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Alterations to Glutathione and Nicotinamide Nucleotides During the Mitochondrial Permeability Transition Induced by Peroxynitrite

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ABSTRACT. Peroxynitrite is a biologically important oxidant that damages mitochondria in a number of ways. We investigated the interaction of peroxynitrite with the mitochondrial glutathione pool by measuring the formation of oxidised glutathione and glutathione–protein mixed disulfides in mitochondria exposed to either peroxynitrite or *tert*-butylhydroperoxide. In contrast to *tert*-butylhydroperoxide, peroxynitrite converts 40–50% of mitochondrial glutathione to products other than disulfides, primarily higher oxidation states of sulfur. These data show that peroxynitrite interacts with mitochondria quite differently from oxidants commonly used in studying mitochondrial oxidative stress. Peroxynitrite also induces a permeability transition in the mitochondrial inner membrane, and here we show that this permeability transition is prevented by the NAD(P)H-linked substrates glutamate and malate and by the thiol reagent dithiothreitol. Glutamate and malate prevented complete oxidation of the NAD(P)H pool by peroxynitrite or *tert*-butylhydroperoxide but did not prevent oxidation of the mitochondrial glutathione pool or the formation of glutathione–protein mixed disulfides. This study is consistent with regulation of the permeability transition by critical protein thiol groups, whose redox state responds to that of the mitochondrial NAD(P)H pool, but which do not equilibrate directly with the mitochondrial glutathione pool. BIOCHEM PHARMACOL 52;7:1047–1055, 1996.

KEY WORDS. mitochondria; peroxynitrite; glutathione; protein mixed-disulfides; nicotinamide nucleotides; mitochondrial permeability transition

Mitochondria accumulate oxidative damage rapidly because they are continually exposed to superoxide formed by the respiratory chain [1–3]. In addition, mitochondria are exposed to the free radical nitric oxide, a widespread biological messenger molecule that crosses membranes easily, affecting mitochondria distant from its site of synthesis [4–6]. Nitric oxide and superoxide react together extremely rapidly ($k = 6.7 \times 10^9\ M^{-1}\ sec^{-1}$ [7]) to form peroxynitrite,† a substantially more reactive and damaging molecule than its precursors [8]. Peroxynitrite is itself a potent oxidant and it also decomposes rapidly (half-life <1 sec) to form products with reactivity similar to that of hydroxyl radical and nitrogen dioxide [9]. Peroxynitrite has been shown to damage protein, DNA, and lipid and may be a major cause of oxidative damage in biological systems [8, 10]. Therefore, peroxynitrite formed by the reaction of nitric oxide with mitochondrially produced superoxide may be an important cause of mitochondrial damage [11].

Because glutathione and protein thiols are major mitochondrial antioxidant defences [12] the oxidation of both protein and low molecular weight thiols by the peroxynitrite anion [13–15] is particularly significant. The mitochondrial and cytosolic glutathione‡ pools are effectively independent [16, 17], and oxidative stress depletes the mitochondrial glutathione pool, thus forming GSSG§ and glutathione–protein mixed disulfides [18, 19]. As well as depleting mitochondrial glutathione, oxidative stress induces a nonspecific permeability transition in the mitochondrial inner membrane that depolarises mitochondria and causes efflux of low molecular weight matrix components [20–22]. This permeability transition is prevented by CsA and is regulated by many other factors (for reviews see [23, 24]). Although its function is unclear, inappropriate induction of the permeability transition contributes to cell death following oxidative stress and ischaemia-reperfusion injury [25–27]. The mechanism of induction of the permeability transition is unknown, but it is associated with the

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§ *Abbreviations:* CsA, Cyclosporin A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidised glutathione; tBHP, *tert*-butylhydroperoxide.

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† Peroxynitrite refers to both the anion and its conjugate acid.

‡ Glutathione refers to the sum of GSH and GSSG.

oxidation of the mitochondrial NAD(P)H pool and of critical protein thiol groups [22, 28, 29].

In this paper, we have studied the interaction of peroxynitrite with mitochondrial glutathione and nicotinamide nucleotide pools. In addition, we have extended our earlier findings [30, 31] by investigating how the interaction of peroxynitrite with the mitochondrial glutathione and NAD(P)H pools contributes to the mitochondrial permeability transition. From these experiments, we are able to draw some conclusions of significance for both the interaction of peroxynitrite with mitochondria and for the mechanism of the mitochondrial permeability transition.

MATERIALS AND METHODS

Materials

CsA was a generous gift from Sandoz Pharma Ltd. (Basel, Switzerland). Silicone fluid (ρ = 1.07 g/mL) was from BDH. Arsenazo III and glutathione reductase (type IV, from baker's yeast) were from Sigma.

Mitochondrial Preparation and Incubation

Liver mitochondria were prepared from fed female Wistar-derived rats (200–300 g) by homogenisation, followed by centrifugation at 4°C in isolation medium containing 70 mM sucrose, 3 mM Tris-HCl (pH 7.2), 220 mM mannitol, 0.1 mM EDTA, and 0.1% (w/v) bovine serum albumin (BSA) [32]. Mitochondria were washed once in this medium and then washed and resuspended in medium without EDTA or BSA and stored at about 50 mg protein/mL on ice. The protein concentration was determined by the biuret assay using BSA as a standard [33]. For all experiments, mitochondria (2 mg protein/mL) were incubated at 25°C in medium containing 195 mM mannitol, 25 mM sucrose, 40 mM HEPES-KOH (pH 7.2), and calcium (65 nmol Ca/Cl_2/mg protein). The high concentration of HEPES buffer was necessary to prevent a pH change with the addition of peroxynitrite to incubations. Incubations also contained succinate (3.3 mM) and rotenone (13 μM), or rotenone was omitted, and succinate, glutamate, and malate (3.3 mM of each) were present. CsA (500 nM) was present for some incubations.

