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IN VIVO MODULATION OF TOTAL AND MITOCHONDRIAL GLUTATHIONE IN RAT LIVER

DEPLETION BY PHORONE AND RESCUE BY *N*-ACETYLCYSTEINE

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Abstract—The aim of the present work was to modulate *in vivo* the level of hepatic mitochondrial glutathione (GSH). Rats were given phorone (diisopropylidene acetone), which *in vivo* becomes enzymatically conjugated to GSH, and were subsequently treated with *N*-acetylcysteine (NAC) to rescue GSH. In liver homogenate, a rapid and biphasic ($T_{1/2} \leq 15$ min and 1.5 hr) drop of GSH was observed upon phorone administration. NAC treatment led to a restoration ($T_{1/2}$ about 1 hr) of GSH in the homogenate above control values within 3 hr. The mitochondrial GSH level decreased with $T_{1/2}$ of about 1.5 hr upon phorone treatment, and was 75% restored by NAC treatment within 3 hr. Hydroperoxide-induced mitochondrial pyridine nucleotide oxidation and Ca^{2+} release were impeded in GSH-depleted organelles, and NAC treatment restored these processes. The GSH status had no influence on mitochondrial pyridine nucleotide oxidation and Ca^{2+} release induced by alloxan, which reacts directly and non-enzymatically with pyridine nucleotides. It is concluded that NAC is able to rescue mitochondrial GSH *in vivo* and restore important mitochondrial functions. The data suggest that NAC may be a useful antidote in oxidative stress-related diseases.

Glutathione (GSH†) is an important cellular antioxidant [1]. As co-substrate of GSH peroxidases it protects aerobic organisms from the ever-present threat posed by ROS, since it provides electrons for the reduction of hydroperoxides to water or alcohols. There are at least two separate cellular GSH pools: one in the cytosol, the other in mitochondria. These organelles do not synthesize their own GSH. Rather, they probably take it up actively from the cytosol, and also extrude GSH actively, as indicated recently by the *in vitro* studies of Martensson and Meister [2].

Although GSH serves important functions in mitochondria [3], its modulation *in vivo* has been studied very little. A moderate depletion of mitochondrial GSH has been achieved in adult rats by exposure to ethylene oxide [4]. Administration to young rats of BSO, an inhibitor of GSH synthetase, and subsequent treatment with GSH monoesters resulted in a decrease and restoration of mitochondrial GSH, respectively [2].

Phorone, a phase II compound, is conjugated enzymatically to GSH [5], thus lowering the *in vivo* levels [6]. NAC is an antioxidant [7] which is believed to increase the intracellular GSH level [8, 9]. Here we show the *in vivo* depletion of rat liver mitochondrial GSH with phorone and subsequent restoration of GSH with NAC. The *in vivo* manipulation of GSH by phorone and NAC is reflected in the modulation of the ability of mitochondria to release Ca^{2+} when exposed to tbh.

MATERIAL AND METHODS

Male Wistar rats (approximately 180 g body wt) received food and water *ad lib*. After starvation over night, they were injected i.p. between 6 and 8 a.m. with 250 mg of phorone/kg, dissolved in sunflower oil, or with the vehicle alone. Where indicated, they received 4 g of NAC/kg, dissolved in water, by stomach tubing. The animals were killed by decapitation at the times indicated in the Figures, the livers were removed and immersed in ice-cold buffer (210 mM mannitol, 70 mM sucrose, 5 mM 4-(hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 1 mM (ethylenenitrilo)tetraacetic acid). About 2 min later, livers were diced and homogenized in the same buffer. No precautions were taken to prevent GSH oxidation since only minimal amounts of oxidized glutathione were found (see Results).

Preparation of liver homogenates and mitochondria, calcium transport, and pyridine nucleotide measurements were done as described previously [10]. Briefly, mitochondria were isolated by differential centrifugation. They were incubated and energized by succinate according to the standard procedure described in Ref. 10. Calcium movements across the inner mitochondrial membrane were monitored in a dual wavelength spectrophotometer in the presence of arsenazo III at 685–675 nm and mitochondrial pyridine nucleotides at 340–370 nm. GSH and oxidized glutathione were determined, immediately after preparation of the homogenate and mitochondia, by the HPLC method of Reed *et*

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† Abbreviations: AIDS, acquired immunodeficiency syndrome; AZT, azidothymidine; BSO, L-buthionine (*S*,*R*)-sulfoximine; GSH, glutathione; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; tbh, *t*-butylhydroperoxide.

