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Effect of Acetaminophen Administration on Hepatic Glutathione Compartmentation and Mitochondrial Energy Metabolism in the Rat

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ABSTRACT. Changes in cell energy metabolism and mitochondrial dysfunction have been observed after acetaminophen administration. Because consumption of hepatic glutathione is closely related to acetaminophen toxicity, we investigated the kinetics of: 1. glutathione depletion in liver mitochondria and cytosol; 2. State 3 and 4 respiratory rates of succinate-supplemented mitochondria; 3. rate of ATP synthesis; 4. oligomycin-sensitive ATP hydrolase activity and passive proton conductivity of inside-out vesicles of the inner mitochondrial membrane; and 5. changes in hepatic and mitochondrial malondialdehyde in the rat after in vivo acetaminophen administration. Two hours after acetaminophen injection, hepatic glutathione decreased and malondialdehyde increased. In the same interval, an increase in both State 3 and 4 respiratory rates of succinate-supplemented mitochondria was observed. This was accompanied by a decrease in the rate of ATP synthesis and the P/O ratio and by an increase in the passive proton permeability of the inner mitochondrial membrane, which was insensitive to oligomycin. No significant change in oligomycin-sensitive ATP hydrolase activity was observed. Four hours after APAP injection, the respiratory rates, as well as the proton conductivity, decreased, the rate of ATP synthesis was restored, and the mitochondrial glutathione started to increase; the cytosolic levels of glutathione were still low and the cytosolic and mitochondrial levels of malondialdehyde remained high for 2 more hr. The concentrations of these indices were completely restored 24 hr postdosing. Our findings suggest that acetaminophen administration selectively depletes (within 2 hr) mitochondrial glutathione, and produces local toxicity by altering membrane permeability and decreasing the efficiency of oxidative phosphorylation. This renders mitochondria more susceptible to oxidative damage, especially during increased free radical production, as in the case of enhanced mitochondrial respiration in State 4. The concomitant restoration of mitochondrial respiration, oxidative phosphorylation, membrane permeability, and glutathione levels is consistent with the importance of the mitochondrial glutathione pool for the protection of the mitochondrial membrane against oxidative damage. BIOCHEM PHARMACOL 52;8:1147–1154, 1996.

KEY WORDS. acetaminophen; glutathione; intracellular glutathione compartmentation; mitochondrial energy metabolism; mitochondrial proton permeability; lipid peroxidation

It is well known that toxic doses of APAP§, a widely used antipyretic and analgesic drug, can cause hepatocellular necrosis [1, 2]. In particular, the mechanism of cell damage appears to be mediated by the metabolic activation of APAP to a highly reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which is able to delete hepatocellular glutathione (GSH) and to covalently bind to cellular macromolecules [3]. The concentration of intracellular GSH, therefore, is a key determinant of the extent of APAP-induced hepatic injury. The binding of GSH to activated APAP appears to be an obligatory step in the detoxification process, and toxicity occurs only when glutathione stores fall below critical concentrations [4]. Because mitochondria continually produce and accumulate reactive oxygen species [5] and contain no catalase [6], they would be largely, if not entirely, dependent on the GSH-peroxidase system. GSH, therefore, may be considered as the principal functional mitochondrial antioxidant [7]. Indeed, GSH depletion has been suggested to markedly enhance the susceptibility to mitochondrial dysfunction from oxidant stress [8, 9] and to induce mitochondrial structural degeneration [10]. Recently, a relationship between changes in intramitochondrial GSH levels and alterations

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§*Abbreviations:* APAP, acetaminophen, EDTA, ethylendiamine tetraacetic acid; ESMP, sonicated submitochondrial particles; FCCP, carbonylcyamid p-(trifluoromethoxy)phenylhydrazone; GSH, total glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde, NAPQI, N-acetyl p-benzoquinone imine; RCI, respiratory control index.

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of mitochondrial energy metabolism, most likely due to free radical accumulation, has been observed in ageing [11] and during liver regeneration [12].

Ultrastructural changes, such as vacuolization and mitochondrial and plasma membrane degeneration, have been observed during the development of APAP hepatoxicity [13], suggesting that early damage to subcellular organelles may play a crucial role in APAP hepatotoxicity. Studies in vivo and in vitro on the effect of APAP and its metabolite NAPQI showed alterations in cellular energy metabolism, with changes in the respiratory rates [14–17], depletion of mitochondrial ATP content [18, 19], and alteration of energy coupling [17].

