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Review

The Role of Nitric Oxide in the Radiation-induced Effects in the Developing Brain

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Abstract. *The immature and adult brain display clear differences in the way they respond to insults. The effects of prenatal irradiation on the developing brain are well known. Both epidemiological and experimental data indicate that ionizing radiation may disrupt developmental processes leading to deleterious effects on post-natal brain functions. A central role of reactive oxygen and nitrogen species (ROS/RNS) as important mediators in both neurotoxicity and neuroprotection has been demonstrated. However, data concerning the role of ROS/RNS in radiation-induced damage in the developing brain are scarce. The goal of this review was to summarize the current studies concerning the role of nitric oxide and its reactive intermediates in activation of signal transduction pathways involved in cellular radiation response, with particular focus on radiation-induced effects in the developing brain.*

The brain is known to be one of the fetal structures quite susceptible to ionizing radiation (IR) and the effects of prenatal exposures to IR are well known to be deleterious. IR may interfere with several processes such as cell proliferation, migration, differentiation and synaptogenesis. Epidemiological studies on humans exposed *in utero* to the atomic bomb explosions at Hiroshima and Nagasaki suggest that even low doses of IR can induce microcephalia, unprovoked seizures, lower intelligence quotient scores with diminished school performance and mental retardation (1, 2).

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The vulnerability of the immature brain to IR has been shown to depend on the developmental stage. Radiosensitivity is maximal from the 8th through the 15th week of gestation, when a rapid production of neurons occurs and migration to their functional sites in the developing cortex takes place (2). The limited anti-oxidant capacity and the particular lipid composition of the cell membranes of the developing brain render it more vulnerable to oxidative stress (3). Important changes in mitochondrial function, closely related to the regulation of cell death and survival, have been shown to occur during brain development (4). Approximately one-half of all neuronal precursor cells in the developing central nervous system (CNS) die by apoptosis before birth. Moreover, apoptosis is the main pathway involved in radiation-induced cell death in the developing brain (5).

Primary ionization events triggered by IR are amplified and propagated by mechanisms involving reactive oxygen and nitrogen species (ROS/RNS), which activate several signal transduction pathways leading to final radiation effects (6). Over the last decade, there has been a substantial body of work implicating a central role for ROS/RNS as important mediators in neurotoxicity in both the developing and adult brain. Recently, it became evident that RNS may also mediate neuroprotection (7, 8).

The goal of this review was to summarize the role of nitric oxide (NO) and its reactive intermediates in the amplification of radiation-induced signaling pathways, with particular focus on effects involving cell death in the developing brain.

Chemistry of nitrogen-active species

NO is an inorganic free radical gaseous molecule which has been shown, over the last decade, to play an unprecedented range of roles in biological systems. In various different contexts it may act as an intracellular signal, as a transcellular signal or as a cytotoxic host defense molecule (9). It can be

formed in cells and tissues in many ways: from endogenous or exogenous NO donors, or from L-arginine by the activity of the enzyme nitric oxide synthase (NOS) (10). The broader chemistry of NO involves a redox array of species with distinctive properties and reactivities: NO^+ (nitrosonium), NO^- (nitroxyl anion) and NO (NO radical) (Figure1). Neutral NO has a single electron in its 2p-π antibonding orbital and the removal of this electron forms NO^+ while the addition of one more electron to NO forms NO^- (11).

NO^+(nitrosonium). The chemistry of NO^+ is characterized by addition and substitution reactions with nucleophiles such as electron-rich bases and aromatic compounds. Nitrosation in aqueous phase can occur at -S, -N, -O and -C centers in organic molecules and appears to involve NO^+ or related NO^+ equivalents. The biological relevance of NO^+ under weakly acidic or physiological conditions had been disputed, however a variety of nitroso-compounds that form effectively under neutral physiological conditions (11) can be interpreted as reactions with NO^+ carriers. Important examples of such compounds are metal-nytrosyl complexes, thionitrites (RS-NO), nitrosamines (RNH-NO), alkyl and aryl nitrites (RO-NO) and dinitrogen tri- and tetra-oxides (N_2O_3 and N_2O_4). In biological systems, there are numerous nucleophilic centers whose potential susceptibility to nitrosative attack has been shown in *in vitro* studies (11).

NO^- (nitroxyl anion). The chemistry of NO^- has received significantly less attention, particularly in aqueous solution. NO^- converts rapidly to N_2O through dimerization and dehydration (12) and is known to react with Fe(III) heme (13). NO^- also undergoes reversible addition to both low molecular weight and protein-associated thiols, leading to sulfhydryl oxidation. Electron transfer and collisional detachment reactions are common and generally yield NO radical (NO) as the major product. S-nitrosothiols are believed to be a (minor) product of the reaction of NO^- with disulfides(11).

