Oxygen Toxicity in the Perfused Rat Liver and Lung under Hyperbaric Conditions

By KATSUYUKI NISHIKI, DANA JAMIESON,* NOZOMU OSHINO† and BRITTON CHANCE
Johnson Research Foundation, University of Pennsylvania School of Medicine, Philadelphia, PA 19174, U.S.A.

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1. In the lung and liver of tocopherol-deficient rats, the activities of glutathione peroxidase and glucose 6-phosphate dehydrogenase were increased substantially, suggesting an important role for both enzymes in protecting the organ against the deleterious effects of lipid peroxides. 2. Facilitation of the glutathione peroxidase reaction by infusing t-butyl hydroperoxide caused the oxidation of nicotinamide nucleotides and glutathione, resulting in a concomitant increase in the rate of release of oxidized glutathione into the perfusate. Thus the rate of production of lipid peroxide and H_2O_2 in the perfused organ could be compared by simultaneous measurement of the rate of glutathione release and the turnover number of the catalase reaction. 3. On hyperbaric oxygenation at 4×10^5 Pa, H_2O_2 production, estimated from the turnover of the catalase reaction, was increased slightly in the liver, and glutathione release was increased slightly, in both lung and liver. 4. Tocopherol deficiency caused a marked increase in lipid-peroxide formation as indicated by a corresponding increase in glutathione release under hyperbaric oxygenation, with a further enhancement when the tocopherol-deficient rats were also starved. 5. The study demonstrates that the primary response to hyperbaric oxygenation is an elevation of the rate of lipid peroxidation rather than of the rate of formation of H_2O_2 or superoxide.

O_2 toxicity in animals has been recognized since the classic work of Bert (1878), and many data about its symptoms and aetiology have been accumulated. However, the underlying mechanism has remained obscure owing to a lack of knowledge of the biochemical processes involved.

There is abundant evidence that the rate of development of O_2 toxicity depends primarily on local variables, such as blood supply (i.e. O_2 delivery), tissue metabolic rate and susceptibility of the cells involved in O_2 poisoning (Winter & Smith, 1972; Gottlieb, 1965; Haugaard, 1968). A conclusive result for a direct toxic effect of O_2 on the pulmonary system was demonstrated by Penrod (1958). The lungs are directly in contact with the ambient air and are exposed to O_2 partial pressures higher than those in any other organ *in situ*; thus it is not surprising that they are severely affected during breathing of pure O_2 (Caldwell *et al.*, 1966; Fisher *et al.*, 1968; Puy *et al.*, 1968; Clark & Lambertsen, 1971).

Studies on the biochemistry of O_2 toxicity have shown a great variation in enzymic resistance to O_2 toxicity, with some systems showing a high sensitivity,

i.e. a rapid inactivation by O_2 (Jamieson & van den Brenk, 1962; Haugaard, 1964, 1968). Some of these sensitive enzyme groups are found in the tricarboxylic acid cycle (Thomas *et al.*, 1963) and in the glycolytic pathway (Horn *et al.*, 1965). On the other hand, Chance *et al.* (1965, 1966) showed that hyperbaric oxygenation caused a rapid oxidation of nicotinamide nucleotides in isolated mitochondria and in a variety of tissues *in situ* of anaesthetized rats in the initial phase of O_2 compression.

An increased production of lipid peroxides under hyperbaric conditions was demonstrated in lung homogenates of tocopherol-deficient rats *in vitro* (Raskin *et al.*, 1971). Boveris & Chance (1973) showed that hyperbaric O_2 increased the formation of H_2O_2 in mitochondria isolated from normal rat livers. After exposure to hyperoxia, an increased H_2O_2 production *in vivo* was detected qualitatively in the erythrocytes (Johnson *et al.*, 1972) and in the brain (Jerret *et al.*, 1973) of tocopherol-deficient rats, but Oshino *et al.* (1975a,b) were unable to detect this phenomenon in perfused liver and liver *in situ* in the normal anaesthetized rat. This discrepancy suggests differences in the biological systems, which make it difficult to determine the primary effect of hyperbaric O_2 .

The enzyme reactions involved in the reduction of peroxide appear to include those of catalase (EC 1.11.1.6) and glutathione peroxidase (EC 1.11.1.9).

* Present address: Roche Research Institute of Marine Pharmacology, P.O. Box 255, Dee Why, N.S.W. 2099, Australia.

† Present address: Nihon Schering K.K., Development and Research Department, 2-6-64 Nishimiyahara-cho, Yodogawa-ku, Osaka 532, Japan.

Whereas catalase preferentially decomposed H_2O_2 produced in normal substrate metabolism (Oshino & Chance, 1977), the glutathione peroxidase system rather specifically reduces lipid peroxide (cf. Scheme 1).

In the perfused liver, the reactions of the NADPH-regenerating system seem to be the rate-determining steps of peroxide metabolism, and hence stimulation of the glutathione peroxidase reaction by an organic peroxide causes corresponding increases in the steady-state concentrations of both NADP^+ and GSSG (oxidized glutathione), which then result in an increased rate of glutathione release into the effluent perfusate (Sies *et al.*, 1974; Oshino & Chance, 1977). Under certain conditions, this latter rate is proportional to the turnover number of the glutathione peroxidase reaction. Therefore, if the rate of glutathione release is compared with the rate of H_2O_2 production as calculated from the turnover number of the catalase reaction (Oshino *et al.*, 1975a), it is possible to study the production of lipid peroxide and H_2O_2 under various conditions. In addition, these measurements may be carried out in the perfused organ under hyperbaric conditions (Oshino *et al.*, 1975b), permitting these events in the aetiology of O_2 toxicity to be evaluated as a function of time.