Several studies have been carried out on the metabolism and compartmentation of hepatic GSH [20–22], but little is known about the time-course of mitochondrial GSH depletion, its re-uptake after APAP intoxication, or the influence of these processes on mitochondrial function in vivo.

In this study, the relationship between the two effects induced by in vivo APAP intoxication, has been investigated by the kinetic analysis of: 1. rat hepatic mitochondrial and cytosolic GSH depletion and replacement; 2. mitochondrial respiration in State 3 and 4; 3. the rate of ATP synthesis; and 4. the passive proton permeability of the inner mitochondrial membrane. Finally, the hepatic cytosolic and mitochondrial oxidative state of lipids and proteins was determined.

MATERIALS AND METHODS

Male Wistar rats (230–250 g) fed ad lib with standard stock diet were injected intraperitoneally with 500 mg/kg body weight of a 0.25 M APAP saline solution. Four to six rats from each group were sacrificed by decapitation, at time 0 (prior to APAP administration) and 2, 4, 6, 8, and 24 hr after APAP injection. The livers were removed and immediately homogenized in 10 volumes of 0.1 M K-phosphate buffer pH 7.4 containing 5 mM EDTA. 4 g of livers were separately homogenized in 40 volumes of MSM buffer pH 7.4 (0.44 M mannitol, 0.07 M sucrose, 5 mM morpholinopropanesulfonic acid) containing 0.1 M Na_2-EDTA for mitochondrial isolation, as previously described [23]; cytosol was separated from other subcellular organelles by ultracentrifugation (105,000 × g, 15 min at 4°C).

Total (GSH) and oxidized (GSSG) glutathione were measured enzymatically in liver homogenate, cytosol, and mitochondria according to the methods of Tietze [24] and Griffith [25], respectively.

From the remaining portion of liver (5–6 grams), mitochondria were prepared to measure the rates of respiration, following the high-yield preparation method described by Bustamante et al. [26].

A portion of the mitochondrial pellet was fixed in 2% osmium tetroxide-phosphate buffer (2 hr at 0°C) for electron microscopy. After dehydration with ethanol, an inclusion in Epon 812 was performed [27]. Thin sections were then stained in 5% uranile acetate (1 hr at 45°C) and in lead hydroxide [28]. For the final observation, a Philips Tem 301 electron microscope (located at the University of Bari, Italy) at 60 kWatt was used. Electron microscopy showed that this mitochondrial preparation exhibited very little contamination with other organelles, in particular, lysosomes (Fig. 3; see also [26]). "Inside-out" submitochondrial particles (ESMP) were prepared by exposure of rat liver mitochondria that had been stored frozen at −70°C for at least 24 hr to ultrasonic energy in the presence of EDTA at pH 8.5 [29].

The respiratory rate was measured following O_2 uptake with Clark oxygen electrode in a 1 mL water-injected chamber with magnetic stirring at 25°C. Mitochondria (0.5 mg protein/mL) were suspended in 1 mL 200 mM sucrose, 3 mM $MgCl_2$, 1 mM EDTA, 10 mM K-phosphate (pH 7.4). After equilibration for 3 min, 20 mM K-succinate was added and the respiratory rate in State 4 was followed for 3–4 min; next, 300 μM Mg-ADP was added, and the respiratory rate in State 3 was followed for 3–4 min. Then, oligomycin (1 $\mu g \cdot mg\ protein^{-1}$) was added, and the oligomycin-inhibited respiration was followed for 3–4 min. Finally, 4 μM of the uncoupler carbonylcyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) was added and the stimulated respiration was measured (Fig. 4). The rate of ATP synthesis was evaluated on mitochondrial suspension (500 $\mu g\ protein \cdot mL^{-1}$) as described in [11].

The ATP hydrolase activity was spectrophotometrically determined in ESMP using a regenerating system containing an excess of lactate dehydrogenase and pyruvate kinase [30]. Passive proton permeability in ESMP was analyzed potentiometrically, following anaerobic release of the respiratory proton gradient [31]. Briefly, ESMP, prepared as described above, were incubated at a concentration of 3 mg/mL for 5 min under nitrogen flux at 25°C in a mixture containing 200 mM sucrose, 10 mM KCl, and 20 mM K-succinate. After this incubation, proton cycles were induced by addition of H_2O_2 in the presence of catalase, and the rate of passive proton conduction was estimated by determining the $t_{1/2}$ of the anaerobic release of the respiratory-induced transmembrane proton gradient [31], which was inhibited by the F_0 inhibitor oligomycin [31].

