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Review

Nitric oxide and cell death

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Abstract

Nitric oxide (NO) has several essential roles in mammals, but unregulated NO production can cause cell death through oxidative stress, disrupted energy metabolism, DNA damage, activation of poly(ADP-ribose) polymerase, or dysregulation of cytosolic calcium. Such disturbances can lead to either apoptotic or necrotic cell death, depending on the severity and context of the damage. Here I review the mechanisms by which NO kills cells and discuss how NO thereby contributes to ischaemia-reperfusion injury and neurodegeneration.

Keywords: Nitric oxide; Cell death; Apoptosis; Necrosis; Neurodegeneration; Ischemia-reperfusion injury; Glutamate toxicity

Contents

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; eNOS, endothelial NOS; iNOS, inducible NOS; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; NO^+, nitrosonium cation; PARP, poly(ADP-ribose) polymerase; PT, mitochondrial permeability transition; $ONOO^-$, peroxynitrite; NO_x, oxides of nitrogen; NMDA, *N*-methyl-D-aspartate; $O_2^{\bullet-}$, superoxide; SOD, superoxide dismutase

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1. Introduction

The controlled production of nitric oxide (NO) has important roles in the human nervous, immune and vascular systems [1–5]. Shortly after its discovery excessive or inappropriate production of NO was found to cause cell death in human pathologies [6]. Whether NO is beneficial or harmful depends on the type of cells exposed to and producing NO, the chemical fate of NO, the amount of NO produced and the site of its production. Here I overview how NO damages and kills cells in human pathologies [5–14].

2. Properties of NO relevant for cell death

2.1. Synthesis and diffusion of NO

NO is produced by three different nitric oxide synthases (NOS), two of which are constitutively expressed, predominantly in neurones (nNOS) and endothelial tissue (eNOS), respectively [5,15,16]. The major function of eNOS is to regulate vascular tone, the main role for nNOS may be retrograde signalling across synapses. An inducible form (iNOS) can be upregulated considerably in immune cells and many other tissues, where it is required for the cellular immune response [17–20]. At normal intracellular calcium levels NO production by iNOS is limited only by the amount of enzyme, substrate or cofactor present [6,17]. In contrast, eNOS and nNOS are inactive at normal calcium concentrations, producing 'puffs' of NO in response to transient increases in cytosolic calcium, but chronic calcium elevation will cause persistent NO production [7].

Being small and hydrophobic, NO can pass easily through membranes and as it persists in vivo for a few seconds, it can diffuse several cell diameters from its site of synthesis [11,21–23]. The transcellular diffusion of NO is more rapid than the rate of its intracellular reactions, consequently the steady-state NO concentration experienced by a cell is determined by the number of NO-producing cells nearby [21–23]. Hence NO is a paracrine messenger, rather than autocrine, and NO-producing cells experience similar NO concentrations to surrounding non-NO-producing cells [21]. As NO cannot be contained, it must be removed by reaction after signalling, rather than by reuptake [24].

2.2. Reactions of NO

In aqueous buffer NO decays to nitrite by a reaction with O_2 that is second order in NO and first order in O_2, consequently its decay rate is largely determined by the NO concentration [24]. At maximum biological concentrations (1–5 μM) the half-life of NO in vitro is several minutes, and it is far longer at more usual physiological concentrations [23–26]. However, its biological half-life is only 1–10 s [25], therefore in vivo NO decays by other pathways and its uncatalysed reaction with oxygen is minimal [26]. As both NO and O_2 are hydrophobic, they accumulate in lipid membranes, and most autoxidation to nitrite in vivo occurs there [27]. NO reacts rapidly with oxyhaemoglobin or oxymyoglobin to form nitrate [23,24,28]. The rapid rate of this reaction, together with the high haemoglobin concentration in blood and the rapid diffusion of NO, makes nitrate the major endpoint of NO metabolism in vivo [23]. Furthermore, the steep NO concentration gradient from tissues to blood lowers tissue NO concentrations by decreasing the reentry of NO that has left the cell [21].

