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SYMPOSIUM: Oxidative Stress in Neurological Disease

Oxidative Stress in Brain Ischemia

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Brain ischemia initiates a complex cascade of metabolic events, several of which involve the generation of nitrogen and oxygen free radicals. These free radicals and related reactive chemical species mediate much of damage that occurs after transient brain ischemia, and in the penumbral region of infarcts caused by permanent ischemia. Nitric oxide, a water- and lipid-soluble free radical, is generated by the action of nitric oxide synthases. Ischemia causes a surge in nitric oxide synthase 1 (NOS 1) activity in neurons and, possibly, glia, increased NOS 3 activity in vascular endothelium, and later an increase in NOS 2 activity in a range of cells including infiltrating neutrophils and macrophages, activated microglia and astrocytes. The effects of ischemia on the activity of NOS 1, a Ca^{2+}-dependent enzyme, are thought to be secondary to reversal of glutamate reuptake at synapses, activation of NMDA receptors, and resulting elevation of intracellular Ca^{2+}. The up-regulation of NOS 2 activity is mediated by transcriptional inducers. In the context of brain ischemia, the activity of NOS 1 and NOS 2 is broadly deleterious, and their inhibition or inactivation is neuroprotective. However, the production of nitric oxide in blood vessels by NOS 3, which, like NOS 1, is Ca^{2+}-dependent, causes vasodilatation and improves blood flow in the penumbral region of brain infarcts. In addition to causing the synthesis of nitric oxide, brain ischemia leads to the generation of superoxide, through the action of nitric oxide synthases, xanthine oxidase, leakage from the mitochondrial electron transport chain, and other mechanisms. Nitric oxide and superoxide are themselves highly reactive but can also combine to form a highly toxic anion, peroxynitrite. The toxicity of the free radicals and peroxynitrite results from their modification of macromolecules, especially DNA, and from the resulting induction of apoptotic and necrotic pathways. The mode of cell death that prevails probably depends on the severity and precise nature of the ischemic injury. Recent studies have emphasized the role of peroxynitrite in causing single-stand breaks in DNA, which activate the DNA repair protein poly(ADP-ribose) polymerase (PARP). This catalyzes the cleavage and thereby the consumption of NAD^+, the source of energy for many vital cellular processes. Over-activation of PARP, with resulting depletion of NAD^+, has been shown to make a major contribution to brain damage after transient focal ischemia in experimental animals. Neuronal accumulation of poly(ADP-ribose), the end-product of PARP activity has been demonstrated after brain ischemia in man. Several therapeutic strategies have been used to try to prevent oxidative damage and its consequences after brain ischemia in man. Although some of the drugs used in early studies were ineffective or had unacceptable side effects, other trials with antioxidant drugs have proven highly encouraging. The findings in recent animal studies are likely to lead to a range of further pharmacological strategies to limit brain injury in stroke patients.

Introduction

Ischemic brain damage was, for many years, regarded simply as the passive outcome of reducing the oxygen supply of neurons and other cells below the threshold for adequate energy production to allow their survival. However, there is now a large body of experimental evidence to indicate that much of the damage is mediated by active processes, many of which lead to or result from the production of free radicals and other highly reactive, oxidizing chemical species. Research using animal models has shown pharmacological intervention in several of these processes to reduce substantially the brain damage and neurological dysfunction that result from ischemia.

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Sources of reactive nitrogen and oxygen species in brain ischemia

Nitric oxide. Nitric oxide (NO•) is a water- and lipid-soluble free radical with diverse biological activities, including vasodilatation, inhibition of platelet aggregation, inhibition of smooth muscle proliferation, modulation of neurotransmission, promotion of synaptogenesis and synaptic remodelling, an involvement in long-term potentiation and depression, and antimicrobial toxicity (45, 51, 107, 115, 124, see also 19, 20, 90). It is produced in the body by the activity of nitric oxide synthases (see box below).

Nitric oxide synthases. The members of the nitric oxide synthase (NOS) family are all large (~300 kDa) protein homodimers that catalyse the conversion of L-arginine first to N-hydroxyl-arginine, and then L-citrulline and NO• (68, 98). These reactions are coupled to the donation of two electrons by NADPH. Activation of NOS requires the binding of calmodulin (CaM). Other necessary cofactors are FAD, FMN, heme and tetrahydrobiopterin. Two of the known types of mammalian NOS (NOS 1 and 3) bind CaM in a reversible Ca^{2+}-dependent manner (hence the designation cNOS for both types). Because the normal intracellular levels of Ca^{2+} are too low to allow the binding of CaM to these types of NOS, they are active only during periods of transient, agonist-induced elevations in intracellular Ca^{2+}. The third type of NOS (NOS 2) binds CaM even at very low concentrations of intracellular Ca^{2+} and is therefore constitutively active. Regulation of NOS 2 activity is primarily mediated by a wide range of transcriptional inducers (including several cytokines, and hypoxia) and inhibitors, although post-transcriptional and post-translational control mechanisms also play a role. Hypoxia has been shown to induce increased transcription of NOS 3 by endothelial cells in culture (2).

Although the preferred terminology for NOS is as indicated above, NOS 1 is still often referred to as neuronal NOS or nNOS, reflecting the fact that it was initially purified from neurons. NOS 3, first purified from endothelial cells, is also known as endothelial NOS or eNOS, and NOS 2 as inducible NOS or iNOS. However, nitric oxide synthases occur in a wide variety of other cell types. Although NOS 1 is the principal neuronal form of NOS and, indeed, the predominant NOS in the normal nervous system, all three forms of NOS have been reported to be expressed in some populations of neurons. In addition, NOS 1, NOS 2 and possibly NOS 3 have been detected in astrocytes, and NOS 1 in oligodendrocytes and microglia (see 24, 25, 69, 89, 98). Three splice variants of NOS 1 (α, β and γ) have been identified, of which NOS 1α accounts for most NOS activity in the brain. NOS 1β may be a significant additional source of NO in some regions, such as the striatum and lateral tegmental nuclei in the pons (32). Two further human NOS genes that show close sequence similarity to the previously identified human NOS 2 gene and are also on chromosome 17 were identified by Bloch *et al* (8) and designated NOS 2B and 2C (NOS 2 being renamed NOS 2A); the biological significance of these additional NOS 2-like genes is still unclear.