The present paper describes the use of the perfused lung and liver of normal and tocopherol-deficient rats for the study of O_2 toxicity, and demonstrates the formation of lipid peroxide and the associated alteration in the cellular redox state which are produced by hyperbaric oxygenation of the perfused organs.

Methods and Materials

Animal preparations

Male albino rats of the Sprague-Dawley strain, weighing 190–250 g, were used throughout these experiments. Control rats were given access to ordinary laboratory rat chow and tap water before the experiments. A tocopherol-free diet (Nutritional Biochemicals, Cleveland, OH, U.S.A.) was given for 14 consecutive days to achieve tocopherol deficiency. The rats to be starved were deprived of food for 40–50 h. Sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL, U.S.A.) was given intraperitoneally (40 mg/100 g body wt.) as an anaesthetic.

Lung and liver perfusion

The trachea was intubated just below the larynx for lung ventilation. The perfusion consisted of two steps: first, removing blood from the vascular system of the lung, and second, perfusing only the lung tissue. Then the portal vein was cannulated and perfused at a flow rate of approx. 25 ml/min. This procedure was followed by ligation of the vena cava

between the hepatic and renal veins. The lung was then perfused through the tube cannulated into the pulmonary artery at a rate of 8 ml/min. This procedure minimized local stasis and resultant trapped blood in the pulmonary tissue. The excision of the lung required the greatest care, since the lobes are readily damaged. The perfusion fluid, Krebs-Ringer phosphate buffer, consisted of 140 mM-NaCl, 5 mM-KCl, 8.6 mM- Na_2HPO_4 , 1.4 mM- NaH_2PO_4 , 1.0 mM- MgSO_4 , 1.3 mM- CaCl_2 , and was adjusted to pH 7.4 with NaOH. In the liver experiment, it was perfused as described previously (Sies & Chance, 1970; Theorell *et al.*, 1972). Erythrocytes were not added to the perfusate because they have enough enzyme activity to interfere with the present results of H_2O_2 production and glutathione release with respect to catalase, glutathione reductase (EC 1.6.4.2) and glutathione peroxidase (Keilin & Hartree, 1945; Mills & Randal, 1958; Cohen & Hochstein, 1963).

Instrumentation

The excised organs, lung and liver, were placed in a hyperbaric chamber (model 1386-HP; Bethlehem Instrument Co., Philadelphia, PA, U.S.A.) equipped with a circulating system containing a peristaltic pump (model 1 203; Harvard Instruments; Millis, MA,

Fig. 1. Schematic diagram of instrumental arrangement for surface fluorimetry

AP, air pump (miniature fish pump); ER, effluent fluid reservoir; FM, flow-meter; HC, hyperbaric chamber; HP, hot plate; IP, infusion port; L, isolated lung; LP, light-pipe; MSV, miniature solenoid ventilator; NC, N_2 cylinder; OC, O_2 cylinder; PG, pressure gauge; PP, peristaltic pump; PR, perfusate reservoir; SP, sampling port. In the flow-through perfusion, the path between PR and ER is closed.

U.S.A.), a flow-meter (model F-1200; Gilmon Instruments, New York, NY, U.S.A.) and the fluorimetric system shown in Fig. 1. The simultaneous recording of the catalase-H₂O₂ compound (660–640 nm), nicotinamide nucleotide fluorescence (excitation at 366 nm, emission measured at 460 nm) and the oxidation/reduction changes of cytochrome *c* (550–540 nm) required the use of a time-sharing spectrophotometer (Theorell *et al.*, 1972; Chance *et al.*, 1975) placed outside the hyperbaric chamber. Optical coupling to the perfused organ was accomplished by means of fibre light-guides penetrating the chamber wall.

The perfusion was performed with the peristaltic pump outside the hyperbaric chamber for reasons of safety. The flow-meter maintained the flow rate of the perfusion fluid constant at 8 ml/min (Oshino *et al.*, 1975a). A closed recirculating or open flow-through perfusion system was chosen as appropriate for each experiment. Metabolic inhibitors and substrates were infused into the pulmonary artery via a three-way connector as desired.

The lung was ventilated at 80 cycles/min with a 2 ml tidal volume and $2\text{--}3 \times 10^2$ Pa end expiratory pressure, by using a miniature explosion-proof solenoid-operated ventilator developed in this laboratory. Hyperbaric oxygenation was accomplished by compression with pure O₂ up to a final pressure of 4.1×10^5 Pa at a rate of 0.4×10^5 Pa/min, preceded by O₂ flushing to expel the air inside the chamber. The surface-fluorimetric method has been described previously (Theorell *et al.*, 1972; Chance *et al.*, 1975; Oshino *et al.*, 1975a).

Biochemical analysis

Glutathione was measured by the procedure of Oshino & Chance (1977) and its concentration was expressed as equivalent to GSH (reduced glutathione). Lactate dehydrogenase activity was determined as soon as possible and without freezing, which leads to serious inactivation of this enzyme. Other details appear in the legend of each Table and Figure.

Materials

t-Butyl hydroperoxide was purchased from Matheson, Coleman and Bell Manufacturing Chemicals (East Rutherford, NJ, U.S.A.); NADP⁺, NAD⁺ and glutathione were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 5,5'-dithiobis-(2-nitrobenzoic acid) and Amytal (ammobarbital) were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The enzymes used in the present work were obtained from Boehringer Mannheim (New York, NY, U.S.A.). The other reagents used were of the highest purity supplied by local distributors.