Peroxynitrite Preparation

Peroxynitrite was prepared at 4°C by a variation [30] of a published procedure [8]. Briefly, 5 mL 0.6 M sodium nitrite was mixed with 5 mL acidified hydrogen peroxide (0.7 M H_2O_2) in 0.6 M HCl) in a simple flow reactor, running into 5 mL rapidly stirred 1.5 M NaOH. Residual hydrogen peroxide was degraded by incubation with MnO_2 (2 g) for 30 min, which was removed by filtration. Freeze fractionation of this solution at −20°C formed an upper yellow band, containing 90–140 mM peroxynitrite (ϵ_{302} = 1670 $M^{-1} \cdot cm^{-1}$ [34], which was stored at −80°C and used within 4 days of preparation. A "late quenched" solution, in which peroxynitrite had degraded, was prepared by reacting acidified peroxide with nitrite for 10 min before addition to NaOH.

Glutathione Measurements

Mitochondria were incubated in 1 mL medium in a 1.5-mL plastic centrifuge tube, and at the indicated times 700 μL was removed and the mitochondria rapidly separated from the extramitochondrial medium by centrifugation through oil. To do this 300 μL of dioctyl phthalate:silicone fluid (42:58) was layered on top of 100 μL 5% (w/v) sulfosalicylic acid containing 250 mM sucrose, 0.5 mM EDTA, and 100 μM diethleneaminepentaacetic acid in a 1.5-mL plastic centrifuge tube, centrifuged to separate the layers, and then cooled on ice. A 700-μL mitochondrial sample was carefully layered on top of the oil, centrifuged (16,250 × g, 1 min), and the liquid above the oil layer retained and assayed for extramitochondrial glutathione equivalents. Most of the oil layer was then carefully removed by aspiration, and the residual oil was removed by freezing in ethanol/dry ice followed by washing with cold hexane. The acid layer was removed and analysed for GSSG and total glutathione equivalents and the protein pellet was washed with 5% (w/v) sulfosalicylic acid and analysed for glutathione-protein mixed disulfides.

Glutathione equivalents (GSH + 2GSSG) in the mitochondrial matrix and in the extramitochondrial fraction were quantified by using the recycling assay [35, 36] adapted for a 96-well plate reader. Briefly, 10–40-μL sample, or GSH standard, was added to a well, made up to 50 μL with water, and then 25 μL 6 mM DTNB and 175 μL buffer (143 mM sodium phosphate, pH 7.5, 6.3 mM EDTA, and 0.25 mg/mL NADPH) were added. After addition of 50 μL glutathione reductase (10 U/mL), the rate of thionitrobenzoic acid formation was measured at 405 nm in a BIO-TEK Bio Kinetics EL 340 plate reader for 2 min and the glutathione equivalents determined from a standard curve. There were typically 4–6 nmol glutathione equivalents/mg protein in our mitochondrial preparations. To measure GSSG, GSH was first derivatised with 2-vinylpyridine, rendering it unreactive, and the remaining GSSG was analysed by the recycling assay [36]. To do this, 70-μL sample, or GSSG standard, was mixed with 3.2 μL triethanolamine (raising the pH to 6.5) and 4 μL 2-vinylpyridine in a 1.5-mL screw-capped plastic centrifuge tube, sealed under argon, and incubated at room temperature for 1 hr with gentle agitation. Samples were then assayed for GSSG by the recycling assay as before, using twice the concentration of glutathione reductase to overcome inhibition by 2-vinyl pyridine.

To determine glutathione–protein mixed disulfides [37], the washed mitochondrial protein pellet was resuspended in 65 μl 8 M urea, and then 15 μL 200 mM Tris-HCL (pH 7.4) and 20 μL 10% (w/v) sodium borohydride were added. After incubation at 40°C for 30 min, residual borohydride

was degraded and protein precipitated by addition of 25 μL of 50% (w/v) sulfosalicylic acid followed by incubation on ice for 30 min and centrifugation (16,000 × *g* for 5 min). The glutathione content of the supernatant was determined by the recycling assay as before.

Mitochondrial Calcium Transport

Efflux of calcium from mitochondria was determined by incubating mitochondria in a stirred and thermostatted cuvette in medium supplemented with 30 μM arsenazo III. Changes in the absorbance of the calcium-sensitive dye were measured in an SLM-Aminco DW2000 double wavelength spectrophotometer by using the wavelength pair 675–685 nm as described elsewhere [30].

Mitochondrial NAD(P)H Redox State

The redox state of the mitochondrial NAD(P)H pool was measured fluorometrically by using a Perkin-Elmer MPF-3L fluorescence spectrophotometer. Incubations were carried out in a 3-mL cuvette by using an excitation wavelength of 366 nm and an emission wavelength of 450 nm [38].