NOS activity and brain ischemia. The activity of all three forms of NOS increases after the initiation of ischemia, NOS 1 and 3 within minutes (see The roles of glutamate in ischemic damage) and NOS 2 after several hours (see 18,61,112). The surge in NOS 1 activity is short-lived, declining to normal by about 60 min, although ischemia has been reported to induce increased transcription of NOS 1 mRNA for up to 7 days (152). NOS 2 activity in ischemic brain tissue probably derives from infiltrating neutrophils, microglia, macrophages and astrocytes as well as vascular endothelial cells, and remains elevated for several days. The sources of NOS 2 vary somewhat according to the nature of the ischemic insult. In frank infarcts caused by sustained focal ischemia, neutrophils and macrophages are probably the principal source of NOS 2. Transient focal and global

Designation	Synonyms	Activity dependent on elevated iCa^{2+}	Time course of activity	Chromosomal location
NOS 1	neuronal NOS, nNOS, ncNOS	yes	transient	12q24.2-q24.31*
NOS 2A	inducible NOS, iNOS	no	continuous	17cen-q11.2†
NOS 2B				17p13.1-q25†
NOS 2C				17p13.1-q25†
NOS 3	endothelial NOS, eNOS, ecNOS	yes	transient	7q36‡

* Reference 145
† Reference 8
‡ References 88 and 116

ischemia have been reported to induce NOS 2 expression by vascular cells and astrocytes respectively. The increase in NOS 3 activity is briefer than that of NOS 2, but elevated levels of NOS 3 can be detected for several hours after transient ischemia, and a delayed increase in NOS 3 occurs at the periphery of the region of infarction and is sustained for several days (5, 153).

Superoxide and hydroxyl radicals. Particularly under conditions of reduced availability of L-arginine or tetrahydrobiopterin, when NOS activity is uncoupled from electron donation by NADPH, the synthesis of NO• is accompanied by the production of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Superoxide is the dissociated form of the weak acid, perhydroxyl radical ($HO_2^{\bullet}$) but at physiological pH only about 1% of is combined with H^+ as $HO_2^{\bullet -}$. The reaction of superoxide with hydrogen peroxide leads to the formation of highly reactive hydroxyl free radicals. Sources other than NOS of superoxide include xanthine oxidase, NADPH oxidase, cyclo-oxygenases and leakage from the electron transport chain.

Xanthine oxidase is a biochemically-modified form of xanthine dehydrogenase. Normally, xanthine dehydrogenase catalyses the oxidation of xanthine and hypoxanthine to uric acid, with NAD^+ as the electron acceptor. Xanthine oxidase, modified from xanthine dehydrogenase by oxidation (eg, after transient ischemia) or limited proteolysis, uses oxygen as the electron acceptor so that the oxidation of xanthine and hypoxanthine generates superoxide and hydrogen peroxide and contributes to brain injury during reperfusion after ischemia (7, 52, 103). NADPH oxidase is present in neutrophils and macrophages and generates superoxide and hydrogen peroxide within the phagocytic vacuoles (35, 50). Studies of mutant mice lacking a functional NADPH oxidase suggest that there is also significant endogenous NADPH oxidase activity within the central nervous system and that this contributes to superoxide generation and tissue damage in ischemic brain injury (140). Some of the superoxide that is produced during brain ischemia and reperfusion can be inhibited by the administration of indomethacin and is probably generated by cyclo-oxygenases (110). Superoxide is also produced by the mitochondrial electron transport chain, particularly under conditions of brain ischemia (27, 108).

Peroxynitrite and related species. Nitric oxide and superoxide rapidly combine to form peroxynitrite, which is much more toxic to DNA and other macromolecules than is either of its precursors (6, 16, 133). Other potentially damaging metabolites of nitric oxide include the nitrogen dioxide radical $\bullet NO_2$ and nitryl chloride (NO_2Cl), formed by reaction of nitrite, an end-product of nitric oxide metabolism, with hypochlorous acid (HOCl), itself produced by the action of myeloperoxidase in neutrophils (29, 30, 139).

Beneficial and deleterious effects of NOS in ischemia

The complex balance of protective and destructive effects of NOS activation in brain ischemia has been the subject of several excellent reviews (eg, 17, 18, 59, 122). In general, the administration of selective inhibitors of NOS 1 or NOS 2 results in a reduction of infarct volume and other measures of ischemic damage (41, 62, 63, 94, 149, 151). As might therefore be expected, disruption of either of the corresponding NOS genes results in amelioration of brain damage after ischemia (48, 58, 60, 150, see 55). There are, as yet, no selective inhibitors of NOS 3, but mice with disruption of the NOS 3 gene have been generated and used to study the effects of this type of NOS in brain ischemia (56, 57, 84). Unlike NOS 1 and NOS 2 knockout mice, the NOS 3 knockouts develop *larger* infarcts than do their wild type counterparts. The analysis of changes in ischemic brain damage in the NOS 3 knockout mice is complicated by the fact that the loss of the normal basal vasodilatation mediated by endothelial production of NOo causes these mice to be hypertensive (56). However, normalization of their blood pressure by the administration of hydralazine did not reduce the size of infarct caused by temporary middle cerebral artery occlusion whereas inhibition of residual NOS 1 and 2 activity by an infusion of nitro-L-arginine did (57). Functional CT scanning has shown the penumbral zone in NOS 3 knockout mice to be narrowed and perfusion in this zone to be reduced (84). These findings are in keeping with earlier observations that intravascular administration of nitric oxide donors during the first 2 h after induction of ischemia improve penumbral blood flow and lessen brain damage (see 17, 18, 59, 122).

The above studies indicate that the initial nitric oxide-mediated vasodilatation and enhanced penumbral perfusion that result from the activation of NOS 3 are neuroprotective, at least during the first 2 h after the ischemic insult. However, the overall effects of enhanced NOS 1 and NOS 2 activity after ischemia are detrimental.

The role of glutamate in ischemic damage

Glutamate is an excitatory neurotransmitter that is widely expressed within the central nervous system. It that binds to two distinct families of receptors, the metabotropic receptors, linked to the activation of phospholipase C and inhibition of adenyl cyclase (125), and the ionotropic receptors, that are linked to ion channels (96, 145). The latter family of glutamate receptors comprises three types, named according to their differential sensitivity to the agonists N-methyl-D-aspartate (NMDA), kainic acid (KA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). The non-NMDA ionotropic glutamate receptors (ie, the KA and AMPA receptors) have much faster kinetics than the NMDA receptors whereas only the latter respond to activation by increasing Ca^{2+} permeability. However, activation of all of the ionotropic glutamate receptors leads to an increase in Na^+ and K^+ permeability and the resulting depolarization can secondarily activate voltage-sensitive Ca^{2+} channels (see 54, 91). Activation of the metabotropic glutamate receptors may also contribute to a rise in intracellular Ca^{2+} in ischemia, as a result of the mobilization of Ca^{2+} from intracellular stores (93, see 91).

Under normal circumstances, the action of glutamate that is released at synapses is terminated by its uptake from the extracellular space by a family of glutamate transporter proteins. The energy that drives this uptake derives from the linked transport of Na^+ and K^+ down their respective electrochemical gradients: Na^+ is co-transported with glutamtate into the cell (glial or neuronal) and K^+ out of the cell (1, 4, see 136). Each cycle of glutamate transporter activity also results in the transfer of one proton-equivalent but it is unclear whether this reflects the transport of H^+ into the cell or OH^- outwards.