NO and its products react with a large number of biological molecules (see [7,29]), contributing to the breadth of its signalling and deleterious effects. Reaction of NO with the haem of guanylyl cyclase leads to cGMP production, thereby mediating many of the

known physiological effects of NO [20]. NO binds directly to and inhibits several other haem enzymes such as cytochrome oxidase, catalase and cytochrome P-450 [24] and reacts with iron to form iron-nitrosyl complexes in vivo [14]. Iron-sulphur proteins are also particularly sensitive to NO damage, probably due to formation of peroxynitrite ($ONOO^-$), a damaging derivative of NO [23,30].

The formation of *S*-nitrosothiols influences the effects of NO signalling and damage [29,31,32]. *S*-Nitrosothiols have longer half-lives in vivo than NO and they are thought to increase the duration of NO signalling by acting as NO reservoirs [29,32]. *S*-Nitrosothiols are widespread in vivo, but do not arise from the direct reaction of NO with thiols [23,26,33]. Instead, the oxidation of NO to a nitrosating species with considerable nitrosonium cation (NO^+) character is required [29]. These can arise from the reaction of NO with oxygen, superoxide ($O_2^{\bullet-}$) or transition metals [6]. Alternatively, *S*-nitrosothiols can be formed by the direct reaction of NO with a thiyl radical [23]. The nitrosonium group can be transferred from the *S*-nitrosothiol to other thiols (transnitrosation), and NO can released from the *S*-nitrosothiol by reduction [29,32,34,35].

The best understood *S*-nitrosylation of a protein thiol is that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), where *S*-nitrosylation both inactivates the enzyme and leads to its ADP-ribosylation by a non-enzymatic reaction [36]. Such *S*-nitrosylations and ADP-ribosylations of thiol proteins may be widespread [29]. The formation of *S*-nitrosothiols, and subsequent oxidation of thiol proteins, might act as switches in signalling pathways [37,35] and *S*-nitrosylation may regulate many thiol-containing enzymes and regulatory proteins, such as the transcription factors NF-κB, AP-1 and CREB [6,38–40], G-proteins [41], ion channels and receptors [42] and kinases [29,34,35,43].

2.3. NO and oxidative stress

Oxidative stress is the major cause of damage associated with elevated NO [6,23], resulting largely from the formation of $ONOO^-$ [23,44–48]. Reaction of $O_2^{\bullet-}$ with NO forms the short-lived $ONOO^-$ (half-life <1 s) which is far more reactive and damaging than its precursors [44]. This reaction is extremely rapid, therefore $ONOO^-$ forms whenever $O_2^{\bullet-}$ and NO are produced simultaneously. As $O_2^{\bullet-}$ is formed continually as a byproduct of oxidative metabolism [49], a major role of superoxide dismutases (SODs) may be to intercept $O_2^{\bullet-}$ and prevent $ONOO^-$ formation [23,44]. A consequence is that whenever the NO concentration increases it will compete with SOD for $O_2^{\bullet-}$, leading to $ONOO^-$ formation and damage [23,47]. $ONOO^-$ oxidatively damages a wide range of biological molecules, including proteins, lipids and nucleic acids, and also oxidises thiols, nitrates protein tyrosine residues and damages mitochondria [23,47,48]. Furthermore, $ONOO^-$, as its conjugate acid ONOOH (pK_a 6.8), can diffuse through membranes and cause damage some distance from its site of synthesis [50].

NO also causes oxidative stress by other pathways. NO reacts with oxygen to produce reactive oxides of nitrogen (NO_x), which may form preferentially within lipid membranes [27], however the amount formed in vivo is uncertain [26]. NO enhances the oxidative stress caused by hydrogen peroxide [51]. The iron liberated from ferritin by NO may contribute significantly to elevated oxidative stress [52]. NO reacts rapidly with other radicals formed during oxidative stress, such as lipid peroxides, but the significance of these reactions in vivo is uncertain [53,54]. Interestingly, NO can also protect against oxidative damage by intercepting reactive species, such as the hydroxyl radical, converting them to less damaging and more easily detoxified products [26]. The relative rates of production of NO and $O_2^{\bullet-}$ are critical in determining whether NO acts as a pro- or antioxidant [55].

