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Interactions of Mitochondrial Thiols with Nitric Oxide

NIKOLA J. COSTA, CHRISTINA C. DAHM, FIONA HURRELL, ELLEN R. TAYLOR, and MICHAEL P. MURPHY

ABSTRACT

The interaction of nitric oxide (NO) with mitochondria is of pathological significance and is also a potential mechanism for the regulation of mitochondrial function. Some of the ways in which NO may affect mitochondria are by reacting with low-molecular-weight thiols such as glutathione and with protein thiols. However, the detailed mechanisms and the consequences of these interactions for mitochondria are uncertain. Here we review mitochondrial thiol metabolism, outline how NO and its metabolites interact with thiols, and discuss the implications of these reactions for mitochondrial and cell function. *Antioxid. Redox Signal.* 5, 291–305.

INTRODUCTION

MITOCHONDRIA play a vital role in energy and intermediary metabolism and are also central to cell death, calcium homeostasis, and free radical generation (6, 123, 141, 174). Consequently disruption of mitochondrial function has a significant impact on the cell. Nitric oxide (NO) has a number of damaging effects on mitochondria that are thought to be of pathophysiological significance (12, 20, 120, 122, 140). These interactions often stem from excessive production of NO through persistent activation of inducible NO synthase (iNOS), with excess NO diffusing into and damaging mitochondria (20, 44, 122, 140). In addition to these disruptions, NO may also play a physiological role in regulating mitochondrial function through the reversible inhibition of cytochrome oxidase, the terminal enzyme of the respiratory chain (25, 38). Although the biological context in which these changes occur is obscure, inhibition of cytochrome oxidase by NO may be a way of regulating mitochondrial ATP synthesis, of decreasing respiration when oxygen concentration is low, or of increasing the production of superoxide ($O_2^{\cdot -}$) by the respiratory chain (25, 38, 120, 150). In support of a possible physiological role for NO within mitochondria, there is a putative mitochondrial NOS (mtNOS) (47, 60, 61, 87, 150, 169). This enzyme is thought to produce NO within mitochondria when stimulated by calcium, but its (patho)physiological significance is unclear.

Apart from the relatively specific interaction of NO with cytochrome oxidase, the other major way in which NO and its derivatives affect mitochondria is through the modification of thiols. These alterations certainly contribute to the mitochondrial damage that occurs in pathological situations and may also regulate mitochondrial function (120, 143). Here we review the nature and function of mitochondrial thiols and thiol redox systems. We then outline the ways in which NO and its derivatives affect mitochondrial thiols and consider how these interactions impact on mitochondrial and cell function. Finally, we discuss recent developments that may help in measuring how NO affects mitochondrial thiols.

MITOCHONDRIAL THIOLS

As the mitochondrial respiratory chain is a major source of reactive oxygen species (ROS), mitochondria have a range of antioxidant defenses, and among the most important of these are thiols (114, 143) (Fig. 1). Mitochondrial thiols can be divided into low-molecular-weight and protein thiols (PrSH). Glutathione (GSH) is the major low-molecular-weight mitochondrial thiol. There are a range of mitochondrial PrSH that

MRC–Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Cambridge, U.K.

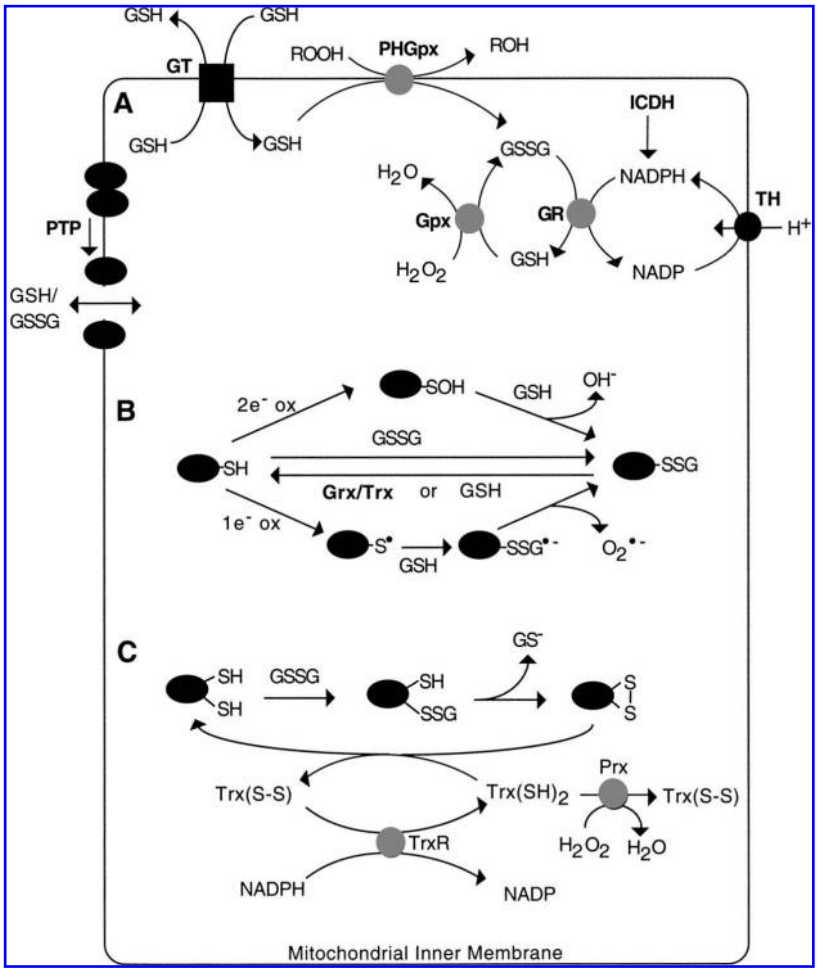

FIG. 1. Mitochondrial thiol system. The gray circles are enzymes, the black ovals are thiol proteins, and the black square is a putative transport protein. Enzyme names are in bold. Gpx, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulfide; GT, mitochondrial glutathione transporters; ICDH, isocitrate dehydrogenase; $O_2^{\cdot -}$, superoxide; PHGPx, phospholipid hydroperoxide glutathione peroxidase; Prx, peroxiredoxin; PTP, permeability transition pore; $RS^{\cdot}$, thiyl radical; RSOH, sulfenic acid; Trx, thioredoxin; TrxR, thioredoxin reductase.

can be broadly divided into the following: essential thiols in the active sites of enzymes; regulatory PrSH, alterations to which modulate protein function; thiols exposed on the surface of proteins; and buried PrSH that may play a structural role.