After the onset of ischemia, anerobic metabolism leads to a fall in pH, and depletion of ATP to slowing and then failure of the Na^+/K^+ pump, resulting in the movement of these ions down their electrochemical gradients across the plasma membrane: Na^+ into the cell and K^+ outwards. This redistribution is initially gradual but after about 2 min occurs more rapidly, as the membranes depolarize. The membrane depolarization and the change in the concentration gradients of Na^+ and K^+ across the plasma membrane cause reversal of the direction of action of the glutamate transporter proteins (see 135, 136), as a result of which glutamate rapidly accumulates extracellularly until it reaches neurotoxic levels.

The accumulation of glutamate is a monophasic, relatively short-lived event after ischemia, lasting no more than 5-10 min. The consequence is, however, a biphasic rise in intracellular Ca^{2+} (127, 128, see 91). The initial, marked rise is closely coupled temporally to the accumulation of glutamate and is largely due to activation of neuronal NMDA receptors. In many experimental systems, transient ischemia also induces a secondary, less marked but sustained rise in intracellular Ca^{2+}, commencing approximately 2-3 h after reperfusion, not associated with elevated levels of glutamate, and usually signifying irreversible cell damage.

As might be expected, the initial rise in intracellular Ca^{2+} can be prevented by pharmacological blockade of the NMDA receptors. Less clearly understood is the physiological basis of the oft-repeated observation that both the secondary rise in Ca^{2+} and much of the cell death in the penumbral region of an infarct can, in many experimental systems, be prevented by NMDA and AMPA receptor antagonists even if these are administered up to 2 h after the ischemic episode (see 54, 130). This is may be partly due to post-ischemic potentiation of Ca^{2+} influx through NMDA and AMPA channels (see 135). The beneficial actions of glutamate receptor antagonists administered after ischemia are probably also attributable to their prevention of recurrent spreading electrical depression, a process that is mediated by vesicular release of glutamate; under normal circumstances this has no long-term deleterious effects but in penumbral tissue that is of marginal viability, the further depletion of oxygen and ATP that results from spreading electrical depression may tip the balance and cause cell death (see 54, 105). The spreading depression also induces NOS 1 expression in astrocytes (11).

Whatever the other contributions of glutamate to ischemic cell death may be, there is little doubt that the elevation of intracellular Ca^{2+} which results from the initial surge in NMDA receptor activation causes the activation of Ca^{2+}-dependent nitric oxide synthases and the production of NO• (see Sources of free radicals and other oxidants in brain ischemia).

Damage to DNA and other macromolecules

Single-strand breaks and base modifications characteristic of oxidative injury to DNA can be detected within minutes of reperfusion after transient brain ischemia, and double-strand breaks within 1 hour (79, 80, 81, 12).

The major base modifications that result from the reaction of peroxynitrite with DNA are the conversion of guanine to 8-nitroguanine and the deamination of guanine to form xanthine but several other base modifications are also produced (39, 62, 132). In addition, peroxynitrite causes single strand breaks in DNA (39, 62,

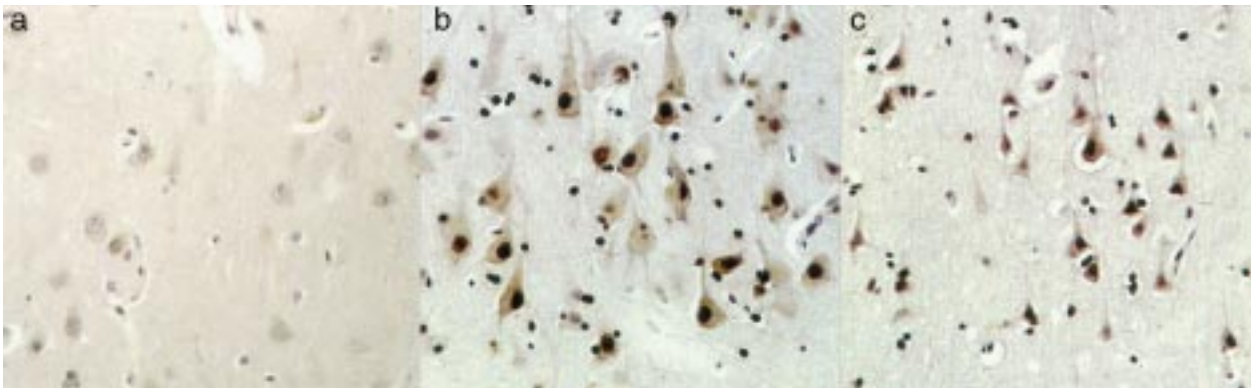


Figure 1. Upregulation of PARP after global brain ischemia in man. **a** Scant PARP immunoreactivity is present in sections of the CA1 field in a non-ischemic control brain. **b** Strong nuclear immunolabelling of CA1 neurons and glia 19h after ischemia. **c** Neurons and and glia in the temporal neocortex are strongly immunolabeled for PARP 24h after ischemia, especially, as shown here, in the deep sulcal cortex. Reproduced with permission from NeuroReport 1998; 9: 955-959, reference 86.

120, 121), as do hydroxyl radicals, superoxide and, to a lesser extent, nitric oxide (22, 38, 40, 99, 131). Nitric oxide is capable of causing other modifications to DNA including its deamination and nitration (82, 99, 144).

