen levels in the heart tissue. The onset of the membrane damage is the first event in hyperbaric O_2 toxicity. Despite a clear induction, activities of protecting enzymes seem to be 'swamped' by raising oxygen concentration above normal.

Responses to hyperbaric oxygenation have been intensively studied in isolated organs, subcellular particles and bacteria under conditions of direct oxygen contact. Thus, toxic effects or changes in enzyme activities could be immediately related to increased oxygen tension within the respective preparation systems. More complex conditions must be taken into account to evaluate the response of inner organs of animals exposed *in vivo* to hyperbaric oxygen. Possible effects may depend on local variables such as the sensitivity of the microvascular system to changes in oxygen tension and the susceptibility of cells to O_2-toxicity. Mitochondria appear to play a key role in hyperbaric oxygen poisoning. One reason for this can be derived from the fact that mitochondrial electron transfer carriers represent the greatest pathway for the reduction of molecular oxygen in mammalian cells. Furthermore, it has been recognized that small amounts of the electron flux through the chain leak off to molecular oxygen prematurely to generate reactive oxygen species [1–3]. These oxygen compounds exert deleterious structural and functional effects at the inner mitochondrial membrane [3–6].

We have tried to evaluate possible consequences of oxygen stress by investigating isolated heart mitochondria from animals exposed to hyperoxia. One approach to this problem was to examine the initiation of lipid peroxidation or the stimulation of enzyme systems involved in the formation and detoxification of hazardous oxygen species. It appears from our experiments that the onset of homeolytic events following exposure to hyperbaric oxygenation is rather fast and not in equilibrium with homeostatic mechanisms which prevent and repair radical initiated damage of the mitochondrial membrane.

MATERIALS AND METHODS

Chemicals. Superoxide dismutase was purchased from Miles Laboratories, Indiana, U.S.A. Cumene hydroperoxide was a gift from Peroxid Chemie München GmbH, Höllriegelskreuth, West Germany. Reduced and oxidized glutathione, glutathione reductase, reduced and oxidized nicotinamide adenine dinucleotides, DL-isocitric trisodium salt (type I), cytochrome *c* (horse heart type III) and L-epinephrine were purchased from Sigma, U.S.A. Other biochemicals were from Boehringer, Mannheim, West Germany. Other chemicals were from Merck, Darmstadt, or from Serva, Heidelberg, West Germany.

Pretreatment and preparation of mitochondria. Male Wistar rats (200–250 g body weight) maintained on Altromin® *ad libitum* were kept in a hyperbaric chamber of our own design for 26–29 hr prior to preparation of mitochondria. Hyperbaric oxygenation was accomplished by compression with pure O_2 up to a final pressure of 1.2×10^5 Pa preceded by O_2 flushing to expel the air inside the chamber. The flow rate of O_2 was adjusted so as to avoid accumulation of CO_2 within the chamber. Rats breathing under normal atmospheric air conditions were taken for control experiments. Heart mitochondria were prepared according to Szarkowska [7].

Assays. The inhibitory effect of superoxide dismutase on O_2-dependent oxidation of epinephrine

* Dedicated to Professor Dr. MAX FRIMMER on the occasion of his 60th birthday.

to adrenochrome (absorption coefficient: 2.86 mM^{-1} cm^{-1}) or on the reduction of ferricytochrome *c* to ferrocytochrome *c* (absorption coefficient: 18.7 mM^{-1} cm^{-1}) was used to follow mitochondrial generation of superoxide radicals. Adrenochrome formation was measured at 480–575 nm, ferricytochrome *c* reduction at 550–575 nm using an Aminco DW-2 spectrophotometer (American Instr. Co., Md, U.S.A.). Oxygen consumption rates of mitochondrial preparations were measured with a micro Clark-type electrode.