NO (NO radical). From a biological point of view the important reactions of NO are those with oxygen and its various redox forms and with transition metal ions. The reaction of NO with O_2 in aqueous solution is a second order reaction in [NO]($v = k [NO]^2 [O_2]$) (11), thus the biological half life of NO, generally assumed to be in the order of seconds, strongly depends on its initial concentration. NO also reacts rapidly with O_2^- in aqueous solution, yielding peroxynitrite (14).

When discussing the chemistry and physiological effects of NO, it should be considered that NO is a highly diffusible second messenger that can elicit effects relatively far from its site of production. The concentration and therefore the source of NO are the major factors determining its biological effects (15). At low concentrations (<1 μM) the direct effects of NO predominate. At higher concentrations (>1 μM), the indirect effects mediated by RNS prevail. Thus, in cell types that contain constitutive NOS isoforms (cNOS), NO is produced in relatively low amounts for short periods of time and elicits mostly direct effects. High concentrations of NO are produced *via* inducible isoforms of NOS (iNOS) and the indirect and often pathological effects of NO are exhibited (16). The direct effects of NO most often involve the interaction of NO with metal complexes. NO forms complexes with the transition metal ions, including those regularly found in metalloproteins. The reactions with heme-containing proteins have been widely studied, particularly in the case of hemoglobin. NO also forms non-heme transition metal complexes and biochemical interest has been focused on its reactions toward iron-sulfur centers in proteins, including several proteins involved in mitochondrial electron transport and enzymes such as aconitase (17). The reactions of NO with heme-containing proteins are the most physiologically relevant and include interactions with soluble guanylate cyclase (18) and cytochrome P_{450} (19). Another established direct effect of NO on proteins is tyrosine nitration, which has been demonstrated by immunohistological staining in numerous human diseases, animal models and also under basal physiological conditions. Tyrosine nitration is selective and reversible and it has been shown that there are peroxynitrite-dependent and independent pathways for the nitration *in vivo* (16). NO also is able to terminate lipid peroxidation (20). The indirect effects of NO, produced through the interaction of NO with either O_2 or O_2^-, include nitrosation (when NO^+ is added to an amine, thiol, or hydroxy aromatic group), oxidation (when one or two electrons are removed from the substrate), or nitration (when NO_2^+ is added to a molecule) (15). In aqueous solution NO can undergo autoxidation (*i.e.* reaction with O_2) to produce N_2O_3 and this compound can undergo hydrolysis to form nitrite (21). Since NO and O_2 are 6-20 times more soluble in lipid layers compared to aqueous fractions, the rate of autoxidation is increased dramatically in the lipid phase (21) and the primary reactions of N_2O_3 are thought to occur mainly in the membrane fraction.

In its reaction with O_2^-, NO generates peroxynitrite ($ONOO^-$) at a rate close to diffusion, and $ONOO^-$ acts as both nitrating agent and powerful oxidant to modify proteins (formation of nitrotyrosine), lipids (lipid oxidation, lipid nitration) and nucleic acids (DNA oxidation and DNA nitration).

In summary, the potential reactions of NO are numerous and depend on many different factors. The site and source of production, as well as the concentration of NO collectively determine whether NO will elicit direct or indirect effects. In addition, a relative balance between oxidative and nitrosative stress exists and it is a main aspect that should be carefully evaluated to understand the complexity of the biological effects of NO.