The content of lipid peroxidation products, evaluated as malondialdehyde-TBA reactive compound, was detected spectrophotometrically [32] both in liver homogenate and mitochondria; 1,1,3,3-tetraethoxypropane was used as a standard. The cytosolic and mitochondrial content of carbonyl proteins was assumed as an index of the protein oxidation process, and evaluated spectrophotometrically as dinitrophenyl-hydrazone derivatives [33]. Cytosolic and mitochondrial protein concentrations were determined by the method of Lowry [34].

All chemicals were purchased from Sigma (Milan) or represented the best available commercial grades.

The study was approved by the State Commission on Animal Experimentation.

Statistical Analysis

Results are expressed as mean ± SE. Data were analyzed by one-way ANOVA; $P < 0.05$ was assumed as the lowest significance of differences.

RESULTS

Table 1 shows that the treatment of rats with APAP (500 mg/kg) does not induce any change either in liver weight or mitochondrial protein content. These data are in agreement with the observation of Katyare and Satav [17]; in that study, no changes in liver weight and yield of mitochondrial proteins were found in rats treated with subtoxic doses of APAP (375 mg/kg), whereas only a small decrease (≤20%) was observed after toxic doses of APAP (750 mg/kg). Figure 1 reports that hepatic GSH content immediately decreased after APAP administration; the maximum depletion was observed 4 hr postdosing (2.13 ± 0.33 vs. 5.32 ± 0.59 μmol/g, $P < 0.001$). The level of GSH started to recover after 6 hr and, at 24 hr, had risen to approximately 73% of the basal value.

Figure 2A shows the kinetics of GSH depletion in cytosolic and mitochondrial fractions. As for liver homogenate, the cytosolic and mitochondrial GSH content immediately decreased. However, mitochondrial GSH reached its lowest value 2 hr after APAP injection, and in the cytosol, maximum GSH depletion was observed only 6 hr after APAP intoxication. In addition, the recovery of GSH was faster in mitochondria (beginning 4 hr after APAP injection) than in cytosol, where the GSH level remained relatively low until 8 hr after APAP injection (Fig. 2A).

The time-course of cytosolic and mitochondrial GSSG content appeared to parallel that of total GSH (Fig. 2B); indeed, GSSG concentrations started to decrease 2 hr after APAP injection and reached their lowest values after 4–6 hr. Thereafter, GSSG levels slowly recovered, reaching the basal values 24 hr after APAP injection (Fig. 2B). The evolution of the GSH/GSSG ratio in both cytoplasm and mitochondria is also shown in Fig. 2B. No significant variation was observed in the cytosolic or mitochondrial fractions.

As shown by electron microscopy, mitochondrial swelling with disoriented cristae and vacuolated mitochondria with loss of matrix were observed 2 hr after APAP intoxication (Fig. 3). The mitochondria, isolated 2 hr after APAP