The determination of superoxide dismutase activity was based on the ability of this enzyme to inhibit oxidation of epinephrine by the xanthine–xanthine oxidase system [8]. The amount of ultrasonicated mitochondrial protein which causes a 50 per cent inhibition of adrenochrome formation was defined as one unit of superoxide dismutase activity. Catalase activity was determined as previously described [9]. Glutathione peroxidase activities were assayed in a coupled optical test as described elsewhere [10]. Reduced glutathione (GSH) was determined enzymatically according to the method of Bernt and Bergmeyer [11]. NADP-dependent isocitrate dehydrogenase activity was determined photometrically by following the decrease of the NADPH absorbance at 340 nm in the presence of 5 mM GSSG, 0.2 mM NADPH and 0.7 mg rat heart mitochondria (RHM) in 1 ml of the isolation buffer. Glutathione reductase activity was assayed as previously described [12]. All enzymic activities were measured at 25°.

Chemical analyses of mitochondrial membrane lipids were performed as previously described [13]. Protein was measured by a modification of the biuret method using KCN in order to circumvent turbidity due to contaminating lipids.

RESULTS

Determination of the Michaelis constant for the autoxidation component for O_2

There is convincing support from earlier investigations [14, 15] that superoxide radical formation is

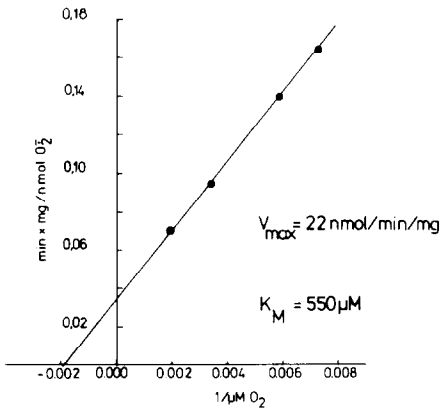

Fig. 1. Determination of Michaelis constant for the monovalent reduction of O_2. Experimental values of the straight line represent means of 5 experiments (S.E.M. ranged between ± 6 and ± 9%). For methodical details see Materials and Methods.

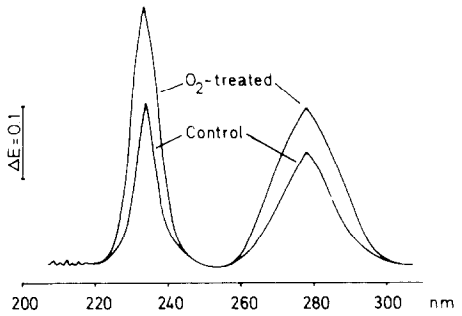

Fig. 2. Spectrophotometric detection of lipid peroxides in the mitochondrial membrane of rats exposed to hyperbaric oxygen. Membrane lipid (0.5 mg) was dissolved in 1 ml chloroform/methanol (1/1 mixture) and absorption spectra were followed between 210 and 310 nm using an Aminco DW-2 spectrophotometer. The traces represent typical spectra similar to those obtained in 7 measurements with preparations for control and 6 O_2-treated animals, respectively.

the first event in reaction sequences leading to highly toxic oxygen species. Since superoxide production appears very likely to occur by autoxidation of reduced electron transferring components [2, 3], monovalent reduction of oxygen may follow first order kinetics with respect to oxygen concentration. Thus, increasing the oxygen concentration increases the rates of superoxide production. This relationship is normally expressed by the Michaelis constant (K_m), and can be determined from the slope of a Lineweaver–Burk reciprocal plot (Fig. 1). Since there is a continuous decrease of oxygen concentration in air saturated suspensions of respiring mitochondria, this natural concentration change was followed and taken as one parameter for the determination of K_m. Simultaneously the corresponding superoxide generation was determined by the sensitivity of cytochrome *c* reduction or cooxidation of adrenalin against added superoxide dismutase. For technical reasons these measurements were performed with separate suspensions but under identical conditions. The maximal velocity of O_2^- formation was calculated to be 22 nmoles min^{-1} mg^{-1}; for the K_m an oxygen concentration of 550 µM was found.