Measurement of S-Nitrosothiols

After incubation with peroxynitrite, mitochondrial matrix extracts were prepared by centrifugation through oil into 200 μL 500 mM sucrose/0.1% (w/v) Triton X-100. The extracts were then assayed for nitrite using the Greiss assay [39] in the presence or absence of $HgCl_2$, which degrades S-nitrosothiols to nitrite [40]. The difference in nitrite content between these samples was taken as the amount of S-nitrosothiols.

RESULTS

Mitochondrial Calcium Efflux

Calcium-loaded mitochondria exposed to peroxynitrite rapidly released calcium by induction of the mitochondrial permeability transition (Fig. 1A, B, traces a), as has been shown elsewhere [30]. In considering the interaction of peroxynitrite with biological systems, it is the exposure to peroxynitrite that is important, not the absolute concentration present [31], because peroxynitrite decays rapidly. When the exposure to peroxynitrite caused by addition of 250 μM is calculated, it is comparable to levels seen *in vivo* under pathological conditions [31]. Calcium efflux was blocked by prior incubation with the thiol-reducing reagent DTT (Fig. 1A, trace b), and addition of DTT after efflux had started reversed the effects of peroxynitrite (Fig. 1A, trace c). Incubation of mitochondria with the NAD(P)H-linked substrates glutamate and malate also prevented calcium efflux by peroxynitrite (Fig. 1B, trace b). In similar experiments, DTT or glutamate and malate prevented mitochondrial calcium efflux caused by tBHP (500 μM; data not shown), in agreement with other reports [21, 38, 41]. Therefore, mitochondrial calcium efflux caused by peroxynitrite is critically dependent on the oxidation state of mitochondrial thiols and NAD(P)H.

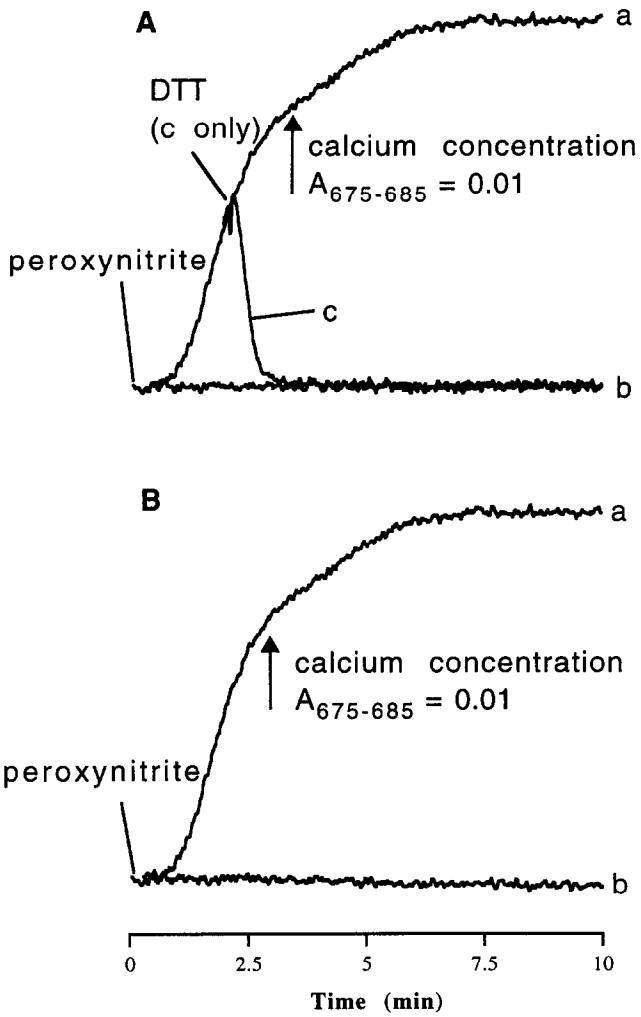

FIG. 1. Mitochondrial calcium efflux caused by peroxynitrite is blocked by DTT or NAD(P)H-linked substrates. Mitochondria were incubated with the calcium-sensitive dye arsenazo III as described in Materials and Methods, and peroxynitrite (250 μM) was added where indicated. (A) Rotenone and succinate were present (trace a), together with 1 mM DTT from the start of the incubation (trace b) or added where indicated (trace c). (B) Succinate and rotenone were present for trace a, and succinate, glutamate, and malate were present for trace b. Traces are typical results of experiments that were repeated on at least three separate mitochondrial preparations.

Alterations to the Mitochondrial Glutathione Pool

The mitochondrial glutathione pool protects mitochondria from oxidative damage by compounds such as peroxynitrite, and depletion of the mitochondrial glutathione pool leaves mitochondrial proteins and lipids vulnerable to oxidative damage. In addition, the permeability transition is significantly affected by the oxidation of mitochondrial thiols and NAD(P)H, both of which interact with the mitochondrial

glutathione pool. We investigated the effects of peroxynitrite and tBHP on the mitocondrial glutathione pool under conditions where calcium efflux occurs and where it is prevented by incubation with CsA or with glutamate and malate. To characterise alterations to mitochondrial glutathione, we measured the amounts of GSH and GSSG remaining within the mitochondria, mitochondrial glutathione–protein mixed disulfides, and glutathione equivalents released from the mitochondria. To facilitate comparison, all data are presented as the amount of glutathione equivalents in these four forms as a percentage of the total amount of glutathione equivalents in the incubation.