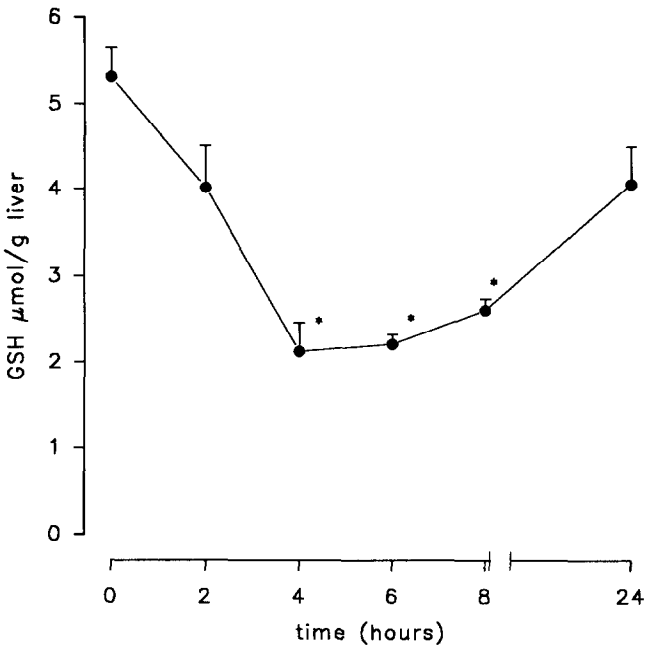


FIG. 1. Effect of APAP administration (500 mg/kg i.p.) on total hepatic GSH content in the rat. GSH was measured at time 0 (before APAP administration) and 2, 4, 6, 8, and 24 hr after APAP injection. Each point represents the mean ± SE of 4–6 rats. *$P < 0.001$ when compared to basal values.

injection, showed an increase in the respiratory rate in State 4 (1.70 times the basal values, $P < 0.002$) and the respiratory rate in State 3, using succinate as a substrate (1.17 times the basal values, $P < 0.02$) (Fig. 4). Therefore, a decrease in the respiratory control index (R.C.I.), given by the ratio between the respiratory rate of States 3 and 4, was observed 2 hr after APAP injection (Fig. 4). However, at 4 hr, the R.C.I. had returned practically to normal values; indeed, State 3 respiration continued to increase but State 4 respiration remained constant between 2 and 4 hr postdosing. Eight hours after APAP administration, both State 3 and 4 rates decreased, and at 24 hr the same values as time 0 were reached. The respiratory rate in State 3 was inhibited in control mitochondria as much as 70% by the ATP synthase inhibitor oligomycin (Fig. 4), indicating that the mitochondria were well coupled. This was confirmed by the stimulation of oligomycin-inhibited respiration by the uncoupler FCCP (Fig. 4). Sensitivity to oligomycin was lower in mitochondria isolated 2–4 hr after APAP intoxi-

TABLE 1. Liver weights and mitochondrial protein content in control and APAP-treated rats

	Control rats	APAP-treated rats			
		2 hr	4 h	8 hr	24 hr
Liver weight (g)	11.7 ± 0.8	11.5 ± 0.4	11.9 ± 0.8	11.6 ± 0.7	11.3 ± 0.9
Mitochondrial protein content (mg/g tissue)	17.8 ± 1.1	17.5 ± 1.1	18.2 ± 0.8	17.9 ± 0.8	18.3 ± 1.0

Male Wistar rats (230–250 g) were injected ip with APAP (500 mg/kg) as described in Materials and Methods. Control animals received only equivalent volumes of saline vehicle. Results are given as mean ± SE of 6 rats.