Glutathione

GSH is a small, hydrophilic molecule formed from glycine, cysteine, and glutamate that is present in high concentrations within the cytoplasm as the predominant free thiol (51, 114, 143, 153). Mitochondria have their own independent GSH pool with a concentration of 5–10 m*M,* about the same as that in the cytosol (71, 85, 143). This GSH pool is the major thiol redox buffer within mitochondria and is also an important protection against oxidative damage, both by direct reaction with ROS and as an electron donor for antioxidant enzymes (114, 143). GSH also protects against toxins through glutathione-*S*-transferases, which convert electrophilic xenobiotics and lipid peroxidation products, such as 4-hydroxynonenal, to thioethers for excretion (57, 134). For all these roles, the reduced thiol of GSH is essential, but in protecting mitochondria against ROS, GSH is oxidized to glutathione disulfide (GSSG) (153). Consequently, glutathione reductase (GR) plays an essential role in continually recycling GSSG back to GSH (132).

Mitochondrial GR is a flavoenzyme that uses NADPH as the electron donor to reduce GSSG to GSH (89, 127, 166, 167). A single gene encodes both cytoplasmic and mitochondrial isoforms of GR through alternative transcription initiation sites (166, 167). The mature mitochondrial GR is a homodimer of 51.7-kDa monomers (166, 167). To maintain sufficient NADPH to supply GR, the mitochondrial NADPH/NADP ratio is kept high by a transhydrogenase that utilizes the proton electrochemical potential difference across the inner membrane to drive electrons from NADH to NADP (19), and by NADP-dependent isocitrate dehydrogenase (84). Through these reactions, the ratio of GSH to GSSG in mitochondria is kept high and the mitochondrial GSH pool is typically 95–99% reduced, except during oxidative stress (143, 153) (Fig. 1A).

GSH is not made in mitochondria, but is synthesized in the cytoplasm and then imported (51, 71). The separation of the mitochondrial and cytosolic GSH pools was demonstrated by the biphasic decline of GSH in isolated hepatocytes, and in the liver *in vivo,* on administration of buthionine sulfoximine, a GSH synthesis inhibitor (71, 115). Cytosolic GSH in isolated hepatocytes was depleted relatively rapidly ($t_{1/2}$ = 2 h) by buthionine sulfoximine, whereas the mitochondrial pool was lost far more slowly ($t_{1/2}$ = 30 h), suggesting that mitochondria can maintain their GSH pool even when that in the

cytosol has been depleted or oxidized (71, 112, 114, 115). A further implication of these findings is that the mitochondrial and cytoplasmic GSH pools can change their redox states independently (95, 152). Even so, the mitochondrial and cytosolic GSH pools must communicate, as GSH is imported into mitochondria from the cytosol (51, 71). Furthermore, there is relatively rapid exchange of cytosolic and mitochondrial GSH, as the specific activity of the two pools equilibrates rapidly when the cytosolic pool is "spiked" with [^{35}S]cysteine (50, 71, 114). This suggests that there are glutathione transporters in the mitochondrial inner membrane that catalyze rapid GSH/GSH exchange, but not the net transport of GSH equivalents from mitochondria to the cytoplasm (114).

The nature of the glutathione transporter(s) in the mitochondrial inner membrane is uncertain, but there does not appear to be a mitochondrial GSSG efflux system (128). There was rapid, membrane potential-dependent uptake of GSH by isolated liver mitochondria by both high- and low-affinity transport systems (95, 112). In contrast, isolated kidney mitochondria had only a low-affinity uptake system that was not membrane potential-dependent, and in this case uptake was by electroneutral exchange with other dicarboxylates (113). A further contrast with liver was that kidney mitochondria took up GSSG; however, the low GSSG affinity of this process makes a physiological role unlikely (113). The efflux of GSH from GSH-loaded liver mitochondria was stimulated by external GSH, although at higher external GSH concentrations there was reuptake of the released GSH, implying sequestration of GSH in the intermembrane space (112, 114). A GSH transport activity was isolated and reconstituted into liposomes by techniques that are selective for the mitochondrial carrier superfamily (34, 113). However, these putative mitochondrial GSH carrier(s) have not been identified definitively, and the uptake mechanisms may be tissue-specific (34, 51, 113).

A further mechanism of GSH transport across the mitochondrial inner membrane is through induction of the mitochondrial permeability transition pore (PTP) (18, 73, 103, 152, 183). This is the formation of a relatively large proteinaceous pore in the inner membrane that leads to mitochondrial depolarization and the exchange of solutes up to 1,200 Da between the mitochondrial matrix and the cytosol (18, 73, 103, 183). Induction of the PTP does lead to the rapid release of GSH and GSSG from mitochondria (151), but whether this occurs *in vivo* as a mechanism to accumulate or release GSH and/or GSSG from mitochondria is not known (Fig. 1A).

Protein-glutathione mixed disulfides

An important response of thiols to oxidative stress is the formation of protein-glutathione mixed disulfides (PrSSG) (55, 153, 170, 182). Exposed PrSH are free to undergo protein *S*-thiolation by thiol–disulfide exchange with low-molecular-weight disulfides to form protein mixed disulfides (41, 170, 182). As GSH is the predominant low-molecular-weight thiol in the cell, this reaction is generally between GSSG and a PrSH to form a PrSSG, a process termed *S*-glutathionylation (41, 55, 153, 170, 182). In addition to thiol–disulfide exchange, another pathway to mixed disulfides is through the formation of a thiyl radical ($RS^{\cdot}$) by the one-electron oxidation of a thiol (170). The thiyl radical can then react with a glutathionylate anion (GS^-) to form a radical mixed disulfide ($RSSG^{\cdot -}$), which will lose an electron to oxygen to form $O_2^{\cdot -}$ leaving a mixed disulfide (178). The final route to mixed disulfides is through the two-electron oxidation of a thiol to a sulfenic acid (RSOH), which will then react with a thiolate to displace OH^- and generate a disulfide (153) (Fig. 1B).