Mitochondria were preincubated, zero-time samples were taken, and then either peroxynitrite or tBHP were added and further measurements taken (Figs. 2, 3). The mitochondrial glutathione pool was largely reduced before addition of oxidants, with small amounts of GSSG, mixed disulfides, and extramitochondrial glutathione equivalents.

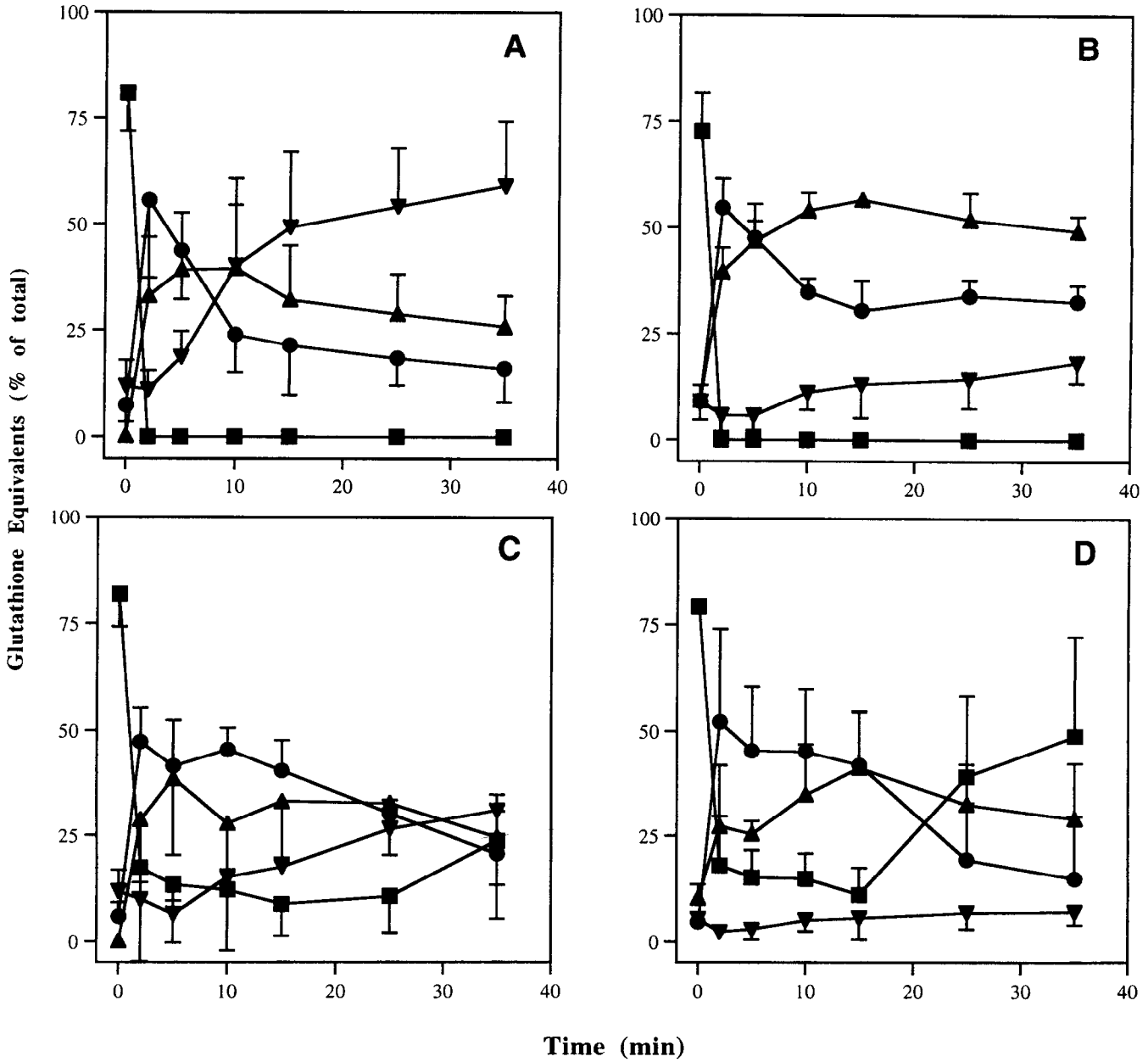

FIG. 2. **Oxidation of the mitochondrial glutathione pool by tBHP. Mitochondria were incubated as described in Materials and Methods, and after a 5-min preincubation a zero-time sample was taken; then tBHP (500 μM) was added and more samples taken at the times indicated. All samples were assayed for GSH (■), GSSG (●), glutathione–protein mixed disulfides (▲), and glutathione equivalents released into the extramitochondrial medium (▼). The following were included in the incubations: (A) succinate and rotenone; (B) succinate, rotenone, and CsA; (C) glutamate and malate; (D) CsA, glutamate, and malate. Data are the percentage of glutathione equivalents in the incubation present in that form and are the means ± SEM of experiments on at least three separate mitochondrial incubations.**