Structural changes of the inner mitochondrial membrane

Isolated RHM from animals treated with hyperbaric oxygen exhibited no change in formation rates of O_2^- and H_2O_2 when compared with RHM from control animals. However, indirect evidence for the *in vivo* existence of toxic oxygen species was obtained from chemical analysis of the inner mitochondrial membrane. Figure 2 shows the effect of oxygen stress on the accumulation of reaction products of reactive oxygen compounds with susceptible material of the inner membrane. Since phospholipids are known to be highly sensitive against free radical attack [16, 17], we investigated Folch extracts of the inner membrane for spectra of peroxidized lipids (Fig. 2). The appearance of diene conjugation in peroxidized, unsaturated fatty acids or their deriva-

Table 1. Changes in the fatty acid composition of membrane lipids following hyperbaric oxygenation*

Fatty acids	Control	O_2-treated
16:0	8.90 (±0.26)	11.90 (±0.51)
18:0	17.70 (±0.90)	22.60 (±1.14)
18:1	6.10 (±0.32)	5.50 (±0.36)
18:2	30.30 (±1.02)	20.40 (±0.98)
20:4	18.20 (±0.59)	20.40 (±0.74)
22:5	2.04 (±0.08)	1.82 (±0.04)
22:6	12.60 (±0.60)	12.20 (±0.71)
Ratio $\frac{\text{unsaturated}}{\text{saturated}}$	2.60 (±0.09)	1.75 (±0.12)

* Values are expressed as percentage of recording area of the total peak areas of fatty acids in gas liquid chromatography (mean ± S.E.M.); n control = 16; n O_2-treated = 8.

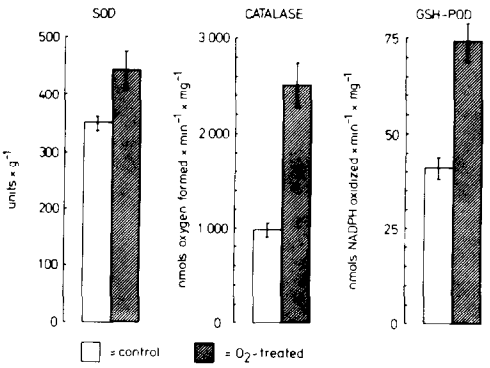

Fig. 3. Influence of hyperbaric oxygen stress on protective mitochondrial enzymes. Deviation bars represent ± S.E.M.; n control = 9; n O_2-treated = 8.

tives is due to resonance following free radical attack on methylene bridges separating the double bond in compounds which absorb light at 235 and 278 nm. RHM from O_2-treated animals exhibit a clear absorption increase at 235 nm due to the formation of conjugated dienes and split products absorbing at 278 nm. The fact that lipids from control rats also accumulated lipid peroxide was not unexpected and had already been subject to earlier discussions [3]. The prooxidant stress of hyperbaric oxygenation further stimulates the decomposition of the organic hydroperoxide groups involving cleavage of double bonds of polyunsaturated fatty acids. As can be seen from gas chromatographic analysis (Table 1), oxygen stress clearly changes the fatty acid pattern of the inner mitochondrial membrane. In O_2-treated animals the ratio of unsaturated to saturated fatty acids decreased mainly due to a drastic fall in linoleic acid (18:2) content. Furthermore, besides saturated fatty acids, the highly unsaturated arachidonic acid (20:4) was also found to be increased.

The peroxidative alterations in lipid architecture causes differences in chromatographic separations. Table 2 demonstrates these differences as obtained by 2-dimensional thin layer chromatography. Hyperbaric oxygenation influences phospholipid pattern by a drastic decrease in the cardiolipin content while lysophosphatides and phosphatidylcholine were found to be increased.

Response of protective enzymes to oxygen toxicity in hyperbaric oxygenation

Superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-POD) have been shown to act synergistically against deteriorative reactions following increasing formation rates of toxic oxygen species in mitochondria [18, 19]. Figure 3 demonstrates that all protective enzymes responded to an elevation of oxygen concentration in the living animal. However, the increase in enzyme activities was not unique. Catalase activity exhibited a 250 per cent increase, GSH-POD activity increased by 83 per cent, while SOD activity under hyperbaric oxygenation was only 25 per cent higher than under normal conditions.