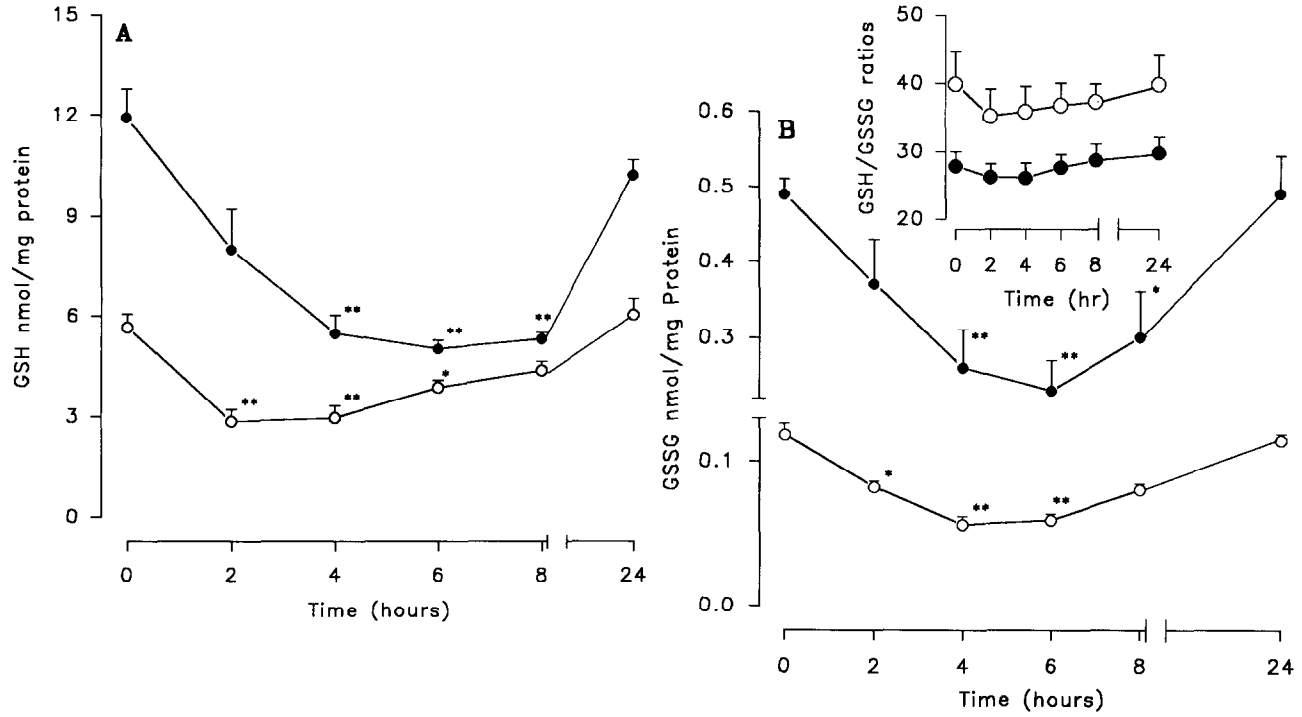


FIG. 2. Time-course of GSH (A) and GSSG (B) concentrations in liver cytosol (●) and mitochondria (○) of rats at time 0 and 2, 4, 6, 8, and 24 hr after APAP administration (500 mg/kg i.p.). In Fig. 2B, the cytosolic and mitochondrial GSH/GSSG ratios throughout the study period are inserted. Each point represents the mean ± SE of 4–6 rats. *$P < 0.01$ and **$P < 0.001$ when compared to basal values.

cation, suggesting a partial uncoupling. This was confirmed by the decrease in the ratio of the rate of ATP formed to that of oxygen consumed ($^{-}$P/O in Fig. 4) ($P < 0.002$).

This picture may reflect the removal of respiratory control by the increase in dissipation, through the inner mitochondrial membrane, of the transmembrane proton gradient set up by the respiratory chain. To verify this hypothesis, we followed the passive release, in anaerobiosis, of the respiratory proton gradient in the inside-out sonic submitochondrial particles. Table 2 shows that, in ESMP obtained 2 hr after APAP injection (when the mitochondria showed a decrease in the R.C.I., see Fig. 4), the rate of passive proton conduction increased. It then started to decrease and, 24 hr after APAP injection, the rate of passive proton conduction was again equal to basal values.

It is noteworthy that the increase in passive proton conduction observed at 2 hr was insensitive to oligomycin, the inhibitor of proton conduction by the membrane sector of ATP synthase. No significant changes were observed in oligomycin-sensitive ATP hydrolase activity.

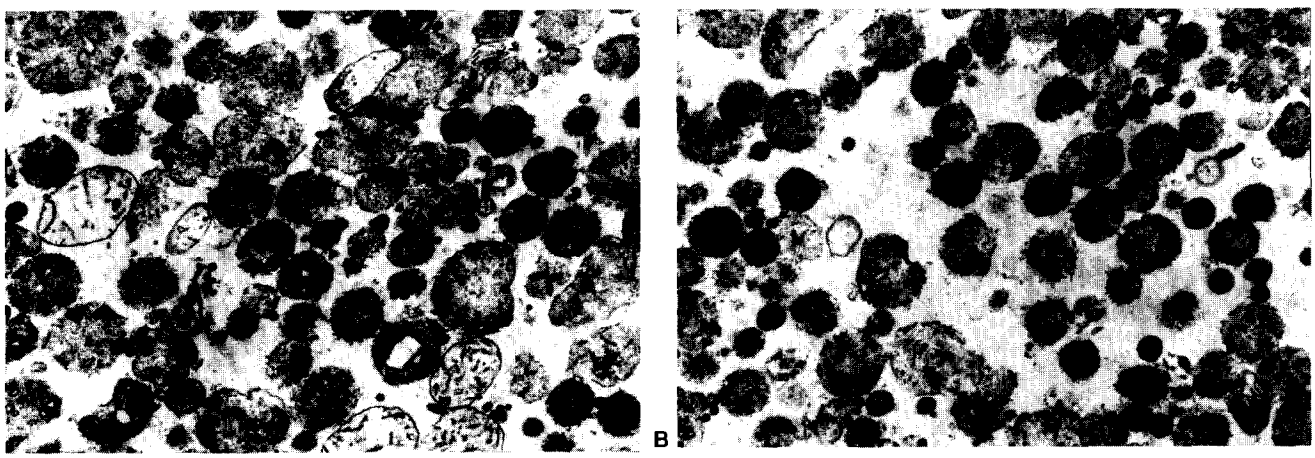
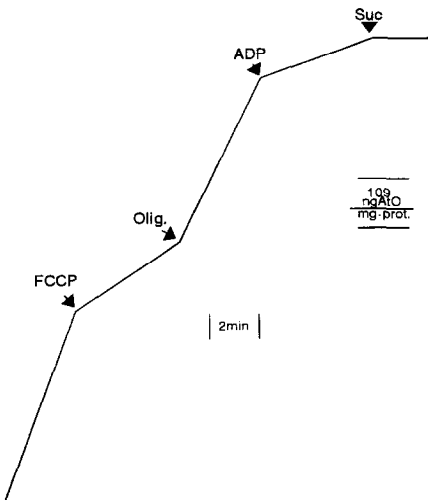


