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Nitric oxide and mitochondria

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TABLE OF CONTENTS

1. ABSTRACT

Nitric oxide (NO) and its derivatives (reactive nitrogen species) have multiple effects on mitochondria that impact on cell physiology and cell death. Mitochondria may produce and consume NO and NO stimulates mitochondrial biogenesis, apparently via cGMP upregulation of transcriptional factors. NO inhibits mitochondrial respiration via: (A) an acute and reversible inhibition of cytochrome oxidase by NO in competition with O_2, and (B) irreversible inhibition of multiple sites by reactive nitrogen species. NO is a potent vasodilator (via cGMP), increasing O_2 and respiratory substrate supply to mitochondria. NO stimulates reactive oxygen and nitrogen species production from mitochondria via respiratory inhibition, reaction with ubiquinol and reaction with O_2 in the membrane. NO can induce apoptosis, mainly via oxidative stress. NO induces necrosis, mainly via energy depletion. Reactive nitrogen species activation of the mitochondrial permeability transition pore may cause apoptosis or necrosis. NO may protect against mitochondria-mediated cell death by multiple mechanisms.

2. INTRODUCTION

Nitric oxide (NO) is synthesized from L-arginine by three isoforms of NO synthase (NOS), two of which (eNOS and nNOS) are constitutively expressed and are acutely regulated by calcium/calmodulin and phosphorylation, while the third (iNOS) is induced during inflammation, and produces higher levels of NO for a longer period (1). NO diffuses very rapidly both through water and membranes, so NO can easily diffuse from one cell to the next.

NO itself at physiological concentrations (unclear, but probably 0.1-100 nM) is relatively unreactive, and most of its physiological actions are mediated by NO binding to Fe^{2+} in the haem of soluble guanylate cyclase causing activation (at about 1 nM NO) and cGMP production (1). However, NO may be converted to a number of more reactive derivatives, known collectively as reactive nitrogen species (RNS). At high concentrations (or within membranes) NO reacts directly with O_2 to produce NO_2, which rapidly reacts with a further NO to

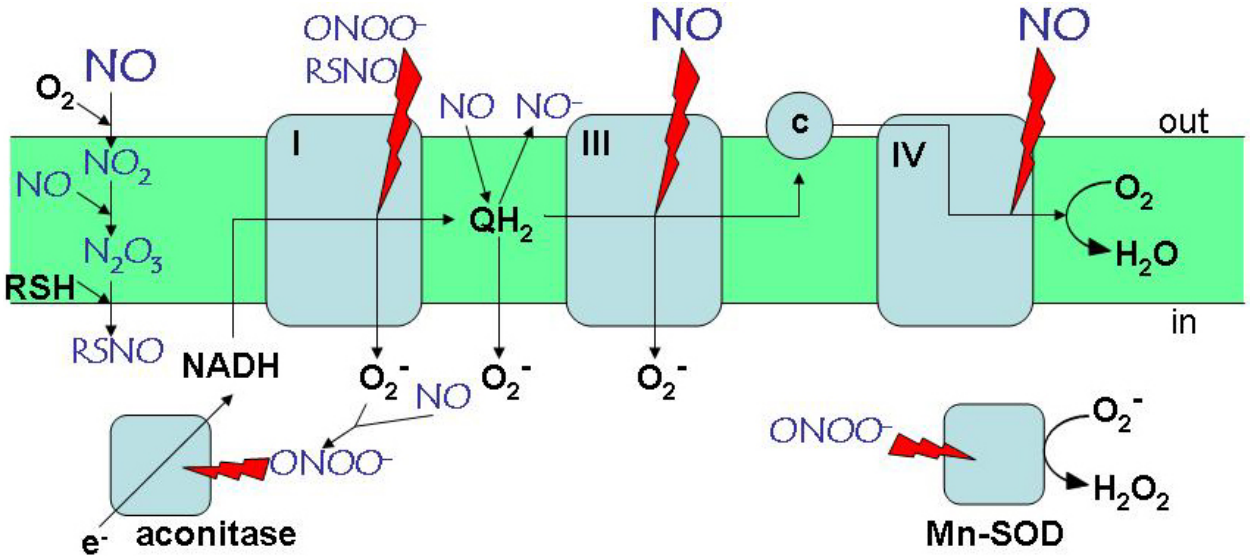

Figure 1. Interactions of NO, reactive nitrogen species, reactive oxygen species and the mitochondrial respiratory chain. NO is converted to RNS in the mitochondrial membrane. NO and RNS inhibit the respiratory chain resulting in superoxide production that reacts with NO to give peroxynitrite ($ONOO^-$), which further inhibits the respiratory chain, aconitase and the Mn-superoxide dismutase (Mn-SOD). Inhibitions are indicated by a red lightning bolt.

