Changes in oxygen consumption induced by t-butyl hydroperoxide in perfused rat liver

Effect of free-radical scavengers

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The addition of t-butyl hydroperoxide to perfused rat liver elicited a biphasic effect on hepatic respiration. A rapid fall in liver oxygen consumption was initially observed, followed by a recovery phase leading to respiratory rates higher than the initial steady-state values of oxygen uptake. This overshoot in hepatic oxygen uptake was abolished by free-radical scavengers such as (+)-cyanidanol-3 or butylated hydroxyanisole at concentrations that did not alter mitochondrial respiration. (+)-Cyanidanol-3 was also able to facilitate the recovery of respiration, the diminution in the calculated rate of hydroperoxide utilization and the decrease in liver GSH content produced by two consecutive pulses of t-butyl hydroperoxide. It is suggested that the t-butyl hydroperoxide-induced overshoot in liver respiration is related to increased utilization of oxygen for lipid peroxidation as a consequence of free radicals produced in the scission of the hydroperoxide by cellular haemoproteins.

Organic hydroperoxides are largely reduced by the selenoenzyme glutathione peroxidase (EC 1.11.1.9) in the liver (Little & O'Brien, 1968; Chance *et al.*, 1979) at rates up to $3\text{--}4\ \mu\text{mol}\cdot\text{min}^{-1}\cdot(\text{g of liver})^{-1}$ (Sies & Summer, 1975). The coupling of this system with glutathione reductase (EC 1.6.4.2) has been shown to produce a number of metabolic perturbations, related to the NADPH–NADP and GSH–GSSG couples (Sies *et al.*, 1972). In fact, when t-butyl hydroperoxide is added to the perfused rat liver, NADPH is markedly oxidized, GSSG is released from the tissue, and a reversible respiratory inhibition develops (Sies *et al.*, 1972; Sies & Summer, 1975; Oshino & Chance, 1977). The inhibition of oxygen uptake by t-butyl hydroperoxide has also been observed in isolated liver cells and mitochondrial preparations and is apparently due to an alteration in the mitochondrial glutathione redox state, leading to thiol oxidation (Sies & Moss, 1978). This, in turn, was

suggested to inhibit the keto acid oxidation systems (e.g., oxidation of pyruvate and 2-oxoglutarate), which are dependent on lipoamide and coenzyme A (Sies & Moss, 1978). Under these conditions, the extensive nicotinamide nucleotide oxidation induced by t-butyl hydroperoxide is followed by an increased lipid peroxidation (Sies & Summer, 1975). In the present work, we have examined the effect of t-butyl hydroperoxide on the oxygen consumption of perfused rat liver in relation to the development of lipid peroxidation, as assessed by the action of free-radical scavengers.

Materials and methods

Haemoglobin-free liver perfusion

Livers from male Wistar rats of 150–200 g body wt., fed a commercial diet *ad libitum*, were perfused essentially as described by Sies (1978). Perfusate flow was 3.34 ± 0.06 (mean $\pm$ S.E.M.; $n = 25$) $\text{ml}\cdot\text{min}^{-1}\cdot(\text{g of liver})^{-1}$ and experiments were carried out at $36\text{--}37^\circ\text{C}$, using a perfusion fluid containing $118\text{ mM-NaCl}/1.2\text{ mM-KH}_2\text{PO}_4/1.2\text{ mM-MgSO}_4/2.5\text{ mM-CaCl}_2/25\text{ mM-NaHCO}_3/$

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

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10mM-glucose, equilibrated with O_2/CO_2 (19/1) to give pH 7.4 (Sies, 1978). The perfusion system was operated with recirculation, and stepwise additions of substances were performed by infusion of solutions (150ml) prepared separately.

Preparation of mitochondrial fractions

Rat liver mitochondria were isolated by a conventional procedure using 0.23M-mannitol/0.07M-sucrose/1mM-EDTA/5mM-Tris/HCl buffer, pH 7.4, as isolation medium (Boveris & Chance, 1973).

