LOSS OF SULFITE OXIDASE ACTIVITY AND OUTER MEMBRANE DAMAGE IN RAT HEPATIC MITOCHONDRIA DURING CCl_4 POISONING

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SUMMARY: The activity of sulfite oxidase increased in intact rat hepatic mitochondria and decreased in solubilized mitochondria during poisoning by CCl_4. During acute damage the sulfite oxidase activity of the post-mitochondrial supernate increased, while the total activity in the hepatocyte declined. Thus, the outer membrane loses its selective permeability to macromolecules coincident with the decline of oxidative phosphorylation. In recovery, normal levels of activity in intact mitochondria and in the hepatocyte were restored, but a large proportion of the total activity remained in the cytoplasm. The data suggest that outer membrane repair and replacement of sulfite oxidase are involved in the restoration of mitochondrial structure and function.

One characteristic of acute carbon tetrachloride poisoning is damage to hepatic mitochondria (1). The damage is indicated by a loss of coupled respiration, a decrease of protein and nucleic acid synthesis (2), and an alteration of morphological appearance (3). Hepatic levels of ATP decline in a manner parallel to the loss of coupled respiration (4). If the exposure to CCl_4 is sub-lethal, a restoration of the damage occurs (2). Because mitochondrial damage occurs after other CCl_4-induced cytoplasmic disturbances are well advanced, the conditions which are responsible for the damage are uncertain as yet. Further, the complexity of oxidative phosphorylation makes it difficult to discern the mitochondrial components involved in the dysfunction. A less complex aspect of mitochondrial integrity is the condition of the outer membrane. This study reports that the outer membrane of the hepatic mitochondrion is damaged during CCl_4 poisoning. This damage is revealed by the release of an intramembranal enzyme, sulfite oxidase (EC 1.8.3.1) from the organelle, and a

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disappearance of the structural-linked latency normally exhibited by the enzyme in intact mitochondria (5).

METHODS AND MATERIALS

Male rats weighing 150-300 g were injected with CCl_4 i.p. at doses from 0.7 to 1.1 ml per kg, depending upon the experiment. Sections of liver were removed, minced and homogenized in isolation media (0.25M sucrose-10mM KCl-1mM EDTA-10mM Tris, pH 7.0). The homogenate was centrifuged at 450 X G for 10 min to remove debri and nuclei. The supernate, designated total homogenate and always containing 84-88% of the initial sulfite oxidase activity, was centrifuged at 8,800 X G for 10 min to obtain the mitochondrial fraction. The pellet was washed twice in 0.25M sucrose by centrifuging for 10 min at 7,200 X G. Four to 6 percent of the sulfite oxidase activity was lost during the washing. The final pellet was resuspended in 1 ml of the isolation media.

Sulfite oxidase activity was assayed by the method of Cohen, with these exceptions (6). Each cuvette contained (final volume, 3 ml) 20mM Tris-1mM EDTA-9mM KCl-225mM sucrose, with or without 0.1% Triton X-100, 0.4mM NaCN, 1.3 mg cytochrome c and sufficient enzyme to cause a change in absorbance of 0.01-0.5 O.D. per min (T=30°C, pH 8.5). The reaction was initiated by the addition of 1mM Na_2SO_3. When the detergent was omitted, the sulfite oxidase activity of the intact mitochondria was obtained. Addition of the detergent allowed the total enzyme activity to be assayed. Before each experiment, a Gilford-Beckman Model 2000 spectrophotometer was finely adjusted to the exact wavelength (ca. 550 nm) at which cytochrome c, reduced with $Na_2S_2O_4$, exhibited the maximum absorbance. Protein determinations were by the methods of Lowry et al. (7) and Gornall (8). Respiratory control ratios were determined on a Biological Oxygen Monitor (Yellow Springs Instruments Co.) as described previously (2). Monoamine oxidase activity was determined exactly as described by Greenawalt and Schnaitman using benzylamine as substrate (9).

Cytochrome c (Sigma Type III), Tris, Triton X-100 and heavy metal-free sucrose were obtained from Sigma Chemical Co. All other chemicals were reagent grade. Chloramphenicol (sodium succinate salt) was a gift from Parke-Davis Co., Detroit, Michigan.