2.4. Interaction of NO with mitochondria

NO interacts with mitochondria in a number of ways that are central to its role in cell death [8,56]. Externally produced NO diffuses easily into mitochondria and NO synthesis may also occur in mitochondria, catalysed by an organelle-specific isoform of NOS [57,58,157,158]. NO affects mitochondria in three principal ways: reversible inhibition of respiration; irreversible inactivation of mitochondrial enzymes; and induction of the mitochondrial permeability transition (PT). In addition, NO may nitrosylate critical thiol residues on creatine kinase, disrupting ATP supply by mitochondria [155,156].

NO binds reversibly to haem a_3 in cytochrome oxidase and extremely low (nM) concentrations of NO are sufficient to inhibit electron transport at physiological oxygen levels [59]. Thus NO may reversibly regulate mitochondrial function in vivo by blocking respiration and ATP synthesis, and by causing the release of mitochondrial calcium [60–63]. Mitochondria are a major source of $O_2^{\bullet-}$ which forms continually by electron leakage from the respiratory chain [49,64]. NO diffusing into mitochondria reacts with $O_2^{\bullet-}$ to form $ONOO^-$ [65]. In addition, $ONOO^-$ formed outside mitochondria can diffuse into the matrix, although interception by cytoplasmic thiols probably limits the damage so caused [66]. Interaction of $ONOO^-$ with the mitochondrial respiratory chain causes further $O_2^{\bullet-}$ formation [67] and $ONOO^-$ inactivates Mn-SOD, increasing $O_2^{\bullet-}$ availability in the mitochondrial matrix [68]. Hence NO may initiate a destructive cascade of $ONOO^-$-mediated mitochondrial damage events [56,68]. Respiratory chain complexes I, II and III, and citric acid cycle enzymes such as aconitase, contain iron-sulphur centres that are irreversibly damaged by $ONOO^-$ [30,66,69–72]. NO also inhibits glycolysis by *S*-nitrosylating and thus inactivating GAPDH [36]. Therefore, NO considerably impairs cellular ATP synthesis.

$ONOO^-$ induces mitochondrial calcium efflux, which can lead to the PT, by oxidising mitochondrial thiols and NAD(P)H [73–76]. The PT is induced by oxidative stress in conjunction with elevated calcium which causes the formation of a 2–3 nm diameter protein pore in the mitochondrial inner membrane, depolarising and swelling the mitochondria, and releasing solutes from the matrix [77,78]. NO might also directly modulate induction of the PT [79] and thereby contribute to both necrotic and apoptotic cell death [77,80].

An important consequence of impaired energy metabolism is the disruption of cellular calcium homeostasis [78,81,82]. Depletion of ATP prevents cells from maintaining transmembrane calcium gradients by inhibiting endoplasmic reticulum and plasma membrane Ca^{2+}-ATPases [78,81,82]. Mitochondria also directly modulate cytosolic calcium and act as a sink for excess cytosolic calcium [78,83]. The decrease in membrane potential and induction of the PT will disrupt these processes, leading to elevated cytoplasmic calcium [60,78].

2.5. DNA damage by NO and activation of PARP and p53

Ribonucleotide reductase is inhibited by NO, blocking the supply of deoxyribonucleotides for DNA synthesis and slowing cell proliferation [84]. Excess NO also damages DNA, with $ONOO^-$ causing single strand breaks [85,86]. Damaged DNA activates poly(ADP-ribose) polymerase (PARP) in the nucleus, probably to assist in DNA repair [87,88], and upregulates p53 [7,13,89,90]. Activation of PARP is particularly important in NO cytotoxicity [91]. Activated PARP transfers up to 100 ADP-ribose moieties from NAD^+ to nuclear proteins including histones and PARP itself [87,92] and the resulting ADP-ribose polymers are then degraded by glycohydrolases. The NAD^+ resynthesis that follows this futile cycle depletes ATP, while the decreased availability of NAD^+ severely compromises ATP synthesis [92]. Hence a major consequence of DNA damage by NO is a cellular energy deficit.