FIG. 3. (A) Electron micrograph of mitochondria isolated from an APAP-treated rat 2 hr after intoxication (15600 ×). Mitochondrial swelling with disoriented cristae and vacuolated mitochondria with loss of matrix are shown. (B) Normal control rat (12500 ×).



Time after APAP injection hr	Rates of oxygen consumption (ngAtO$_2$/min/mg prot.) State III	State IV	Oligomycin	Rate of ATP synthesis nmolATP / min·mg prot.	R.C.I. (III/IV)	~P/O
0	119±4	23±2	38±2	179±10	5.2±0.3	1.48±0.02
2	139±4**	39±1 *	58±3	146±6	3.5±0.1	1.04±0.01*
4	181±5	38±2	70±3	213±11	4.7±0.3	1.17±0.04*
8	143±7	31±1	46±5	179±7	4.6±0.1	1.25±0.02
24	117±5	22±2	31±2	160±7	5.3±0.2	1.36±0.03

FIG. 4. Effect of in vivo APAP administration on succinate-supported mitochondrial respiration and ATP synthesis. Rat liver mitochondria were prepared at 0, 2, 4, 8, and 24 hr after APAP administration (500 mg/kg i.p.). The trace on the left represents a typical polarographic determination of oxygen consumption by succinate-supplemented control mitochondria (see Materials and Methods). Where indicated, oligomycin (1 μg · mg protein^{-1}) and FCCP (4 μM) were added. The data reported in the table are the mean ± SE of 4 experiments. The R.C.I. was obtained by the ratio between the respiratory rates in State 3 and State 4. ~P/O represents the ratio of the rate of ATP synthesis to the respiratory rate in State 3. *$P < 0.002$, **$P < 0.02$ when compared to basal values.

The depletion of GSH and the increment of passive proton conduction were accompanied by an increase in MDA production, both in liver homogenate (Fig. 5A) and mitochondria (Fig. 5B). MDA levels started to increase 2 hr after APAP injection, remained high until 6 hr, and gradually returned to basal values within 24 hr.

No increased production of protein carbonyl groups was observed in cytoplasm and mitochondria throughout the study period (data not shown).

DISCUSSION

Several reports [14–17] suggest that alterations in mitochondrial function may be of great importance in APAP-induced hepatoxocity and that depletion of mitochondrial GSH seems to be a major mechanism inducing imbalance of mitochondrial function [18].

Despite the fundamental role of GSH, mitochondria do not contain the enzyme activities required for GSH synthesis and effectively transport this tripeptide from the cytosolic compartment with an ATP-dependent mechanism [35, 36].

The importance of GSH for mitochondrial function is well established, and several investigations have shown that a marked decrease in GSH sensitizes mitochondria to oxidative modifications by drugs or xenobiotics [37, 38].

APAP and its metabolite NAPQI can directly interact with mitochondria [3], thereby inducing depletion of GSH content or local toxicity by altering membrane permeability [18, 39].

