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Hepatic antioxidant-sensitive respiration

Effect of ethanol, iron and mitochondrial uncoupling

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The addition of the antioxidants (+)-cyanidanol-3, butylated hydroxyanisole and ascorbate to the perfused rat liver resulted in a decrease in the rate of oxygen consumption. This basal antioxidant-sensitive respiration of 110–130 nmol·min^{-1}·(g of liver)$^{-1}$ represents 5–7% of total respiration. Increased antioxidant-sensitive respiratory rates are found after the infusion of increasing concentrations of ethanol (1.8–72.2 mM) or iron (35.5–248.5 μM). This respiratory component exhibits a dependence on ethanol or iron concentration, with maximal rates of 200–255 and 330 nmol·min^{-1}·(g of liver)$^{-1}$ respectively. After the addition of 100 μM-2,4-dinitriphenol, an antioxidant-sensitive respiratory component of 230 nmol·min^{-1}·(g of liver)$^{-1}$ is found, which is not observed at lower concentrations of the uncoupler (5–50 μM). The lack of effect of the antioxidants used on mitochondrial respiration [the preceding paper, Videla, Villena, Donoso, Giulivi & Boveris (1984) *Biochem. J.* **223**, 879–883] and on the glycolytic rate of the perfused liver suggests that the basal and chemically induced antioxidant-sensitive respiration observed are related to oxygen required for one-electron transfer reactions associated with the generation of active species of oxygen and lipid peroxidation in the liver cell.

Oxidative metabolism in aerobic cells is often associated with the generation of oxygen-related species with intermediate reduction states, e.g., superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) (Fridovich, 1978; Singh, 1982). These species can interact with cellular components, producing structural and functional alterations (Tappel, 1973; Kappus & Sies, 1981). Among them, lipid peroxidation is a process related to aerobic metabolism normally kept at a level compatible with cellular life by different antioxidant defensive mechanisms (Forman & Fischer, 1981). In certain situations, however, lipid peroxidation can be drastically enhanced and the defensive mechanisms of the cell might be overcome, leading to widespread pathological consequences (Recknagel *et al.*, 1982). Of particular interest are a variety of toxigenic substances that have a lipid-peroxidative action associated with their metabolism (carbon tetrachloride, ethanol, acetaminophen etc.) (Wendel & Feuerstein, 1981; Recknagel *et al.*, 1982; Videla & Valenzuela, 1982), whereas other agents, such as iron, are able to induce cellular lipid peroxidation by acting as direct catalysts of the process (Svingen *et al.*, 1979), and have been shown to potentiate the peroxidative response of the liver to other xenobiotics, e.g. ethanol (Valenzuela *et al.*, 1983).

The chemistry of lipid peroxidation indicates a requirement for oxygen in the reaction pathways leading to hydroperoxide and endoperoxide generation (Recknagel *et al.*, 1982; Singh, 1982). However, the assessment of the respiratory component associated with cellular lipid peroxidation is difficult, owing to the presence of an enzymic mechanism that can dismutate $O_2^{-\cdot}$ and H_2O_2, the initial species involved in the univalent pathway of oxygen reduction (Hamilton, 1974), in which O_2 is evolved. Results given in the preceding paper (Videla *et al.*, 1984) showed that the addition of (+)-cyanidanol-3 or butylated hydroxyanisole to the perfused rat liver system was able to produce a diminution in hepatic oxygen consumption. Since these agents are known to be effective free-radical scavengers (Slater & Eakins, 1975; Videla, 1983; Labuza, 1971) and they did not alter mitochondrial

Abbreviation used: LDH, lactate dehydrogenase.

respiration at the concentration used (Videla *et al.*, 1984), it is likely that this respiratory component is representing oxygen uptake related to the lipid-peroxidative process. The present work deals with the assessment of this basal antioxidant-sensitive respiratory rate in the non-recirculating haemoglobin-free perfused rat liver and its modification by agents known to enhance lipid peroxidation (e.g. ethanol and iron) or by mitochondrial uncoupling (2,4-dinitrophenol).