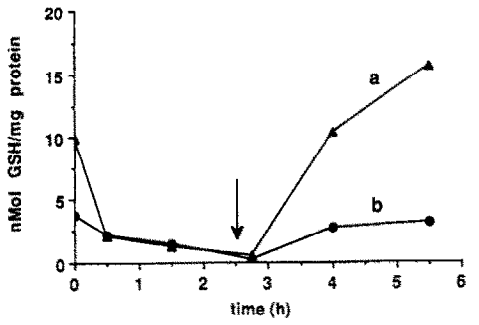

Fig. 1. GSH content of rat liver homogenate and mitochondria after phorone administration. Rats were injected with phorone (time 0 hr) and killed at the times indicated. The animals killed at 4 and 5.5 hr additionally received NAC at 2.75 hr (arrow). Livers were removed and homogenized, mitochondria were isolated, and GSH was determined as described in Materials and Methods. Curve a, GSH in homogenate; curve b, GSH in mitochondria. The results are from one experiment typical of five.

al. [11] with modifications as described by Fariss *et al.* [12].

RESULTS

Manipulation of GSH in vivo

Administration of phorone to rats lead to a drastic, biphasic drop in GSH when measured in liver homogenate (Fig. 1, curve a). The two phases of the decrease could be distinguished: one with $T_{1/2} \leq 15$ min, the resolution limit of the present investigation, the other with a $T_{1/2}$ of about 1.5 hr. In mitochondria isolated from these homogenates, a monophasic decrease in GSH was observed, with a $T_{1/2}$ of about 1.5 hr (Fig. 1, curve b). No increase in the level of oxidized glutathione was observed upon phorone treatment. Indeed, the amount of oxidized glutathione found in the homogenates and in mitochondria was always less than 5% of GSH. Sunflower oil, used as vehicle for phorone, did not influence the hepatic GSH content (not shown).

When NAC was given to rats whose hepatic GSH level was < 5% of control values, total GSH was restored and even increased above control values within 2 to 3 hr (Fig. 1, curve a). Restoration of GSH in mitochondria was slower and less extensive than in the homogenate, resulting in a recovery to about 75% of the control value within 3 hr (Fig. 1, curve b). Without NAC treatment the GSH level in homogenate and mitochondria remained low during this time (not shown).

Prooxidant-induced mitochondrial pyridine nucleotide oxidation and Ca^{2+} *release.*

Figure 2 shows uptake of Ca^{2+} by mitochondria and its prooxidant-induced release. When tbh was used to induce Ca^{2+} release from mitochondria in the organelles isolated from phorone-treated rats (Fig. 2A, curve c), release was retarded strongly compared to that from mitochondria isolated from control (Fig. 2A, curve a) or phorone *plus* NAC-treated (Fig. 2A, curve b) animals. In the absence of tbh, no Ca^{2+} release was observed. When alloxan was used (Fig. 2B) to induce Ca^{2+} release the various mitochondrial preparations behaved virtually identically.

Various prooxidants cause Ca^{2+} release from mitochondria by promoting pyridine nucleotide oxidation and hydrolysis, and the prooxidant tbh but not the prooxidant alloxan is linked to pyridine nucleotides by glutathione-dependent enzymes [13]. The metabolic link of tbh to mitochondrial pyridine nucleotides via GSH was confirmed by measurements of the redox state of the nucleotides (Fig. 3). In mitochondria isolated from control animals (Fig. 3A, curve a), tbh caused extensive pyridine nucleotide oxidation, whereas in mitochondria isolated from phorone-treated rats (Fig. 3A, curve b) tbh caused negligible oxidation. Alloxan, which is known to oxidize pyridine nucleotides non-enzymatically and directly [10], induced oxidation in the mitochondria of control as well as of phorone-treated animals (Fig. 3A, curves b, c, d). It should be noted that the photometric determination of pyridine nucleotides in the presence of alloxan is complicated by spectral overlap with some newly formed, unidentified compounds. The increase in absorbance at 340–370 nm observed a few minutes after alloxan addition was not due to pyridine nucleotide reduction [10]. The responsiveness to tbh of mitochondria isolated from phorone-treated animals was restored by NAC administration (Fig. 3B).