Results

Activation of the peroxide-metabolizing enzymes by tocopherol deficiency

As discussed below in detail, the development of O₂ toxicity seems to be closely correlated with the production of lipid peroxides. The system for detoxifying lipid peroxides comprises glutathione reductase and glutathione peroxidase, and is linked to the NADPH-generating system. The reducing equivalents delivered to NADP⁺ are primarily supplied by the operation of the pentose phosphate shunt, which is regulated by glucose 6-phosphate dehydrogenase (EC 1.1.1.49) activity (Eggleston & Krebs, 1974). To estimate their participation in the mechanism that protects against peroxide poisoning the activities of these enzymes were compared in the lung and liver of normal and tocopherol-deficient animals. No significant change was found in glutathione reductase, but glutathione peroxidase and glucose 6-phosphate dehydrogenase showed a remarkable enhancement of activity in organs from tocopherol-deficient rats over the control values: in lung, 74 and 230% respectively, and in liver, 35 and 154% (see Table 1).

Since stimulation of lipid peroxidation has been observed in tocopherol-deficient animals *in vitro* (Raskin *et al.*, 1971; Riely *et al.*, 1974) and *in vivo* (Johnson *et al.*, 1972; Jerret *et al.*, 1973), the observed increases in the activity of glucose 6-phosphate dehydrogenase and glutathione peroxidase indicate their adaptive response to an enhanced production of lipid peroxides and suggest key roles for these enzymes in lipid-peroxide metabolism in lung and liver.

Fluorimetric detection of the accelerated oxidation of NADPH during reduction of t-butyl hydroperoxide in perfused lung

t-Butyl hydroperoxide, which is a specific substrate for glutathione peroxidase (Little & O'Brien, 1968), was infused to mimic a situation of stimulated production of lipid peroxides, so that the initial phases of the consequences of lipid peroxidation could be studied. During the infusion of t-butyl hydroperoxide, there is a phase associated with the reduction of the peroxide to t-butyl alcohol by glutathione peroxidase in which the fluorescence of reduced nicotinamide nucleotides decreases (Fig. 2a); this decrease is accompanied by glutathione release from the lung tissue (Fig. 2b). Both responses occur immediately after t-butyl hydroperoxide infusion, indicating operation of the sequential enzyme reactions of the alkyl peroxide-metabolizing system (Scheme 1). After cessation of the infusion, the fluorescence recovers to the initial value, indicating that the observed reactions are not due to non-specific cell damage. These phenomena are similar to those observed in the perfused liver (Sies *et al.*, 1974;

Table 1. Comparisons of the activities of glutathione reductase, glutathione peroxidase and glucose 6-phosphate dehydrogenase in the lung and liver of control and tocopherol-deficient rats

The activities are expressed in terms of NADPH ($\mu\text{mol}/\text{min}$ per g wet wt. of tissue). Each value represents the mean $\pm$ S.E.M. of n separate experiments. The erythrocytes were removed from the tissue by perfusion with ice-cold Krebs-Ringer phosphate buffer. The activities were assayed at 25°C in the supernatant obtained from a 10% homogenate by centrifugation at $480g_{av}$ for 10 min. The buffer solution for homogenizing the tissues consisted of 2% (w/v) Triton X-100, 50 mM-Tris/HCl and 20 mM-methanol (pH 7.4). Glutathione reductase was measured in a reaction mixture consisting of 0.1 M-potassium phosphate buffer, pH 7.1, 0.1 mM-NADPH, 0.25 mM-GSSG and 1 mM-EDTA; GSH peroxidase was measured in 0.1 M-potassium phosphate buffer, pH 7.1, 0.1 mM-NADPH, 0.25 mM-GSH, 1 mM-EDTA and an excess of glutathione reductase. Glucose 6-phosphate dehydrogenase was determined in 50 mM-triethanolamine hydrochloride, pH 7.5, 0.5 mM-NADP $^+$, 0.5 mM-glucose 6-phosphate and 1 mM-EDTA. Each assay was performed in a final volume of 2.5 ml and was initiated by the addition of the appropriate substrate.

Organ	Enzyme	n ...	Activity		Increment (%)
			Control (7)	Tocopherol-deficient (7)	
Lung	GSSG reductase		3.48 ± 0.23	3.49 ± 0.12	0.3
	GSH peroxidase		2.15 ± 0.21	3.75 ± 0.75	74.4
	Glucose 6-phosphate dehydrogenase		0.27 ± 0.04	0.89 ± 0.12	229.6
Liver		n ...	(5)	(8)	
	GSSG reductase		7.93 ± 0.73	9.19 ± 0.40	16.0
	GSH peroxidase		12.69 ± 1.01	17.18 ± 0.95	35.4
	Glucose 6-phosphate dehydrogenase		1.44 ± 0.41	3.54 ± 0.38	145.8

Fig. 2. Changes in the oxidation-reduction state of nicotinamide nucleotide and in the glutathione release caused by *t*-butyl hydroperoxide

The lung from a normally fed rat was perfused with Krebs-Ringer phosphate buffer, pH 7.4, at 30°C in an open (i.e. flow-through) perfusion system at atmospheric pressure (air). The perfusion flow rate was maintained constant at $8\text{ ml}/\text{min}$ per g wet wt. of lung; 0.1 M-*t*-butyl hydroperoxide and Amytal were infused via an infusion pump to achieve the desired concentration in a given time. Trace (a) is that of the fluorescence of reduced nicotinamide nucleotide excited at 366 nm and measured at around 460 nm. The fluorescence was calculated regarding an initial fluorescence intensity as 100%. Trace (b) illustrates the changes in glutathione release from the lung tissue to the effluent fluid. The traces shown are representative of four experiments.