give N_2O_3. NO_2 may oxidise or nitrate (add an NO_2^+ group to) a variety of molecules (including tyrosine), while N_2O_3 can nitrosate/nitrosylate (add an NO^+ group to) amines or thiols. NO reacts at the diffusion-limited rate with O_2^- to produce peroxynitrite ($ONOO^-$), which can oxidise or nitrate other molecules, or can decay producing other damaging species (possibly, hydroxyl radical ($OH^\bullet$) and NO_2). NO may indirectly (possibly via N_2O_3) nitrosate thiols (*e.g.* in proteins or glutathione) to give S-nitrosothiols (RSNO, *e.g.* S-nitroso-glutathione and S-nitroso-albumin). The NO^+ group is directly transferable between different S-nitrosothiols, a process known as transnitrosation or transnitrosylation. S-Nitrosated or tyrosine-nitrated proteins may have altered function. S-Nitrosothiols breakdown and release NO in the presence of either light, reduced thiols or metal ions, such as Cu^+. NO and each of its derivatives have different properties (and have different properties at different concentrations), which act by different means, but particular species may (or may not) be interconvertible in particular conditions.

3. NO PRODUCTION AND CONSUMPTION BY MITOCHONDRIA

The NOS isoforms eNOS, nNOS or iNOS may be found attached to or within mitochondria, in which case the NO synthase is referred to as mitochondrial NOS (mtNOS) (2-4). For example, NO production from individual mitochondria was shown, which was absent in nNOS-knockout mice (2) and NOS isolated from mitochondria was found to be identical in sequence to the main isoform of nNOS, but covalently modified (4). A proportion of nNOS was subsequently found to physically interact with cytochrome c oxidase (5). However, the existence of mtNOS remains controversial because a number of research groups can not find any NOS activity on mitochondria (6). A putative arginine-dependent NO synthase, unrelated to mammalian NO synthases, has been identified in plants, and is located in mitochondria (7).

In the absence of O_2, mitochondria may reduce nitrite to NO, but the rate is low compared to NO synthases in mammals (8) but may be significant in plants (9). Mitochondrial aldehyde dehydrogenase may be responsible for releasing NO from nitroglycerin (10), but this is of pharmacological rather than physiological relevance.

Isolated mitochondria consume/breakdown NO at some rate, but whether this rate significantly contributes to cellular or in vivo NO consumption is unclear. Part of the consumption appears to be due to the direct, unmediated reaction of NO with O_2 in the phospholipid bilayer (11). In the presence of respiratory substrate, there is an increased NO consumption by mitochondria, which is partly reduced by adding superoxide dismutase (SOD), and thus is probably due to superoxide production by mitochondria (see below), which rapidly reacts with NO to give $ONOO^-$ (12). Other contributions to this respiration-dependent NO consumption are proposed to be: NO reduction by ubiquinol (13) and NO reduction or oxidation by cytochrome oxidase (14,15) (Figure 1).

4. NO STIMULATES MITOCHONDRIAL BIOGÉNESIS

NO (or NO donors) have been found to increase the concentration of mitochondrial proteins and mtDNA in some cells in culture (16). NO stimulation of

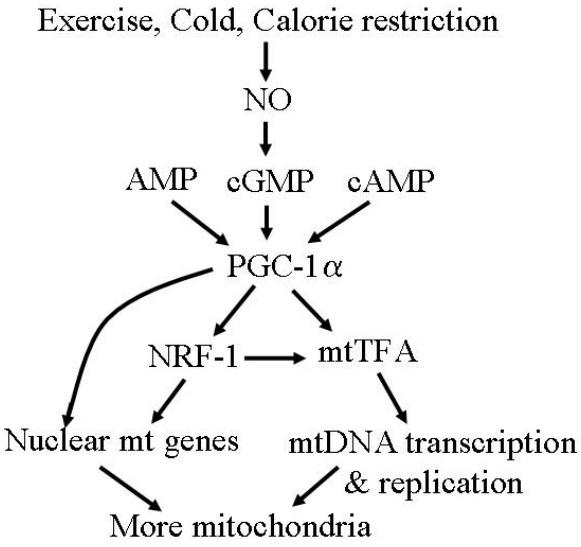

Figure 2. Possible roles of NO in mitochondrial biogenesis. Exercise, cold exposure or calorie restriction can activate or increase the expression of eNOS resulting in increased NO production that increases PGC-1alpha expression via cGMP. PGC-1alpha stimulate mitochondrial biogenesis by promoting the expression of nuclear-encoded mitochondrial genes, nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (mtTFA).