Protein glutathionylation is a common early consequence of various forms of oxidative stress (55). One role of protein glutathionylation is to buffer the GSH concentration during transient oxidative stress by releasing GSH from GSSG and thus partially restoring the GSH pool (153, 170). The rapid reaction between protein thiyl radicals and GSH to form a radical mixed disulfide also has an important antioxidant role because it prevents thiyl radicals from reacting with oxygen to form higher oxidation states such as sulfinic (RSO_2H) and sulfonic (RSO_3H) acids. This may be a major pathway for the repair of thiyl radicals *in vivo,* diverting the radical to $O_2^{\cdot -}$, which can then be detoxified by superoxide dismutase (178).

In addition to antioxidant functions, there is growing evidence of a role for the formation of PrSSG in signaling and regulatory pathways (41, 55, 170). This could occur through the glutathionylation of proteins causing a change in their activity, and as PrSSG can be easily reduced, this may lead to reversible alteration of protein function, analogous to phosphorylation (55, 170). Supporting such a role, a large number of enzymes and proteins undergo alterations in activity on glutathionylation (41, 55, 170). However, one significant difference between this process and phosphorylation is that glutathionylation can occur by thiol–disulfide exchange between PrSH and the bulk GSH pool, and is therefore less selective than residue-specific phosphorylation and dephosphorylation by kinases and phosphatases. One corollary of this is that protein glutathionylation may be a general response to oxidative stress (41, 170, 182). However, it is also likely that particular thiol proteins are modulated selectively by *S*-glutathionylation. This can occur because the susceptibility of a particular PrSH to glutathionylation can be modified by altering its pK_a and/or exposure to the ambient GSH pool (153). In support of this, it has been shown that there are different classes of proteins with a range of propensities to form protein mixed disulfides within cells under oxidative stress (155). Furthermore, glutathionylation/deglutathionylation can be catalyzed by glutaredoxin (Grx), thioredoxin (Trx), or protein disulfide isomerase (PDI), which may help to form or degrade mixed disulfides at particular PrSH and thus modulate the lifetime of critical PrSSG (86, 133).

PrSSG may act as redox-sensitive "switches" in protein function in response to changes in the ambient GSH redox state (46, 153). Although glutathionylation of a single PrSH could alter protein function in this way, another class of PrSH, vicinal dithiols [$Pr(SH)_2$], may enable proteins to respond to smaller changes in thiol redox state than those required to affect a lone PrSH (153). The formation of a PrSSG on one thiol of a vicinal dithiol can lead to the formation of an internal protein disulfide (PrSS) through the free thiol displacing GSH from the adjacent mixed disulfide (64) (Fig. 1C). That the formation of an internal disulfide is more sensitive to changes in the GSH redox state than the formation of a

mixed disulfide can be illustrated by comparing the equilibrium for the formation of a mixed disulfide of a single thiol (153):

$$GSSG + PrSH \rightleftharpoons PrSSG + GSH \qquad K_{eq} = \frac{[GSH][PrSSG]}{[GSSG][PrSH]}$$

with that of a vicinal dithiol, which will form an internal disulfide:

$$Pr(SH)_2 + GSSG \rightleftharpoons PrSS + 2GSH \qquad K_{eq} = \frac{[GSH]^2[PrSS]}{[GSSG][Pr(SH)_2]}$$

In the latter case, the ratio of oxidized to reduced protein is proportional to $[GSSG]/[GSH]^2$, whereas that for a simple mixed disulfide is proportional to $[GSSG]/[GSH]$. Consequently, the formation of an internal disulfide will be more sensitive to changes in the GSSG/GSH ratio (153). Consistent with this, vicinal dithiols are widespread *in vivo* (64), and there is a correlation between the reduction potential of the cell's GSSG/2GSH redox couple and cell state, with cell proliferation occurring at approximately −240 mV, differentiation at approximately −200 mV, and apoptosis at −170 mV (31, 153). Although the proteins that the cell uses to sense and respond to these changes in GSH redox state are not known, it is tempting to speculate that they may be vicinal dithiol proteins.

The glutathionylation of mitochondrial thiol proteins is well established (128, 142, 152). As there is no specific mechanism for the release of GSSG from mitochondria, during oxidative stress GSSG builds up, leading to spontaneous thiol–disulfide exchange between PrSH and GSSG that glutathionylates PrSH (128, 142, 152). If the mitochondrial GSH/GSSG ratio is restored, then these mixed disulfides will be gradually reduced, displacing GSH from the protein (152). Although this process does not require enzyme catalysis, both Trx and Grx can catalyze glutathionylation and deglutathionylation, as will be considered later. One potential role for the formation of protein mixed disulfides within mitochondria is to buffer GSH, as the reaction of PrSH with GSSG will regenerate GSH (143). The formation of glutathione mixed disulfides also has pathological significance for mitochondria as they disrupt the activity of respiratory complexes (82), may lead to the cross-linking of membrane proteins, and can also induce the PTP (49, 102, 171). Whether induction of the PTP is due to the formation of mixed disulfides alone or to their subsequent conversion to internal and intermolecular disulfides is unclear; however, a role for vicinal dithiols is supported by the induction of the PTP by the vicinal dithiol reagent phenylarsine oxide (18, 135) (Fig. 1A). Whether there are other regulatory roles for the formation of mixed and internal disulfides in the modulation of mitochondrial function is not known.