Materials and methods

Male albino rats (Wistar strain) weighing 240–250g with a liver/body wt. ratio of 3.57 ± 0.05 (mean ± S.E.M.) ($n = 62$) were kept on a standard chow diet and water *ad libitum*. Before surgical preparation, the animals were anaesthetized with 50mg of nembutal/kg, intraperitoneally. The perfusion technique was that described by Sies (1978), with a perfusion fluid containing 118mM-NaCl, 1.2mM-KH_2PO_4, 1.2mM-$MgSO_4$, 2.5mM-$CaCl_2$, 25mM-$NaHCO_3$ and 10mM-glucose, equilibrated with a O_2/CO_2 mixture (19:1) to give pH 7.4. Perfusate flow rates [3.72 ± 0.08 ml·min^{-1}·(g of liver)$^{-1}$ ($n = 62$)] were maintained constant throughout individual experiments. The effluent perfusion fluid was collected via a cannula placed in the vena cava and allowed to flow past a Clark-type oxygen electrode. Perfusions were carried out at 36–37°C without recirculation of the perfusate. Stepwise additions of substance were performed by infusion of 150–200ml of different solutions, prepared separately and continuously oxygenated. Rates of oxygen consumption were calculated from arterio–venous concentration differences, referred to the corresponding flow rate and wet liver weight. Basal antioxidant-sensitive respiration corresponds to the difference in oxygen uptake in the absence and presence of antioxidant. Antioxidants were employed at the following concentrations: (+)-cyanidanol-, 2mM; butylated hydroxyanisole, 100μM; or ascorbate, 100μM. Chemically induced antioxidant-sensitive respiration corresponds to the difference in oxygen consumption by livers perfused with increasing concentrations of pro-oxidant agents such as ethanol or iron (iron/dextran/Imferon; Fisons), and 2,4-dinitrophenol as a mitochondrial uncoupler, in the absence and presence of antioxidants. In these calculations the basal antioxidant-sensitive respiration has been discounted. The concentration of ethanol in the influent perfusion fluid was determined enzymically (Hawkins *et al.*, 1966) and that of non-haem iron was measured by the bathophenanthroline assay (Diagnostica Merck, Merckotest no. 3307). In separate control experiments, samples of the effluent perfusion fluid were collected every 5min for 30min and analysed for lactate (Gutmann & Wahlefeld, 1974) and pyruvate (Czok & Lamprecht, 1974). Lactate dehydrogenase activity released from the perfused livers into the effluent perfusate was routinely measured (Bergmeyer & Bernt, 1974) as an integrity parameter (Sies, 1978) and was compared with the activity in the tissue. All chemicals used were obtained from Sigma (St. Louis, MO, U.S.A.). Values shown represent the mean ± S.E.M. for the number of perfusions indicated.