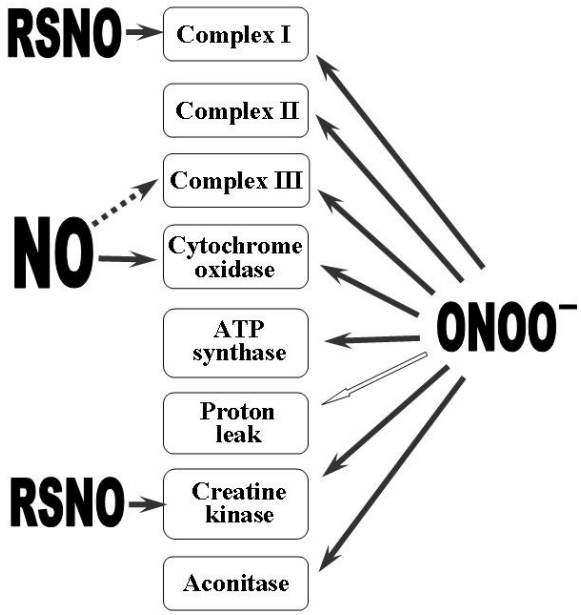

Figure 3. Main actions of NO and reactive nitrogen species on mitochondrial oxidative phosphorylation. NO specifically and reversibly inhibits cytochrome oxidase (complex IV); peroxynitrite ($ONOO^-$) inactivate multiple respiratory complexes (I, II, III, IV), the ATP synthetase (ATPase), creatine kinase and aconitase. $ONOO^-$ stimulates proton leak through mitochondrial inner membrane. Nitrosothiols (RSNO) inhibit complex I and creatine linase.

mitochondrial biogenesis was accompanied by and apparently caused by an increase in cGMP, PGC-1 alpha (peroxisome proliferator-activated receptor gamma coactivator-1 alpha), NRF-1 (nuclear respiratory factor-1), mtTFA (mitochondrial transcription factor A) and mtDNA (mitochondrial DNA), thus biogenesis was blocked by an inhibitor of soluble guanylate cyclase (ODQ) or downregulation of PGC-1alpha (16). eNOS knockout mice had lower mitochondrial levels in many tissues indicating that NO from eNOS physiologically regulates mitochondrial density in tissues (16). Calorie-restricted mice had increased eNOS expression and increased mitochondrial density and oxidative phosphorylation, and this increase was prevented in eNOS knockout mice (17) (see Figure 2).

5. REVERSIBLE NO INHIBITION OF CYTOCHROME OXIDASE

Nitric oxide inhibits mitochondrial respiration by different means: (A) NO itself causes rapid, selective, potent, but reversible inhibition of cytochrome oxidase, and (B) RNS cause slow, non-selective, weak, but irreversible inhibition of many mitochondrial components (14-20) (see Figure 3).

The reversible NO inhibition of cytochrome oxidase occurs at nanomolar levels of NO (18,20), so that NO is potentially a physiological regulator of respiration. NO inhibits cytochrome oxidase apparently by two different means involving NO binding to two different components of the O_2 binding site, which in both cases blocks O_2 binding. The O_2-binding site consists of two metals, the iron of haem a_3 and the copper of the Cu_B centre, and O_2 binds between them (and is rapidly reduced by them) when both metals are reduced (a_3^{2+} and Cu^+). NO can either (1) bind to reduced cytochrome a_3 to give cytochrome a_3^{2+}-NO, or (2) NO can bind and reduce oxidised Cu_B^{2+} to give Cu_B^+-NO^+, and the NO^+ can rapidly hydrate to give nitrite (NO_2^-) (14,15,21). Both forms of inhibition are rapid and reversible, due to debinding of NO in (1) and debinding of nitrite in (2). The first form of inhibition is competitive with O_2 and reversible by light whereas the second is not, and these characteristics may be used to distinguish between them (14). It seems that at least *in vitro* both forms of inhibition may occur simultaneously, but the first form is favoured at high levels of cytochrome reduction and low O_2, whereas the second form is favoured by the opposite conditions (14,21). However, the inhibition observed in cells in response to NO or iNOS expression appears to be largely competitive with O_2 (20,22,23) and reversible by light (24), suggesting that inhibition due to reversible binding to haem a_3^{2+} may be more important in cells.