Assays

The oxygen uptake of the perfused liver and of mitochondrial preparations were measured polarographically (Estabrook, 1967), at 36–37°C and 30°C, respectively, with a Clark-type platinum electrode. In the perfused liver, oxygen consumption was calculated from the influent–effluent concentration difference and the corresponding flow rate and liver weight. Mitochondrial respiration was measured in a reaction medium containing 0.23M-mannitol, 0.07M-sucrose, 0.5mM-EDTA, 5mM- KH_2PO_4 , 4mM- $MgCl_2$ and 20mM-Tris/HCl buffer, pH 7.3. Either 5mM-malate + 5mM-glutamate or 10mM-succinate were used as substrates. State-3 respiration was elicited by adding 1.5mM-ADP. The mitochondrial protein concentration in the reaction media was 1.2–1.4mg/ml.

The concentration of hepatic GSH was determined in tissue samples (50–80mg) obtained from the perfused livers. GSH was measured with 5,5'-dithiobis-(2-nitrobenzoate) at 412nm in the supernatants from 2% (w/v) liver homogenates prepared in cold 0.5M- $HClO_4$ and centrifuged at 2500g for

10min at 4°C. Samples were preincubated at 60°C for 5min with 0.5M-phosphate buffer, pH 6.8, containing 5.4mM-glyoxylic acid to remove cysteine and 5.7mM-ascorbic acid to prevent GSH oxidation (Ball, 1966).

Protein was determined as described by Lowry *et al.* (1951). All chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results and discussion

Fig. 1 shows the effect of *t*-butyl hydroperoxide on hepatic respiration as seen when the hydroperoxide is added to the recirculated perfused rat liver. It can be observed that *t*-butyl hydroperoxide, added to the liver-perfusion system, elicited a biphasic effect on hepatic oxygen consumption (Fig. 1) as found previously (Sies *et al.*, 1972). This effect was characterized by an initial rapid fall in the rate of oxygen uptake, which was found to be dependent on the concentration of the hydroperoxide added in the range 0.25–1.0mM (Fig. 1). The inhibition of hepatic respiration by *t*-butyl hydroperoxide was followed by a recovery phase in which oxygen uptake increased above the original steady-state values of oxygen consumption (Figs. 1 and 2). Furthermore, the addition of two consecutive pulses of 0.5mM-*t*-butyl hydroperoxide to the perfused liver produced oxygen-uptake overshoots of 0.11 and 0.26 $\mu\text{mol of oxygen} \cdot \text{min}^{-1} \cdot (\text{g of liver})^{-1}$ respectively (Fig. 2). These increases in hepatic oxygen consumption were completely abolished when the pulses of the hydroperoxide were carried out in the presence, in the perfusate, of 2mM-(+)-cyanidanol-3 or 100 μM -butylated hydroxyanisole (Fig. 2), two effective free-radical scavengers (Slater, 1979; Videla, 1983;

Fig. 1. Changes in oxygen consumption after the addition of *t*-butyl hydroperoxide (tBH) to perfused rat liver. Experimental conditions were as described in the Materials and methods section. The effect of different concentrations of *t*-butyl hydroperoxide added as a single pulse in four separate experiments is shown. Numbers adjacent to the traces indicate decreases (–) or increases in oxygen uptake with respect to initial values, expressed as $\mu\text{mol of oxygen} \cdot \text{min}^{-1} \cdot (\text{g of liver})^{-1}$.

Fig. 2. Effect of (+)-cyanidanol-3 (Cy3) and butylated hydroxyanisole (BHA) on the changes in oxygen consumption elicited by pulses of t-butyl hydroperoxide (tBH) in recirculated perfused rat livers

Experimental conditions were as described in the Materials and methods section. The traces represent the effect of two consecutive pulses of 0.5 mM-t-butyl hydroperoxide in three separate perfusions carried out either with no additions or in the presence of 2 mM-(+)-cyanidanol-3 or 100 μ M-butylated hydroxyanisole. Numbers adjacent to the traces indicate the increases in oxygen uptake after the pulses of t-butyl hydroperoxide with respect to the initial value, expressed as μ mol of oxygen $\cdot$ min $^{-1}$ $\cdot$ (g of liver) $^{-1}$.

Fig. 3. Effect of (+)-cyanidanol-3 and butylated hydroxyanisole on mitochondrial respiration

Experimental conditions were as described in the Materials and methods section. The values shown represent three separate mitochondrial preparations incubated in the absence (○) or presence of different concentrations of (+)-cyanidanol-3 (●) or butylated hydroxyanisole (■) under State-4 or State-3 (1.5 mM-ADP) conditions, as defined by Chance & Williams (1956).