Results and discussion

Liver basal antioxidant-sensitive respiration

Hepatic oxygen consumption is known to be dependent on the metabolic performance of the organ (Scholz & Bücher, 1965; Brauser *et al.*, 1972). The state of the respiratory chain in the stationary respiring liver from fed rats perfused with substrates approaches the controlled state (i.e. State 4) of coupled mitochondria *in vitro* (Brauser *et al.*, 1972). It has been estimated that about 80% of this stationary oxygen consumption depends on mitochondrial respiratory-chain activity (cyanide-sensitive respiration) (Scholz & Bücher, 1965), in which cytochrome oxidase elicits the tetravalent reduction of oxygen without the generation of intermediates of oxygen reduction (Fridovich, 1978). However, univalent reduction of oxygen occurs in several cytosolic, microsomal and mitochondrial enzymic reactions, with the concomitant release of superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and, indirectly, hydroxyl radicals ($HO^{\cdot}$) (Fridovich, 1978; Chance *et al.*, 1979; Kappus & Sies, 1981). The generation of these intermediates has been postulated to play a role in cellular oxygen toxicity which includes the oxygen-dependent process of lipid peroxidation (Kappus & Sies, 1981). Results presented here show that the addition of different antioxidant substances to the perfused rat liver under steady-state conditions resulted in a consistent decrease in the rate of oxygen consumption. In fact, as can be observed in Fig. 1, 2mM-(+)-cyanidanol-3 elicited a diminution in hepatic oxygen uptake of 130nmol·min^{1-}·(g of liver)$^{-1}$ over an experimental period of 70min. Data collected from 18 different perfusions (Figs. 1, 2*a*, 4*b* and 6*a*) indicate a basal (+)-cyanidanol-3-sensitive respiration of 110 ± 10nmol·min^{-1}·(g of liver)$^{-1}$. Moreover, the addition of 100μM-butylated hydroxyanisole or 100μM-ascorbate resulted in basal antioxidant-sensitive respiration of 130 ± 20nmol·min^{-1}·(g of liver)$^{-1}$ ($n = 7$; from Fig. 2*a*) and 120 ± 15 ($n = 5$) [oxygen uptake without additions = 1.88 ± 0.04 (5) μmol·min^{-1}·(g of liver)$^{-1}$; with 100μM-ascorbate = 1.76 ± 0.04 (5)] respectively. During liver perfusions, LDH activity in the

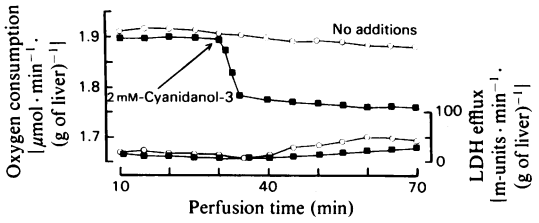

Fig. 1. *Effect of (+)-cyanidanol-3 on hepatic oxygen consumption by the non-recirculated haemoglobin-free perfused rat liver*

The values for oxygen uptake (upper curves) and LDH efflux (lower curves) shown are from two separate infusion experiments. ■, +2mM-Cyanidanol-3; ○, no additions.

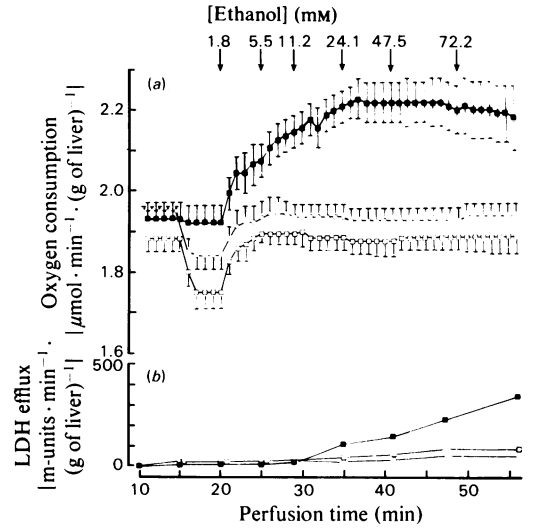

Fig. 2. *Oxygen consumption by perfused rat liver as a function of the concentration of ethanol, added alone* (■) *(n = 7) or in the presence of 2mM-(+)-cyanidanol-3* (○) *(n = 7) or 100 μM-butylated hydroxyanisole* (□) *(n = 7)*

Results represent means ± S.E.M. for oxygen uptake (*a*) and LDH efflux (*b*).

perfusate was routinely measured as an integrity parameter (Sies, 1978). As shown in Fig. 4(*b*), the time course in control experiments resulted in LDH effluxes of 10–50 m-units·min^{-1}·(g of liver)$^{-1}$, which correspond to less than 0.1% of the activity in the tissue [181.6 ± 5.1 ($n = 6$) units·(g of liver)$^{-1}$] for up to 70 min perfusion. This LDH efflux was not altered by the addition of 2mM-(+)-cyanidanol-3 (Figs. 1 and 4*b*), 100 μM-butylated hydroxyanisole (Fig. 2*b*) or 100 μM-ascorbate (results not shown).