Endogenously produced NO may tonically inhibit respiration at cytochrome oxidase in some cells (22,25,26). Inhibition is generally in competition with O_2, so that NO can dramatically increase the apparent K_M of respiration for O_2 (20,22,27,28). In synaptosomes (isolated nerve terminals) half-inhibition of respiration occurred at 250 nM NO when the oxygen level was about 150 microM (roughly

the arterial level), but the K_i was about 50 nM NO at 30 microM O_2 (a median tissue level) (20). According to this data then the presence of 50 nM NO would raise the apparent K_M of respiration for O_2 from below 1 microM to 30 microM O_2, well into the physiological range. A variety of cells (including macrophages, microglia, astrocytes, endothelial cells and aorta), when inflammatory activated to express iNOS, have been shown to produce sufficient NO to not only inhibit their own respiration but also that of surrounding cells via reversible NO inhibition of cytochrome oxidase (23,27-29).

Whether NO can regulate mitochondrial respiration *in vivo*, either physiologically or pathologically, remains an important unresolved problem. Haemoglobin and myoglobin both have very high capacities and rates of NO scavenging, which may protect tissues from significant NO inhibition of respiration in vivo. The sensitivity of soluble guanylate cyclase to NO appears to be at least two orders of magnitude higher than that of cytochrome oxidase, which might indicate that the latter is not a significant physiological target for NO. However, inhibition of NOS *in vivo* has been shown to cause substantial increases in organ and whole-body oxygen consumption, apparently not due to changes in blood flow, consistent with a tonic inhibition of mitochondrial respiration *in vivo* (26). On the other hand, it is still not known whether such effects of NO on O_2 consumption are mediated by cGMP, cytochrome oxidase or other processes.

There is some evidence that NO may acutely and reversibly inhibit respiratory complex III (the cytochrome bc_1 complex) at relatively high concentrations (K_i 300 nM) resulting in superoxide production (12), but the nature of this inhibition is unclear.

6. IRREVERSIBLE INHIBITION OF RESPIRATION BY NO

Cells exposed to NO (or NO-producing cells) show immediate but reversible inhibition of respiration at cytochrome oxidase. However, after several hours of exposure to NO an irreversible inhibition develops, probably due to conversion of NO to RNS that inhibit respiration at multiple sites (18,30-32). One of the more rapid effects is an inactivation of complex I, possibly due to S-nitrosation of the complex (24,31), followed by inhibition of aconitase and complex II, possibly due to removal of iron from iron-sulphur centers under conditions where peroxynitrite may be formed (33,34). Peroxynitrite can inhibit complex I, complex II/III, cytochrome oxidase (complex IV), the ATP synthase, aconitase, Mn-SOD, creatine kinase, and probably many other proteins (18,34). Peroxynitrite is a strong oxidant and can also cause DNA damage, induce lipid peroxidation and increase mitochondrial proton (and other ions) permeability (probably by lipid peroxidation or thiol cross-linking) (35).

NO and RNS inhibit mitochondrial complex I by several different mechanisms that are not well characterised (36). There is an inactivation by NO, peroxynitrite and S-nitrosothiols that is reversible by light or reduced thiols, and therefore may be due to S-nitrosation of the complex. There is also an irreversible inhibition by peroxynitrite, other oxidants and high levels of NO, which may be due to tyrosine nitration, oxidation of residues or damage of FeS centres. The S-nitrosothiols, S-nitrosoglutathione and S-nitrosoacetylpenacillamine, can rapidly inactivate complex I when added to isolated mitochondria, even in conditions when little or no NO is released, and such inhibition is reversed by light or reduced thiols (glutathione or DTT), suggesting that the inactivation is mediated by transnitrosation (24). Indeed S-nitrosoglutathione was shown to cause S-nitrosation of complex I in association with inhibition (37). However, the inhibition of complex I induced by peroxynitrite (which is a poor nitrosating agent) is also partly reversed by light and reduced thiols (24) (as is the inhibition induced by NO treatment of cells (31)), suggesting that mechanisms other than nitrosation might be involved. It has been suggested that peroxynitrite inhibits complex I by tyrosine nitration (38), and NO-induced inhibition of complex I in isolated mitochondria was prevented by peroxynitrite scavengers (32). An alternative (but not exclusive) target might be one or more iron-sulphur centres in complex I. High concentrations of NO can destroy iron-sulphur centres by binding and displacing the iron, and there is EPR evidence for such damage in complex I (39). Damage must start with NO binding/reacting with the iron and/or cysteine residues that bind the iron, and this initial phase of inhibition may be reversible by light or reduced thiols. Whatever the mechanism, NO inhibition of complex I may be important in cell dysfunction or death. Inactivation of complex I by NO or RNS is seen in cells or tissues expressing iNOS, and may be relevant to inflammatory pathologies, such as septic shock and Parkinson's disease. However, NO-induced thiol depletion seems to precede inhibition of complex I, and the reduced thiols reverse the inhibition (31). NO-induced inactivation of complex I may be enhanced by hypoxia (40).