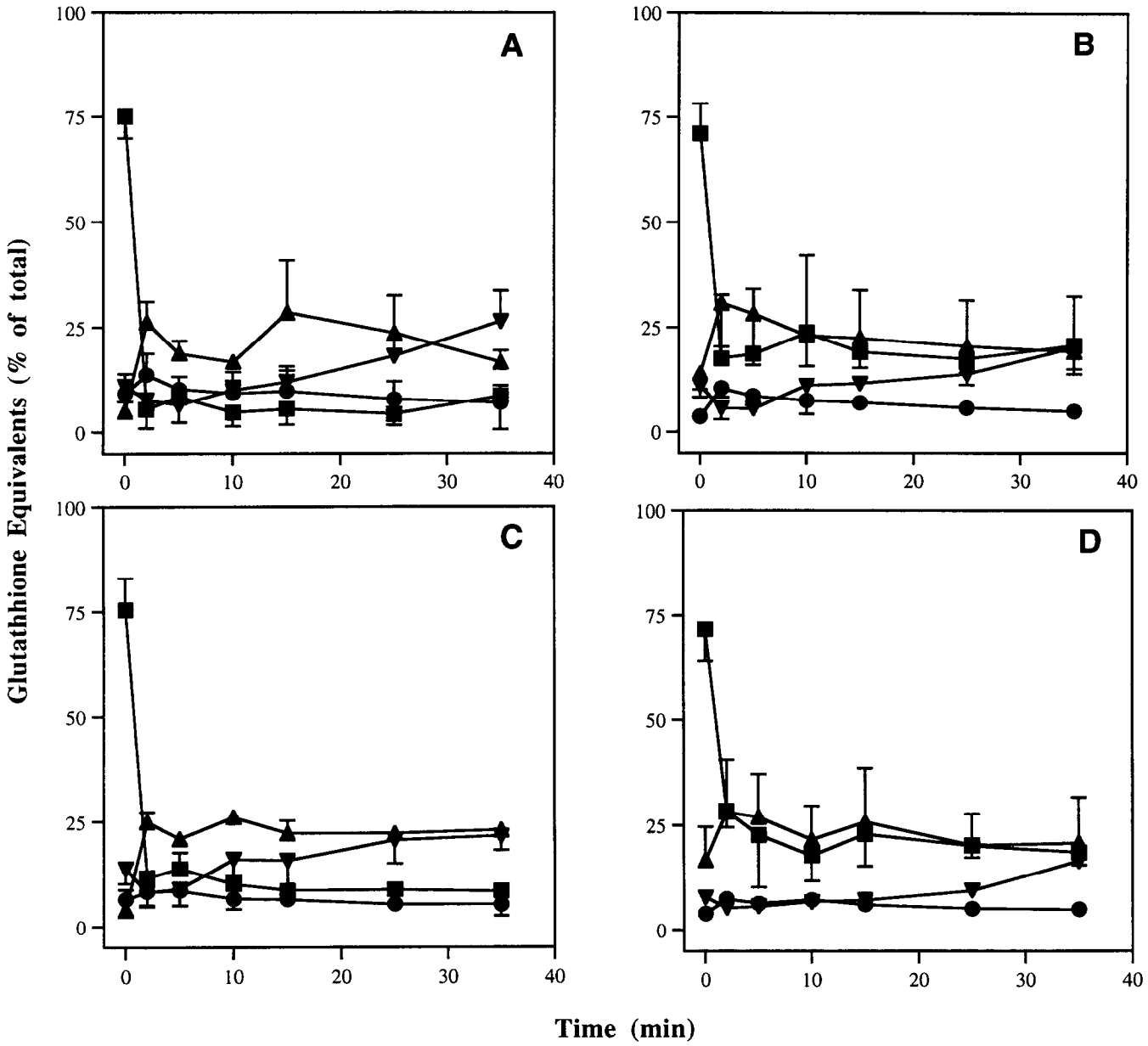

FIG. 3. Oxidation of the mitochondrial glutathione pool by peroxynitrite. Mitochondria were incubated as described in Materials and Methods, and after a 5-min preincubation a zero-time sample was taken; then peroxynitrite (250 μM) was added and more samples taken at the times indicated. All samples were assayed for GSH (■), GSSG (●), glutathione–protein mixed disulfides (▲), and glutathione equivalents released into the extramitochondrial medium (▼). The following were included in the incubations: (A) succinate and rotenone; (B) succinate, rotenone, and CsA; (C) glutamate and malate; (D) CsA, glutamate, and malate. Data are the percentages of glutathione equivalents present in that form expressed as a percentage of the total number of glutathione equivalents present in the zero-time incubation. Data are the means of experiments on at least three separate mitochondrial incubations. Control experiments in which "late quenched" peroxynitrite was used did not show any oxidation of glutathione.

Addition of tBHP (Fig. 2A) caused the GSH to decrease and led to a substantial increase in the amount of GSSG and mixed disulfides. This increase was followed by a gradual efflux of GSSG from the mitochondria due to induction of the permeability transition [42]. Preventing the permeability transition with CsA (Fig. 2B) or glutamate and malate (Fig. 2C) decreased the efflux of glutathione equivalents from the mitochondria substantially. In the presence of CsA (Fig. 2B), tBHP still completely oxidised the mitochondrial glutathione pool, but instead of being released from the mitochondrial (Fig. 2A), the GSSG gradually formed mixed disulfides, presumably by disulfide exchange between protein thiols and GSSG. The NAD(P)H-linked substrates glutamate and malate prevented the permeability transition (data not shown) but did not prevent tBHP from substantially oxidising the mitochondrial pool and forming glutathione–protein mixed disulfides (Fig. 2C), even in the presence of CsA (Fig. 2D).