The tumour suppressor protein p53 [93] is also upregulated in response to DNA damage caused by NO [89,90], although depletion of ribonucleoside triphosphate pools may also contribute [93]. DNA damage increases the amount of p53 present by promoting its phosphorylation, thus slowing its normally rapid proteolysis [93]. This upregulation of p53 causes growth arrest by blocking the G_1/S transition in the cell cycle giving time for DNA repair; however, p53 will induce apoptosis if the DNA damage is extreme [93]. In both cases p53 functions as a transcription factor, increasing the expression of proteins involved in DNA repair or apoptosis [93,94]. In addition, p53 may interact directly with other proteins, leading to apoptosis independently of its transcriptional action [93].

2.6. Summary

Excessive NO production can arise from upregulation of iNOS, or by exposing eNOS or nNOS to chronically elevated calcium. The NO produced easily diffuses several cell diameters from its site of synthesis. The local concentration of NO is a function of its rate of production and loss by both reaction and diffusion. Elevated NO can cause damage by disrupting haem enzymes or forming *S*-nitroso-

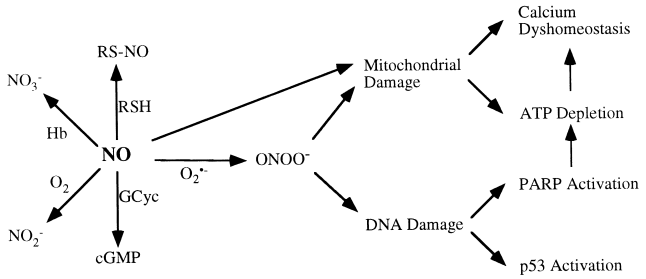

Fig. 1. Damaging reactions of NO. GCyc, guanylyl cyclase; Hb, Haemoglobin; RS-NO, *S*-nitrosothiols; RSH, thiols.

thiols, but most damage probably follows reaction with $O_2^{\bullet-}$ to form $ONOO^-$. The resulting oxidative stress damages DNA, mitochondria and a range of iron-containing enzymes; activates PARP and p53; and disrupts energy metabolism and calcium homeostasis (Fig. 1).

3. How does NO kill cells?

Cell death is generally considered to occur by two distinct pathways: apoptosis, in which cells deliberately activate an in-built death programme over several hours, and necrosis (sometimes called oncosis) which is the uncontrolled and rapid death of the cell [95]. These two extremes are useful models for cell death, but the mechanism of cell death in vivo probably involves a continuum from necrosis after extreme cellular damage, to apoptosis following mild damage or specific extracellular signals [96].

3.1. Cell death following damage

As outlined above, excess NO causes oxidative stress, DNA damage and disruption of energy metabolism, calcium homeostasis and mitochondrial function. All of these disturbances can lead to cell death by apoptosis or necrosis, depending on the context and severity of the damage [95,97,98]. Chronic elevation of cytosolic calcium activates a wide range of cell damage pathways [81,99–102]. Small decreases in ATP levels lead to apoptosis, while large decreases rapidly cause necrosis [98,103]. Mild oxidative stress leads to apoptosis whereas severe oxidative stress leads to extensive cellular damage and necrotic cell death [96,99]. Mechanisms for such extreme damage include activation of PARP, depletion of cellular ATP levels, and the induction of the mitochondrial PT [104]. These events rapidly lead to necrosis by disrupting ion gradients, swelling the cell and rupturing the plasma membrane [95,105]. In contrast, if the damage is insufficient to cause necrosis, the upregulation of p53 stops cell division and provides the opportunity for damaged DNA to be repaired [93,97]. Beyond a certain damage threshold the cell will undergo apoptosis, but how it makes such a decision to die rather than to undergo repair is uncertain (Fig. 2).