Oshino & Chance, 1975a, 1977) and have been attributed to increased intracellular concentrations of both NADP $^+$ and GSSG.

The maximal fluorescence change is less than 3% of the initial fluorescence, and the rate of glutathione release is $14.6\text{ nmol}/\text{min}$ per g wet wt. of lung tissue during an infusion rate of *t*-butyl hydroperoxide of approx. $30\text{ }\mu\text{mol}/\text{min}$ per g wet wt. of lung. The extent of fluorescence decrease observed under these conditions is compatible with that of the fluorescence increase due to the reduction of nicotinamide nucleotides resulting from inhibition of the mitochondrial respiratory chain by infusion of Amytal in subsequent experiments. The 3% change in fluorescence is small compared with that seen in various metabolic transitions in other tissues (Chance *et al.*, 1966; Theorell *et al.*, 1972), but appropriate to lung tissue (Fisher *et al.*, 1974), which exhibits a high background fluorescence. The change in fluorescence is always compared with the initial fluorescence, and the latter is large in the lung owing to white structural components of lung tissue.

The increased release of glutathione by Amytal may be ascribed to a combination of the known actions of this barbiturate; Amytal inhibits mitochondrial respiration (Chance & Williams, 1956) and is metabolized via the microsomal drug-hydroxylation system (Axelrod, 1955; LaDu *et al.*, 1955; Junge & Brand, 1975). In perfused liver (Oshino & Chance, 1977), acceleration of the drug-metabolizing reaction results in an increased release of GSSG owing to the oxidation of NADPH, with a subsequent shift of the cellular redox state of glutathione in the

direction of oxidation. The decrease in the energy-dependent transhydrogenation from NADH to NADP⁺, which results from the Amytal inhibition of energy production, may further enhance the effect of this substance on glutathione release.

Fig. 3. Relationship between the rate of glutathione release and infused t-butyl hydroperoxide in the lung

Each lung received a series of infusions of t-butyl hydroperoxide where the concentration increased in stepwise fashion, and was weighed after blotting on a filter paper to eliminate water. The perfusion was performed under the same conditions as those of Fig. 2. ○, Control; ●, tocopherol-deficient fed; □, tocopherol-deficient starved. Each point represents the mean $\pm$ S.E.M. of three to six rat lungs.

Table 2. Lactate dehydrogenase release from lung tissue under hyperbaric oxygenation

The isolated lung was perfused with Krebs-Ringer phosphate buffer, pH 7.4, supplemented with glucose, via the pulmonary artery in a hyperbaric chamber. The effluent fluid was collected at various time-intervals after pressurization. A recirculation system was used for the lung perfusion. Each value represents nmol of NADH formed/min per 0.1 ml of effluent perfusate, and is the mean $\pm$ S.E.M. for five rat lungs. In the control experiment, the lungs were subjected to hyperbaric N₂. The assay mixture consisted of 0.5 M-glycine, 0.4 M-hydrazine hydrate and 4 mM-NAD⁺, pH 9.0. The final volume was 2.5 ml, and the reaction temperature was 23°C.

Time exposed to O ₂ at 4.1 $\times$ 10 ⁵ Pa (min)	Enzyme released	
	Control	Tocopherol-deficient
Before exposure	3.3 $\pm$ 0.8	1.7 $\pm$ 0.4
0	4.3 $\pm$ 1.4	3.3 $\pm$ 1.0
30	5.6 $\pm$ 1.5	5.9 $\pm$ 1.7
90	7.0 $\pm$ 1.4	6.1 $\pm$ 1.7

Relationship between the rates of t-butyl hydroperoxide reduction and of glutathione release

A comparison of the influent and effluent concentrations of t-butyl hydroperoxide in the perfusate (not shown) reveals that lung is able to decompose almost all of the infused peroxide up to a rate of approx. 1.2 μ mol/min per g wet wt. of lung in the normal control. The rate of glutathione release was determined as a function of the rate of infusion of the peroxide in the lungs of control, tocopherol-deficient fed and tocopherol-deficient starved rats (Fig. 3). The rate of glutathione release increases linearly with the rate of reduction of the peroxide by the lung. The slope of the linear plot is greater in the lung of tocopherol-deficient rats than in the control (Fig. 3).

The observation that tocopherol deficiency results in a decrease of the relative activities of glutathione reductase to glucose 6-phosphate dehydrogenase compared with that of glutathione peroxidase (Table 1) coincides with the increased release of glutathione induced by a given rate of peroxide reduction (Fig. 3). In addition, the decreased capability of NADPH generation induced by starvation causes a further increase in the susceptibility of the system to peroxide infusion. From the results presented in Fig. 3 it is concluded that the mechanism underlying the phenomenon of glutathione release in perfused lung is, in principle, the same as that proposed for the perfused liver. Thus the rate of glutathione release may be regarded as an indicator proportional to the turnover of the glutathione peroxidase reaction under these particular conditions.