RESULTS AND DISCUSSION

Sulfite oxidase displays structure-linked latency. Maximum activity in this assay occurs only when the outer mitochondrial membrane is removed (5). The latency is attributed to the failure of one substrate, cytochrome c, to pass the intact outer membrane (10). This latency can be observed in Fig. 1, as the detergent-released (total) activity is 17-fold greater than that of the intact mitochondria at zero time. Comparison of intact versus detergent-released sulfite oxidase activity provides an index for assessing the continuity of the outer mitochondial membrane.

The levels of intact and total sulfite oxidase activity in the mitochondrial fraction during the course of damage elicited by CCl_4 poisoning is shown

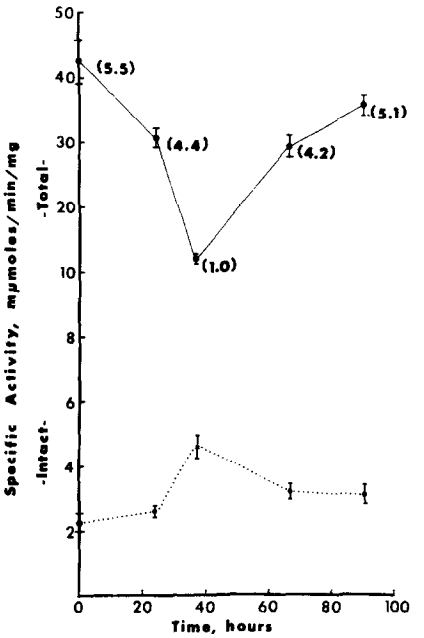

Fig. 1. Sulfite oxidase activity in intact (·····) and Triton-solubilized mitochondrial (Total ——) during poisoning by CCl_4. Rats were injected with 0.7 ml/kg CCl_4 and the mitochondrial fraction prepared and assayed at the indicated times. The average respiratory control ratios are enclosed in parentheses beside the appropriate time point. Each time period represents 3-16 animals, assayed in duplicate. Standard error is indicated.

in Fig. 1. The specific activity of sulfite oxidase of intact mitochondria appeared to be highest 40 hours after poisoning, the time of the most severe disruption of oxidative phosphorylation (respiratory control ratios were 1.0). It is also apparent that the solubilized (total) specific activity of sulfite oxidase in the mitochondrial fraction had declined during this time. Both the increase of specific activity in the intact, and the decrease of specific activity in the solubilized mitochondrial fraction, suggest that the outer membrane has been disrupted. No change from the control level of monoamine oxidase (a marker enzyme for the outer membrane [9]) was apparent until 40 hours after CCl_4, when a 10% decline was found (control = 4.5 $\pm$ 0.4; 40 hr = 4.1 $\pm$ 0.2 mmoles min^{-1} mg^{-1}). This suggests that no gross loss of outer mem-

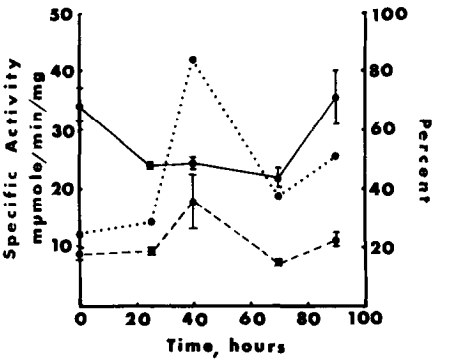

Fig. 2. Sulfite oxidase activity in the total hepatic homogenate (_____) and post-mitochondrial supernate (-----) during poisoning by CCl_4. The percent of sulfite oxidase recovered in the post-mitochondrial supernate is also displayed (·····). Recovery of enzyme in all fractions totaled 103-112%. Data from the same experiments as in Fig. 1.

brane had occurred. This was supported by the comparison of electron micrographs of damaged and normal mitochondria which indicated that some of the damaged mitochondria were swollen, with rupture of the outer membrane, although the fragments were still attached to the swollen structures (11).

When the distribution of enzyme activities in mitochondrial and post-mitochondrial fractions was determined (Fig. 2), a marked increase of activity was found in the post-mitochondrial fraction 40 hours after administration of CCl_4, suggesting that some of the enzyme lost from the mitochondria appeared in this fraction. The release of enzyme from damaged mitochondrial appearded to be a specific result of damage and not due to desolution of some proportion of the organelle population. That is, the recovery of protein in the mitochondrial fraction did not change (protein of the mitochondrial fraction of all experiments ranged 37-43%; total protein recovery, all fractions, was 103-109%). Previous work has shown that no depletion of mitochondrial cytochrome c occurred after poisoning by CCl_4 (2).