The physiological activity of GSH-POD is dependent on the availability of its cosubstrate GSH. Thus, if one evaluates the activity of GSH-POD in a certain biological structure, determination of steady state levels of reduced glutathione is also required in this system. Via GSH-reductase the equilibrium of the GSH redox state is linked to the redox state of pyridine nucleotides. NADP-dependent isocitrate dehydrogenase is the main mitochondrial generator for NADPH. Despite a simultaneous increase in the activities of isocitrate dehydrogenase, GSH-reductase and GSH-POD the GSH content was found to be decreased during oxygen stress (Fig. 4).

Table 2. Changes of phospholipid pattern in mitochondrial membranes from rats exposed to hyperbaric oxygen*

Phospholipids	Control	O_2-treated
Cardiolipin	13.03 (±0.54)	9.68 (±0.74)
Phosphatidic acid	1.72 (±0.22)	1.78 (±0.21)
Phosphatidylethanolamine	39.64 (±0.26)	39.78 (±1.01)
Phosphatidylcholine	38.56 (±0.48)	42.10 (±0.82)
Phosphatidyl serine	5.35 (±0.25)	5.34 (±0.51)
Sphingomyelin	0.79 (±0.06)	1.15 (±0.23)
Phosphatidylglcerol	0.29 (±0.09)	0.21 (±0.07)
Lysophosphatides	0.14 (±0.01)	0.52 (±0.02)

* Phospholipids were analysed by two dimensional thin layer chromatography as described [13]. The data represent per cent values of total membrane lipids (mean ± S.E.M.); n control = 10; n O_2-treated = 8.

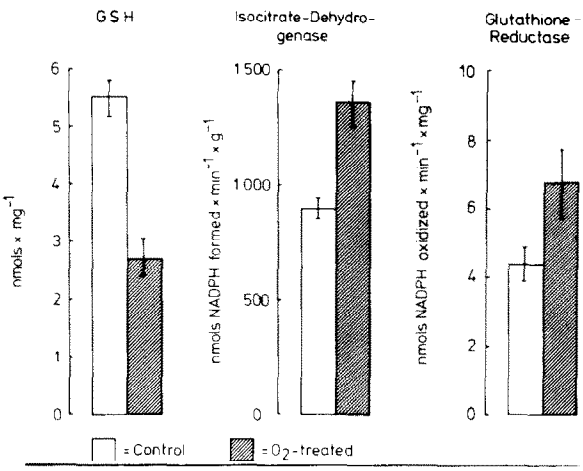

Fig. 4. Response of hyperoxia on mitochondrial GSH-content and activities of GSH regenerating enzymes. *n* Control = 12; *n* O_2-treated = 9.

DISCUSSION

The present paper provides indirect evidence that toxic oxygen species are formed in heart cells during hyperbaric oxygenation of the whole animal. Mitochondrial membrane lipids have been shown earlier to be highly susceptible to reactive oxygen species [3, 4]. The accumulation of typical reaction products of radical-induced lipid peroxidation in the inner mitochondrial membrane may therefore be regarded as an indicator for the existence of steady state concentrations of toxic oxygen species. SOD, which together with catalase completes a cooperative system in controlling the formation of hazardous oxygen compounds, exhibits only a small activity increase under conditions of hyperoxia. Since enzymatic dismutation is first order with respect to O_2-radical concentration it must be concluded that a given concentration of SOD will intercept variable fractions of O_2-radicals as the steady state level changes. Thus, within a certain range of SOD levels one cannot infer the efficiency of its protective activity. However, the small increase of dismutating activity may be interpreted in terms of the existence of O_2-radicals above physiological concentrations.