Therefore, NAD(P)H-linked substrates do not prevent the permeability transition by preventing oxidation of the mitochondrial glutathione pool or the formation of glutathione–protein mixed disulfides.

When peroxynitrite was used to oxidise the mitochondrial glutathione pool instead of tBHP (Fig. 3), the situation was quite different. After addition of peroxynitrite, 40–50% of the total glutathione was not recoverable (Figs. 3, 4). In all cases (Fig. 3A–D), the addition of peroxynitrite substantially decreased the amount of GSH and increased glutathione-protein mixed disulfides but did not lead to a substantial increase in GSSG. The presence of glutamate and malate (Fig. 3C,D) did not protect the mitochondrial glutathione pool from oxidation by peroxynitrite, again indicating that glutamate and malate do not prevent the permeability transition by blocking oxidation of the mitochondrial glutathione pool. The data shown in Figs. 2 and 3 indicate that peroxynitrite interacts with the mitochondrial glutathione pool very differently than tBHP and that the prevention of the mitochondrial permeability transition by NAD(P)H-linked substrates is not because of their effects on the mitochondrial glutathione pool.

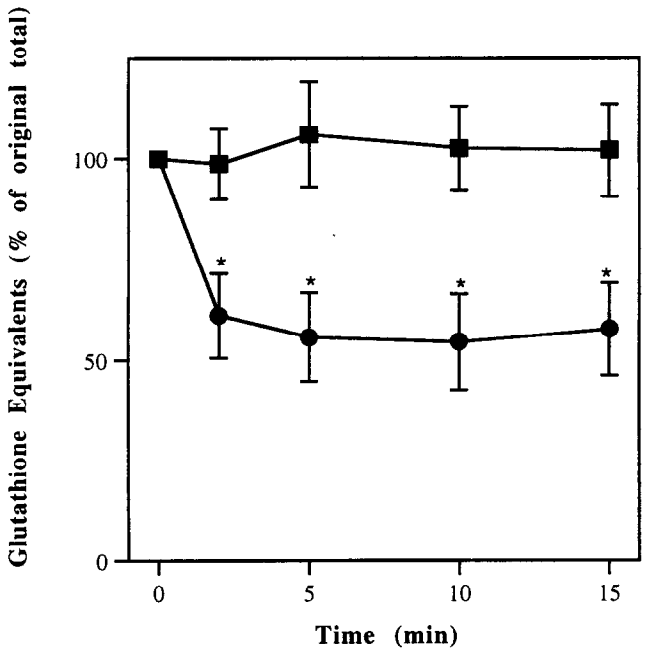

FIG. 4. Loss of mitochondrial glutathione equivalents after treatment with peroxynitrite. Data are taken from the experiments in the presence of CsA, rotenone, and succinate described in the captions to Figs. 2 and 3. After preincubation, a zero-point sample was taken and then either tBHP (500 μM; ■) or peroxynitrite (250 μM; ●) was added to the incubation. At subsequent time points, the total amount of glutathione equivalents present in the incubation (present as oxidised or reduced glutathione, or as mixed disulfides) were determined, summed, and are expressed as a percentage of the total number of glutathione equivalents present at the start of the incubation. Data are the means ± SEM of experiments on at least three separate mitochondrial incubations. *P < 0.05 by Student's *t*-test for unpaired data.

Oxidation of Glutathione by Peroxynitrite

Exposure to peroxynitrite decreased the amount of recoverable glutathione found in the experiments described by Fig. 3. This decrease is shown clearly in Fig. 4 where addition of peroxynitrite converts about 40–50% of glutathione equivalents to a product that is neither GSSG nor glutathione–protein mixed disulfides. A similar disappearance of glutathione was found when peroxynitrite was added directly to purified GSH (data not shown), suggesting a direct reaction between GSH and peroxynitrite. These findings are consistent with peroxynitrite reacting with thiols to form higher sulfur oxidation states such as sulfinic or sulfonic acids [13], which would not be detected by our assays. To test this possibility, we incubated glutathione samples isolated from peroxynitrite-treated mitochondria with borohydride to reduce sulfur oxidation products to a thiol detectable by the recycling assay (Fig. 5). A large proportion of the glutathione equivalents lost following treatment with peroxynitrite are regained by borohydride treatment, indicating that peroxynitrite converted GSH to higher sulfur oxidation states. To determine the nature of these oxi-