Glutathione peroxidase

The $O_2^{\cdot -}$ produced by the respiratory chain is converted to hydrogen peroxide (H_2O_2) through the action of manganese superoxide dismutase (MnSOD), or by spontaneous dismutation (54, 141). Thus, mitochondria continually produce H_2O_2, which can react with Fe^{2+} or Cu^{+} to produce the damaging hydroxyl radical ($^{\cdot}OH$), and therefore it is important for mitochondria to degrade H_2O_2. With the exception of the heart (138), mitochondria from most tissues do not have catalase; consequently, they rely on glutathione peroxidases (Gpx) and peroxiredoxin (Prx) to detoxify peroxides (132, 143). Gpx catalyze the reduction of H_2O_2 and alkyl peroxides (ROOH) using GSH as the reducing agent. Mitochondria have their own soluble Gpx, Gpx1, which is a 22-kDa selenoenzyme that is highly expressed in mitochondria from the liver and kidney, but poorly expressed in heart and muscle (48, 132). In mice lacking this enzyme, the rate of H_2O_2 efflux from liver mitochondria increases and their mitochondria become more oxidatively damaged, but heart mitochondrial H_2O_2 efflux is unaffected (48). Phospholipid hydroperoxides (PlOOH) are a common consequence of oxidative damage, particularly to phospholipids with a large proportion of unsaturated fatty acids, such as cardiolipin (43). To degrade PlOOH, there is a specific phospholipid hydroperoxide glutathione peroxidase (PHGPx: *Gpx4*), a selenoenzyme that directly reduces lipid hydroperoxides to water and a hydroxylated lipid (1). There is a short isoform of this enzyme in the cytoplasm and a longer, mitochondrial isoform that has an N-terminal targeting peptide that is processed to give a mature membrane-bound enzyme of 20 kDa (1, 66). PHGPx protects against the peroxidation of cardiolipin in mitochondria (126), and may also directly detoxify other ROS, such as peroxynitrite ($ONOO^-$) (3, 4) (Fig. 1A).

Glutaredoxin

Grx, also known as thioltransferases or thiol/disulfide oxidoreductases, are small proteins with an active-site dithiol that can reduce internal protein disulfides with the concomitant oxidation of its dithiol to an internal disulfide (7, 70, 149). The disulfide form of Grx is then reduced back to its dithiol form by equilibration with the ambient GSH pool. Grx can also catalyze the formation of PrSSG; hence, they have the potential to accelerate the formation of mixed disulfides in response to an increased GSSG/GSH ratio (7, 70, 149). Grx2 has a mitochondrial isoform (Grx2a) generated by differential splicing; processing of the mitochondrial targeting sequence yields a 14.8-kDa mature protein (65, 109). Grx is generally more effective than other thiol enzymes, such as Trx or PDI, at reversing PrSSG, but the relative effectiveness of catalysis varies considerably with the particular protein substrate (86, 153) (Fig. 1B).

Thioredoxin and thioredoxin reductase

Trx, like Grx, are small thiol proteins that have an active-site dithiol that reduces internal disulfides within proteins, leaving an internal disulfide on Trx (76). The disulfide form of Trx is returned to its active dithiol form by thioredoxin reductase (TrxR) (76), which may also have $ONOO^-$ reductase activity (3, 4). Mitochondria contain their own Trx (Trx2) a 12.2-kDa mature protein (76, 159, 168), and their own TrxR (TrxR2) which is a homodimer of 55-kDa subunits that is abundant in heart, liver, kidney, and adrenal gland mitochondria (58, 101, 117, 176). TrxR2 is an FAD-containing selenoenzyme that uses matrix NADPH to reduce the disulfide form of Trx2 (58, 101, 117, 176). As well as reducing protein disulfides, Trx can also remove GSH from glutathionylated proteins, although with markedly different rates for different

protein mixed disulfides (133). Interestingly, the activity of cytosolic Trx is decreased by glutathionylation on a regulatory thiol that is not part of its active site (32). Within mitochondria, there is evidence that TrxR2 may be involved in regulating the PTP (145), and that TrxR2 is inhibited by calcium concentrations in the physiological range (104) (Fig. 1C).

Peroxiredoxins

Prx, formerly known as thiol-specific antioxidant, or Trx-specific peroxidase, are peroxidases that utilize Trx as a reducing agent (144). Prx act as dimers with a thiol on one of the monomers reacting with H_2O_2 to form a RSOH, which then reacts with a thiol on the other monomer to displace OH^- and form an intermolecular disulfide (144). This disulfide is returned to its active dithiol form by Trx (144). Mitochondria have their own Prx, PrxIII (176), which is a member of the two cysteine-containing Prx group (144). In addition to detoxifying peroxides, Prx can inactivate protein thiyl radicals before they can react with oxygen (180), and may also act as $ONOO^-$ reductases (27) (Fig. 1C).

Protein disulfide isomerase

PDI is a protein-thiol oxidoreductase that catalyzes the oxidation, reduction, and isomerization of protein disulfides and acts as a chaperone in protein folding and maturation (63, 175). It primarily operates in the oxidized thiol environment of the endoplasmic reticulum where its two thiol/disulfide active sites facilitate the forming and breaking of internal disulfides within proteins during their folding and maturation (63). PDI also catalyzes the formation and degradation of PrSSG, can accelerate protein aggregation (63), and also catalyzes transnitrosation (181). A mitochondrial isoform of PDI has been purified, but not yet cloned, and appears to be a 54-kDa protein that is bound primarily to the outer membrane (146, 147). This PDI can be reduced by both Trx and TrxR, and although its function is unclear, it may be involved in pore formation in the outer membrane in response to cytosolic thiol oxidation (147). Whether there is also a mitochondrial matrix PDI, which might be predicted to assist in the maturation of both mitochondrially encoded and imported proteins, is unclear.

Sulfhydryl oxidase

Sulfhydryl oxidases are flavoproteins that oxidize thiols to disulfides (17, 92, 97, 100). The primary electron acceptor is a dithiol motif that is then reoxidized by FAD, which in turn passes two electrons to oxygen to form H_2O_2 (92, 97). The mitochondrial sulfhydryl oxidase (Erv1p) is an essential protein in yeast and has a close mammalian homologue, ALR (97).These proteins are found in the mitochondrial intermembrane space and are involved in the biogenesis of cytosolic iron sulfur clusters (97). Mammalian mitochondrial sulfhydryl oxidase may also be involved in mitochondrial biogenesis, at least in sperm (92), but how it does this is not known.

Overview of mitochondrial thiol metabolism

Mitochondria have a comprehensive array of interacting thiol metabolizing systems (Fig. 1). The major function of these is to protect mitochondria from oxidative stress. If these systems prove ineffective, then the consequent alterations to thiol redox state will disrupt mitochondrial function through inhibition of enzymes (37, 82) and by changes to the permeability of the inner membrane, such as through induction of the PTP. As induction of the PTP can release cytochrome *c* and induce apoptosis, this suggests that mitochondrial thiol redox state is one of the triggers by which cells sense when they are damaged irreversibly and thus commit to apoptosis (18, 39, 157, 158). This would also explain how GSH and the overexpression of mitochondrial thiol antioxidant enzymes such as PrxIII and TrxR2 prevent cell death (93).