Different mechanisms may be taken into consideration when evaluating the stimulation of superoxide radical formation during oxygen stress. Induction of autoxidizing side reactions of the respiratory chain must be ruled out as a possibility since we did not observe any change in the kinetic of monovalent electron leak to oxygen after isolation of the mitochondria. The K_m detected for generation of O_2-radicals ranged by a factor of 11 above the physiological oxygen concentration normally found in heart tissue. Therefore, if the tissue concentration of oxygen is elevated as a result of hyperbaric treatment, monovalent oxygen reduction should be linearly increased as well. It should be added that the determination of K_m was performed with the complex enzyme set of intact mitochondria and not with the isolated autoxidizing components which still remain unidentified. Thus, it cannot be excluded that oxygen exerts its effect differently from a mechanism following Michaelis–Menten kinetics. A generalized increase of oxidative capacity of the respiratory chain under hyperbaric conditions was not observed by previous experiments of others [20]. That means that an increased leak of electrons parallel to enhanced electron transport through the cytochrome chain can be eliminated as a rationale to explain stimulation of radical formation. Wilson *et al.* [21] recently reported that the cellular (ATP)/(ADP) (P_i) ratio is regulated by oxygen tension in that an increase in oxygen concentration is followed by an increase in this phosphorylation ratio. From these findings it must be concluded that mitochondrial respiration will switch more and more to state 4 conditions as soon as oxygen tension is elevated. This metabolic condition has been shown to be a prerequisite for the formation of O_2-radicals [2, 3] and may therefore be regarded as another mechanism which could account for radical-induced changes during hyperbaric oxygen stress.

It is now unequivocally accepted that O_2-radical species cannot account for radical attack at the methylene bridge of polyunsaturated fatty acids. The necessity for H_2O_2 which would be formed by dismutation of O_2-radicals has been repeatedly demonstrated [4, 22]. The drastic increase of catalase activity at mitochondria from O_2-treated animals indicates the existence of non-physiologically high levels of H_2O_2. Together with O_2-radicals these compounds may be due to membrane damage via the formation of the highly reactive OH-radicals [12, 22]. The existence of this type of radical species in RHM has been recently demonstrated [15].

The first reaction step in the sequence of radical induced lipid damage leads to the generation of conjugated dienes which decompose to ketones and further propagate lipid damage by oxidative cleavage of the polyunsaturated fatty acids. Our results have shown that oxygen stress is due to initiation of pathophysiological lipid autoxidation since we observed accumulation of conjugated dienes, destruction of polyunsaturated fatty acids and changes in phospholipid pattern of the mitochondrial membrane. The increase in arachidonic acid (20:4) is not in harmony with the concept as discussed above. Similar changes in fatty acid pattern have been reported to occur during paraquat intoxication [23] and as a result of biological ageing [13]. The development of both phenomena also includes enhanced formation rates of superoxide, the radical species which was shown to be involved in prostaglandin synthesis from arachidonic acid [24]. Elevated levels of arachidonic acid might refer to stimulation of its formation at the expense of linoleic acid (18:2), the compound which was found to be drastically diminished during oxygenation.

The increase in phosphatidylcholine content during oxygen stress may point to its function as an antioxidant, exerting this kind of activity by its ability to form tightly bound ligands around catalytic metal ions [25].

Enzymes effective in protecting mitochondria from hazardous radical attack, such as SOD and catalase, have been shown to respond with an activity increase upon oxygenation. The increase in the

activity of GSH-POD indicates the presence of lipid hydroperoxides [12, 18], the main substrate for GSH-POD. The simultaneous decrease in GSH levels following hyperbaric oxygenation occurs despite increased activities of enzymes replenishing GSH by reducing its oxidized form at the expense of NADPH. Thus the repair function of GSH-POD at the level of peroxidized membrane lipids seems to be controlled by the availability of free GSH. Oxidation of reduced glutathione is not exclusively related to the reduction of lipid peroxides by GSH-POD but may occur as well by enzymatic and nonenzymatic reactions linked to mixed disulfides involving thioltransferases. It has been reported that hyperbaric oxygen is also due to perturbation of the redox state of free thiols [26–28]. From this finding it can be concluded that the repair mechanism of radical-induced lipid peroxidation by GSH-POD has to compete for reduced glutathione with repair mechanism related to oxidized thiol compounds. It appears that the onset of homeolytic events following exposure to hyperbaric oxygen is rather fast and not in equilibrium with homeostatic mechanisms which prevent and repair radical-initiated membrane damage. The importance of the mitochondrial membrane for the regulation of cellular metabolism suggests that O_2 poisoning is further established by perturbation of cellular metabolism. This, however, should be subject to further investigations in this field.

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