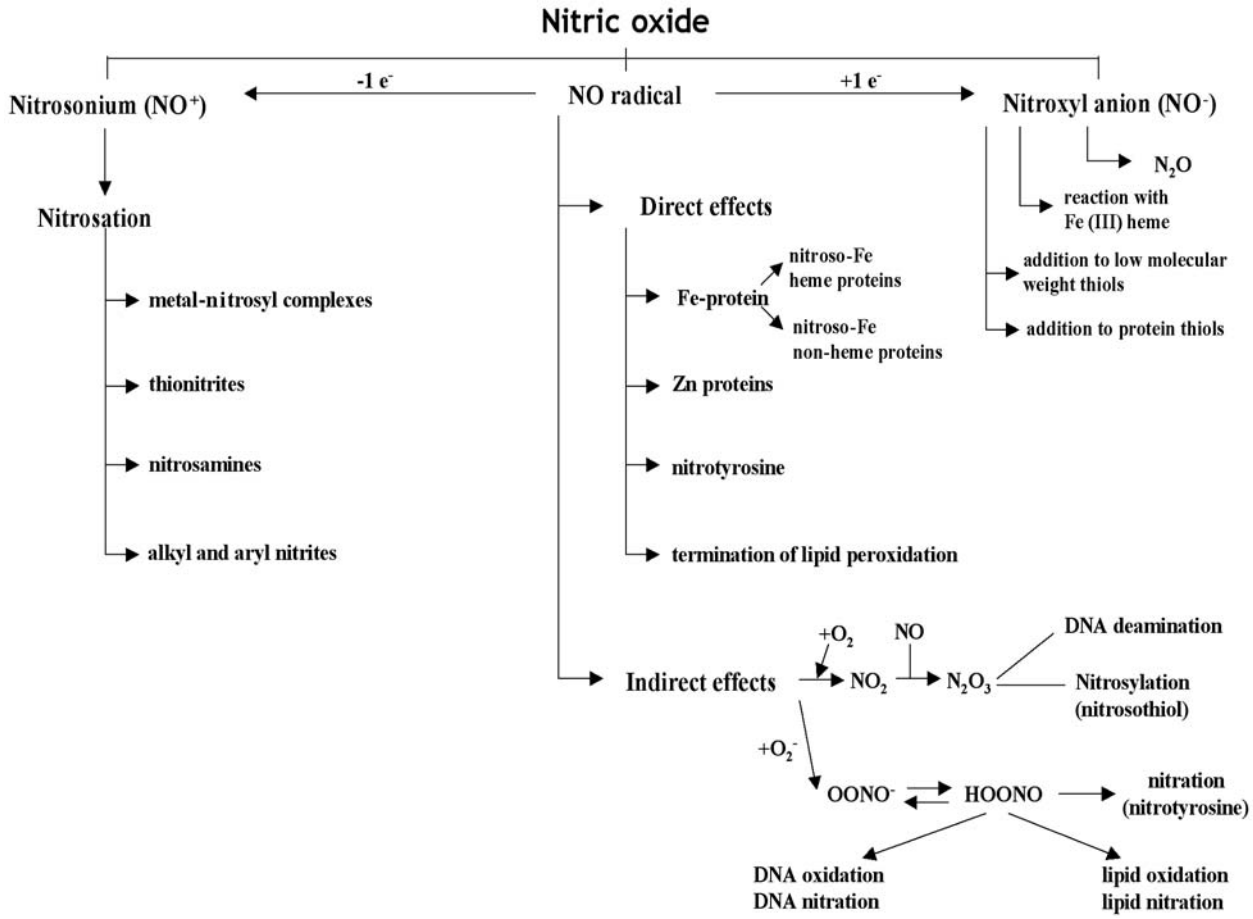

Figure 1. *Summary of the chemistry of the redox-interrelated forms of NO of potential biological significance. Interconversion of the redox-related forms of NO of potential biological significance involve metal nitrosyl complexes, charge transfer to electron acceptors and coupling to thiol/disulfide redox reactions. The direct effects of NO are usually elicited at low concentrations (< 1 μM), whereas the indirect effects of NO are elicited at high concentrations of NO (>1 μM). Taken and modified from Stamler et al. (11) and Davis et al. (16).*

Nitric oxide and ionizing radiation

IR may disrupt chemical bonding in the molecule of DNA inducing both single- (SSBs) and double-strand breaks (DSBs), either by depositing energy directly (direct effect) or by generating free radicals which, in turn, will attack the DNA molecule (indirect effect). A protein kinase cascade, whose roles in radiation response may be diverse and even dual, connects the detection of DNA damage to the implementation of an appropiate response: either DNA-repair or cell death (reviewed in 6 and 22).

Two superfamilies of protein kinases have a key role in radiation response: mitogen-activated protein kinases (MAPK) and phosphatidylinositol-3 kinases (PI3K). The MAPK includes the mitogen-activated extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK/SAPK) and p38 MAPK (23). The MAPK superfamily participates in cell survival and apoptosis pathways.

PI3K and its downstream effectors, protein kinase C (PKC) and B (PKB/Akt), promote cell survival. Up-regulation of telomerase, an enzyme activated *via* PKB/Akt, has been observed following exposure to IR (24). The DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia-mutated kinase (ATM) and ataxia telangiectasia-related kinase (ATR), are PI3K-related kinases closely connected to DNA repair (25, 26).

The p53 protein plays a pivotal role in determining whether a radiation-damaged cell will survive or undergo apoptosis (27). Within the first minutes following IR exposure, p53 is stabilized by ATM/ATR and activates the transcription of regulatory genes such as p21, GADD45, 14.3.3, Bax, Fas/APO1 and others (28). p53 activity is inhibited by murine double minute 2 (Mdm2), which induces its proteasome-mediated degradation (29).