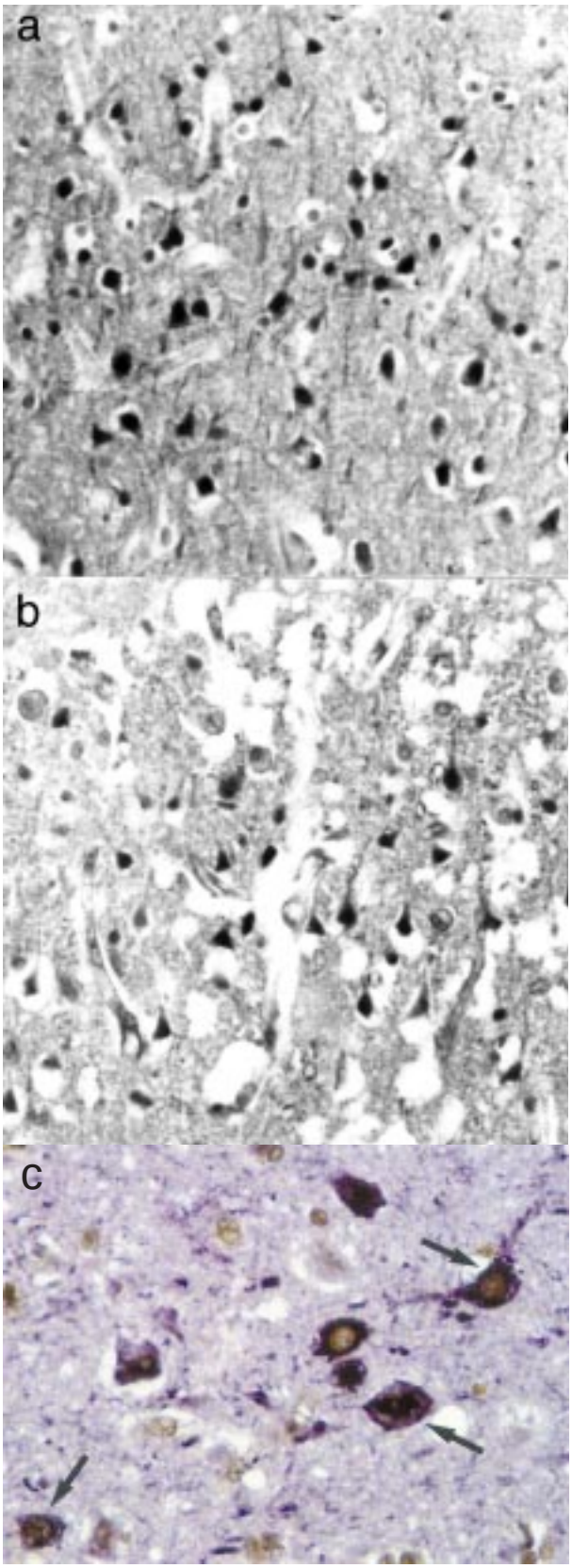
Reactive oxygen and nitrogen species have many other damaging effects on respiration and cell viability. These include disruption of electron carriers and other enzymes involved in mitochondrial respiration, binding of NO• to cytochrome oxidase raising the effective K_m for oxygen of mitochondrial respiration, lipid peroxidation and membrane damage (10, 114, see 46).

Notwithstanding the wide range of mechanisms of free radical-mediated toxicity described above, the generation of single strand breaks in DNA, particularly by peroxynitrite, is probably of key importance in compromising the viability of cells after ischemia of brain tissue (see below, and Apoptosis).

The role of PARP in ischemic damage

PARP is a zinc-finger DNA-binding protein that is activated by single- and double-strand breaks in DNA (74, 134, 143). PARP catalyses the cleavage of NAD^+ into adenosine 5'-diphosphoribose (ADP-ribose) and nicotinamide, and the covalent attachment of polymers of up to 200-300 ADP-ribose groups to nuclear proteins, including PARP itself (21, 123, 133). Nuclear proteins that become poly(ADP-ribosyl)ated include histone H1, nucleosomal core histones, DNA polymerases α and β, proliferating cell nuclear antigen, DNA ligase 2, HMG (high-mobility-group) proteins, and topoisomerases I and II. Poly(ADP-ribosyl)ation of nuclear enzymes generally causes a decrease in their catalytic activities, and inhibits transcription and replication in the presence of damaged DNA (31, 73, 106). Poly(ADP-ribosyl)ation of PARP itself inhibits PARP-mediated NADase activity and poly(ADP-ribose) chain extension (23). PARP associates with several other nuclear proteins during DNA replication, recombination and repair (73, 129). The function of the poly(ADP-ribose) groups, which are rapidly degraded, is still unclear. Several physiological roles have been ascribed to PARP, including recovery from DNA damage, maintenance of genomic stability, prevention of DNA recombination, prevention of transcription or replication of damaged DNA, protection of free ends of DNA from exonuclease action, and unraveling of chromatin structure to allow access of DNA repair enzymes (21, 64, 73, 118, 126). It has also been suggested that the principal role of PARP may be to provide a transcription-independent mechanism to prevent the survival of mutated, possibly apoptosis-incompetent, cells after acute DNA damage (95).

The consumption of NAD^+ that results when PARP is activated makes a substantial contribution to brain damage after ischemia, as shown by several studies in which PARP has either been inhibited or the gene for PARP disrupted. Takahashi *et al* (137) produced focal brain ischemia in rats by a combination of cauterization of a middle cerebral artery and 90 min occlusion of the carotid arteries and found that intraperitoneal injection of the PARP inhibitor 3,4-dihydro 5[4-(1-piperdinyl)butoxy]-1(2H)-isoquinolinone before and after the induction of ischemia reduced infarct volume by up to 53%. A similar reduction in infarct volume was noted in mice given the PARP inhibitor 3-aminobenzamide intracerebroventricularly 10 min before temporary occlusion of one middle cerebral artery (36). *PARP*-knockout mice (141) have also been used to study the contribution of PARP to ischemic brain damage. Both



Eliasson *et al* (33) and Endres *et al* (36) observed a marked decrease in the size of infarcts produced by temporary middle cerebral artery occlusion in *PARP*-knockout mice compared to that in littermates, with infarct volumes and neurological deficits being more substantially reduced in homozygous knockouts than in heterozygotes. A study by Lo *et al* (83) confirmed the beneficial action of 3-aminobenzamide in reducing ischemic brain damage (caused in this study by temporary middle cerebral artery occlusion in the rat) but also provided evidence that the deleterious effects of PARP activation in ischemia may not be due solely to energy depletion. The authors found that local perfusion of the cerebral cortex of rats with NMDA, by means of microdialysis probes, caused large elevations of glutamate and that these could be limited by prior administration of 3-aminobenzamide, suggesting that PARP activation may somehow augment glutamate release.

We have shown that PARP is upregulated within hours of global brain ischemia in man and is strongly expressed in neurons and glia in the frontal and temporal cortex for several days afterwards, particularly in regions of susceptibility to ischemic neuronal degeneration (Fig. 1) (85, 86). The activation of PARP causes intranuclear accumulation of poly(ADP-ribosyl)ated proteins, particularly during the first 2 days after global brain ischemia due to cardiac arrest (Fig. 2a,b) (87). Double immunolabeling for poly(ADP-ribose) and MAP2 showed most of the cells with early accumulation of poly(ADP-ribose) to be neurons (Fig. 2c) (87). During the first 1-2 d after ischemia, poly(ADP-ribose) accumulates in cells throughout the ischemic cortex. Thereafter poly(ADP-ribose) accumulation is largely confined to variable numbers of cells adjacent to frank infarcts or regions of ischemic cell damage. Poly(ADP-ribose) accumulation in neurons and glia may also be evident during the first 1-2 days after focal atherothrombotic infarction, predominantly around the margin of the

Figure 2. (Left) Intranuclear accumulation of poly(ADP-ribosyl)ated proteins after global brain ischemia due to cardiac arrest. **a** Intense nuclear poly(ADP-ribose) immunoreactivity in neurons in the frontal cortex, 19 h after cardiac arrest. **b** By 24 h after cardiac arrest, when the morphological changes of early ischemic injury are quite pronounced, many neurons still show nuclear accumulation of poly(ADP-ribose) immunoreactivity. **c** Neuronal accumulation of poly(ADP-ribose) in temporal cortex adjacent to a zone of infarction (not shown), 37 h after cardiac arrest. The section has been immunolabeled for both poly(ADP-ribose) (brown reaction product) and the neuronal microtubule-associated protein, MAP2 (dark purple reaction product). Several of the nuclei that contain poly(ADP-ribose) are surrounded by cytoplasm with strong MAP2 labelling (arrows). Reproduced with permission from Neuropathol Appl Neurobiol, in press, reference 87.

infarct, implying continuing consumption of NAD^+ in tissue that may be of marginal viability (Fig 3). These findings suggest that early administration of PARP inhibitors may limit brain damage due to focal arterial occlusion or transient global brain ischemia.