An attempt was made to determine if damage to the outer membrane was relevant to damage and dysfunction of the inner membrane. If the induction

Table 1: Sulfite oxidase levels in mitochondria during poisoning by CCl_4 and protection by chloramphenicol.

		Sp. Act., Sulfite Oxidase[b]	
	R.C.R.[a]	intact	solubilized
CCl_4 - chloramphenicol (n=6)	5.4 ± 0.1	3.9 ± 0.3	86.1 ± 5.0
control (n=5)	5.0 ± 0.4	3.8 ± 0.5	87.4 ± 11.1

[a]Respiratory control ratio, succinate as substrate.

[b]mμmoles/min/mg. Mitochondria were assayed as described in Methods and Materials except Lubrol (0.16 mg/mg protein) was used for solubilization. Standard error indicated. Chloramphenicol (100 mg/kg) and CCl_4 (1.1 ml/kg) were administered 40 hours prior to assay.

of damage in each membrane was distinct, it should be possible to inflict damage on one, but not the other, membrane. We attempted this separation of damage by the administration of chloramphenicol. Chloramphenicol effectively prevents the loss of respiratory control by the mitochondria during CCl_4 poisoning (12), but does not relieve the loss of cytochrome P-450, or the fatty infiltration characteristic of the first 10 hours of CCl_4 poisoning (13). When mitochondria ware assayed from CCl_4-poisoned rats protected by chloramphenicol administration, a slight activation of sulfite oxidase in intact mitocoondria was evident, while the total enzyme activity of the solubilized mitochondria was normal, as were the respiratory control ratios of these mitochondria (Table 1). It would therefore seem that the ability of chloramphenicol to prevent loss of oxidative phosphorylation after poisoning by CCl_4 also extends to prevent the loss of sulfite oxdiase activity and damage to the outer membrane. Thus, damage to the two membranes may either be a concomitant event, or causally related.

The specific activity of sulfite oxidase in the whole homogenate was considerably depressed at 24, 40 and 68 hours (Fig. 2). The loss of enzyme activity indicates that enzyme was being activated, as well as released from the damaged mitochondria. It can not be distinguished whether the inactivation of enzyme occurred at the intermembranal site of function, or after its release

from the mitochondrion. However, the 40% decline of solubilized sulfite oxidase activity at 24 hours, occurred before the respiratory control values and the level of intact sulfite oxidase activity were drastically altered (Fig. 1).

Seventy hours after CCl_4, when respiratory control ratios were returning to normal, the level of sulfite oxidase activity of intact mitochondria was near that of control. At this time the total mitochondrial and cellular levels of sulfite oxidase activity were 67% and 65% of controls, respectively (cf. Fig. 1 and Fig. 2). Since the level of sulfite oxidase activity in intact mitochondria reflects the permeability of the outer membrane, this implies that restoration of the outer membrane preceded replacement of sulfite oxidase. At a later period of recovery (90 hours), total cellular levels of enzyme activity had been restored to normal, but a considerable proportion of that activity was in the post-mitochondrial supernate (50% vs 12%, cf. Fig. 2). We can speculate that this distribution may represent cytoplasmic accumulation of newly synthesized enzyme prior to its transfer to the mitochondrion, if, consistent with other soluble mitochondrial enzymes, the synthesis of this protein occurs in the cytoplasm (15).

The consequences of outer membrane damage for inner membrane function are not completely obvious. Hackenbrock and Miller have reported points of fusion between the two membranes, although the functional significance of such fusion points was not established (16). However, if the outer membrane is removed by digitonin dissolution, the resulting inner membrane-matrix fraction is susceptible to uncoupling by physiological levels (5mM) of Mg^{++} (17). The ability of chloramphenicol to prevent outer membrane damage and dysfunction of the inner membrane suggests that the outer membrane is critical to the normal operation of inner membrane activities.

We conclude that the outer membrane of the hepatic mitochondria was damaged, possibly perforated or ruptured, during poisoning by the hepatotoxin, CCl_4. This conclusion is based on the dissappearance of latency normally associated with sulfite oxidase and loss of the enzyme from the damaged cell.

Further, the recovering cell restores the outer membrane before the mitochondrion is replenished with its normal complement of sulfite oxidase. We are presently investigating the further significance of outer membrane damage to mitochondrial function and the mechanisms of its induction and repair in the traumatized hepatocyte.

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