Poly(ADP-ribose) polymerase (PARP) is a highly sensitive detector of DNA damage which plays a key role in the early phase of cell response to IR (30). Nuclear factor-kappaB (NF-Î B) and AP-1 are transcription factors whose activation is relevant in early radiation-induced signaling events (31,32). Ceramide participates in mitochondria collapse and caspase activation involved in radiation-induced apoptotic cell death (33).

The role of NO and its reactive intermediates in radiation response remains an open question. However, several authors have reported the up-regulation of NOS following exposure to IR. Enhanced NO production was demonstrated in liver, lung, kidney, intestine, brain, heart and bone marrow after γ-irradiation (34-37). Radiation-induced enhancement of NO production was also reported in chorioallantoic membrane, chondrocytes, CHO cell line and tumor cells (38-42). Significant increases of NO blood concentration and nitrite plasma levels were reported following IR exposure of rats (43, 44). NO has been proposed as mediator in radiation-induced nephritis, pneumonitis, endothelial and intestinal dysfunction (45-49). It has been suggested that IR may enhance tumor blood flow and oxygenation by activating NOS and restoring NO-mediated vasorelaxation (50).

There have been several reports that NO production triggered by interferon γ (IFN-γ) or lipopolysaccaride (LPS) is enhanced in macrophages previously exposed to IR (51, 52). This phenomenon was also observed in liver cells (53). "Bystander" is the term used to describe the biological effects observed in non-irradiated cells that are neighbors of cells that have been irradiated. Co-cultivation assays demonstrated that NO released by irradiated cells may induce changes in non-irradiated cells, suggesting that radiation-induced NO production could be involved in this type of effect (36).

Few authors have reported NO production following irradiation of CNS (34, 54). Concerning the developing brain, an early increase in NOS activity with a further increase in NO concentration was observed after prenatal exposure of rats to γ-radiation (55).

In summary, the available data concerning nitric oxide and IR indicate that:

a) IR may induce up-regulation of NOS with further increase in NO production in mammalian cells.
b) IR could act as a priming signal which synergizes with a second signal (such as IFN-γ or LPS) to induce NO production.
c) NO may be involved in "bystander" effects following IR exposure.

Nitric oxide in the developing brain

Neuronal NOS (nNOS) has many isoforms due to alternative splicing of some of the 29 exons present in the gene. In neurons both soluble and membrane-bound forms are present. The activation of constitutive isoforms is calcium-calmodulin-dependent (56). Biochemical studies indicate that nNOS can be phosphorylated by calcium/calmodulin-dependent protein kinase, cAMP-dependent protein kinase, cGMP-dependent protein kinase, PKC, among others, and that phosphorylation by all these enzymes decreases the catalytic activity of nNOS (57). Calcineurin, a protein phosphatase, is capable of dephosphorylating nNOS then increasing its catalytic activity (58). The amount of NO produced by nNOS is strongly correlated with the local free calcium level. The interference with the Ca^{2+}- calmodulin activation of the enzyme is a mechanism of nNOS regulation, although other mechanisms may occur downstream to this step, as has been demonstrated for some kinases (59).

The short half-life and diffusion properties of NO led to the idea, further confirmed, that NO plays a key role in CNS morphogenesis and synaptic plasticity (60). The presence of nNOS in cortical plate neurons and other structures of the developing brain such as optic tectum, retinal axons and developing olfactory epithelium has been broadly described (61). In cerebral cortical plate, nNOS expression occurs in the majority of cells at embryonic day E15 to E19 in rats while the ventricular zone and cortical subplate display diminished NOS immunoreactivity (62). At E14 staining it is confined to the Cajal-Retzius cells, while at E15 to E19 the great majority of cortical cells stain positively. NOS stain is also prominent in the intermediate zone. The increase in NOS activity in rat correlates with the development of the estrogen receptor population and precedes the development of synaptic structures, supporting the hypothesis that NO formation may have a role in the developmental physiology of synapses (63). After birth, the intense NOS labeling decreases rapidly and vanishes by the 15th postnatal day, coinciding with a lower activity of nNOS and a decreased expression of nNOS mRNA (64, 65).

However, in some cases substantial expression overlaps with other periods of neurogenesis and there is some indication for a role of NO in the regulation of neural proliferation (59). The fact that the survival of cerebellar granule neurons *in vitro* significantly decreases when cells are exposed chronically to the NOS inhibitor L-NAME from the beginning of the culture (66) supports the role of NO in neural cell survival during development. Since NO is easily diffusible, it could exert its properties not only in the cell that produces it, but in the adjacent cell as well. Thereby, it is plausible that NO could contribute to the synchronic development of the neighboring committed cells to form a particular structure (67). It has been proposed that NO could regulate cell survival *via* telomerase activity (TA) modulation (68). TA is present in neuroblasts and progressively diminishes during neuron differentiation, sharply decreasing during the period when synapses form and programmed cell death occurs (69).