The decrease in hepatic oxygen consumption by (+)-cyanidanol-3, butylated hydroxyanisole and ascorbate were observed at concentrations that did not modify mitochondrial respiration (Videla *et al.*, 1984). Further, the possibility that the rate of oxygen consumption could be modified by changes in the glycolytic rate of the liver (Yuki & Thurman, 1980) produced by the addition of the antioxidants under study can be discarded, since the production of lactate + pyruvate by the perfused liver was not significantly changed under these conditions [control: 1.70 ± 0.16 ($n = 4$) μmol·min^{-1}·(g of liver)$^{-1}$; 2mM-(+)-cyanidanol-3: 1.65 ± 0.18 ($n = 4$); 100 μM-butylated hydroxyanisole: 1.78 ± 0.15 ($n = 4$); 100 μM-ascorbate: 1.68 ± 0.12 ($n = 4$)]. Thus, in view of these observations and the powerful free-radical-scavenging capacity exhibited by (+)-cyanidanol-3 (Slater & Eakins, 1975; Videla, 1983), butylated hydroxyanisole (Labuza, 1971) and ascorbate (Forman & Fisher, 1981), it is suggested that the respiratory component of hepatic oxygen uptake, which is abolished by these agents, could probably be related to the oxygen required in either one-electron transfer reactions, in the maintenance of a basal lipid peroxidative rate, or both. However, further studies are needed to clarify this view. If basal antioxidant-sensitive respiratory rates [110–130 nmol·min^{-1}·(g of liver)$^{-1}$] are referred to the respective stationary rates of oxygen consumption [1.76–1.96 μmol·min^{-1}·(g of liver)$^{-1}$] (Figs. 1, 2, 4 and 6) the antioxidant-sensitive component can account for 5–7% of the total hepatic respiration. This is in agreement with the previous suggestion that oxygen utilization via superoxide-radical generation must represent a minor pathway during cellular aerobic metabolism (Fridovich, 1978).

Liver chemically induced antioxidant-sensitive respiration

The administration of a variety of xenobiotics to experimental animals has been shown to produce an enhancement in hepatic free-radical-induced lipid peroxidation (Plaa & Witschi, 1976; Videla & Valenzuela, 1982; Recknagel *et al.*, 1982). Among them, ethanol and iron treatments increased malonaldehyde production by the liver and its biliary release, effects that were additive when both agents were administered simultaneously (Valenzuela *et al.*, 1983). In the present experiments, the addition of increasing concentrations of ethanol (from 1.8 to 72.2mM) to perfused rat livers elicited an increase in the rate of oxygen consumption over the steady-state value, which was maximal at about 24mM-ethanol (Fig. 2*a*). Perfusions with ethanol carried out in the presence of 2mM-(+)-cyanidanol-3 or 100 μM-butylated hydroxyanisole also exhibit a similar effect on hepatic respiration, although of a smaller magnitude, reaching respiratory rates comparable with the initial steady-state values (Fig. 2*a*). From these data, ethanol-induced antioxidant-sensitive re-

spiratory rates were calculated as described in the Materials and methods section and are presented in Fig. 3. As can be observed, there is a dependence of this respiratory component on ethanol concentration (Fig. 3), the analysis of which resulted in maximal rates of 203 and 255 nmol·min^{-1}·(g of liver)$^{-1}$ when assessed with butylated hydroxyanisole and (+)-cyanidanol-3 respectively (Fig. 3). In both situations, half-maximal rates were found at about 8 mM-ethanol (Fig. 3). These results indicate that the increase in oxygen consumption by ethanol infusion in the perfused liver has two components: (*a*) a respiratory component [80–130 nmol·min^{-1}·(g of liver)$^{-1}$; Fig. 2*a*] not abolished by the presence of antioxidants and which is probably reflecting the introduction of ethanol as an alternative oxidizable substrate to the perfused liver supplied only with glucose, and (*b*) a component sensitive to the antioxidants used, which more than doubles the basal antioxidant respiration (Figs. 1 and 2) and represents a net 12% of the total oxygen uptake of the liver. The sensitivity of hepatic oxygen consumption to agents with antioxidant capacity suggests the involvement of free-radical species during ethanol and/or acetaldehyde