In addition to responding to oxidative damage, the intricacy of their thiol systems suggests that mitochondria have the potential to regulate the redox state of their protein thiols selectively, although whether this occurs *in vivo* is not known. Even so, the growing evidence for a role for thiol modification in cell signaling and the central position of mitochondria in metabolism suggests that this is a promising area of investigation. In support of this, changes in mitochondrial thiol redox state occur early during apoptosis (111), and the extrusion of GSH from the cell that occurs during receptor-activated apoptosis leads to mitochondrial changes and cytochrome *c* release (39, 62, 172). In addition, the cytokine tumor necrosis factor α can cause changes in mitochondrial thiol redox state (67, 154). Depletion of mitochondrial GSH leads to increased efflux of ROS from mitochondria to the cytoplasm with the consequent activation of the transcription factor nuclear factor-κB (NFκB) (56). These and other findings suggest that mitochondrial thiol alterations may be part of the process by which the cell commits to apoptosis. One possibility is that these thiol systems may modulate the sensitivity of mitochondria to apoptotic stimuli, and such a role for the redox poise of mitochondrial thiols may help resolve conflicting data on the role of oxidative stress and antioxidants in apoptosis. The potential role of mitochondria in redox signaling is also being explored, and the possibility of a link between the redox state of mitochondrial thiols and the production of ROS such as H_2O_2 in signaling to transcription factors in the cytoplasm is under investigation (52, 53, 79). Any such mitochondrial thiol changes are likely to occur through the formation of mixed and internal disulfides at particular thiols, with the further modulation of their redox state by Trx2, Grx2, and other enzymes. However, the particular PrSH involved, the signaling pathways, and the biological significance of these changes are far from certain. In the next section, we discuss how NO and its derivatives may interact with mitochondrial thiols.

INTERACTION OF NO WITH MITOCHONDRIAL THIOLS

Reactions of NO and its derivatives with thiols

The free radical NO is produced by the activity of various NOS. Its physiological concentration is in the nanomolar range, with micromolar concentrations being pathological (90, 122) (Fig. 2). Physiological concentrations of NO are stable in water under anaerobic conditions, but in the presence of oxygen NO decomposes by a third-order reaction:

$$2NO + O_2 \rightarrow 2 \cdot NO_2$$

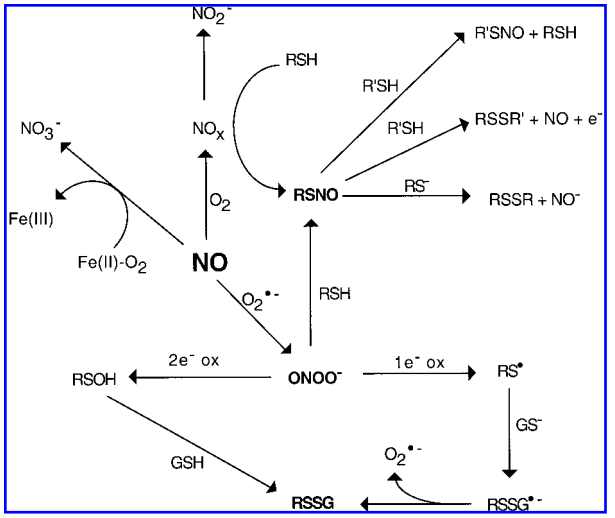

FIG. 2. Reactions of NO and its derivatives with thiols.

$^{\cdot}NO_2$ then reacts further with NO to form N_2O_3, which is hydrolyzed to HNO_2 (12, 30, 177):

$$^{\cdot}NO_2 + NO \rightarrow N_2O_3$$
$$N_2O_3 + H_2O \rightarrow 2HNO_2$$

The reaction of NO with O_2 to form $^{\cdot}NO_2$ is, however, too slow to explain the decay of NO *in vivo,* where the NO and O_2 concentrations are low; instead, the diffusion of NO to oxyhemoglobin or oxymyoglobin to form NO_3^- is likely to predominate (12, 177). Although NO is relatively soluble in water, reaching saturation at ~2 m*M,* it is about ninefold more soluble in hydrophobic solvents, and consequently dissolves and diffuses rapidly within lipid bilayers (107, 156). As O_2 is also more soluble in lipid bilayers, membranes may act as a "lens" focusing the reactivity between NO and O_2 so that these reactions mostly take place within lipid bilayers (107, 156).

Another major reaction of NO is with $O_2^{\cdot -}$ to form $ONOO^-$, which is itself a particularly reactive oxidizing and nitrating agent that can also interact with molecules such as CO_2 to form further reactive metabolites (11–15, 124). As the pK_a of $ONOO^-$ is ~6.8, it protonates readily and its conjugate acid decays to nitrate within a second or so at physiological pH (12, 13, 124). There are many other reactions that may be relevant to NO (patho)physiology, but as their significance is currently unclear, they will not be considered further here.

NO itself is generally unreactive with most nonradicals at physiological concentrations, and its rate of reaction with thiols in the absence of oxygen is low (12, 177). The only significant pathway by which NO can react directly with thiols is through thiyl radicals, and this radical–radical reaction leads to the formation of *S*-nitrosothiols (RSNO), which are far longer lived than NO (12). This reaction may also have a protective role in preventing thiyl radicals from reacting with O_2 to form $RSOO^{\cdot}$ and further oxidation products. Another possible route to RSNO is by transnitrosation of thiols by transition metal–NO complexes such as dinitrosyl iron, which may form on exposure of iron sulfur centers to NO (30). In the presence of oxygen, NO does react with thiols to form RSNO and this reaction is thought to occur through the reaction of the thiol with intermediates (NO_x) that occur in the autooxidation of NO to NO_2^- by O_2 (30, 75, 91). Because of the greater solubility of NO and O_2 in hydrophobic solvents, such intermediates may be present in higher concentrations in and around the lipid bilayers (107, 156). All other pathways by which thiols can react with NO to form RSNO require electron acceptors, such as the transition metal ions Fe^{3+} or Cu^{2+}, or O_2, which would form $O_2^{\cdot -}$ (12, 68, 177).