Apoptosis

Oxidative injury of sufficient severity to DNA and other macromolecules causes cell death that may be either necrotic or apoptotic. Multiple factors probably determine which mode of cell death prevails, amongst them: the type of cell, severity and precise nature of oxidative injury, and possibly the basal level of NAD^+ (9, 15, 28, 67, 78, 102, 104). Although the mode of cell death after brain ischemia is predominantly necrotic, apoptosis of neurons and glia has been demonstrated in numerous animal models of temporary or permanent brain ischemia (see, for example, 12, 14, 26, 79, 80).

Expression of caspase-3, an interleukin-1β-converting enzyme-like protease that plays a key role in the initiation of apoptosis (42, 101, 117), is enhanced during the first few hours after transient ischemia (13, 97, 100). Caspase-3 cleaves and inactivates PARP at the onset of apoptosis (75, 117). Inhibition of caspase-3 and consequent prevention of PARP cleavage also prevents apoptosis (101). Conversely, over-expression of caspase-3 induces apoptosis (42). Observations supporting the importance of caspase-3 in mediating post-ischemic brain damage include: (i) caspase-3 mRNA and protein expression were particularly enhanced in CA1 pyramidal neurons, that subsequently underwent apoptosis, in mouse and rat models of transient focal ischemia (13, 100), and (ii) inhibitors of interleukin-1β-converting enzyme-like proteases reduced infarct size and improved clinical outcome, even when administered several hours after ischemia, especially after ischemia that was relatively brief (13, 49, 37).

The possible role of PARP itself in apoptosis is controversial. Although over-activation of PARP results in cell necrosis not apoptosis, several researchers have reported that PARP inhibitors reduce apoptosis *in vitro* (71, 138, 148) and *in vivo* (67, 72). The apoptosis that results from oxidative injury is generally preceded by increased poly(ADP-ribosyl)ation of nuclear proteins (71, 117, 148). Yoon *et al* (148) suggested that the poly(ADP-ribosyl)ation of histone H1 protein may facilitate subsequent internucleosomal DNA fragmentation during apoptosis by increasing the susceptibility of chromatin to endonuclease. Apoptosis does, however, occur perfectly well in cells from PARP-knockout mice (77, 142).

In summary, activation of PARP and the resulting depletion of energy due to consumption of NAD^+ make a substantial contribution to necrotic cell death after global or focal brain ischemia. PARP is inactivated by caspase-3-mediated cleavage prior to apoptosis and is not needed for apoptosis to occur. Apoptosis may, however, be facilitated by the prior poly(ADP-ribosyl)ation of some nuclear proteins, including histone H1. Enhanced expression of caspase-3, with resulting apoptosis, accounts for a clinically significant proportion of neuronal death in some animal models of transient focal brain ischemia.

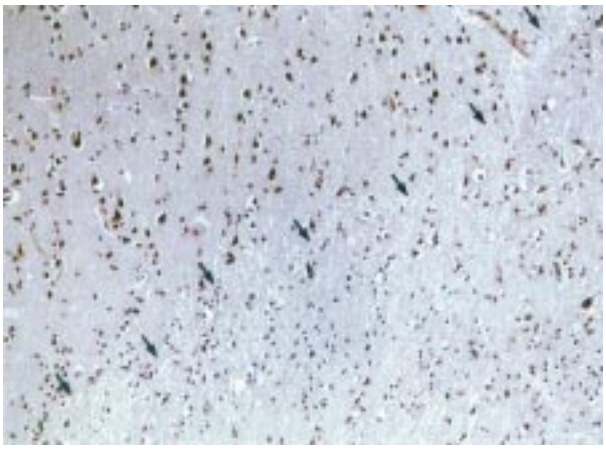


Figure 3. Poly(ADP-ribose) accumulation after focal atherothrombotic infarction. A line of early demarcation is visible (arrows) between acutely infarcted cortex (towards bottom right of figure) and adjacent, preserved cortex (towards top left). Many neurons in the preserved cortex adjacent to the infarct are strongly immunolabeled for poly(ADP-ribose).

Therapeutic approaches to preventing oxidative stress and its consequences in brain ischemia in man

Reference has already been made to the numerous studies of glutamate antagonists, non-selective and selective inhibitors of NOS, and inhibitors of PARP and of interleukin-1β-converting enzyme-like proteases (including caspase-3) in experimental brain ischemia. Several therapeutic approaches to reducing oxidative stress after brain ischemia are being tested in stroke patients. Tirilazad mesylate, a lazaroid (21-aminosteroid) that acts as a free radical scavenger, has been investigated as a neuroprotective agent in patients with subarachnoid hemorrhage (SAH). The two large trials to date have yielded conflicting findings. In a multicenter European, Australian and New Zealand trial, tirilazad reduced mortality and significantly improved functional outcome in men with SAH, although not in women (66). However, the same dose of tirilazad did not improve the outcome after SAH in patients participating in a multi-

center North American trial (47). More recently, the administration of ebselen, a seleno-organic compound with antioxidant activity, was shown to reduce delayed ischemic neurological deficits after SAH (119) and to improve outcome after stroke (147). Other antioxidants that have shown some promise in the treatment of acute myocardial infarction but have not been evaluated in stroke patients include N-acetylcysteine (given with nitroglycerin and streptokinase) (3) and a combination of selenium and coenzyme Q10 (70).

Several NMDA antagonists have been tested in stroke patients. The clinical experience with these drugs was reviewed by Muir and Lees (92) and Lees (76). Most of the NMDA antagonists are poorly tolerated when administered in doses sufficient to achieve neuroprotective drug levels. Side effects include hypertension, sedation, confusion, hallucinations and, at high doses, catatonia. Studies with selfotel and eliprodil were discontinued because of the unacceptable side effects at higher doses and lack of evidence of benefit at doses that were clinically tolerable. The ratio of benefit to side effects may be more favorable for aptiganel, trials of which are still in progress.

PARP inhibitors have not yet been tested in stroke patients but have been used as adjunctive therapy for several other diseases in which oxidative damage to DNA and over-activity of PARP have been implicated. Nicotinamide (niacinamide), in particular, has been found to be of benefit in delaying the progression of recent-onset insulin-dependent diabetes mellitus (34,44,111-113). There are also anecdotal reports of its benefit in bullous pemphigoid (53,109), lichen planus pemphigoides (43) and osteoarthritis (65).