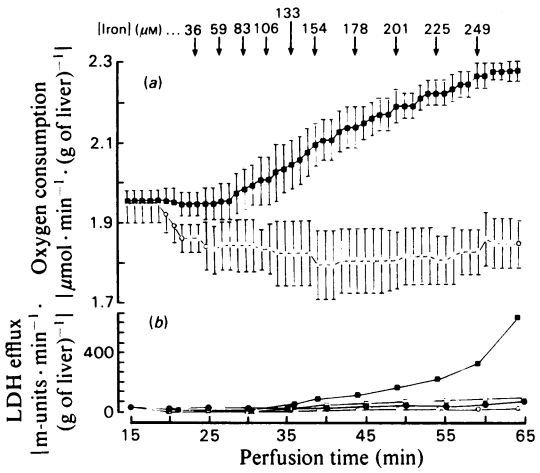

Fig. 4. *Oxygen uptake by perfused rat liver as a function of the concentration of iron added alone* (■) ($n = 5$) *or in the presence of 2 mM-(+)-cyanidanol-3* (○) ($n = 5$)
Results represent means ± S.E.M. for oxygen uptake (*a*) and LDH efflux (*b*). In addition, in (*b*): ●, no additions; □, cyanidanol-3 alone.

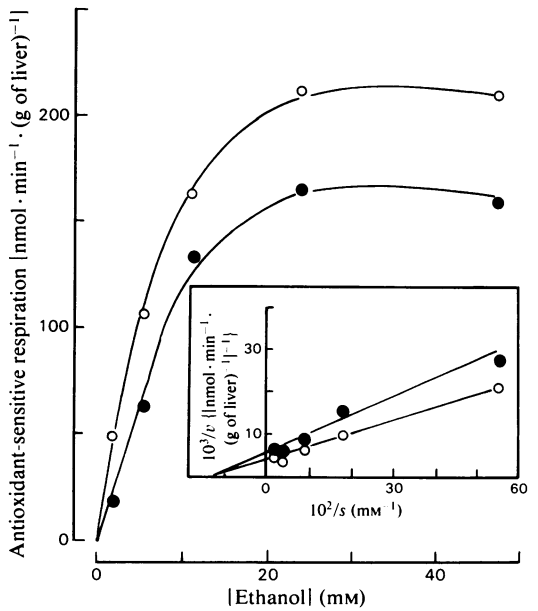

Fig. 3. *Calculated rates of hepatic antioxidant-sensitive respiration induced by ethanol infusion as a function of ethanol concentration (inset: Lineweaver–Burk plot of ethanol-induced antioxidant respiration)*
Ethanol-induced antioxidant-sensitive respiratory rates were calculated as described in the Materials and methods section, from the average values shown in Fig. 2(*a*). ○, Cyanidanol-3 (2 mM); ●, butylated hydroxyanisole (100 μM).

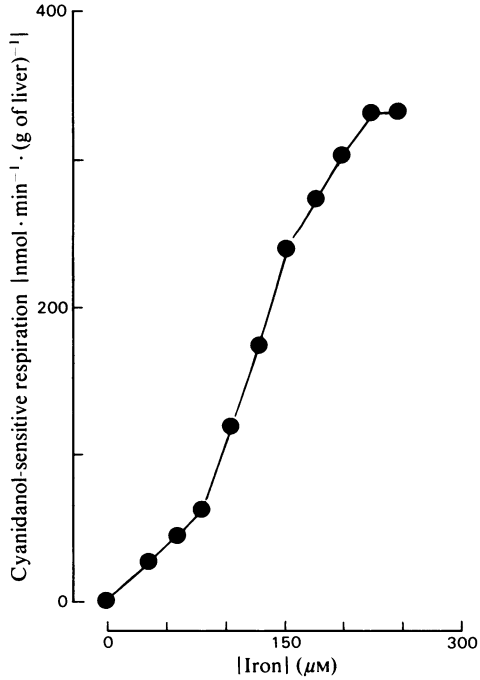

Fig. 5. *Calculated rates of a hepatic (+)-cyanidanol-3-sensitive respiration induced by iron infusion as a function of iron concentration*
Iron-induced (+)-cyanidanol-3-sensitive respiratory rates were calculated as described in the Materials and methods section, from the average values shown in Fig. 4.