The other significant reaction pathway between NO and thiols is through $ONOO^-$ (137). Two reactions of $ONOO^-$ with thiols predominate, a one-electron oxidation to form a thiyl radical and a two-electron oxidation to form an RSOH (59, 137). Thiyl radicals react rapidly with oxygen to form $RSOO^{\cdot}$, which can decay to oxidation end products such as RSO_2H and RSO_3H (88, 161). However, *in vivo* the high GSH concentrations would cause GSH to react with the thiyl radical to form $RSSG^{\cdot -}$, which will lose the electron to oxygen to form $O_2^{\cdot -}$, leaving a mixed disulfide (178). The RSOH will also react with thiols, primarily GSH, to displace water and form a mixed disulfide (161). One other possible reaction of $ONOO^-$ with thiols is the direct formation of an RSNO (173, 179). Interestingly, the *S*-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by $ONOO^-$ required the presence of the thiol-reducing reagent dithiothreitol (DTT), probably to prevent excess $ONOO^-$ oxidizing PrSH to higher oxidation states; in the presence of DTT, *S*-nitrosylation of DTT itself may occur, which then transfers NO to the PrSH (118).

So, a major product of the reactions of NO and its derivatives with thiols will be mixed disulfides, which have already been considered in detail above. Another significant end product will be higher thiol oxidation states, such as RSO_2H and RSO_3H, which are generally regarded as an irreversible form of damage *in vivo.* The other possible products are RSNO, whose formation within cells on exposure to NO is of particular interest. RSNO are known to arise *in vivo,* for example, exposure of neutrophils to NO leads to the formation of *S*-nitrosoglutathione (35, 94, 125, 164), and there is basal *S*-nitrosylation of thiol proteins *in vivo* that increases when endogenous NO synthesis is stimulated (69, 80).

The formation of RSNO by NO may be simply of pathological significance, depleting and inactivating thiols in a manner akin to other forms of oxidative stress. Alternatively, there are several potential physiological roles for RSNO (160, 161). As RSNO generally have a longer lifetime than NO, they have been considered as a more persistent or buffered pool of NO that can be released when or where it is required for signaling (162). For example, such a role for an RSNO on serum albumin has been postulated to increase the duration of NO signaling (162). For RSNO to act in this way requires the selective decay of the RSNO to release NO; this could occur by homolytic fission to generate $RS^{\cdot}$ and NO, but homolytic fission of nitrosothiols *in vivo* is unlikely (2). Alternatively, RSNO can accept an electron from a donor such as Cu^+ to release NO and a thiolate anion (30). In related mechanisms, the electron donor can be a thiol, and RSNO are known to react with thiol proteins such as Trx to release NO (125). Alternatively, the RSNO can react in ways that will not generate free NO, for example, by reaction with a thiolate to displace the nitroxyl anion (NO^-), leaving a disulfide and NO^-, which

is unstable and rapidly decays by a number of pathways, including reaction with O_2 to form $ONOO^-$ (2, 12, 75).

In addition to acting as an NO store, the formation of RSNO on proteins may alter their function and is thought to be a significant posttranslational modification for reversibly regulating proteins, akin to phosphorylation (74, 80, 161, 163). There is evidence for extensive *S*-nitrosylation of proteins *in vivo* (69), and a number of proteins are known, such as GAPDH (119), hemoglobin (83), AP-1 (165), and $p21^{ras}$ G (41), where *S*-nitrosylation affects their activity. Furthermore, there may be particular amino acid motifs around certain cysteine residues that favor *S*-nitrosylation (161). The mechanism of protein *S*-nitrosylation may be by the direct reaction of NO derivatives with the PrSH. Alternatively, the nitrosonium (NO^+) group can be passed from thiolate to thiolate, by reaction of a thiolate with an RSNO to generate a new RSNO, a process called transnitrosation (116). *In vivo* it is likely that this will be initiated by the *S*-nitrosylation of abundant thiols such as GSH to form *S*-nitrosoglutathione, which then rapidly passes on NO^+ to PrSH (116). As different RSNO have a range of different lifetimes and reactivities, the net effect would be the selective *S*-nitrosylation of those particularly reactive thiols that form stable RSNO.

For protein *S*-nitrosylation to be a regulatory mechanism, the decay of the *S*-nitrosylated thiol must be regulated, in a similar way to that of phosphorylated proteins by phosphatases. Protein RSNO will spontaneously lose their NO to bulk thiols, particularly GSH, through transnitrosation. The *S*-nitrosoglutathione thus formed can react to form glutathione disulfide *S*-oxide [GS(O)SG], which then reacts with GSH to form GSSG (32, 105). Alternatively, the nitrosylated protein thiol can react with a thiolate to displace NO^-, leaving a mixed or internal disulfide, or hydrolysis of PrSNO will give RSOH and NO^-, with the RSOH then reacting with GSH to give a mixed disulfide (125, 153, 161). Thus, the end point for most *S*-nitrosylated thiols will be to form a mixed or internal disulfide that will be processed by endogenous thiol-reducing systems. It is also possible that the decay of *S*-nitrosylated proteins can be regulated by Trx and Gpx, which can release NO from *S*-nitrosoglutathione to modulate selectively the lifetime of *S*-nitrosylated proteins (78, 94, 125). It may also be of relevance that NO can inactivate a number of thiol-metabolizing enzymes such as GR (10), Gpx (5), and Trx, which is specifically *S*-nitrosylated at Cys69 (72).

The reactions of NO and its derivatives with thiols are summarized in Fig. 2. These reactions will lead to irreversibly oxidized thiols, disulfides, or RSNO. All three sets of reactions may contribute to the cell damage associated with nitrosative stress. More intriguingly, these interactions can also lead to the selective formation of mixed and internal disulfides and RSNO in proteins, all of which may reversibly regulate protein function.