Glutathione release from the perfused lung under hyperbaric oxygenation

Fig. 4 shows the concentration of glutathione in the effluent from the isolated perfused lungs of normal and tocopherol-deficient rats under hyperbaric oxygenation. In the control, the rate of glutathione release is less than 1.0 nmol/min per g wet wt. of lung from normal rats; with N₂ rather than O₂, as shown in Fig. 4, almost no change in glutathione release is induced even by pressurization at 4.1 $\times$ 10⁵ Pa for 90 min. With hyperbaric oxygenation of the normally fed rat, a slight increase in the rate of glutathione release, which rose to 1.5 nmol/min per g wet wt. of lung, is detected immediately after initiation of pressurization.

The effect of hyperbaric oxygenation on glutathione release, calculated from experimental points obtained within 20 min after initiation of pressurization, is intensified by 9.0 nmol/min per g wet wt. of lung in tocopherol-deficient rats in which the initiation and propagation of the radical chain reaction of lipid peroxidation may readily take place because of the lack of the natural anti-oxidant, tocopherol. Starving the tocopherol-deficient rats causes a further enhancement of the rate of glutathione release in

Fig. 4. Glutathione release from normal and tocopherol-deficient rat lungs under hyperbaric oxygenation as a function of time

The lung was perfused in a recirculating system containing 200 ml of perfusate under the same conditions as those of Fig. 2. Glucose supplements (5.5 mM) were added to the perfusion fluid for organs of fed but not of starved rats. Before pressurization, the hyperbaric chamber was flushed with O_2 for 10 min to clear out the air, and pressurization then continued throughout the experiments. Each point represents the mean value $\pm$ S.E.M. for seven rats. Δ , Normal lung, not pressurized; the results of N_2 pressurization were similar to this trace. $\circ$, Normal lung of fed rat, pressurized with pure O_2 ; the lung from normal starved rats showed no significant difference from the fed. $\bullet$, Lung from tocopherol-deficient fed rats. Δ , Lung from tocopherol-deficient starved rats.

response to hyperbaric oxygenation, by 13.2 nmol/min per g wet wt. of lung. The decrease subsequent to the maximal release is probably caused by the degradation of glutathione by molecular oxygen under hyperbaric oxygenation (Gilbert *et al.*, 1958).

To confirm that the observed glutathione release resulted from an acceleration of the glutathione peroxidase reaction and not from destruction of cell integrity and/or alteration in the permeability of the cell membrane, the leakage of lactate dehydrogenase into the perfusate was measured (Table 2). The total activity of this enzyme in the perfusate increased slightly as the perfusion time was prolonged and after 90 min was equivalent to 7 μ mol of NADH oxidized/min, a value corresponding to less than 0.1% of the total activity in the lung tissue. No sig-

nificant differences were detected between the time-course of the leakage from the perfused lungs of control and tocopherol-deficient rats under normal partial pressure of O_2 and hyperbaric conditions. No oedematous profile was found in the lungs under these conditions, i.e. the amount of water accumulated in the lung was less than 17 mg/g wet wt. of lung throughout these experiments.

Assuming that the observed increase in the rate of glutathione release on hyperbaric oxygenation is due to the acceleration of the glutathione peroxidase reaction, increments in the turnover number of this reaction under hyperbaric oxygenation may be expressed in terms of the rate of reduction of t-butyl hydroperoxide. The increased rate of glutathione release observed under hyperbaric oxygenation at

Table 3. Summary of the rates of glutathione release, H₂O₂ generation and lipid peroxide formation in the isolated perfused lung and liver of normal and tocopherol-deficient rats under hyperbaric oxygenation

Each value represents the mean ± s.e.m. of the maximal rates (nmol/min per g wet wt. of organ) for the number of experiments in parentheses after each value. For lipid-peroxide formation, the rate is expressed in terms of nmol of t-butyl hydroperoxide equivalents/min g wet wt. of organ. The calculation of intracellular H₂O₂ generation was based on the formation of the catalase-H₂O₂ intermediate, as described in detail in Chance & Oshino (1971) and Oshino *et al.* (1973). Perfusion was as described in the Methods and Materials section. The rates of lipid peroxide formation were derived from the difference between glutathione release under the pressures given here, by using the calibration curves of Fig. 3; the calibration curve for the liver is not shown. The statistical *P* values refer to the difference of the glutathione release and that of H₂O₂ generation between 1 × 10⁵ Pa and 4 × 10⁵ Pa of O₂.

O ₂ pressure (10 ⁵ Pa) ...	Glutathione release			H ₂ O ₂ generation			Lipid peroxide formation
	1	4.1	<i>P</i>	1	4.1	<i>P</i>	
Lung							
Normal starved	0.9 (4) ±0.2	1.5 (7) ±0.2	<0.05	—	—		91.7
Tocopherol-deficient fed	1.0 (5) ±0.7	9.0 (5) ±1.8	<0.01	—	—		533.3
Tocopherol-deficient starved	1.0 (4) ±0.4	13.2 (5) ±2.1	<0.01	—	—		610.0
Liver							
Normal starved	4.3 (4) ±1.2	8.2 (3) ±1.6	<0.01	47.3 (4) ±3.1	55.5 (4) ±4.0	>0.1	91.8
Tocopherol-deficient starved	5.2 (3) ±1.3	18.9 (4) ±2.4	<0.01	55.5 (4) ±4.2	78.1 (4) ±8.1	>0.05	322.5

Fig. 5. Effect of hyperbaric oxygenation on H₂O₂ generation in the normal perfused rat liver

O₂ compression up to 4.1 × 10⁵ Pa took place as shown in the Figure, and was maintained constant at that pressure throughout the experiment. The perfusion was performed under the conditions of Fig. 2 except that the flow rate was decreased to 3–5 ml/min per g of liver wet wt. In (a), an increase in the concentration of the catalase-H₂O₂ intermediate corresponds to an upward deflexion at 660 – 640 nm. In (b), cytochrome c reduction at 550 – 540 nm is shown by an upward deflexion. The glutathione released into the perfusate appears in (c). (a) and (b) are simultaneously recorded on a strip chart recorder, and the effluent is sampled to assay the glutathione content shown in (c). Changes in the concentration of infused methanol are indicated by the arrows. Time proceeds from left to right. MeOH, methanol; EtOH, ethanol. This Figure represents the results of five separate experiments.