Cells can commit to apoptosis without inducing new gene expression or protein synthesis, so the apoptotic machinery is constitutively expressed within the cytoplasm [106]. However, changes in transcription can also initiate apoptosis [93]. Once apoptosis is initiated the cell gradually self-destructs, dismantling itself and preparing the cell remnants for removal by phagocytosis [97]. ATP is required for this apoptotic pathway and if ATP becomes limiting before its completion the mechanism will switch to necrosis (in effect, aborting apoptosis) [98,103].

Irreversible commitment to apoptosis involves caspases, cytoplasmic cysteine proteases that are activated by proteolytic cleavage [107,108]. Caspases are able to cleave a wide range of enzymes and proteins, leading to the characteristic changes of apoptosis, including PARP inactivation, phosphatidylserine exposure on the outer leaflet of the plasma membrane, DNA fragmentation and the formation of cellular remnants termed apoptotic bodies [97,108,109]. Caspases can be inactivated by oxidation or *S*-nitrosylation of a critical thiol residue [118,119], perhaps partially explaining why excessive oxidative stress generally leads to necrotic cell death, not apoptosis.

How can NO-induced cell damage activate the cas-

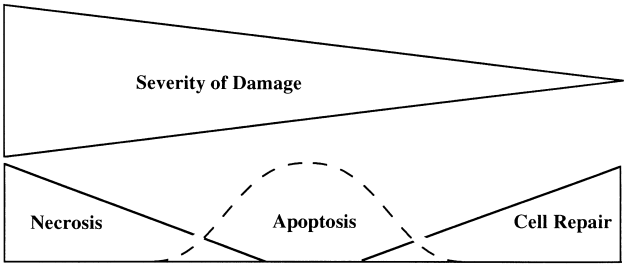

Fig. 2. Dependence of cell response on severity of damage.

pase cascade? One means of activating caspases is the release of cytochrome *c*, and possibly other factors, from the mitochondrial intermembrane space into the cytoplasm [106,110–112] (Fig. 3). This release is prevented by the anti-apoptotic proteins Bcl-2 and Bcl-X_L and activated by the pro-apoptotic protein Bax [113]. Once in the cytoplasm, cytochrome *c* binds to two proteins, Apaf 1 and procaspase 9, leading to the sequential activation of caspase 9 and caspase 3, thereby committing the cell to apoptosis [114]. The mechanism of cytochrome *c* release is uncertain, but one possibility is that induction of the mitochondrial PT swells the matrix and ruptures the outer membrane to release cytochrome *c* [106, 115,116,137]. Therefore induction of the PT by $ONOO^-$ and elevated calcium is one possible mechanism by which NO activates apoptosis [56,137]. However, cytochrome *c* release often occurs without apparent induction of the PT, therefore there may be additional specific mechanisms of cytochrome *c* release from mitochondria [110,111,113]. How this might occur is uncertain, but pro-apoptotic proteins such as Bax and Bak interact with the mitochondrial outer membrane and thus may cause the release of cytochrome *c*, which is opposed by the anti-apoptotic proteins Bcl-2 and Bcl-X_L. NO could activate this pathway by altering the recruitment of these regulatory proteins to the mitochondrial outer membrane [117], or by affecting their relative amounts [93,113] (Fig. 3).