metabolism in the liver. This is in agreement with previous studies showing an inhibitory effect of (+)-cyanidanol-3 on hepatic lipid peroxidation, determined either as ethane release from the perfused rat liver during the infusion of 32mM-ethanol (Müller & Sies, 1982) or as formation of hepatic diene conjugates after the administration *in vivo* of 5g of ethanol/kg (Videla *et al.*, 1981).

The effect of increasing concentrations of iron (35.5–248.5 μM) on the rate of oxygen consumption by the perfused rat liver is shown in Fig. 4(*a*). As can be seen, iron addition also enhanced the hepatic oxygen uptake as found with ethanol (Fig. 2*a*). However, in the presence of 2mM-(+)-cyanidanol-3, iron was unable to increase hepatic respiration, which is most likely due to the fact that iron is not a metabolic substrate for the liver (Fig. 4*a*). Fig. 5 shows the calculated iron-induced (+)-cyanidanol-3-sensitive respiratory rate during perfusion. This rate was found to depend on iron concentration, to have a maximal value of 336nmol·min^{-1}·(g of liver)$^{-1}$ (17% of total oxygen uptake) and an apparent K_m of 120μM-iron (Fig. 5).

The antioxidant-sensitive respiration induced when hepatic oxygen consumption was increased

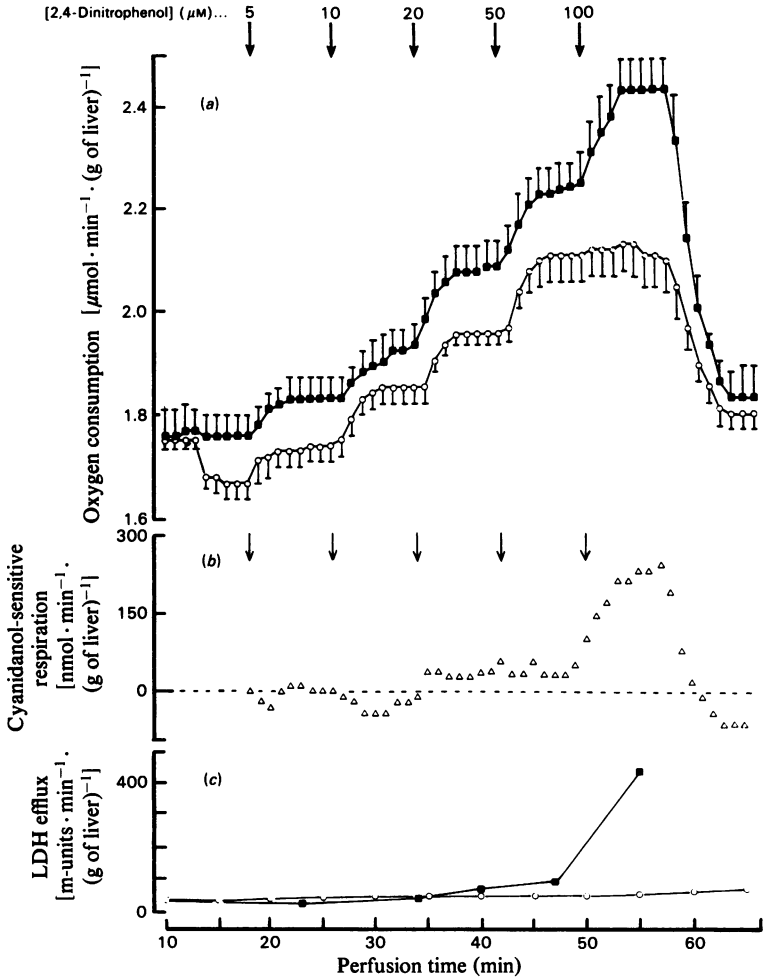

Fig. 6. *Oxygen consumption by perfused rat liver as a function of the concentration of 2,4-dinitrophenol, added alone* (■) *(n = 6) or in the presence of 2mM-(+)-cyanidanol-3* (○) *(n = 6)*