Redox state and early events in radiation response

Historically, the redox homeostasis system has been implicated in the generation and persistence of the radiation-induced effects (70). It has been pointed out that the great susceptibility of the developing brain to IR is in part due to the low status of its redox system (3). Redox status is a term used to describe the ratio of the interconvertible oxidized and reduced form of a specific redox couple and more generally to describe the redox environment of the cell. Most of the energy from IR is delivered in water, leading to (·OH) hydroxyl radicals which can subsequently react with bases and sugar in DNA (71).

An important question concerns how a few primary ionization events in the order of 2000/Gy/cell can be sufficiently amplified to account for the relatively rapid and robust activation of signal transduction pathways (6). Related to this, extranuclear amplification mechanisms involving ROS/RNS have been demonstrated relatively long (>15 min) after irradiation (72).

Redox state and radiation-induced oxidative stress. Through radiolytic processes IR is able to generate primary and secondary ROS which can persist and diffuse within cells, giving origin to delayed effects (73). Overproduction of ROS is able to disrupt the delicate pro-oxidant/ antioxidant balance leading to protein, lipid and DNA damage (74). Persistent oxidative stress after radiation exposure was associated with long delayed effects such as chromosomal instability (75) and inherited radiosensitivity (76).

Alterations in the redox equilibrium are precipitated by changing either the glutathione/glutathione disulfide ratio (GSH/GSSG) and/or the reduced /oxidized thioredoxin (Trx) ratio. GSH is the principal thiol responsible for scavenging ROS and mantaining the oxidative balance in tissues (77). The majority of GSH in cells is found in the cytosol, the most important site of GSH biosynthesis, in the range of 1-11 mM. Another important component in the redox control is Trx, which is translocated to the nucleus when cells are exposed to radiation-induced oxidative stress (78). IR elicits an increase in nuclear Trx that is accompanied by an increase in AP-1 binding activity (79).

It has been proposed that the redox state is able to determine the cytoprotective and cytotoxic potential of NO (80) and that GSH depletion switches both effects in dopaminergic neurons (81). The redox state may have a direct influence on the disposition of NO. Interestingly, GSH-transferases can exert functions of NO carriers increasing the availability of the molecule in cases of impairment of the GSH concentration (82). One signalling pathway described in mid brain cultures involving the interaction GSH-NO is related to the arachidonic acid metabolism through 12-lipoxygenase (81). In the developing brain, impaired redox state may inhibit neuronal migration in the corticogenetic stage (83).

NO and redox regulation of cell signaling. Several cellular signaling pathways are regulated by the intracellular redox environment. Both oxidative and reductive stress can trigger redox cascades changing the thiol status of proteins. Redox state has been implicated in the post-translational regulation of p53, AP-1 activator protein, NF-Î B, Sp-1 transcription factor, c-myb proto-oncogene and others (84-89). Several proteins related to apoptosis, such as signal-regulating kinase-1, protein-tyrosine phosphatase 1-B and thioredoxin-peroxidase, have been identified as components of redox signaling that act downstream of the generation of ROS/RNS, having stimulative or inhibitory actions.

Owing to its redox chemistry, NO is able to stimulate signaling through protein kinases as well as through its reactions with ROS and redox metals. Its major intracellular target sites are the sulphydryl and metal-containing proteins such as protein-kinases, phosphatases and transcription factors containing either sulphydryls or redox metals located at either allosteric or active sites (11). The diversity of the NO-modulated tyrosine posphorylation/dephosphorylation signaling pathway is highly dependent on the cell type (90). Along with the modulation of the kinases and the broadly recognized activation of guanylyl cyclase, other functions have been described to be regulated by NO. For example, by inducing a conformational change in p21 *via* S-nitrosylation, NO can increase the nucleotide exchange operated by this G protein family member (91). Moreover, NO is able to regulate the activity of different members of the MAPK superfamily such as p38 and JNK/SAPK (92). Recently, evidence was presented that NO regulates, either positively or negatively, the JNK/SAPK signaling pathway in microglial cells (93). Members of the PI3K superfamily are also targets of NO (94).