TABLE 2. Effect of APAP injection on oligomycin-sensitive proton conduction and ATP hydrolase activity in sonicated submitochondrial particles (ESMP)

Time after APAP inject. (hr)	Anaerobic H$^+$ conduction [1/t (sec^{-1})] Control	+Oligomycin	ATP hydrolase activity [mol ATP hydr. (min^{-1}/mg prot)] Control	+Oligomycin
0	0.7 ± 0.1	0.32 ± 0.07	1.58 ± 0.15	0.12 ± 0.2
2	1.22 ± 0.09†	1.00 ± 0.10†	1.36 ± 0.11	0.13 ± 0.01
4	1.05 ± 0.07*	0.62 ± 0.05†	1.38 ± 0.10	0.12 ± 0.01
8	0.8 ± 0.1	0.46 ± 0.06	1.60 ± 0.15	0.16 ± 0.02
24	0.66 ± 0.08	0.43 ± 0.06	1.52 ± 0.10	0.12 ± 0.01

ESMP, prepared as reported in Materials and Methods, were suspended (3 mg/mL) in: 200 mM sucrose, 20 mM KCl, 20 mM K-succinate, pH 7.2. The proton conduction was analyzed as described in Materials and Methods. After oxygen pulses, ~50 g of proteins (17 L) were taken during anaerobiosis and used to test the ATP-hydrolase activity. Where indicated, oligomycin (0.2 g/mg protein particles) was added and an incubation of 5 min was run before addition of oxygen. The reported data are the mean ± SE of 5 experiments. *$P < 0.01$ and †$P < 0.001$ compared to Time 0.

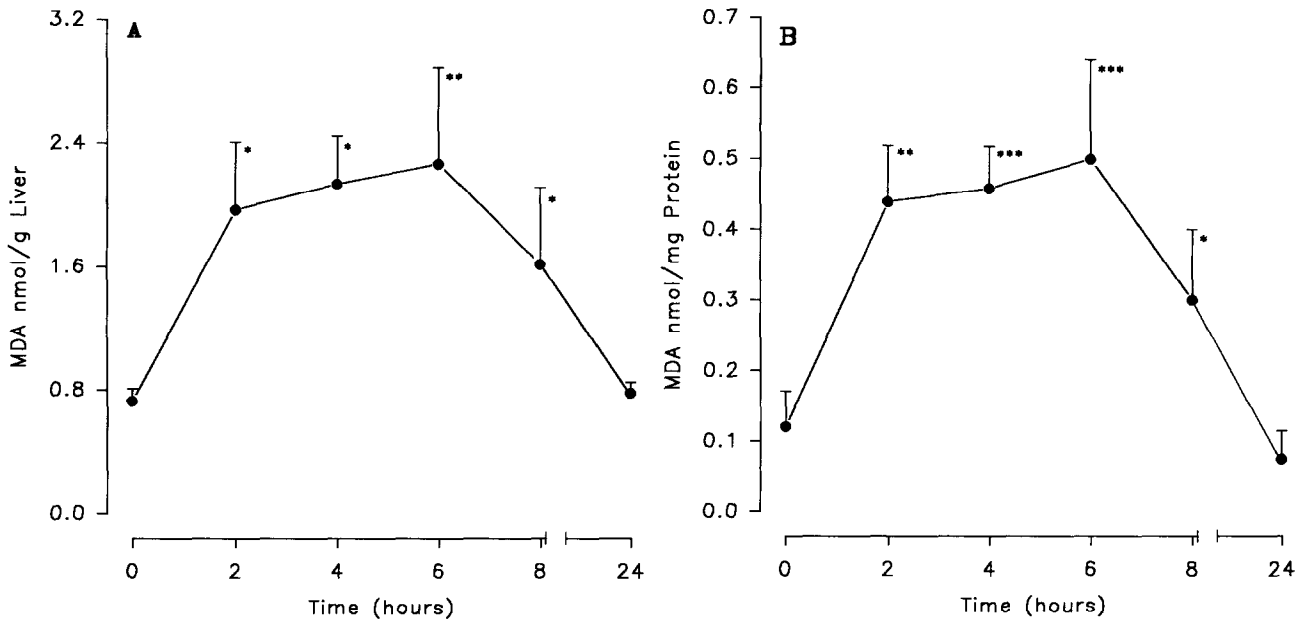


FIG. 5. Time-course of MDA concentrations in liver homogenate (A) and mitochondria (B) of rats at time 0 and 2, 4, 6, 8, and 24 hr after APAP administration (500 mg/kg i.p.). Each point represents the mean ± SE of 4–6 rats. *$P < 0.01$, **$P < 0.005$ when compared to basal values.