Labuza, 1971). It is important to point out that the suppression of t-butyl hydroperoxide-induced overshoot in hepatic respiration by the free-radical scavengers was observed at concentrations that did not modify the mitochondrial oxygen uptake, assessed in isolated rat liver mitochondria under State 4 and State 3, with either succinate or malate plus glutamate as substrates (Fig. 3). This fact eliminates a possible inhibition of mitochondrial respiration by these agents. These data suggest that the t-butyl hydroperoxide-induced overshoot in oxygen consumption could be related to a lipid-peroxidation process derived from the interaction of the hydroperoxide with cellular haemoproteins. In fact, when t-butyl hydroperoxide is present in the hepatocyte, it can react with microsomal cytochrome *P*-450 (Cadenas & Sies, 1982) and mitochondrial cytochrome *c* (Cadenas *et al.*, 1980), in addition to its utilization by the glutathione peroxidase system. Both cytochromes can catalyse the homolytic scission of the hydroperoxide, producing hydroxyl and t-butyl alkoxy radicals, cytochrome *P*-450 being about 70 times more effective than cytochrome *c* (Cadenas & Sies, 1982). The generation of these free-radical species will promote the radical chain reaction of lipid peroxidation, thus increasing oxygen utilization. The observation of an overshoot in hepatic respiration by t-butyl hydroperoxide which is sensitive to free-radical scavengers is in agreement with the lipid-peroxidation process observed in isolated hepatocytes (Sies & Summer, 1975) or in microsomal fractions (Cadenas & Sies, 1982) on addition of the hydroperoxide. In the latter study, microsomal

Fig. 4. Recovery time ($t_{i,off}$) for the inhibition of oxygen consumption in recirculated perfused liver on addition of *t*-butyl hydroperoxide

Experimental conditions were as described in the Materials and methods section. Results shown were collected from 16 different perfusions from traces similar to those shown in Figs. 1 and 2. $t_{i,off}$ values were calculated for either one (●) or two (■) pulses of *t*-butyl hydroperoxide under normal conditions or from one (○) or two (□) pulses in the presence of 2mM-(+)-cyanidanol-3. The lines shown were obtained by regression analysis.

malonaldehyde accumulation was found to occur concomitantly with enhanced light emission and oxygen uptake (Cadenas & Sies, 1982).

The existence of a biphasic effect of *t*-butyl hydroperoxide on hepatic respiration allows the calculation of the recovery time ($t_{i,off}$) (Chance, 1957; Boveris *et al.*, 1971), i.e., the interval from half-inhibition to half-recovery of oxygen uptake, a parameter that has been evaluated for the NADPH oxidation-reduction changes produced by *t*-butyl hydroperoxide in isolated hepatocytes, monitored by absorbance-difference spectrophotometry (Sies & Summer, 1975). Fig. 4 shows the plots of different concentrations of *t*-butyl hydroperoxide added to recirculated perfused livers versus the respective $t_{i,off}$ values, collected from 16 different perfusions. As the Figure shows, there is a linear relationship between these two parameters when assessed after the first or second pulses of *t*-butyl hydroperoxide, either in absence or presence of 2mM-(+)-cyanidanol-3 (Fig. 4). The slopes of the straight lines obtained by regression analysis express the 50% utilization of *t*-butyl hydroperoxide by the perfused liver and can be con-