Results represent means ± S.E.M. for oxygen uptake (*a*). 2,4-Dinitrophenol-induced (+)-cyanidanol-3-sensitive respiratory rates (*b*) were calculated as described in the Materials and methods section, from the average values shown in (*a*). Values shown in (*c*) correspond to the respective LDH effluxes.

by ethanol (Figs. 2 and 3) or iron (Figs. 4 and 5) was compared with a condition in which liver oxygen uptake was enhanced by mitochondrial uncoupling. As can be observed in Fig. 6(*a*), the addition of 5–100 μM-2,4-dinitrophenol to the perfused liver resulted in a progressive increase in hepatic respiration, which readily returned to the initial steady-state values on cessation of the infusion of the uncoupler. In the presence of 2 mM-(+)-cyanidanol-3, the addition of 5–50 μM-2,4-dinitrophenol was also able to stimulate respiration (Fig. 6*a*) so that the (+)-cyanidanol-3-sensitive respiration induced by the uncoupler at these concentrations was approximately zero (Fig. 6*b*). However, a (+)-cyanidanol-3-sensitive respiration was observed at a 2,4-dinitrophenol concentration of 100 μM (Fig. 6*a* and 6*b*). This 2,4-dinitrophenol-induced (+)-cyanidanol-3-sensitive respiratory component reached a maximal value of about 230 nmol·min^{-1}·(g of liver)$^{-1}$ (13% of total oxygen uptake) and it readily ceased after the infusion of the uncoupler was ended (Fig. 6*b*). The existence of this respiratory component at high concentrations of 2,4-dinitrophenol could be due to the metabolic state imposed in this condition. It has been suggested that a destabilization of some of the components of the respiratory chain could occur in mitochondria with a reduced respiratory control (Dryer *et al.*, 1980). This, in turn, would enhance one-electron reduction of oxygen, the production of superoxide radicals and that of hydrogen peroxide via superoxide dismutase (Dryer *et al.*, 1980). In agreement with this view, it has been reported that mitochondrial respiratory chain can generate superoxide radicals at the NADH dehydrogenase as well as at the ubiquinone–cytochrome *b* sites (Turrens & Boveris, 1980).

The present results indicate that the addition of agents with antioxidant activity [e.g. (+)-cyanidanol-3, butylated hydroxyanisole and ascorbate] to the perfused rat liver produces a basal antioxidant-sensitive respiration of 110–130 nmol·min^{-1}·(g of liver)$^{-1}$, which corresponds to 5–7% of the total oxygen uptake by the tissue. The infusions of pro-oxidant agents such as ethanol or iron were able to enhance the antioxidant-sensitive respiration up to rates of 200–330 nmol·min^{-1}·(g of liver)$^{-1}$, which are comparable with those observed after mitochondrial uncoupling with high concentrations of 2,4-dinitrophenol. Thus, during chemical-induced enhancement of hepatic oxygen consumption, total antioxidant sensitive respiration can amount to up to rates of 310–460 nmol·min^{-1}·(g of liver)$^{-1}$, i.e. 16–24% of total hepatic respiration.

Finally, it is important to point out that the increased rates of hepatic oxygen consumption induced by high concentrations of ethanol, iron and 2,4-dinitrophenol were found to occur concomitantly with increased LDH effluxes from the perfused livers (Figs. 2, 4 and 6). Since these changes were not observed in the presence of antioxidants, it is suggested that the development of high rates of antioxidant-sensitive respiration might affect the integrity of the liver, altering the permeability properties of the plasma membrane of the hepatocytes.

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