Conclusions

There is increasing evidence that a substantial proportion of the cell death that occurs after brain ischemia results directly or indirectly from oxidative injury to DNA and other macromolecules. The injury is mediated through a complex cascade of metabolic events that involves glutamate and its transporter proteins, NMDA and probably also AMPA and KA receptors, nitric oxide synthases, several other enzymes responsible for the generation of superoxide and other reactive oxygen species, caspase-3, and PARP. The mode of cell death may be apoptotic or necrotic, although the latter probably predominates, at least in human brain ischemia. Experimental observations and preliminary data from clinical studies in stroke patients suggest that a large part of the cell death due to oxidative injury may be preventable by the early administration of antioxidants and, possibly, by use of selective NOS inhibitors and glutamate antagonists. Results from recent experimental studies indicate that the degree of neuroprotection afforded by inactivating PARP is greater than that resulting from the use of free radical-scavenging agents, NMDA antagonists, or from inhibiting NOS. This is presumably because PARP activation is a final common pathway of several processes that contribute to DNA damage and cell death after brain ischemia. The 'window of opportunity' for clinically effective use of PARP inhibitors after cardiac arrest or stroke may be wider than is the case for interventions that act 'upstream' of PARP in the cascade of events that follows an ischemic episode. PARP inhibitors are already used clinically in other contexts and data from trials in stroke patients should soon be forthcoming. The development of specific caspase-3 inhibitors is still at a relatively early stage but in time these may also prove useful, possibly in preventing delayed neuronal apoptosis after the immediate ischemic damage.

As individual steps have been elucidated in the oxidative injury cascade that follows brain ischemia, each has become a potential target for therapeutic intervention. The multiplicity of pathways and processes involved suggests that there is considerable potential for additive or synergistic benefit from combined therapies. We have certainly not reached the end of the line in this field of investigation and can expect to see the identification of several further therapeutic targets and much refinement of existing treatments.

Acknowledgement

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References

1. Amato A, Barbour B, Szatkowski M, Attwell D (1994) Counter-transport of potassium by the glutamate uptake carrier in glial cells isolated from the tiger salamander retina. *J Physiol Lond* 479: 371-380
2. Arnet UA, McMillan A, Dinerman JL, Ballermann B, Lowenstein CJ (1996) Regulation of endothelial nitric-oxide synthase during hypoxia. *J Biol Chem* 271: 15069-15073
3. Arstall MA, Yang J, Stafford I, Betts WH, Horowitz JD (1995) N-acetylcysteine in combination with nitroglycerin and streptokinase for the treatment of evolving acute myocardial infarction. Safety and biochemical effects. *Circulation* 92: 2855-2862
4. Barbour B, Brew H, Attwell D (1988) Electrogenic glutamate uptake is activated by intracellular potassium. *Nature* 335: 433-435

5. Beasley TC, Bari F, Thore C, Thrikawala N, Louis T, Busija D (1998) Cerebral ischemia/reperfusion increases endothelial nitric oxide synthase levels by an indomethacin-sensitive mechanism. *J Cereb Blood Flow Metab* 18: 88-96
6. Beckman JS, Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 271: C1424-C1437
7. Beetsch JW, Park TS, Dugan LL, Shah AR, Gidday JM (1998) Xanthine oxidase-derived superoxide causes reoxygenation injury of ischemic cerebral endothelial cells. *Brain Res* 786: 89-95
8. Bloch KD, Wolfram JR, Brown DM, Roberts JD Jr, Zapol DG, Lepore JJ, Filippov G, Thomas JE, Jacob HJ, Bloch DB (1995) Three members of the nitric oxide synthase II gene family (NOS2A, NOS2B, and NOS2C) colocalize to human chromosome 17. *Genomics* 27: 526-530
9. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci USA* 92: 7162-7166
10. Brown GC (1977) Nitric oxide inhibition of cytochrome oxidase and mitochondrial respiration: implications for inflammatory, neurodegenerative and ischaemic pathologies. *Mol Cell Biochem* 174: 189-192
11. Caggiano AO, Kraig RP (1998) Neuronal nitric oxide synthase expression is induced in neocortical astrocytes after spreading depression. *J Cereb Blood Flow Metab* 18: 75-87
12. Chen J, Jin K, Chen M, Pei W, Kawaguchi K, Greenberg DA, Simon RP (1997) Early detection of DNA strand breaks in the brain after transient focal ischemia: implications for the role of DNA damage in apoptosis and neuronal cell death. *J Neurochem* 69: 232-245
13. Chen J, Nagayama T, Jin K, Stetler RA, Zhu RL, Graham SH, Simon RP (1998) Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J Neurosci* 18: 4914-4928
14. Chopp M, Li Y (1996) Apoptosis in focal cerebral ischemia. *Acta Neurochir* 66(suppl): 21-26
15. Coppola S, Nosseri C, Maresca V, Ghibelli L (1995) Different basal NAD levels determine opposite effects of poly(ADP-ribosyl)polymerase inhibitors on H2O2-induced apoptosis. *Exp Cell Res* 221: 462-469
16. Crow JP, Beckman JS (1996) The importance of superoxide in nitric oxide-dependent toxicity: evidence for peroxynitrite-mediated injury. *Adv Exp Med Biol* 387: 147-161
17. Dalkara T, Moskowitz MA (1994) The complex role of nitric oxide in the pathophysiology of focal cerebral ischemia. *Brain Pathol* 1994; 49-57
18. Dalkara T, Moskowitz MA (1997) Neurotoxic and neuroprotective roles of nitric oxide in cerebral ischemia. Int *Rev Neurobiol* 40: 319-336
19. Dawson TM, Dawson VL (1995) Nitric oxide: actions and pathological roles. *Neuroscientist* 1: 7-18
20. Dawson TM, Snyder SH (1994) Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *J Neurosci* 14: 5147-5149
21. de Murcia JM, Niedergang C, Trucco C, Ricoul M, Dutrillaux B, Mark M, Oliver FJ, Masson M, Dierich A, LeMeur M, Walztinger C, Chambon P, de Murcia G (1997) Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. *Proc Natl Acad Sci USA* 94: 7303-7307
22. Delaney CA, Green IC, Lowe JE, Cunningham JM, Butler AR, Renton L, D'Costa I, Green MH (1997) Use of the comet assay to investigate possible interactions of nitric oxide and reactive oxygen species in the induction of DNA damage. *Mutation Res* 375: 137-146
23. Desmarais Y, Menard L, Lagueux J, Poirier GG (1991) Enzymological properties of poly(ADP-ribose)polymerase: characterization of automodification sites and NADase activity. *Biochim Biophys Acta* 1078:179-186
24. Dinerman JL, Dawson TM, Schell MJ, Snowman A, Snyder SH (1994) Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. *Proc Natl Acad Sci USA.* 91: 4214-4218
25. Doyle CA, Slater P (1997) Localization of neuronal and endothelial nitric oxide synthase isoforms in human hippocampus. *Neuroscience* 76: 387-395
26. Du C, Hu R, Csernansky CA, Hsu CY, Choi DW (1996) Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? *J Cereb Blood Flow Metab* 16: 195-201
27. Dugan LL, Sensi SL, Canzoniero LM, Handran SD, Rothman SM, Lin TS, Goldberg MP, Choi DW (1995) Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J Neurosci* 15: 6377-6388
28. Duriez PJ, Shah GM (1997) Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem Cell Biol* 75: 337-349
29. Eiserich JP, Cross CE, Jones AD, Halliwell B, van der Vliet A (1996) Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. A novel mechanism for nitric oxide-mediated protein modification. *J Biol Chem* 271: 19199-19208
30. Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A (1998) Formation of nitric oxide derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391: 393-397
31. Eki T (1994) Poly (ADP-ribose) polymerase inhibits DNA replication by human replicative DNA polymerase α, δ and ϵ in vitro. *FEBS Lett* 356:261-266
32. Eliasson MJ, Blackshaw S, Schell MJ, Snyder SH (1997) Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. *Proc Natl Acad Sci USA* 94 :3396-3401
33. Eliasson MJL, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang ZQ, Dawson TM, Snyder SH, Dawson VL (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nature Med* 3: 1089-1095