Fig. 6. Effect of hyperbaric oxygenation on the methanol titration of H_2O_2 generation in the liver of tocopherol-deficient rats

The liver of the tocopherol-deficient rat was perfused with Krebs-Ringer phosphate buffer supplemented with 0.3 mM-pyruvate, 1 mM-lactate, 0.3 mM-acetoacetate and 0.1 mM- β -hydroxybutyrate under the conditions of Fig. 5. An increase in the concentration of the catalase- H_2O_2 intermediate at 660–640 nm is shown as an upward deflexion (a), as is an increase in the fluorescence of reduced nicotinamide nucleotide (excitation at 366 nm, fluorescence at 460 nm) in the lower trace (b). The glutathione release, lactate/pyruvate ratio and β -hydroxybutyrate/acetoacetate ratio in the effluent are illustrated in (c), (d) and (e) respectively. The traces (a) and (b) were simultaneously recorded on a strip chart recorder, while the effluent was sampled to assay the glutathione and other metabolite contents. Lactate was measured by the procedure of Gutmann & Wahlefeld (1974), pyruvate by the procedure of Czok & Lamprecht (1974), β -hydroxybutyrate by the procedure of Williamson & Mellanby (1974) and acetoacetate by the procedure of Mellanby & Williamson (1974). Abbreviations are as in Fig. 5. The lower rate of GSSG release compared with other Figures is due to the reduction of glutathione by added substrates. This Figure represents the result of five separate experiments. Time proceeds from left to right.

4×10^5 Pa is equivalent to the rate obtainable with the reduction of t-butyl hydroperoxide at 92, 533 and 610 nmol/min per g wet wt. of lung from normal, tocopherol-deficient fed and tocopherol-deficient starved rats respectively (Table 3).

Determination of the rate of H_2O_2 production in perfused liver under hyperbaric O_2

It has been demonstrated above that hyperbaric oxygenation causes the release of GSSG from the perfused lung, indicating an enhancement of the turnover of the glutathione peroxidase reaction under such conditions. Whereas H_2O_2 is a substrate for both catalase and glutathione peroxidase, lipid peroxides are specifically decomposed by the latter enzyme (Little & O'Brien, 1968; Flohé & Schlegel, 1971). Thus the question arises as to whether H_2O_2 or lipid peroxide is responsible for the observed acceleration of the glutathione peroxidase under hyperbaric O_2 .

The rate of H_2O_2 production can be determined by measuring the turnover number of the catalase reaction spectrophotometrically (Oshino *et al.*, 1973; Chance & Oshino, 1973). This technique has been extensively used on the liver but not, as yet, in lung. In agreement with previous results (Oshino *et al.*, 1975b), the rate of H_2O production measured by methanol titration of the catalase- H_2O_2 intermediate in control rat (cf. Figs. 5 and 6) was 47.3 ± 3.1 nmol/min per g at 1×10^5 Pa of oxygen, and 55.5 ± 4.0 nmol/min per g wet wt. of liver under hyperbaric oxygenation at 4×10^5 Pa (mean $\pm$ S.E.M., $n=4$). The differences are within the experimental error of the assay method. The rate of H_2O_2 production under hyperbaric oxygenation was increased from 55.5 ± 4.2 to 78.1 ± 8.1 nmol/min per g wet wt. of liver in the tocopherol-deficient starved rat (Table 3).

However, small but significant changes in the rate of glutathione release associated with O_2 compression were observed. The rate changed from 4.3 ± 1.2 to 8.2 ± 1.6 nmol/min per g wet wt. of liver from normal rats ($n=3-4$) during 15 min of hyperbaric oxygenation at 4.1×10^5 Pa of O_2 . This increment corresponds to that observed with infusion of t-butyl hydroperoxide at a rate of approx. 92 nmol/min per g wet wt. of liver under similar conditions. In the tocopherol-deficient starved rat, the extent of the change with O_2 compression was greatly intensified, reaching a value comparable with that found with infusion of t-butyl hydroperoxide at a rate of 323 nmol/min per g wet wt. of liver under similar conditions (Table 3). These results suggest that hyperbaric oxygenation of the liver tissue preferentially activates lipid peroxidation and not the formation of O_2^- or H_2O_2 . Further evidence should be provided by the slight stimulation by starvation of glutathione release and the remarkable stimulation by tocopherol deficiency (Fig. 7 and Table 3).

Fig. 7. Glutathione release from the control and tocopherol-deficient rat liver under hyperbaric oxygenation

The liver was perfused in an open system under the conditions of Fig. 5. Each plot depicts the mean value $\pm$ S.E.M. of four separate experiments. ■, Normal fed, not pressurized; ○, normal starved rat, pressurized; ●, tocopherol-deficient starved rat, pressurized.