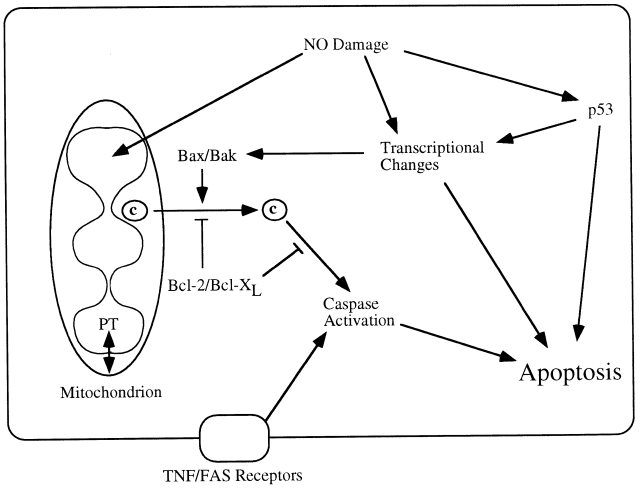

Fig. 3. Possible mechanisms of induction of apoptosis by nitric oxide. PT, permeability transition; TNF/FAS, tumour necrosis factor/FAS receptor pathways of caspase activation; c, cytochrome *c*.

Cytochrome *c* release and subsequent caspase activation may enable cells to gauge whether the damage sustained is sufficient to merit destruction by apoptosis. Each cell contains several hundred mitochondria and release of cytochrome *c* from only a few may favour release from others, because caspases cleave anti-apoptotic proteins such as Bcl-X_L, converting them to pro-apoptotic proteins [120]. This would favour further release of cytochrome *c*, and would also inhibit the other anti-apoptotic activities of Bcl-2 and Bcl-X_L whereby they block caspase activation by cytochrome *c* [120,121]. This potential feedback loop may enable cells to integrate various damage signals and undergo apoptosis when a critical proportion of their mitochondria have released cytochrome *c*.

While caspase activation is not dependent on changes in gene transcription or protein synthesis, such changes in expression can contribute to apoptotic cell death. Activation of p53 increases the expression of Bax [93,97], and other proteins that increase oxidative stress and thus favour apoptosis [94]. Other responses to NO-induced damage include modulation of the activity of the transcription factors AP-1 [39], NF-κB [122] and CREB [123], and of immediate early genes, but the mechanistic significance of these for apoptotic cell death remains unclear. Extracellular signals such as tumour necrosis factor and FAS activate caspases by receptor-mediated pathways which also commit the cell to apoptosis [97], so NO could contribute to apoptosis by modulating these pathways.

3.2. Cell death in vitro in response to NO

Treatment of various cultured cells with excessive NO leads to their death [7]. Since cells lacking Cu,Zn-SOD are more susceptible to NO-induced cell death, and $ONOO^-$ causes cell death in a range of cell types [124,125], NO appears to kill cells by forming $ONOO^-$ [126]. Large amounts of $ONOO^-$ lead rapidly to necrotic cell death [96], whereas smaller amounts promote apoptosis [96]. Since the latter can be blocked by over-expressing Bcl-2 [126,127], apoptosis induced by NO or $ONOO^-$

probably follows release of cytochrome *c* and activation of caspases [137].

The activation of PARP by excessive NO also contributes to cell death in vitro, particularly in neurones [91]. Glutamate in excess, or *N*-methyl-D-aspartate (NMDA), causes neurotoxicity by activating the NMDA receptor, increasing calcium uptake into the cell and thus activating nNOS to produce excess NO and $ONOO^-$. The consequent damage to DNA [91] leads to activation of PARP, depletion of ATP and cell death that can be blocked by selective PARP inhibitors [7,88,91,128]. In addition, cultured cerebral cortical neurones from mice lacking the PARP gene are resistant to the neurotoxic effects of glutamate, NMDA and NO [88,91]. Similarly, in a macrophage-like cell line NO caused activation of PARP, accumulation of p53 [7,127] and apoptosis, but in this case PARP inhibitors did not prevent cell death [90], suggesting that PARP activation was not central to cell death. Mitochondrial ATP synthesis has a critical role in deciding whether a cell dies by necrosis or apoptosis [81,129], because ATP is required for completion of the apoptotic programme. Neurones will undergo apoptosis if mitochondrial ATP synthesis is retained during glutamate excitotoxicity, but the cell will die by necrosis if mitochondrial ATP synthesis is disrupted [81,129].