34. Elliott RB, Pilcher CC, Fergusson DM, Stewart AW (1996) A population based strategy to prevent insulin-dependent diabetes using nicotinamide. *J Pediatr Endocrinol Metab* 9: 501-509

35. Ellis JA, Cross AR, Jones OT (1989) Studies on the electron-transfer mechanism of the human neutrophil NADPH oxidase. *Biochem J* 262: 575-579

36. Endres M, Wang ZQ, Namura S, Waeber C, Moskowitz MA (1997) Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. *J Cereb Blood Flow Metab* 17: 1143-1151

37. Endres M, Namura S, Shimizu-Sasamata M, Waeber C, Zhang L, Gomez-Isla T, Hyman BT, Moskowitz MA (1998) Attenuation of delayed neuronal death after mild focal ischemia in mice by inhibition of the caspase family. *J Cereb Blood Flow Metab* 18: 238-247

38. Epe B, Ballmaier D, Adam W, Grimm GN, Saha-Moller CR (1996) Photolysis on N-hydroxpyridinethiones: a new source of hydroxyl radicals for the direct damage of cell-free and cellular DNA. *Nucleic Acids Res* 24: 1625-1631

39. Epe B, Ballmaier D, Roussyn I, Briviba K, Sies H (1996) DNA damage by peroxynitrite characterized with DNA repair enzymes. *Nucleic Acids Res* 24: 4105-4110

40. Epe B, Pflaum M, Haring M, Hegler J, Rudiger H (1993) Use of repair endonucelases to characterize DNA damage induced by reactive oxygen species in cellular and cell-free systems. *Toxicol Lett* 67: 57-72

41. Escott KJ, Beech JS, Haga KK, Williams SC, Meldrum BS, Bath PM (1998) Cerebroprotective effect of the nitric oxide synthase inhibitors, 1-(2-trifluoromethylphenyl) imidazole and 7-nitro indazole, after transient focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab* 18: 281-287

42. Fernandes-Alnemri T, Litwack G, Alnemri ES (1994) CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *J Biol Chem* 269: 30761-30764

43. Fivenson DP, Kimbrough TL (1997) Lichen planus pemphigoides: combination therapy with tetracycline and nicotinamide. *J Am Acad Dermatol* 36: 638-640.

44. Gale EA (1996) Molecular mechanisms of β-cell destruction in IDDM: the role of nicotinamide. *Hormone Res* 45 Suppl 1: 39-43

45. Garthwaite J, Boulton Cl (1995) Nitric oxide signaling in the central nervous system. *Annu Rev Physiol* 57: 683-706

46. Gross SS, Wolin MS (1995) Nitric oxide: pathophysiological mechanisms. *Annu Rev Physiol* 57: 737-769

47. Haley EC Jr, Kassell NF, Apperson-Hansen C, Maile MH, Alves WM (1997) A randomized, double-blind, vehicle-controlled trial of tirilazad mesylate in patients with aneurysmal subarachnoid hemorrhage: a cooperative study in North America. *J Neurosurg* 86 :467-474

48. Hara H, Huang PL, Panahian N, Fishman MC, Moskowitz MA (1996) Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after transient MCA occlusion. *J Cereb Blood Flow Metab* 16: 605-611

49. Hara H, Friedlander RM, Gagliardini V, Ayata C, Fink K, Huang Z, Shimizu-Sasamata M, Yuan J, Moskowitz MA (1997) Inhibition of interleukin 1β converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. *Proc Natl Acad Sci USA* 94: 2007-2012

50. Henderson LM, Chappell JB, Jones OT (1987) The superoxide-generating NADPH oxidase of human neutrophils is electrogenic and associated with an H+ channel. *Biochem J* 246: 325-329

51. Hibbs Jr JB, Vavrin Z, Taintor RR (1987) L-arginine is required for expression of the activated macrophage effector mechanism causing selective inhibition in target cells. *J Immunol* 138: 550-565

52. Hille R (1994) The conversion of xanthine dehydrogenase to xanthine oxidase and the role of the enzyme in reperfusion injury. *Biochim Biophys Acta* 1184: 143-169

53. Hornschuh B, Hamm H, Wever S, Hashimoto T, Schroder U, Brocker EB, Zillikens D (1997) Treatment of 16 patients with bullous pemphigoid with oral tetracycline and niacinamide and topical clobetasol. *J Am Acad Dermatol* 36: 101-103

54. Hossmann K-A (1994) Glutamate-mediated injury in focal cerebral ischemia: the excitotoxin hypothesis revisited. *Brain Pathol* 4: 23-36

55. Huang PL, Fishman MC (1996) Genetic analysis of nitric oxide synthase isoforms: targeted mutation in mice. *J Molec Med* 74: 415-421

56. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC (1995) Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377: 239-242

57. Huang Z, Huang PL, Ma J, Meng W, Ayata C, Fishman MC, Moskowitz MA (1996) Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J Cereb Blood Flow Metab* 16: 981-987

58. Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA (1994) Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 265: 1883-1885

59. Iadecola C (1997) Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci* 20: 132-139

60. Iadecola C, Zhang F, Casey R, Nagayama M, Ross ME (1997) Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J Neurosci* 17: 9157-9164

61. Iadecola C, Zhang F, Xu X (1995) Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. *Am J Physiol* 268: R286-R292

62. Inoue S, Kawanishi S (1995) Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide. *FEBS Lett* 371: 86-88

63. Izumi Y, Benz AM, Clifford DB, Zorumski CF (1996) Nitric oxide inhibitors attenuate ischemic degeneration in the CA1 region of rat hippocampal slices. *Neurosci Lett* 210: 157-160