Redox state of nicotinamide nucleotides under hyperbaric oxygenation

The oxidation of nicotinamide nucleotides as an early response to hyperbaric oxygenation of tissue has been detected fluorimetrically *in vivo* as well as in biochemical assays (Chance *et al.*, 1966). To clarify the relationship between the oxidation of nicotinamide nucleotides and the enhancement of glutathione peroxidase reaction in response to hyperbaric O_2 , the redox state of the nicotinamide nucleotides in the perfused liver of the tocopherol-deficient rat was further investigated, by using simultaneous measurements of the intensity of nicotinamide nucleotide fluorescence and the extent of changes in the ratios of lactate to pyruvate and β -hydroxybutyrate to acetoacetate in the effluent perfusate.

A representative result of these experiments is shown in Fig. 6. On compression with O_2 , the initial increase in the rate of glutathione release is accompanied by a progressive decrease in the fluorescence intensity of NAD(P)H. The extent of the fluorescence decrease is comparable with that of the fluorescence increase caused by infusion of 2 mM-ethanol. The absolute extent of the observed changes in the glutathione release and in the fluorescence intensity are smaller than those observed in other series of experiments reported here, owing to the oxidation/reduction buffering by substrate supplements. It is noteworthy that whereas the ratio of lactate to pyruvate increased slightly and monotonically, that of β -hydroxybutyrate to acetoacetate decreased in the initial phase of hyperbaric oxygenation and then rose slowly. It is also noteworthy that whereas the change

in fluorescence intensity resulting from hyperbaric oxygenation is accompanied by a decrease in the β -hydroxybutyrate/acetoacetate ratio, the ethanol-induced change in fluorescence intensity is associated with a large increase in the lactate/pyruvate ratio. Because the former and the latter substrate couples represent respectively the oxidation/reduction states of the mitochondrial and cytosolic compartment (Lindros *et al.*, 1974), the former changes seem to follow hyperbaric oxygenation more clearly.

Our tentative interpretation of these phenomena is that the acceleration of lipid peroxidation by hyperbaric oxygenation results in the oxidation of glutathione and NADPH, which in turn causes a shift in the oxidation/reduction state of the NADH/NAD⁺ couple, probably through the energy-linked NADH/NADP⁺ transhydrogenase system, to overcome an increased demand for cellular NADPH.

Discussion

The results presented in this paper show that there is an increased rate of glutathione release in the lung and liver of the tocopherol-deficient rat during hyperbaric oxygenation, and that the glutathione oxidation/reduction enzyme system is functional in detoxifying lipid peroxides produced in the cells. GSH can be released across the cell membrane after it has been oxidized by glutathione oxidases (Srivastava & Beutler, 1969). The glutathione release induced by hyperbaric oxygenation results from intracellular biochemical reactions and not from general, irreversible cellular damage such as membrane destruction, for the following reasons. (1) Nitrogen compression up to 4×10^5 Pa caused no increased release of glutathione from the lung of tocopherol-deficient rats. (2) No difference in the release of lactate dehydrogenase into the perfusion fluid was observed between the control and tocopherol-deficient groups (Table 2). (3) The maximal amount of glutathione release, approx. 20% of the total content of intracellular glutathione, was seen in starved tocopherol-deficient rats. (4) No evidence of gross damage to the tissue cells in terms of release of cell contents etc. was observed under these conditions.

Homogenates of most animal tissues produce thiobarbituric acid reactants (Haugaard, 1968), i.e. malonaldehyde is formed by oxidation of unsaturated fatty acids, lipid constituents of the tissues, a process which is probably initiated via H₂O₂ (Holman, 1954). However, the results on the formation of lipid peroxide *in vivo* after hyperoxic exposure are controversial because of the difficulty of reliable analysis of peroxide *in vivo* (Philpot, 1963). Although the question of how much lipid peroxides are formed *in vivo* is unanswered, H₂O₂ formation in the intact cell can be followed directly by monitoring steady states of the catalase-H₂O₂ intermediate by means of a time-

sharing spectrophotometer (Theorell *et al.*, 1972; Chance *et al.*, 1975), which records the difference in absorption at 660 and 640 nm due to this intermediate (Chance & Oshino, 1971; Theorell *et al.*, 1972; Sies *et al.*, 1973; Oshino *et al.*, 1973, 1975a; Chance *et al.*, 1975). H₂O₂ measured with the catalase system is mainly metabolized by catalase in preference to glutathione peroxidase (Oshino & Chance, 1977). However, the distribution of H₂O₂ metabolism between the two enzymes depends on the site of generation of H₂O₂ within the cell, in peroxisomes, in mitochondria etc. Under conditions when H₂O₂ is generated near catalase, it is the main participant in H₂O₂ metabolism. When the glutathione peroxidase pathway is active, the GSSG that effuses from the cell is indicative of lipid peroxidation. Thus assays for both H₂O₂ and lipid peroxidation are feasible. The remainder of the discussion considers the separate and combined activities of the two pathways.

Livers from normal and tocopherol-deficient rat did not show a statistically significant increase in the rate of H₂O₂ production after hyperbaric oxygenation (see the legend of Table 3 for the measurement of H₂O₂ production). However, an enhanced rate of glutathione release, which is potentiated by tocopherol deficiency, is observed, namely a 1.9-fold increase in the normal rat and 3.6-fold increase in the tocopherol-deficient rat. In the lung also, although the H₂O₂ production was not determined, nutritional stress intensified the rate of glutathione release in hyperbaric oxygenation, and an additional starvation in the rat evoked a further acceleration of glutathione release: approx. 1.5-fold increase in the normal rat, 9.0-fold increase in the tocopherol-deficient and 13-fold increase in the tocopherol deficient starved rat (Table 3). In the liver, where no increase of H₂O₂ was observed, and probably in the lung as well, the greater part of the glutathione release depends on the reductive action of glutathione peroxidase on the lipid peroxides generated in the cell under hyperbaric oxygenation.