Effects of NO and its derivatives on mitochondria

Mitochondria are exposed to NO on activation of NOS in surrounding cells or tissues, with NO or its metabolites such as $ONOO^-$ diffusing into mitochondria (45, 96, 129) (Fig. 3). In addition, there is growing evidence for the production of NO within liver and heart mitochondria through the stimulation of mtNOS by calcium (47, 60, 87, 169). This 125-kDa

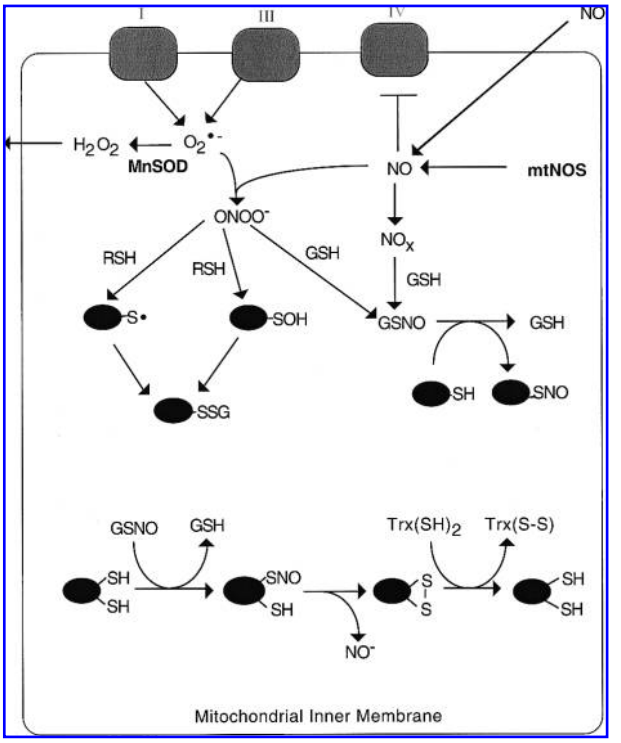

FIG. 3. Interactions of NO and its derivatives with the mitochondria. NO is produced within mitochondria by the action of mtNOS, or diffuses into mitochondria from outside. The inhibition of cytochrome oxidase (IV) by NO leads to increased superoxide ($O_2^{\cdot-}$) production by respiratory complexes I and III. This $O_2^{\cdot-}$ is dismutated to H_2O_2 by MnSOD, and the H_2O_2 can then diffuse out of mitochondria. NO can also react with O_2 to form NO_x, which reacts with mitochondrial glutathione (GSH) to form *S*-nitrosoglutathione (GSNO), which can react with thiol proteins to form *S*-nitrosylated thiol proteins. Alternatively, NO can react with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$), which can oxidize thiols to sulfenic acids (RSOH) or thiyl radicals ($RS^{\cdot}$). The lower section shows the reaction of GSNO with a vicinal dithiol protein to form an *S*-nitrosylated protein, which then loses NO^- to form an internal disulfide. This disulfide can be reduced back to its dithiol form by thioredoxin [$Trx(SH)_2$].

enzyme is associated with the mitochondrial inner membrane as a dimer and is now thought to be the brain NOSα isoform, although its mechanism of import into mitochondria is still unclear (47, 87, 169). mtNOS is myristoylated, may also be phosphorylated, and is widely expressed in various tissues, although so far there is only evidence for enzyme activity in heart and liver mitochondria (47). NO production by mtNOS leads to the formation of S-nitrosoglutathione and to the inhibition of cytochrome oxidase (150, 164), which results in the reduction of the respiratory chain and an increase in $O_2^{\cdot-}$ production by complexes I and III (23, 24, 26, 156). The $O_2^{\cdot-}$ is dismutated to H_2O_2 within mitochondria by MnSOD, and some of the H_2O_2 can then diffuse into the cytosol to act as a potential redox signal (23, 150). An important further consequence of increased $O_2^{\cdot-}$ production is its reaction with NO to form $ONOO^-$ (131). As $ONOO^-$ inhibits MnSOD, this may lead to an increased local concentration of $O_2^{\cdot-}$ and a spiral of further $ONOO^-$ formation (110). $ONOO^-$ also irreversibly

inhibits respiratory complexes I and II, further increasing $O_2^{\cdot -}$ formation (20, 33, 136, 139, 140). Finally, $ONOO^-$ can activate the PTP, thus contributing to cell death (129, 130). In all these processes, a major role for mitochondrial thiols, particularly GSH, is to protect mitochondria from the oxidative damage caused by $ONOO^-$ and NO (8, 20, 81, 108).

In addition to inhibiting cytochrome oxidase and damaging respiratory complexes I and II, NO and $ONOO^-$ oxidize mitochondrial GSH and form mixed and internal disulfides on mitochondrial proteins (16, 82, 140, 152). It is known that exposure of mitochondria to $ONOO^-$ or to NO donors modifies mitochondrial thiols by the formation of PrSSG and by *S*-nitrosylation (16, 21, 36, 106, 152). In particular, the activity of complex I is decreased by *S*-nitrosylation (21, 36). The oxidation of mitochondrial thiols is an early event in some forms of apoptosis (111), and there are thought to be critical thiols on the adenine nucleotide carrier that may affect its incorporation into the PTP and thus modulate cell death (40). These alterations to mitochondrial thiols also lead to induction of the PTP, and consequently this may lead to cell death through disrupting mitochondrial function (98, 99, 148). The interaction of NO and its derivatives with these thiols may be critical, as NO is thought to induce apoptosis by activating the PTP (22, 77), and Trx2 is involved in regulating the mitochondrial apoptosis pathway (168). Furthermore, stimulation of mtNOS increases cytochrome *c* release, although this was not by activating the PTP, but through $ONOO^-$ increasing lipid peroxidation (61). What is uncertain is whether all these alterations due to changes in mitochondrial thiols are solely of pathophysiological significance, or whether they have more selective effects on mitochondrial function. For example, such changes could be involved in activating mitochondrial redox signaling to factors in the cytoplasm, and consistent with this, alterations to the mitochondrial GSH pool can influence the activation of the transcription factor NFκB (56).

In summary, the exposure of mitochondria to NO and the consequent stimulation of $O_2^{\cdot -}$ production will lead to increased $ONOO^-$ formation in mitochondria. Thiols, particularly GSH, will protect against this oxidative damage. Some of the thiol changes that occur on exposure to oxidative stress will promote induction of the PTP and commitment to apoptosis. Finally, the specific modulation of mitochondrial function may occur through the selective glutathionylation or *S*-nitrosylation of thiol proteins.