Nitric oxide as antioxidant. While NO in some conditions may stimulate O_2^- lipid peroxidation, under other conditions it mediates protective reactions in membranes by inhibiting O_2^- and $ONOO^-$-induced lipid peroxidation. These effects are highly dependent on the relative concentration of individual reactive species. NO can act as an inhibitor of chain propagation reaction *via* radical-radical reaction with lipid peroxyl radicals (LOO•) at near diffusion limited rates (95). The termination of LOO• by NO will be significantly more favored than either of the following reactions: the reaction of LOO• with alpha-tocopherol and the secondary propagation reaction of LOO• with vicinal unsaturated lipids (96). A summary of the mechanisms involved in the termination of lipid peroxidation may be as follow: a) NO trapping of alkyl, aloxyl and peroxyl lipid-derived radicals, b) NO regulation of the activity of enzymes such as cycloxygenase, lipoxygenase and cytochrome P_{450}, c) NO regulation of cell signaling not directly associated with lipid oxidation, and d) NO binding to redox-active metal centers. Additionally NO may act regenerating reduced α-tocopherol by direct reduction of α-tocopheryl

radical (97). With the exception of sodium-nitroprusside, NO donors are able to inhibit the iron-catalyzed Fenton reaction reducing the generation of •OH (98).

The antioxidant actions of NO could be summarized as: a) inhibiting iron-stimulated •OH generation by Fenton reaction b)terminating lipid peroxidation; c) augmenting the antioxidative potency of GSH; d) mediating the neuroprotective effect of brain-derived neurotrophins (99).

Nitric oxide and mitochondria

The brain is dependent on the mitochondrial energy supply to support its normal functions. Impairment of one or more components of the respiratory chain may lead to important alterations of cellular ATP synthesis and enhanced production of secondary ROS. Mitochondria are both a source and a target for ROS and, as the main source of O_2^-, they may be target for the deleterious action of $ONOO^-$ in the presence of NO.

The mitochondrial membrane potential ($\Delta\psi_m$) is at the center of the cell interaction controlling ATP synthesis, the mitochondrial Ca^{2+} and consequent generation of ROS/RNS. In this sense, the mitochondria exert a delicate balance between the generation of ROS and the maintenance of a reduced environment favored by a high $\Delta\psi m$ (100).

Leach *et al.* (40) have found that IR is able to induce an early reversible mitochondrial permeability transition with further production of ROS/RNS and that this amplification system appears necessary for radiation-induced activation of downstream signal pathways.

One of the main points concerning the action of NO in mitochondria is related to the effects on the components of the respiratory chain. Complex I (NADH ubiquinone reductase) is inhibited following prolonged exposure of cells to NO (101). S-nitration of a critical thiol in a Fe-S center of the complex is the original mechanism thought to be involved. However, no correlation was found between oxygen consumption and electron paramagnetic resonance-detectable iron-sulphur-dinitrosyl complexes (102). It seems that $ONOO^-$ rather than NO is responsible for loss of complex I activity, at least in the isolated mitochondria (103,104). Nevertheless, complex I functions can be partly reversed by reduced thiol, suggesting that mechanisms other than nitration might be involved (101). In addition, GSH depletion inhibits mitochondrial complex I (105). Complex II (succinate ubiquinone reductase) is the only system that serves as a direct link between the tricarboxylic acid cycle and the electron transport chain. *In vitro* incubation of oligodendrocytes with NO donors results in loss of activity of a component of complex II (succinate dehydrogenase component) (106). Complex III (ubiquinol cytochrome c reductase) transfers electrons from ubiquinol to cytochrome C (cyt C) and this is coupled to the pumping of protons across the mitochondrial membrane. It has been communicated that $ONOO^-$ is able to inhibit complex II with no effect on complex III (107), whereas other authors find complex III affected and complex II unaffected (108). Moreover, $ONOO^-$ inactivates mitochondrial aconitase, a component of Kreb's cycle with a 4Fe4S iron-sulphur center that can be removed by NO (109). Another potential cause of inhibition of glycolysis is the depletion of NAD^+ due to the activation of PARP by radiation-induced DNA damage (110). Complex IV (cytochrome oxidase) couples the reduction of oxygen to the pumping of electrons out of the mitochondrial matrix. ONOO- has been shown to cause irreversible inhibition of neuronal complex IV (111). Moreover, cytochrome oxidase avoids the release of ROS during the reduction of O_2 to water. A binuclear heme-copper ion center of the protein cytochrome a_3 shows high affinity for O_2 and for NO. Because NO competes with oxygen at this level, it increases the apparent Km of the cytochrome oxidase for oxygen (112). The reactions of NO and the specific cytochrome oxidase activity are critically dependent on NO, O_2 and cytochrome oxidase levels. It is to be noted that NO exposures may increase complex IV mRNA and protein levels *in vitro* (113).