verted into full utilization rates by multiplying by 2 and referring to liver weight. The assumption is made that the recovery of respiration correlates with the degradation of *t*-butyl hydroperoxide (Sies & Summer, 1975). Data for pulses of 0.5mM-*t*-butyl hydroperoxide are presented in Table 1, showing that the first pulse of the hydroperoxide gives a $t_{i,off}$ of 3.05 min and a calculated rate of hydroperoxide utilization of $3.68 \mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{g of liver})^{-1}$, which is similar to the values previously reported by Sies & Summer (1975). The concentration of hepatic GSH before the first pulse of *t*-butyl hydroperoxide was $4.18 \mu\text{mol} \cdot (\text{g of liver})^{-1}$, and it decreased to $1.00 \mu\text{mol} \cdot (\text{g of liver})^{-1}$ after the hydroperoxide had been metabolized and a new steady-state respiration had been reached (Table 1; Fig. 2). In this situation, the addition of a second equal pulse of the hydroperoxide resulted in a marked prolongation of the $t_{i,off}$ and a diminished rate of hydroperoxide utilization (Table 1). However, if these experiments are carried out in the presence of 2mM-(+)-cyanidanol-3, the flavonoid is able to abolish 50% of the decrease in the GSH content of the perfused liver induced by the first pulse of *t*-butyl hydroperoxide (Table 1). Under these conditions the $t_{i,off}$ for the second pulse of the hydroperoxide and its calculated utilization rate are significantly less affected (Table 1). These observations indicate that GSH utilization by the liver on *t*-butyl hydroperoxide addition seems to be diverted into two pathways: (a) a (+)-cyanidanol-insensitive mechanism probably related to hydroperoxide reduction catalysed by the glutathione peroxidase system, and (b) a (+)-cyanidanol-3 sensitive mechanism in which GSH could directly scavenge the free radicals (Videla, 1983) generated in the interaction of the hydroperoxide with cellular haemoproteins and/or those produced by the stimulation of lipid peroxidation observed in this situation (Sies & Summer, 1975; Cadenas & Sies, 1982). The presence of (+)-cyanidanol-3 in the liver would scavenge these free radicals by virtue of its powerful antioxidant activity (Slater, 1979; Videla, 1983), thus sparing cellular GSH.

In conclusion, the addition of *t*-butyl hydroperoxide to the perfused liver elicited a reversible inhibition on the oxygen consumption, leading to a respiratory overshoot. The latter phenomenon seems to be related to an enhanced oxygen demand for the lipid-peroxidation process that might set in by the free radicals arising from the interaction of the hydroperoxide with cellular haemoproteins. This view is supported by the abolishment of the respiratory overshoot by free-radical scavengers, which inhibit lipid peroxidation, and also by their sparing effect on hepatic GSH. Finally, the addition of 2mM-(+)-cyanidanol-3 or 100 μM -butylated hydroxyanisole to perfused rat liver elicited a de-

Table 1. Recovery times ($t_{1,off}$) and hepatic GSH levels after one or two pulses of t-butyl hydroperoxide in recirculated perfused rat liver in the absence and presence of (+)-cyanidanol-3

Results represent means $\pm$ S.E.M. for the number of different perfusion experiments shown in parentheses. $t_{1,off}$ corresponds to the interval from half-inhibition to half-recovery of oxygen uptake after the addition of 0.5 mM-t-butyl hydroperoxide. The hepatic GSH contents shown represent the values found in liver samples obtained immediately before the first or second addition of t-butyl hydroperoxide to livers perfused with or without 2 mM-(+)-cyanidanol-3, as indicated in Fig. 2. Statistical analysis for all parameters shown was carried out by Student's *t* test for unpaired results: C versus A, B and D ($P < 0.01$); D versus A and B ($P < 0.02$).

t-Butyl hydroperoxide (0.5 mM)	(+)-Cyanidanol-3 (2 mM)	$t_{1,off}$ (min)	Calculated rate of t-butyl hydroperoxide utilization [$\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{g of liver})^{-1}$]	[Liver GSH] [$\mu\text{mol} \cdot (\text{g of liver})^{-1}$]
First pulse A	—	3.05 ± 0.08 (8)	3.68 ± 0.18 (8)	4.18 ± 0.35 (4)
B	+	3.11 ± 0.20 (4)	3.15 ± 0.37 (4)	4.53 ± 0.24 (4)
Second pulse C	—	6.74 ± 0.11 (5)	1.68 ± 0.28 (5)	1.00 ± 0.11 (4)
D	+	4.54 ± 0.32 (4)	2.29 ± 0.30 (4)	2.95 ± 0.14 (4)

crease in the basal oxygen consumption of about 0.10 to 0.12 $\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{g of liver})^{-1}$ (Fig. 2). The characteristics of this basal antioxidant-sensitive respiration and its modification by pro-oxidant agents are dealt with in the following paper (Videla, 1984).

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