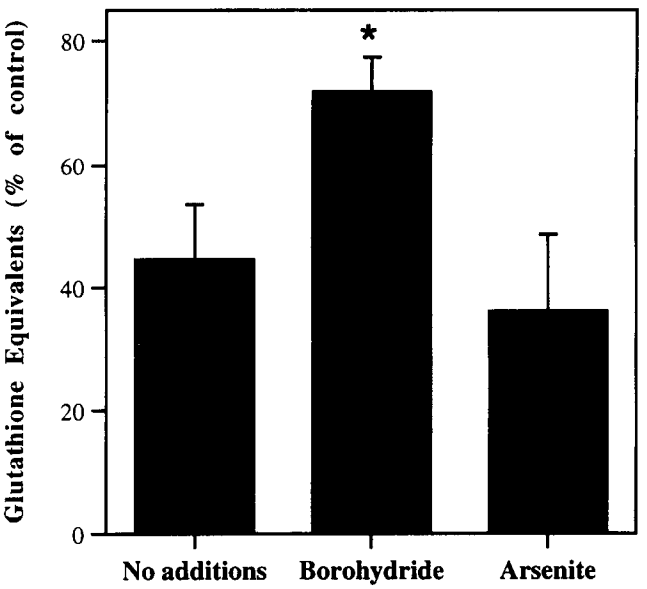

FIG. 5. Recovery of mitochondrial glutathione equivalents by reduction with borohydride. Mitochondria were incubated with CsA as described in Materials and Methods, then treated with peroxynitrite (250 μM), and the mitochondrial matrix glutathione equivalents were extracted by centrifuging the mitochondria through oil into acid as described in Materials and Methods. The acid layer (200 μL) was added to 80 μL 200 mM TRIS-HCl (pH 7.4) and 10 mg K_2CO_3 and incubated at 40°C for 30 min with no further additions, with sodium borohydride (1% w/v), or with arsenite (25 mM). All samples were then acidified with 20 μL sulfosalicylic acid (50% w/v) and assayed for glutathione equivalents by using the recycling assay. Data are expressed as a percentage of the glutathione equivalent content of control incubations that were not exposed to peroxynitrite and are the means ± SEM of experiments on at least three separate mitochondrial preparations. *P < 0.05, by Student's *t*-test for unpaired data.

dation products, the glutathione samples from peroxynitrite-treated mitochondria were also incubated with arsenite (Fig. 5), which reduces sulfenic acid, but not higher oxidation states such as sulfinic or sulfonic acid, back to a thiol [13]. Arsenite treatment did not recover any glutathione equivalents, suggesting that sulfenic acid was not one of the peroxynitrite oxidation products, which is in agreement with an earlier report [13]. Another possibility is that peroxynitrite reacts with GSH to form an S-nitrosothiol [43]. To test this, we treated glutathione isolated from peroxynitrite-treated mitochondria with mercuric chloride, which decomposes S-nitrosothiols, releasing nitrite, and then assayed for nitrite. These experiments showed that about (mean ± SD) 0.5 ± 0.2 nmol S-nitrosothiol/mg protein was formed by peroxynitrite (n = 3). This is about 10% of the total pool of glutathione equivalents in the mitochondrial matrix; therefore, S-nitrosothiol formation occurs in our experiments but accounts for less than 20% of the glutathione depletion shown in Fig. 4, 80% of which is probably due to the formation of higher sulfur oxidation states.

Oxidation of the Mitochondrial NAD(P)H Pool

To determine the mechanism by which the NAD(P)H-linked substrates prevented the mitochondrial permeability transition and to characterise further the interaction of peroxynitrite with mitochondria, we measured the effects of peroxynitrite and tBHP on the mitochondrial NAD(P)H pool. Addition of tBHP rapidly oxidised the mitochondrial NAD(P)H pool (Fig. 6A, trace b), and this oxidation was substantially decreased in the presence of glutamate and malate (Fig. 6A, trace a), suggesting that these substrates prevent the permeability transition by maintaining mitochondrial NAD(P)H. When peroxynitrite was used as the oxidant, there was a rapid oxidation of the mitochondrial NAD(P)H pool (Fig. 6C,D, trace a); however, about 50% of the NAD(P)H pool rapidly reestablished itself. This transient oxidation of NAD(P)H by peroxynitrite is due to the short half-life of peroxynitrite, and the fact that the permeability transition is maintained even after the oxidation has decreased suggests that the critical changes are not rapidly reversed by the NAD(P)H pool. Glutamate and malate prevented the complete oxidation of the NAD(P)H pool by peroxynitrite (Fig. 6D), again suggesting that they prevented the permeability transition by maintaining mitochondrial NAD(P)H. However, even in the presence of glutamate and malate, the NAD(P)H pools did not recover to their original levels.