"Prooxidants" and "antioxidants" effects related to the interaction of NO and complex IV have been proposed by Sarti *et al.* (114). Depending on the metabolic electron flux through the respiratory chain, the scenarios are diverse. When the flux of electrons is high NO is able to bind heme a_3^2 of the complex IV, inhibiting it and being released as such. When the electron flux is low, NO binds Cub^{2+} of the complex IV, inhibits the complex and is thereafter degraded to nitrite (114). After blockade of complex IV, the cell would respond with a defense mechanism that at the earliest stages involves an increased hydrolysis of glucolytic ATP. Contrary to the widely accepted view that NO induces a collapse in $\Delta\psi_m$, it has been found in Jurkat cells that exposure to NO has protective actions including an increase in $\Delta\psi_m$ (115).

Considering the diverse and sometimes paradoxical roles of NO and $ONOO^-$ in regulating apoptosis, mitochondria are a key point related to the cytoprotective or cytotoxic effects of radiation-induced generation of ROS/RNS. The Bcl-2 family proteins, whose activity is in turn under the control of the redox state, regulate the functions of the mitochondrial transition pore (MTP), releasing cyt C (116). Recently it has been reported that NO at low and transient fluxes prevents MTP opening and cyt C release (117). It has been proposed that lipid-peroxidation may also be a key point in the control of the permeabilization of the inner mitochondrial membrane (118). Since NO prevents lipid peroxidation, this may represent an additional anti-apoptotic mechanism of NO at mitochondrial level.

Nitric oxide in apoptosis

NO is endowed with the unique ability to initiate and to block apoptosis (119). The mechanisms governing these effects are

being elucidated. They depend on the cell type, the rate of NO production, the redox state and the interaction with biological molecules such as thiol proteins and ROS, among others (120-122). Pro-apoptotic effects are mainly associated with high NO production *via* iNOS (123, 81), whereas the continuous low-level production of NO mainly through cNOS seems to confer neuroprotection (55).

To understand the role of NO the defining characteristics of apoptosis should be recapitulated. One of the keys is the activation of caspases, that propagate and amplify two types of death pathways: one initiated by death receptors when bound by specific death ligands and the other involving internal and external stressors *via* the mitochondria. There are strong data indicating that a caspase-3-like group (caspase-3 and-7), caspase-2 and caspase-9 are central components in a cascade triggered by NO (124). On the other hand, apoptosis mediated by cytochrome c release and caspase-9 and -3 activation appears to play a particularly significant role in the developing CNS (125, 126). The following paragraphs will summarize the main effects of NO on neural cell apoptosis and particularly on embryonic neuron death.

NO and DNA damage. It was recently reported that neurons in the developing brain die *via* apoptosis after DNA damage, while neurons in the adult brain are generally resistant to these insults (127). ROS/RNS can damage DNA through different chemical mechanism. NO and peroxynitrite can cause SSBs in DNA and inhibit DNA repair mechanisms (121). Although PARP facilitates DNA repair, its excessive activation can lead to significant NAD and ATP depletion resulting in cell dysfunction or necrotic death, since apoptosis is an energy-requiring pathway. However, oxidative damage to cellular proteins and nucleic acids can trigger an apoptotic cascade involving activation of PARP, MPT opening, release of cyt C and activation of caspases that execute the cell death process (128, 129).

NO and p53. It was shown that low-dose irradiation leads to p53 gene induction in the embryonic rat brain and that all apoptotic cells also expressed high levels of p53 protein, suggesting an important role for p53-induced apoptosis in the developing brain (130). In some cell types where NO exerts a pro-apoptotic affect, exposure of cells to NO can lead to up-regulation of p53 (121, 131). Recent studies confirmed p53 stabilization under the impact of NO, accumulation of a transcriptionally active serine15-phosphorylated p53 and predominant nuclear localization. Additionally, a transient and reversible down- regulation of Mdm2 by NO is claimed to contribute to the activation of p53 (124). p53 positively transactivates p21, an inhibitor of cyclin-dependent kinases (CDK) and pro-apoptotic genes such as Bax. Conversely, p53 negatively transactivates the expression of the anti-apoptotic gene Bcl-2 (131). Studies of Bax and p53 deficient mice have indicated roles for both p53-dependent/Bax-dependent and p53-dependent/Bax-independent neuronal apoptosis in response to ionizing radiation (132).

NO and MAP kinases. The MAP kinase superfamily is involved in the cell survival/death decision in many pathological or physiological settings. Cheng *et al.* (133) demonstrated that NO can induce apoptotic death of neural progenitor cells by a p38 MAP kinase-dependent mechanism: p38 MAP kinase acts at an early step prior to dysfunction of mitochondria and caspase activation, and Bcl-2 significantly attenuates the activation of p38 MAP kinase and protects neural progenitor cells against NO-induced death. JNK/SAPK, ERK-1 and ERK-2 were not activated in neural progenitor cells exposed to NO. In primary cultures of cortical neurons, p38 MAP kinase activity plays a critical role in NO-mediated cell death by stimulating Bax translocation from the cytosol to the mitochondria, thereby activating the death pathway. Taking into account the ability of radiation to activate MAP kinases (23), these could represent an attractive pathway to link radiation and NO-induced apoptosis, but the complexity of signaling networks make it difficult to predict causation.