The speed of the onset of glutathione release and presumably lipid peroxidation is remarkable. Glutathione release is observed at the earliest times after O₂ compression, measurable with the flow-through technique in the liver. Chance *et al.* (1965, 1966) found a very rapid response to hyperbaric oxygenation in terms of an oxidative shift of mitochondrial nicotinamide nucleotides, which they attributed to an inhibition of reverse electron transfer. Thus there seem to be two early events in hyperbaric O₂ toxicity. The induction of more serious cell damage, such as modification of amino acid residues or thiol groups of membrane components, might not occur in these initial phases, fortifying the hypothesis that NADH oxidation and lipid peroxidation are the initial steps of damage.

The fraction of the damage caused directly by

superoxide anion (Fridovich, 1975) may be difficult to ascertain because of its extremely low concentration (Oshino & Chance, 1975b). If one is to take the induction of superoxide dismutase activity as evidence of participation of an hyperbaric O_2 response, it should be noted that 7 days are required for its elevation (Crapo & Tierney, 1974). Further, the oedema of the perfused lung under hyperbaric O_2 (Krebs-Ringer bicarbonate buffer + 5.5 mM-glucose was recirculated under 4.1×10^5 Pa of O_2 for 20 min at 30°C) is not significantly affected by infused superoxide dismutase (6 units/ml) to the perfusion fluid (K. Nishiki, N. Oshino & B. Chance, unpublished work), but the diminution of superoxide anion concentration by the perfused protein remains dubious, owing to the probable impermeability of the cells to the dismutase. On the other hand, these free-radical reactions are effectively interrupted by tocopherol as a natural scavenger (Tappel, 1962). Free-radical chain processes, well established for the autoxidation of hydrocarbons via hydrocarbon radicals are sensitive to O_2 ($k = 10^7$ M/s). Thus high concentrations of O_2 may serve effectively to trap radical species of unsaturated fatty acids and thereby initiate the chain reaction of peroxidation.

Two systems afford protection against free-

radical chain reaction involving lipid peroxidation in hyperbaric O_2 toxicity, glutathione (Jamieson & van den Brenk, 1962) and anti-oxidants (Jamieson & van den Brenk, 1964). These protective mechanisms may be functional under a variety of stresses, which include not only hyperbaric oxygenation but also exposure to NO_2 (Thomas *et al.*, 1969; Roehm *et al.*, 1971), CCl_4 (Recknagel, 1967), ethanol (DiLuzio & Hartman, 1967) and other free-radical-inducing agents (Cohen & Heikkila, 1974). If, however, the animal is not in a nutritionally stressed condition as a result of tocopherol deficiency, the increase of lipid peroxides (Tappel, 1962; Riely *et al.*, 1974) may still be under control of the glutathione peroxidase/glutathione reductase system. Thus failure to detect increased amounts of lipid peroxides under these conditions in an intact animal could explain the results of Bunyan *et al.* (1968), as well as those of Gómez *et al.* (1975), who showed hepatic necrosis in the absence of increased lipid peroxides in CCl_4 -poisoned mice.

Scheme 1 indicates a possible mechanism for O_2 toxicity based on these results. The primary action of O_2 is to initiate the free-radical chain reaction, which then propagates spontaneously with the consequent formation of lipid peroxides. The early period of

Scheme 1. Hypothetical reaction sequence related to O_2 toxicity in the cell

See the text for details. Step: 1, free-radical chain reaction; 2, lipid peroxide production; 3, glutathione peroxidase; 4, glutathione reductase; 5, NADPH-generating system; 6, energy-requiring transhydrogenase; 7, NADH-linked dehydrogenase reactions; 8, mitochondrial respiratory chain; 9, direct deleterious action of lipid peroxides, such as oxidation of thiol groups etc.; 10, maintenance of cellular thiol groups; 11, energy supply for the physiological function. Open thick arrows relate these reactions to their physiological functions. Closed thick arrows illustrate increases and decreases in the concentration of intracellular substances, upward and downward directions represent increasing and decreasing concentrations respectively.

hyperbaric oxygenation involves the metabolism of the lipid peroxide itself, which is reduced to the corresponding hydroxy fatty acid through the glutathione peroxidase reaction; the latter is linked to the reaction of glutathione oxidation and causes a partial release of GSSG. This process may result in a lack of thiol groups in the cell, leading to the inactivation of thiol-containing enzymes. In turn, intracellular GSSG is reduced via the glutathione reductase reaction, which is linked to NADPH oxidation. The extreme oxidation of NADPH may be accompanied by the oxidation of NADH via the energy-requiring transhydrogenation. As a result, an unphysiological decrease in the difference in oxidation/reduction potential between the NADH/NAD⁺ and O₂/water couples, which determines the phosphate potential, leads to a decrease in the mitochondrial respiration rate (Chance & Williams, 1956; Klingenberg & Schollmeyer, 1961; Wilson *et al.*, 1974). The accumulated effect of these events may give rise to an intracellular amount of lipid peroxide. Then all of these alterations may cause the pathological disruption of physiological functions, such as osmotic integrity and biosynthesis.

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