DETECTION OF CHANGES TO MITOCHONDRIAL PROTEIN THIOLS CAUSED BY NO AND ITS DERIVATIVES

The pathological and regulatory changes brought about by NO and its derivatives involve the formation of protein mixed disulfides and *S*-nitrosylated proteins within mitochondria. In investigating these, it is vital to identify those PrSH that are particularly susceptible to alteration and to determine the lifetime, fate, and consequences for the cell of these post-translational protein modifications. In addressing these issues, a range of techniques are becoming available.

To identify PrSSG the cellular GSH pools can be ^{35}S-labeled by incubating cells with [^{35}S]cysteine in the presence of protein synthesis inhibitors (32, 41, 55). This procedure incorporates ^{35}S into both the cytoplasmic and mitochondrial GSH pools to a high specific activity. The cells can then be exposed to the stimulus of interest, proteins isolated by non-reducing electrophoresis, and glutathionylated proteins visualized by autoradiography (55). When this approach is applied to oxidatively stressed cells, a large number of glutathionylated proteins are found (32, 41, 55). The particular thiols modified by GSH can be identified by isolating the protein by electrophoresis and then analyzing tryptic digests by mass spectrometry (149). An alternative approach to identify modified thiols is to isolate proteins, block all free thiols with an alkylating reagent, and then reduce the remaining mixed disulfides or internal disulfides before labeling them with a thiol-specific tag (9, 41). However, this approach cannot distinguish between mixed and internal disulfides. In a related method, vicinal dithiol-containing proteins can be labeled selectively using the specific reaction of phenylarsine oxide with vicinal dithiols (64).

The conventional way to detect *S*-nitrosylated thiols has been to photolyze the RSNO and then react the released NO with ozone to generate chemiluminescence (162). However, when working with a tissue or cell extract, this approach requires the prior isolation of the protein so it can only be applied easily to abundant proteins. An alternative approach to detect unknown *S*-nitrosylated proteins in tissues is by using antiserum against *S*-nitrosylated proteins (69). This approach has been used to detect *S*-nitrosylated proteins by immunohistochemistry, and showed that there are basal levels of *S*-nitrosylated proteins in tissues and that the amount increased on stimulating NOS activity (69). These antisera can also detect *in vitro S*-nitrosylated bovine serum albumin on immunoblots, so it may be possible to extend this approach to detect *S*-nitrosylated proteins in tissue homogenates (69). A further approach is to use the fact that *S*-nitrosylation blocks the reactivity of the thiol that it modifies. Therefore, a protein sample containing *S*-nitrosylated proteins can be treated with a reagent that reacts with all remaining exposed thiols. The reagent is then removed, and the sample is treated with ascorbate, which reduces RSNO but not disulfides, and the resulting free thiols are reacted with a thiol reagent that can be detected by immunoblotting (80). As the only thiols available to react are those that were initially *S*-nitrosylated, this method can detect novel *S*-nitrosylated proteins in tissue homogenates (80).

All of the methods discussed above can be applied to isolated mitochondria, or to mitochondria within cells by rapid fractionation. In addition, mitochondrially targeted thiol reagents can be used to investigate thiols within intact cells (28, 29, 42). These reagents comprise thiol-reactive moieties covalently coupled to a lipophilic cation, which directs their selective accumulation by mitochondria within the cell, driven by the large mitochondrial membrane potential (121). Two such reagents have been developed to date (42). One is 4-iodobutyltriphenylphosphonium, which selectively labels mitochondrial thiols within cells (106). The labeled thiol proteins can then be identified on immunoblots using specific antisera against the triphenylphosphonium moiety (106). This approach was used to show that a range of mitochondrial PrSH are blocked by *S*-nitrosating reagents and by oxidation of GSH (106). Interestingly, this technique was also able to

follow changes in the redox state of thiols on respiratory complexes (106). The other mitochondrially targeted compound is 4-thiolbutyltriphenylphosphonium (TBTP) (28, 29). In this case, a free thiol on TBTP equilibrates with mitochondrial GSH and forms mixed disulfides with thiol proteins during oxidative stress, enabling these proteins to be identified on immunoblots (28, 29). Further applications of these and related mitochondrially targeted thiol reagents should help identify the specific mitochondrial thiol proteins that alter during oxidative stress.

CONCLUSIONS

The mitochondrial thiol system is complex. This reflects the requirements of mitochondria to assemble proteins, maintain a reduced thiol environment, and protect themselves from oxidative damage. The pathological interactions of NO with mitochondria involve significant changes to mitochondrial thiols, and these alterations are essential in protecting mitochondria from nitrosative stress. In addition, evidence is accumulating to suggest that these thiol systems may also regulate mitochondrial function, and in this the modulation of mitochondrial thiols by forming mixed disulfides and/or *S*-nitrosylated proteins is likely to prove critical. How and why these changes are brought about by exogenous NO, or by NO produced by mtNOS, are currently unclear, but the recent development of novel techniques to identify modified PrSH should facilitate progress.

ABBREVIATIONS

DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gpx, glutathione peroxidase; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; MnSOD, manganese superoxide dismutase; mtNOS, mitochondrial nitric oxide synthase; NFκB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; $O_2^{\cdot -}$, superoxide; $ONOO^-$, peroxynitrite; PDI, protein disulfide isomerase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; PlOOH, phospholipid hydroperoxides; PrSH, protein thiol; PrSSG, protein-glutathione mixed disulfide; Prx, peroxiredoxin; PTP, permeability transition pore; ROS, reactive oxygen species; RSNO, *S*-nitrosothiol; RSOH, sulfenic acid; RSO_2H, sulfinic acid; RSO_3H, sulfonic acid; TBTP, 4-thiobutyltriphenylphosphonium; Trx, thioredoxin; TrxR, thioredoxin reductase.

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Address reprint requests to:
Dr. Michael P. Murphy
MRC–Dunn Human Nutrition Unit
Wellcome Trust/MRC Building
Hills Road
Cambridge CB2 2XY, U.K.

E-mail: mpm@MRC-dunn.cam.ac.uk

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