DISCUSSION

BSO and GSH monoesters have been used previously to manipulate GSH levels *in vivo*, and administration of GSH monoesters increases substantially mitochondrial GSH levels in BSO-treated animals (Ref. 2, and references therein). We show a rapid and extensive *in vivo* modulation of rat liver GSH, analysed in both homogenate and mitochondria. GSH depletion is achieved with phorone, a substrate of GSH transferases, and subsequent GSH rescue is accomplished with NAC, a putative precursor of GSH. To our knowledge, this is the first report of *in vivo* restoration of mitochondrial GSH by a compound which may serve as a substrate in GSH biosynthesis. Depletion of GSH by phorone treatment still allows GSH resynthesis as suggested in the present study with NAC, in contrast to BSO treatment, which blocks glutathione synthetase. Use of phorone in combination with NAC is thus a promising approach for *in vivo* investigation of GSH biosynthesis as well as of GSH transport from the cytosol into mitochondria. Our study indicates that the mitochondrial GSH pool does not readily equilibrate with the cytosolic pool. Dose–response and time-course studies with phorone and NAC *in vivo* will further elucidate this and other questions. For example, it will be of great interest to see if the cysteine moiety of NAC indeed ends up in GSH.

GSH is important for proper mitochondrial functioning [3]. We have investigated two related

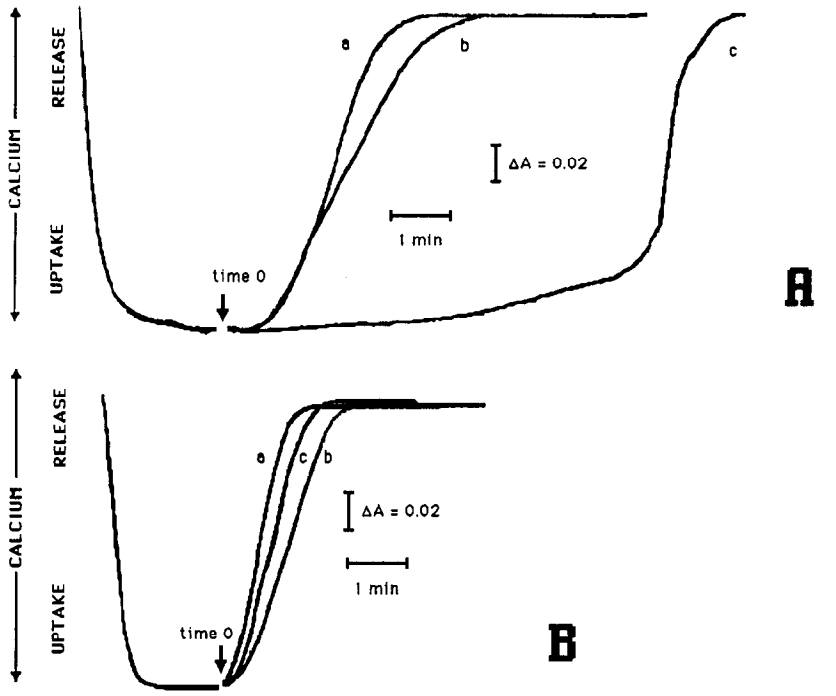

Fig. 2. Prooxidant-induced Ca^{2+} release from rat liver mitochondria. Rats were injected with phorone, NAC or the vehicle (control) as in Fig. 1. Mitochondria were isolated at 5.5 hr. In the presence of arsenazo III, mitochondria were loaded with 30 nmol of Ca^{2+}/mg of protein. At the arrow, Ca^{2+} efflux was initiated by the addition of 100 μM tbh (Panel A) or of 1.4 mM alloxan (Panel B). Curve a, mitochondria obtained from control animals; curve b, mitochondria obtained from phorone *plus* NAC-treated animals; curve c, mitochondria obtained from phorone-treated animals. The results are from one experiment typical of three.