There is some controversy concerning inhibition of complex II and complex III by peroxynitrite: some authors show that peroxynitrite (and NO) inhibit complex II with little or no effect on complex III (18,39), whereas others find complexes I and III inhibited but complex II unaffected by peroxynitrite (41).

Peroxynitrite has relatively little effect on the V_{max} of cytochrome oxidase when added to mitochondria at levels that inhibit the other complexes (18,41). However, it does have various damaging effects on isolated cytochrome oxidase, including particularly increasing the K_M for oxygen (42). High concentrations (>1 microM) of NO (possibly via NO_2 or N_2O_3) can also induce an irreversible rise in K_M for oxygen both in isolated cytochrome oxidase or in cells treated with NO (42,43).

Peroxynitrite and possibly NO itself inhibit cytosolic and mitochondrial aconitase, the latter being a component of the Krebs cycle, and thus required for cellular respiration. Aconitase has a Fe_4S_4 iron-sulphur centre, one particular iron atom of which can be removed

by peroxynitrite and other oxidants (34). Mitochondrial creatine kinase aids the export of ATP from mitochondria in muscle and nerves, and is inhibited by peroxynitrite and S-nitrosothiols, probably by transnitrosation of a critical thiol (44).

7. NO STIMULATES REACTIVE OXYGEN AND NITROGEN SPECIES PRODUCTION FROM MITOCHONDRIA

The mitochondrial respiratory chain can produce superoxide radical (O_2^-), which dismutates to H_2O_2, and inhibition of the respiratory chain may enhance the production of these ROS (reactive oxygen species). At moderate levels NO can acutely increase O_2^- and H_2O_2 production by inhibiting mitochondrial respiration, while at higher levels it inhibits H_2O_2 production by scavenging the precursor superoxide, resulting in peroxynitrite production (12,25,45). NO may also apparently react with ubiquinol (QH_2) to produce NO^- (which may react with O_2 to produce $ONOO^-$) and ubisemiquinone (QH^-) (part of which may react with O_2 to produce O_2^-) (13). S-nitrosothiol-inactivation of complex I can also reversibly increase ROS production from complex I several fold (46), which may have physiological or pathological relevance (see Figure 1).

Thus, NO may cause O_2^-, H_2O_2 and $ONOO^-$ production, but in other conditions it may produce NO, NO_2 or N_2O_3. Both NO and O_2 are more soluble in lipid bilayers than in aqueous solution, and thus reach higher concentrations within cell membranes that in cell cytosol, and as the reaction rate between NO and O_2 is proportional to the square of the NO concentration and proportional to the O_2 concentration this reaction is much more rapid within cell membranes, including mitochondrial membranes, than in the aqueous phases (11). Thus at high NO and O_2 levels, high rates of NO_2 and N_2O_3 production may occur within mitochondrial membranes, and N_2O_3 may S-nitrosate membrane proteins or small thiols to give S-nitrosothiols (see Figure 1).

Reversible NO inhibition of respiration may result in local peroxynitrite production (due to local superoxide production) causing irreversible inhibition of respiration and further oxidant production, a vicious cycle that might contribute to cell death (32,45). In addition to stimulating H_2O_2 production, NO or RNS can also inhibit catalase, deplete cellular glutathione and inhibit glutathione peroxidase, thus increasing H_2O_2 levels in cells (1,25,45).