3.3. Summary

Elevated NO increases oxidative stress, damages DNA and activates PARP, disrupting cellular energy metabolism, mitochondrial function and calcium homeostasis. All of these events occur simultaneously to varying degrees and will be associated with changes in gene expression and p53 activation. The cell then has three options: to repair the damage, to die by apoptosis, or to die by necrosis (Fig. 2). If the damage is sufficiently extensive to deplete ATP or inactivate caspases, apoptosis is impossible and the cell will die by necrosis. If the damage is milder the cell may repair itself during the growth arrest induced by p53 activation, or deliberately induce apoptosis. In some situations the cell may initiate apoptosis, but then switch to necrosis following ATP depletion or caspase inactivation.

4. NO and cell death in clinical situations

Over our lifetimes guanylyl cyclase is routinely activated by low (nM) NO concentrations without harmful consequences in a range of tissues [130]. The concentration of NO can be increased 10–100-fold over normal levels (1–10 nM) causing both acute and chronic damage [131]. For example, NO increases up to 2–4 μM during cerebral ischaemia [132] and can reach 4–5 μM above a layer of stimulated endothelial cells in vitro [133]. Such increases in NO concentration occur in both acute and chronic situations in vivo and probably contribute to a range of human diseases by the damage mechanisms outlined above. However, NO can also have protective effects, for example by increasing blood flow and inhibiting platelet aggregation [10], complicating the interpretation of NO effects in intact animals.

4.1. Acute NO injury

Both cerebral and peripheral neurones are particularly susceptible to cell death from acute NO overproduction during stroke and ischaemic brain injury, with glutamate neurotoxicity being a major effector [8,91,134,135]. In ischaemia after a stroke the supply of oxygen and glucose to the brain are blocked, leading to a decrease in plasma membrane potential and release of glutamate from glutamatergic neurones. The consequential stimulation of NMDA receptors to take up calcium, promotes further glutamate release and a vicious cycle of NMDA receptor overstimulation (excitotoxicity) [8,134,135] and increased NO production. During middle cerebral artery occlusion in rats, cerebral NO rapidly increased to about 2–4 μM, returning to baseline levels after about 1 h of ischaemia, and increasing again on reperfusion [132]. Ischaemia will inevitably lead to cell death if blood supply is not restored, however, reperfusion of ischaemic tissue can also be damaging [136]. Likely mechanisms of reperfusion injury include production of $O_2^{\bullet -}$ by oxygen-rich blood interacting with reduced cell contents, activation of xanthine oxidase on endothelial cells and numerous responses to disturbed intracellular calcium homeostasis [136].

The use of selective nNOS inhibitors, or nNOS

'knock-out' mice, led to substantially lower infarct volumes during cerebral ischaemia, suggesting that nNOS is responsible for producing the damaging levels of NO [10,138]. How does activated nNOS cause damage? It is not by stimulation of guanylyl cyclase, as inhibitors do not affect NMDA or NO neurotoxicity [10]. Overexpression of Cu,Zn-SOD in mice protects against cerebral ischaemic damage [139] and mice lacking the Cu,Zn-SOD gene are more susceptible to stroke damage [11,126]. Furthermore, NO alone is not neurotoxic in mouse models of stroke [11]. Therefore, the reaction of $O_2^{\bullet-}$ with NO to form $ONOO^-$ is required for neurotoxicity [10]. It appears that DNA damage by $ONOO^-$ leading to PARP activation is of particular importance mechanistically [91]. Mice lacking the PARP gene had 80% less tissue damage following middle cerebral artery occlusion [91], and ADP-ribose polymer formation was elevated in the ischaemic brain tissue of normal mice [91]. PARP activation led to ATP depletion and necrotic cell death, depending on the severity of the damage [91,92].