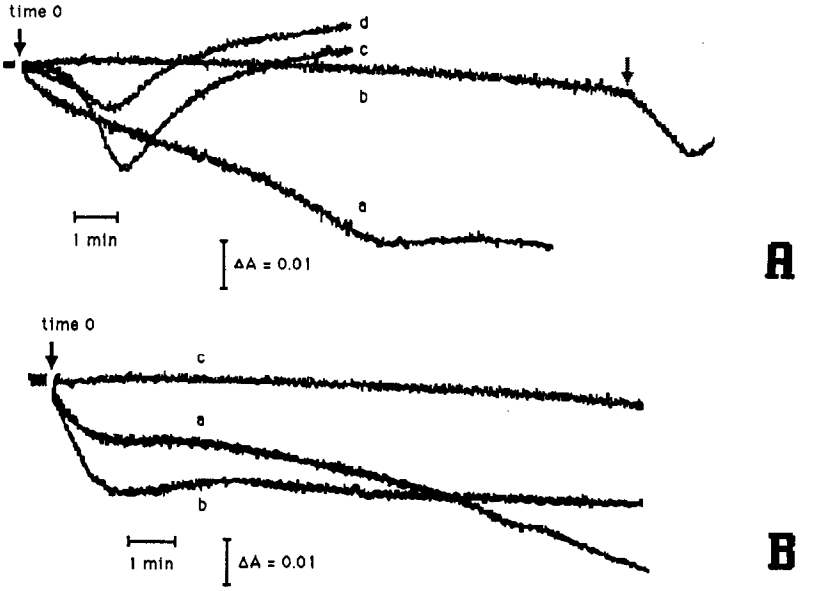

Fig. 3. Changes in the redox level of rat liver mitochondrial pyridine nucleotides. Rats were injected with phorone, NAC or the vehicle (control) as in Fig. 1. Mitochondria were isolated at 5.5 hr. The redox level of mitochondrial pyridine nucleotides was monitored spectrophotometrically at 340–370 nm. Mitochondria were energized with succinate and loaded with 30 nmol of Ca^{2+}/mg of protein. Panel A; at the solid arrow, 100 μM tbh (curves a and b) or 1.4 mM alloxan (curves c and d) were added. At the dashed arrow, 1.4 mM alloxan was added. Curves a and c, mitochondria obtained from control animals; curves b and d, mitochondria obtained from phorone-treated animals. Panel B: At the arrow, 100 μM tbh was added. Curve a, mitochondria obtained from control animals; curve b, mitochondria obtained from phorone *plus* NAC-treated animals; curve c, mitochondria obtained from phorone-treated animals. The results are from one experiment typical of three.

mitochondrial processes, namely prooxidant-induced pyridine nucleotide oxidation and Ca^{2+} release, in GSH-adequate and -depleted organelles. Prooxidants induce Ca^{2+} release from intact mitochondria by a specific and probably physiologically relevant process, which requires pyridine nucleotide oxidation and hydrolysis (reviewed in Ref. 13). The prooxidant tbh is linked to mitochondrial pyridine nucleotides via GSH peroxidase, glutathione reductase and the energy-linked transhydrogenase, whereas the prooxidant alloxan oxidizes pyridine nucleotides essentially without engaging enzymes [10]. As shown here, *in vivo* depletion of mitochondrial GSH retards drastically the onset of the tbh- but not the alloxan-induced Ca^{2+} release, consistent with previous notions of Bellomo *et al.* [14] and ourselves [13]. The drastic depletion of mitochondrial GSH by phorone treatment should allow further probing into the importance of mitochondrial GSH in other mitochondrial activities.

Under normal conditions mitochondria produce copious amounts of ROS. For example, a rat liver mitochondrion generates about 3×10^7 superoxide radicals/day [15]. Although most of the ROS are dealt with adequately by non-enzymatic and enzymatic antioxidants and may even regulate important mitochondrial reactions such as Ca^{2+} release (see above), oxidative damage is an ever present threat to mitochondria, particularly to their DNA [15, 16]. Under non-physiological conditions ROS can overwhelm the antioxidative capacity of mitochondria and cause severe damage. Pathological states such as Parkinson's or Alzheimer's disease, and mitochondrial myopathies have been ethiologically linked to oxidative damage of mitochondria. Indeed, the mitochondrial myopathy classified as Kearns-Sayre syndrome responds positively to treatment with the antioxidant ubiquinol [17]. Very recently Hayakawa *et al.* reported massive oxidative damage to liver mitochondrial DNA of mice treated with the anti-AIDS drug AZT [18]. AZT treatment of humans is accompanied by time- and dose-related toxicity including the acquisition of myopathies [19, 20]. Whether this side-effect of AZT treatment and other AIDS-related phenomena (possibly conferred by ROS [21]) respond positively to a maintenance or rescue of mitochondrial GSH needs to be studied urgently.

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