In the body, NO may promote ROS production simply by increasing tissue O_2 levels. It could do this because NO is a potent vasodilator, increasing O_2 supply to tissues, but also inhibits mitochondrial respiration, which is responsible for 90% of tissue oxygen consumption. Arterial O_2 levels are about 150 microM O_2, while median tissue oxygen levels are roughly 30 microM, and mitochondrial levels may be still lower. Thus NO (at least the high levels coming from iNOS) may increase tissue O_2 levels, which may itself increase mitochondrial ROS production, as the latter is proportional to O_2 level over this range.

8. NO AND MITOCHONDRIAL PERMEABILITY TRANSITION

Reactive nitrogen species, S-nitrosothiols or ROS cause MPT in isolated, calcium-preloaded mitochondria (47-49). MPT is a dramatic increase in permeability of the inner mitochondrial membrane to small (up to 1.5 kDa) molecules. Mitochondrial membrane potential and matrix calcium are known to determine the ability of other compounds to induce MPT, thus inhibition of respiration by NO and subsequent decrease in membrane potential should favour MPT opening. On the other hand, NO itself can inhibit MPT due to the direct inhibition of respiration, preventing calcium accumulation in mitochondria (50). cGMP (which is formed by soluble guanylyl cyclase after stimulation by NO) may also inhibit MPT in cells via protein kinase G (51). However, oxidants (such as tert-butyl hydroperoxide and phenylarsine oxide) at high concentrations can induce MPT even in the absence of calcium and this effect is probably related to direct reaction of these compounds with functional thiols. Thus, NO at high concentrations can promote MPT probably due to either (a) the production of peroxynitrite, nitrosothiols or NO_2/N_2O_3, or (b) depletion/oxidation of glutathione levels. NO/RNS may also directly oxidise the protein thiols that regulate opening of the MPT pore (52).

MPT plays an important role in both necrotic and apoptotic cell death. MPT dissipates the protonmotive force, causing uncoupling of oxidative phosphorylation, and reversal of the ATP synthase, potentially hydrolysing cellular ATP, resulting in necrosis. MPT also causes rapid swelling of the mitochondria, such that the outer membrane can be ruptured releasing intermembrane proteins, such as cytochrome *c* (48-50). However, in cells MPT-related cytochrome *c* release can occur by other mechanisms that do not involve mitochondrial swelling and membrane rupture. Release of cytochrome *c* and matrix components, such as NADH, inhibits respiration, thus potentially causing necrosis. But release of cytochrome *c* and other apoptogenic intermembrane proteins such as AIF and SMAC/Diablo potentially triggers apoptosis. Transient MPT opening may be a physiological process, and usually does not cause cell damage, while longer, sustained MPT opening may cause either apoptosis or necrosis. The mode of cell death after MPT opening is likely to depend on additional factors such as activation of Bid/Bax/Bad pathway or availability of ATP (ATP depletion probably favouring necrosis). Calcium has been suggested to cause cytochrome *c* release from mitochondria and subsequent apoptosis by stimulating mtNOS to produce peroxynitrite, which then induces MPT or related processes (53) (see Figure 4).

9. NO-INDUCED NECROSIS AND EXCITOTOXICITY MEDIATED BY MITOCHONDRIA

Nitric oxide and RNS kill cells by a variety of different mechanisms, which differ depending on the cell type and conditions (1,54-56). Two main types of NO-induced cell death can be distinguished: (1) energy

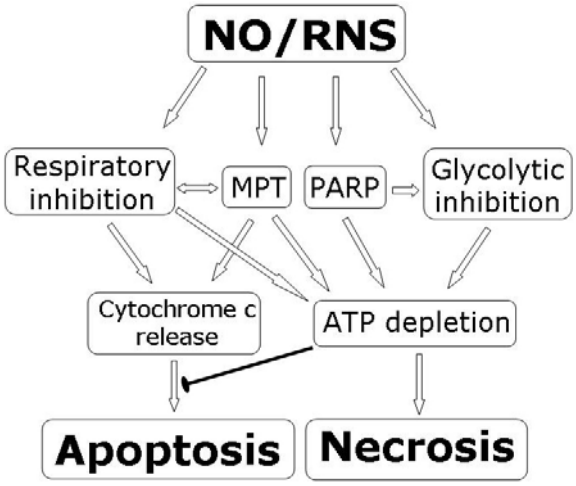

Figure 4. Main mitochondrial actions of NO and reactive oxygen species leading to cell death. Peroxynitrite and S-nitrosothiols can activate mitochondrial permeability transition (MPT), which may result in apoptosis or necrosis, depending on ATP levels. Peroxynitrite can, via DNA damage, activate PARP-1 (peroxisome proliferator-activated receptor gamma coactivator-1 alpha), which can inhibit glycolysis, deplete cellular ATP and promote AIF (apoptosis inducing factor) release from mitochondria.

depletion-induced necrosis, and (2) oxidant-induced apoptosis. In addition, in neurons, NO induces excitotoxic cell death (see Figure 4).