NO and proteasome. Proteasome inhibition plays a causal role in several neuropathological processes and the induction of neuronal apoptosis *via* the release of cytochrome c and activation of caspase-3-like process was reported (134). In other cell types, NO production resulted in inhibition of proteasome activity. Moreover, NO donors inhibited *in vitro* the degradation of polyubiquitinade p53 by the 26S proteasome and prevented the degradation of pro-apoptotic Bax protein, providing an additional way to stimulate apoptosis (131).

NO and caspases. Although indirect stimulation of procaspases by NO has been described (119), NO inhibition of caspase through S-nitrosylation of the active site cysteine has been widely studied (135, 136). S-Nitrosylation of the active site cysteine of the pro-caspase-activating enzyme has also been reported (137). Nevertheless, as pointed out by Brüne (124), whether inhibition of apoptosis as a result of NO generation is restricted to S-nitrosylation of active caspase, or whether assembly of the apoptosome is attenuated, remains to be shown .

NO and cyclic nucleotides. A protective mechanism for NO proceeding *via* a cGMP-mediated blockage upstream of cytochrome c release in neural cells has been shown (138,139). In human neuroblastoma cells, NO inhibits apoptosis through its ability to activate guanylate cyclase, which in turn activates the cGMP-dependent protein kinase (PKG) (140).

NO and Bcl-2. Members of the Bcl-2 protein family have been found to be involved with pro-apoptotic effects (Bax, Bid) or

apoptosis inhibition (Bcl-2, Bcl-X_L). Down-regulation of Bcl-2 and up-regulation of Bax protein levels followed by caspase-3-like activation were described in NO-induced neuronal apoptosis (129). Protection of neural progenitor cells against NO-induced apoptosis by Bcl-2 has been reported (133). Bcl-2 is expressed at high levels during neurogenesis and Bcl-X_L critically regulates immature neuron survival (141). It is conceivable that signals that regulate NO production and Bcl-2 expression might interact in the regulation of neural progenitor cell survival in the developing CNS.

Nitric oxide and apoptosis vs excitotoxicity. Excitotoxity is thought to play a prominent role in a variety of neuronal injuries resulting from the excessive activation of glutamate acting on NMDA and non-NMDA receptors. A number of studies demonstrate a prominent role for NO in excitotoxicity both *in vivo* and *in vitro* (142, 143). However, apoptosis has been identified as a major mechanism that can contribute to neurodegenerative processes during development. MK801, a drug that powerfully blocks NMDA Glu receptors protecting against excitotoxic death, causes a worsening of the apoptotic cell death process (144).

Final considerations

Several relevant features related to radiation-induced effects on the developing brain can be pointed out: a) radiation-induced cell death follows the apoptotic pathway in a dose-dependent manner; b) this process involves a caspase-3-dependent mechanism; c) IR induces an early increase in NO production; d) ROS/RNS play a pivotal role in the signaling pathway toward cell death.

A model can be proposed for the temporal behavior of these events during the first hours following IR exposure: in the first minutes IR generates a transient increase of ROS/RNS and an early reversible mitochondrial $\Delta\psi$ depolarization. A reversible release of cyt C may occur at this stage and even small amounts may be sufficient to activate caspases, which in turn may cause a feedback amplification of cyt C release (145). At a later stage, a further up-regulation of nNOS activity increases the NO content with neither evidence of ROS enhancement nor lipid peroxidation. At this moment NO could be exerting an antioxidant role and, at the same time, it could eventually lead to a reversible inhibition of the mitochondrial chain transport. A subsequent decrease in NOS activity and NO content, coincident with a progressive second rise of ROS, correlates with the activation of caspase-3 and a massive release of cyt C. This later release of cyt C may be the cause of the second wave of ROS generation (146). At this stage apoptosis becomes an irreversible event .

We have shown that inhibition of NOS increases radiation-induced apoptosis in neural precursor cells. In addition, inhibition of caspase-3 leads to a significant decrease in the second wave of ROS generation (unpublished data). These findings suggest that, despite its initial antioxidant action, NO could not prevent radiation-induced apoptosis although it clearly modulates the response. The increased activity of caspases might account for the burst of ROS production, as previously proposed (146). Whether or not NO plays pro-oxidant and pro-apoptotic actions in some stages of this model remains an open question.

Since the NO molecule exerts crucial roles during brain maturation, the elucidation of the involvement of NO in radiation-induced signaling will open an important road in the radiobiology of prenatal exposures.

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