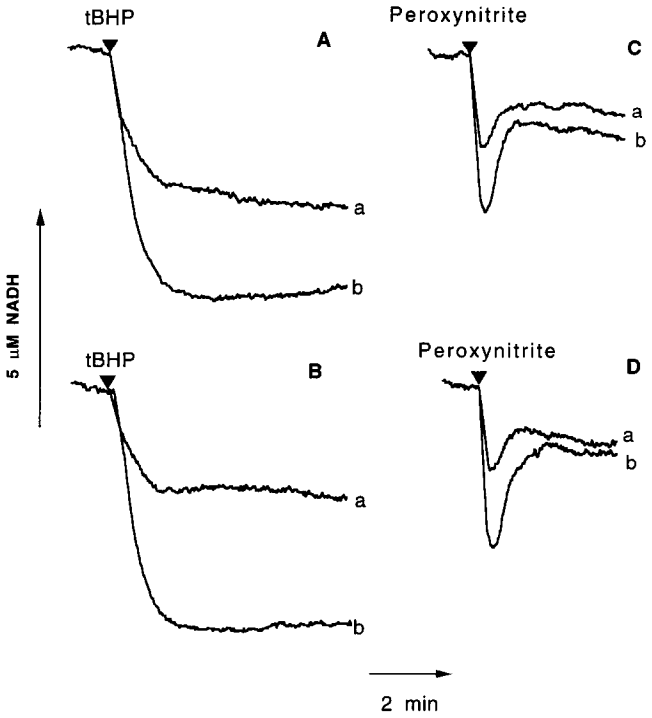

FIG. 6. Oxidation of mitochondrial NAD(P)H by peroxynitrite or tBHP. The relative amounts of NAD(P)H in mitochondrial suspensions were estimated fluorometrically as described in Materials and Methods. For all panels (A–D), mitochondria were incubated with glutamate and malate (upper trace in each panel) or succinate and rotenone (lower trace in each panel). For B and D, CsA was present. After preincubation, tBHP (500 μM) or peroxynitrite (250 μM) were added where indicated. Separate control incubations showed that addition of "late quenched" peroxynitrite (i.e. a control solution that did not contain any peroxynitrite) did not affect NAD(P)H. Other incubations (data not shown) with acetoacetate and β-hydroxybutyrate showed that the NAD(P)H pool was essentially completely reduced prior to addition of oxidants and completely oxidised after addition of tBHP (A). Addition of "late quenched" peroxynitrite to the mitochondrial suspension did not alter the NAD(P)H fluorescence. Addition of tBHP or "late quenched" peroxynitrite to 10 μM NADH did not affect its fluorescence, but addition of peroxynitrite caused a small (9%) stable increase in fluorescence.

DISCUSSION

Peroxynitrite is an important biological oxidant that may significantly damage mitochondria *in vivo* [11,31]. Here we have shown that peroxynitrite rapidly and directly oxidises mitochondrial GSH to glutathione–protein mixed disulfides rather than to GSSG. Higher oxidation states of glutathione, probably sulfinic or sulfonic acids, were also formed and may be significant in the pathology of peroxynitrite because they deplete mitochondrial glutathione, and it is unclear whether mitochondria can recycle these compounds back to GSH. Peroxynitrite reacts directly with protein thiols and glutathione to form thiyl radicals [14, 15], which probably react further with oxygen to form sulfinic or sulfonic acids [15]. A competing reaction for thiyl radicals is that with other thiols to form a $-S-S^{\cdot-}-$ bond, which may lose an electron to oxygen, thus forming a disulfide bond and superoxide [44]. This reaction may account for the glutathione–protein mixed disulfides found in our experiments because the relatively low GSSG formation by peroxynitrite in our experiments makes mixed disulfide formation by thiol-disulfide exchange less likely. A lower oxygen concentration would have caused more of the

initial thiyl radicals formed by peroxynitrite to react with other thiols to form glutathione–protein mixed disulfides and protein–protein cross links rather than sulfinic or sulfonic acid derivatives [15]. However, in our experiments peroxynitrite did not increase mitochondrial protein cross linking, as demonstrated by polyacrylamide gel electrophoresis of mitochondrial proteins under nonreducing conditions (data not shown). In addition to depleting mitochondrial glutathione, peroxynitrite also leads to oxidation of the mitochondrial NAD(P)H pool and this may also contribute to the damage caused by peroxynitrite exposure.

Both NAD(P)H-linked substrates and thiol reagents prevented the mitochondrial permeability transition caused by peroxynitrite, as is the case for other oxidants. Furthermore, induction of the permeability transition by tBHP and peroxynitrite did not correlate with oxidation of mitochondrial GSH or with the formation of glutathione–protein mixed disulfides. In particular, the permeability transition was prevented by glutamate and malate, with the glutathione pool remaining largely oxidised and while there was a large amount of glutathione–protein mixed disulfides; this result agrees with and extends most earlier findings [29, 41, 45] but contrasts with one report [46]. NAD(P)H prevents the permeability transition independently of mitochondrial glutathione, suggesting regulation of the transition by the redox state of the mitochondrial NAD(P)H pool. This finding is consistent with current models for the regulation of the permeability transition, in which the oxidation state of critical protein thiols are central [29, 41]. Specifically, the redox state of vicinal dithiols may modulate the response of the permeability transition to mitochondrial membrane potential [22] and calcium concentration [47]; supporting this, reagents that crosslink vicinal dithiols induce the permeability transition without oxidising NAD(P)H [47, 48]. A reasonable working hypothesis is that the mitochondrial NAD(P)H pool maintains critical vicinal dithiols in a reduced state and that oxidation of these thiols to a disulfide favours induction of the permeability transition. Alternatively, peroxynitrite itself could directly oxidise these critical thiols or could lead to the nitration or nitrosation of other critical components of the calcium efflux pathway. The mechanism by which the NAD(P)H redox state regulates these putative thiols is unclear, but mediation by the thioredoxin/thioredoxin reductase system [38], which is present in mitochondria and does not equilibrate with the mitochondrial glutathione pool, is one possibility. Supporting this, the activity of mitochondrial thioredoxin reductase may be inhibited by calcium, suggesting a possible mechanism by which calcium could modulate the permeability transition [49].

In summary, we have shown that peroxynitrite can damage mitochondria by depleting the mitochondrial glutathione and NAD(P)H pools, which removes a major protective system against oxidative stress and would thus lead to mitochondrial damage. An additional way of damaging mitochondria by peroxynitrite is through induction of the mitochondrial permeability transition. Both of these processes may contribute to the cell damage that follows the formation of peroxynitrite from nitric oxide and superoxide in a number of pathological situations.

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