Energy depletion-induced necrosis results from NO and/or its derivatives causing ATP depletion via four different mechanisms: (a) inhibition of mitochondrial respiration, (b) induction of MPT, (c) inhibition of glycolysis at glyceraldehyde-3-phosphate dehydrogenase, and (d) activation poly-ADP ribose polymerase (PARP, or synthetase: PARS).

It is important to note that inhibition of mitochondrial respiration in cultured cells or *in vivo*, induced for example by anoxia, cyanide or carbon monoxide, is alone sufficient to cause rapid death of a wide range of cells, including particularly neurons and cardiomyocytes, thus NO inhibition of respiration in these cells should also be sufficient to cause rapid death (45,48,57). However, respiratory inhibition does not kill other cell types, such as skeletal muscle or cell lines derived from tumour cells, due to their high glycolytic capacity. An important implication of NO raising the K_M of cytochrome oxidase for O_2 is that NO should sensitise cells to hypoxia-induced cell death. This has found to be true in isolated aorta and in co-cultures of neurons and glia, where exposure to NO from NO donors or from iNOS greatly sensitised to hypoxia-induced cell death (27,28).

Nitric oxide does not inhibit glycolysis directly, however, peroxynitrite and S-nitrosothiols modify the active site cysteine residue of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), causing inhibition of its dehydrogenase activity and inducing an acyl phosphatase activity in the enzyme (58-60). This results in uncoupling of glycolytic flux from ATP synthesis by substrate level phosphorylation. NO-producing macrophages or cells exposed to NO donors exhibit reduced GAPDH activity, increased glycolysis and decreased ATP content and turnover (60), potentially leading to ATP-depletion-induced necrosis (45).

In cells exposed to NO, glycolytic generation of ATP is critical to cell survival because moderate levels of NO invariably inhibit mitochondrial respiration and thus mitochondrial ATP production. In the absence of glucose or sufficient glycolytic capacity moderate levels of NO cause necrosis simply due to respiratory inhibition and consequent ATP depletion (and apoptosis is prevented by the lack of ATP (57,61)). Thus in many cells necrosis induced by NO (or NO-producing cells) is prevented by glucose (30,57). The rate at which ATP is being consumed by the cell may also be important in determining the sensitivity to energy-depletion induced necrosis, as suggested by the synergy between NO and impulse frequency in neuronal death (62).

If MPT is chronically stimulated by NO derivatives then respiration may be inhibited, oxidative phosphorylation uncoupled, and glycolytic ATP hydrolysed by reversal of the ATPase, thus necrosis might proceed even in the presence of rapid glycolysis. However, there is as yet little direct evidence that MPT is involved in NO-induced necrosis.

Another potential cause of inhibition of glycolysis is depletion of NAD^+ due to activation of poly(ADP-ribose) polymerase-1 (PARP-1). PARP is a nuclear protein, which when activated by DNA strand breaks (which may be caused by peroxynitrite or NO_2/N_2O_3) catalyses multiple ADP-ribosylation (using NAD^+ as substrate) of proteins including particularly itself (63). If DNA damage is extensive then PARP activity is so high that cytosolic NAD^+ is depleted (causing inhibition of glycolysis) and adenine nucleotides may also be depleted (either because they are substrates for NAD^+ synthesis or because glycolysis is inhibited). Thus PARP inhibitors or PARP-1 knockouts can protect against NO-induced cell death in some cell types and conditions, however these inhibitors are ineffective in other cells and conditions (63-65). But activation of PARP may contribute to energy depletion of the cell, possibly in synergy with inhibition of respiration, inhibition of glycolysis and/or activation of MPT. There is evidence that PARP-1 activation can cause mitochondrial permeabilisation and release of factors such as AIF to cause a form of apoptosis that is not caspase mediated (66). It has been suggested that a population of PARP found within mitochondria mediates such death (67).