Ischaemia-reperfusion injury in the brain leads to a number of changes in gene expression and transcription factor activity (for a review see [140]): these include c-*fos*, c-*jun*, AP-1, NF-κB, CREB and p53. Such changes will contribute to cell repair and apoptosis, depending on context [141]. A mixture of necrosis and apoptosis is observed in animal models of ischaemia-reperfusion injury, with the proportion depending on the severity of the initial insult. Necrotic cell death usually occurs at the centre of an ischaemic lesion, with increased amounts of apoptosis towards its periphery [140]. The dramatically reduced infarct size in mice lacking the PARP gene suggests that PARP activation and consequent ATP depletion are major contributors to necrotic cell death in ischaemic lesions [91]. Apoptotic cell death in response to relatively mild damage (either at the periphery of the lesion or in response to a less severe insult) probably occurs by a combination of caspase activation and transcriptional alterations, as discussed earlier, but the details are uncertain.

The expression of iNOS in astrocytes and microglial cells may also contribute to cell death following ischaemia-reperfusion injury. While most neurones express only nNOS, expression of iNOS can be induced in glial cells and astrocytes by trauma or infection [11,142] and increases in the brain a few hours after ischaemia [8,10]. This upregulation of iNOS may be required for phagocytosis of cells killed during ischaemia. The NO produced by iNOS can contribute to the cell death that occurs several hours after the initial injury, and preliminary evidence suggests that transgenic mice lacking iNOS have less brain damage following ischaemia-reperfusion injury [10].

4.2. Chronic NO damage

Mitochondrial damage and oxidative stress are elevated in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease, and chronic overproduction of NO has been suggested as a cause of this damage [8,143]. Evidence supporting a role for NO in the pathophysiology of neurodegenerative diseases comes from the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease. Inhibition of nNOS prevents MPTP toxicity in baboons, and mice lacking nNOS are resistant to MPTP toxicity [144,145]. Mice with elevated Cu,Zn-SOD are also less susceptible to MPTP damage [146], and 3-nitrotyrosine, a marker for $ONOO^-$ formation, is found in the brains of mice exposed to MPTP [147], suggesting that the reaction of NO with $O_2^{\bullet-}$ is the cause of the damage. This damage may kill cells by a slow excitotoxic process, in which mitochondrial dysfunction disrupts the maintenance of transmembrane ion gradients, leading to chronically elevated calcium and activation of nNOS. The ensuing oxidative stress and mitochondrial damage would promote greater susceptibility to excitotoxic cell death. The production of NO by iNOS may also contribute to the mitochondrial dysfunction. Expression of iNOS increases in astrocytes and microglial cells in response to cytokines and inflammatory stimuli [8,134] and the NO produced can diffuse to neurones and disrupt their mitochondria [148–150]. As gliosis commonly occurs in neurodegenerative diseases, this mechanism may contribute to the mitochondrial dysfunction [151]. An interesting aspect of neuronal cell death in both neurodegenerative diseases and acute brain injury is that nNOS containing neurones, which comprise about 2% of neurones in the CNS, selectively survive cell death [4]. The reason for this is uncertain, but one possibility is that nNOS

expressing neurones have more effective antioxidant defenses than other neurones [8].

5. Conclusion

Excessive production of NO contributes to cell death in ischaemia-reperfusion injury, neurodegenerative disorders and inflammatory diseases. NO damages cells in a range of ways, but the most important is by the production of $ONOO^-$ which damages DNA leading to PARP activation and ATP depletion, and also directly disrupts mitochondrial function. These events can result in cell death by either necrosis or apoptosis, or in the repair of the damage and cell survival, depending on the severity and context of the damage.

While NO-induced neuronal cell death is understood in outline, more detailed temporal and spatial knowledge of the molecular events in this and other systems is required. In particular, a better understanding of why different cell types have altered susceptibilities to NO toxicity and die by a range of mechanisms following exposure to NO would be informative [7,66,152]. Recent work suggests that alterations in cellular glutathione concentration may contribute to these differences [153]. It is also important to determine how NO can protect against cell death, and the role of cellular redox state in this process [42,55]. In addressing these questions, the subtle modulation of gene expression and signal transduction by NO is likely to prove central [123,154].

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