In vitro experiments indicate that APAP can induce inhibition of mitochondrial respiration supported by NADH-dependent substrates [14, 39]. By contrast, very little [39] or no inhibition [14] occurs when succinate is used as a respiratory substrate. In addition, in vivo treatment of mice with APAP seems to result in an increase in State 4 respiration and a decrease in the R.C.I. These findings, however, do not completely clarify the role of the increase in State 4, with succinate as substrate, of alteration of the organelles in response to energy demand [14].

The data reported in the present study show that in vivo treatment of rats with APAP produces an immediate (within 2 hr) depletion of cytosolic and mitochondrial GSH and GSSG levels that is accompanied by: 1. an increase in the respiratory rate of succinate-supplemented mitochondria in State 4, as previously observed in APAP-treated mice [14, 16]; 2. an increase in the respiratory rate of ADP-stimulated State 3; 3. a decrease in the rate of ATP synthesis; and 4. an increase in the passive proton permeability of the inner mitochondrial membrane.

It should be pointed out that mitochondria isolated from mice [16] and rats [17] treated in vivo with toxic doses of APAP (750 mg/kg) and also using succinate as a substrate showed inhibition of respiratory rates in State 3. However, our finding of a stimulation of the respiratory rate in State 3, using the same substrate, is in agreement with the previous reports of Katyare and Satav [17], who observed similar results after 375 mg/kg APAP. It is likely that APAP treatment induces inhibition or stimulation of the respiratory rate in State 3, according to the dosage used. In any event, the major finding of our studies appears to be the decrease in the R.C.I. (see also [16]) and the ~P/O ratio, which represents an impairment of oxidative phosphorylation. However, the structure of the mitochondrial ATP synthase complex does not seem to be damaged by APAP treatment, as shown by the fact that oligomycin-sensitive ATP hydrolase activity in sonic submitochondrial particles is unchanged (Table 2). Thus, it is suggested that, following APAP administration in vivo, the increased oligomycin-insensitive passive proton permeability of the inner mitochondrial membrane (Table 2) results in a partial uncoupling of oxidative phosphorylation. The stimulation of State 3 could represent a compensatory mechanism for restoration of the efficiency of oxidative phosphorilation, which began to be restored 4 hr after APAP injection and was completely restored at 24 hr postdosing.

The increase in MDA levels observed in liver homogenate and mitochondria might be viewed as an additional index of alteration of membrane permeability, suggesting that oxidative modifications of unsaturated lipids are also detectable in vivo after APAP administration.

These findings are in agreement with the increase in the respiratory rate in State 4, which should increase the production of oxygen free radicals by the respiratory chain [40]. As shown by electron microscopy, 2 hr after APAP administration (time of the maximum impairment of mitochondrial function), remarkable morphological alterations of mitochondria are also detectable.

The faster recovery of GSH in the mitochondria compared to the cytosol may suggest that the ATP synthetized by mitochondrial oxidative phosphorylation is used to favor the intramitochondrial transport of cytosolic GSH. Mitochondria, indeed, can take up GSH even in the presence of very low cytosolic GSH levels [6]. This hypothesis could also explain why cytosolic GSH starts to recover only after the restoration of most of the mitochondrial GSH (at 8 hr).

As a possible mechanism to be tested in vitro, it could be suggested that APAP or its metabolite NAPQI could directly interact with the inner mitochondrial membrane, causing changes in its fluidity. This could result in a transient increase in permeability followed by GSH and GSSG depletion; experiments to test this hypothesis are in progress in our laboratories.

In conclusion, we observed that in vivo APAP administration led to mitochondrial proton permeability damage that, in turn, could be bound to early GSH depletion. The latter may enhance the susceptibility of mitochondria to oxidative damage, especially during conditions of increased free radical production, as in the case of enhanced mitochondrial respiration. The concomitant restoration of mitochondrial energy metabolism and GSH levels is consistent with the important role of GSH compartmentation for mitochondrial function.

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