Neurons are exceptionally sensitive to NO or NO-producing cells (64,68). One reason is that NO inhibition of neuronal respiration causes glutamate release and subsequent excitotoxic death of neurons (68). NO, hypoxia and specific respiratory inhibitors all cause rapid ATP depletion of neurons and glutamate release, apparently due to ATP-limitation of the Na^+ pump leading to run down

of plasma membrane potential and Na^+ gradient, causing reversal of the Na^+-coupled glutamate uptake carrier (68). High concentrations of extracellular glutamate (the main excitatory neurotransmitter in the brain) can kill neurons via activation of one type of glutamate receptor, the NMDA receptor, and such neuronal death is known as excitotoxic death. Respiratory inhibitors can strongly potentiate such death by depolarising the plasma membrane, as depolarisation is required together with glutamate for activation of NMDA receptors, which once activated act as ion channels allowing both Na^+ and Ca^{2+} into the neuron (68).

In some cases inhibition of nNOS blocks glutamate-induced death of neurons, and cell death has been attributed to NO and calcium-induced mitochondrial depolarisation, although mechanisms remain unclear (69,70). NO and NO-producing astrocytes cause irreversible damage to respiratory complexes of neuronal mitochondria (55), but this now appears to be secondary to glutamate release and excitotoxicity (71).

10. NO-INDUCED APOPTOSIS AND PROTECTION MEDIATED BY MITOCHONDRIA

NO can induce apoptosis in cell or conditions where respiratory or glycolytic ATP production is sufficient (see Figure 4). Although specific respiratory inhibitors can induce apoptosis in such conditions, induction is weaker and slower, so respiratory inhibition cannot fully explain NO-induced apoptosis (48,57). NO-induced apoptosis is mediated by downstream caspases (such as caspase-3), and is blocked by caspase inhibitors (48,57,72). Cytochrome *c* is generally released, suggesting that NO-induced apoptosis is normally mediated by mitochondria, but in some cell types early activation of caspase-8 or caspase-2 is observed indicating that NO-induced apoptosis may be triggered not through the mitochondrial pathway (72).

How NO induces apoptosis is still poorly characterised (54,56), but the main routes may include: (A) MPT (48,49,54), (B) oxidation of mitochondrial phospholipids (73,74), (C) upregulation of proapoptotic proteins such as p53 and Bax, or downregulation of antiapoptotic Bcl-2 (74,75), (D) activation of MAP kinase pathways (76), or (E) activation of the endoplasmic reticulum stress-response pathway. NO by itself usually does not induce MPT, however, peroxynitrite, S-nitrosothiols, NO_2 and/or N_2O_3 may cause apoptosis by directly activating MPT, leading to cytochrome *c* release and caspase activation, and these events can be blocked with the MPT inhibitor cyclosporin A (48,49,54). Apoptosis induced by "pure" NO donors such as the NONOates is less susceptible to inhibition by cyclosporin A, but is inhibited by antioxidants, such N-acetyl-L-cysteine, catalase, superoxide dismutase, ascorbate and Trolox, and thus may require NO-induced ROS or RNS (48,72,77).

NO treatment of cells can cause oxidant-induced degradation of the mitochondrial phospholipid cardiolipin (probably due to peroxynitrite- or NO_2-induced peroxidation), associated with irreversible inhibition of the respiratory chain and apoptosis (73). Cytochrome *c* is normally bound to cardiolipin on the inner mitochondrial membrane, but peroxidation causes the release of cytochrome *c*. Addition of peroxynitrite to isolated mitochondria causes lipid peroxidation and thiol cross-linking associated with increased proton and ion permeability, depolarisation and rapid swelling, which in the absence of calcium may be not mediated by MPT (35). It has also been reported that addition of calcium to isolated mitochondria stimulates mtNOS, causing peroxynitrite production and (cyclosporin-insensitive) cytochrome *c* release associated with peroxidation of mitochondrial lipids (53).

NO can also inhibit apoptosis or cell death induced by other agents. Several mitochondrial mechanisms have been suggested: (a) cGMP/PKG-mediated inhibition of permeability transition (51), (b) cGMP/PKG-mediated activation of the mitochondrial K_{ATP} channel, which stimulates mitochondrial ROS production (78), (c) energy depletion, preventing apoptosis (57,62), (d) mitochondrial hyperpolarisation (79-82), and (e) NO-induced permeability transition resulting in calcium activation of the ER stress response (83). There have been reports that NO can cause an increase in mitochondrial membrane potential in some cells (astrocytes, peritoneal macrophages) but not in others (rat cortical neurons, thymocytes) (79-81), and this increase has been suggested to inhibit apoptosis (79,80,82).

11. ACKNOWLEDGEMENTS

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