64. Jeggo PA (1998) DNA repair: PARP - another guardian angel? *Curr Biol* 8:R49-R51

65. Jonas WB, Rapoza CP, Blair WF (1996) The effect of niacinamide on osteoarthritis: a pilot study. *Inflammation Res* 45: 330-334

66. Kassell NF, Haley EC Jr, Apperson-Hansen C, Alves WM (1996) Randomized, double-blind, vehicle-controlled trial of tirilazad mesylate in patients with aneurysmal subarachnoid hemorrhage: a cooperative study in Europe, Australia, and New Zealand. *J Neurosurg* 84: 221-228

67. Klaidman LK, Mukherjee SK, Hutchin TP, Adams JD (1996) Nicotinamide as a precursor for NAD^+ prevents apoptosis in the mouse brain induced by tertiary-butylhydroperoxide. *Neurosci Lett* 206: 5-8

68. Knowles RG, Moncada S (1994) Nitric oxide synthases in mammals. *Biochem J* 298: 249-258

69. Kugler P, Drenckhahn D (1996) Astrocytes and Bergmann glia as an important site of nitric oxide synthase I. *Glia* 16: 165-173

70. Kuklinski B, Weissenbacher E, Fahnrich A (1994) Coenzyme Q10 and antioxidants in acute myocardial infarction. *Mol Aspect Med*15 (Suppl): s143-s147

71. Kuo ML, Chau YP, Wang JH, Shiah SG (1996) Inhibitors of poly(ADP-ribose) polymerase block nitric oxide-induced apoptosis but not differentiation in human leukemia HL-60 cells. *Biochem Biophys Res Comm* 219: 502-508

72. Lam TT (1997) The effect of 3-aminobenzamide, an inhibitor of poly-ADP-ribose polymerase, on ischemia/reperfusion damage in rat retina. *Res Comm Molec Pathol Pharmacol* 95: 241-252

73. Lautier D, Lagueux J, Thibodeau J, Menard L, Poirier GG (1993) Molecular and biochemical features of poly (ADP-ribose) metabolism. *Mol Cell Biochem* 122: 171-193

74. Lautier D, Poirier D, Boudreau A, Alaoui JM, Castonguay A, Poirier G (1990) Stimulation of poly(ADP-ribose) synthesis by free radicals in C3H10T1/2 cells: relationship with NAD metabolism and DNA breakage. *Biochem Cell Biol* 68: 602-608

75. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371: 346-347

76. Lees KR (1997) Cerestat and other NMDA antagonists in ischemic stroke. *Neurology* 49: S66-9

77. Leist M, Single B, Kunstle G, Volbracht C, Hentze H, Nicotera P (1997) Apoptosis in the absence of poly-(ADP-ribose) polymerase. *Biochem Biophys Res Comm* 233: 518-522

78. Leist M, Volbracht C, Kuhnle S, Fava E, Ferrando-May E, Nicotera P (1997) Caspase-mediated apoptosis in neuronal excitotoxicity triggered by nitric oxide. *Molec Med* 3: 750-764

79. Li Y, Chopp M, Jiang N, Yao F, Zaloga C (1995) Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab* 15: 389-397

80. Li Y, Chopp M, Jiang N, Zhang ZG, Zaloga C (1995) Induction of DNA fragmentation after 10 to 120 minutes of focal cerebral ischemia in rats. *Stroke* 26: 1252-1257

81. Liu PK, Hsu CY, Dizdaroglu M, Floyd RA, Kow YW, Karakaya A, Rabow LE, Cui JK (1996) Damage, repair, and mutagenesis in nuclear genes after mouse forebrain ischemia-reperfusion. *J Neurosci* 16: 6795-6806

82. Liu RH, Hotchkiss JH (1995) Potential genotoxicity of chronically elevated nitric oxide: a review. *Mutation Res* 339: 73-89

83. Lo EH, Bosque-Hamilton P, Meng W (1998) Inhibition of poly(ADP-ribose) polymerase: reduction of ischemic injury and attenuation of N-methyl-D-aspartate-induced neurotransmitter dysregulation. *Stroke* 29: 830-836

84. Lo EH, Hara H, Rogowska J, Trocha M, Pierce AR, Huang PL, Fishman MC, Wolf GL, Moskowitz MA (1996) Temporal correlation mapping analysis of the hemodynamic penumbra in mutant mice deficient in endothelial nitric oxide synthase gene expression. *Stroke* 27: 1381-1385

85. Love S, Barber R, Wilcock GK (1998) DNA fragmentation and repair in ischaemic brain injury in man. *Neuropathol Appl Neurobiol* 24: 134-135

86. Love S, Barber R, Wilcock GK (1998) Apoptosis and expression of DNA repair proteins in ischaemic brain injury in man. *NeuroReport* 9: 955-959

87. Love S, Barber R, Wilcock GK (In press) Neuronal accumulation of poly(ADP-ribose) after brain ischaemia. *Neuropathol Appl Neurobiol.*

88. Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Lamas S, Michel T (1992) Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett.* 307: 287-293

89. Merrill JE, Murphy SP, Mitrovic B, Mackenzie-Graham A, Dopp JC, Ding M, Griscavage J, Ignarro LJ, Lowenstein CJ (1997) Inducible nitric oxide synthase and nitric oxide production by oligodendrocytes. *J Neurosci Res* 48: 372-384

90. Moncada S, Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43: 109-142

91. Morley P, Hogan MJ, Hakim AM (1994) Calcium-mediated mechanisms of ischemic injury and protection. *Brain Pathol* 4: 37-47

92. Muir KW, Lees KR (1995) Clinical experience with excitatory amino acid antagonist drugs. *Stroke* 26: 503-513

93. Murphy SN, Miller RJ (1988) A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. *Proc Natl Acad Sci USA* 85: 8737-8741

94. Nagafuji T, Sugiyama M, Muto A, Makino T, Miyauchi T, Nabata H (1995) The neuroprotective effect of a potent and selective inhibitor of type I NOS (L-MIN) in a rat model of focal cerebral ischaemia. *Neuroreport* 6: 1541-1545

95. Nagele A (1995) Poly(ADP-ribosyl)ation as a fail-safe, transcription-independent, suicide mechanism in acutely DNA-damaged cells: a hypothesis. *Radiat Environ Biophys* 34:251-254

96. Nakanishi S. (1992). Molecular diversity of glutamate receptors and implications for brain function. *Science* 258: 597-603

97. Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J, Moskowitz MA (1998) Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J Neurosci* 18: 3659-3668

98. Nathan C, Xie Q (1994) Regulation of biosynthesis of nitric oxide. *J Biol Chem* 269: 13725-13728

99. Nguyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR (1992) DNA damage and mutation in human cells exposed to nitric oxide in vitro. *Proc Natl Acad Sci USA* 89: 3030-3034

100. Ni B, Wu X, Su Y, Stephenson D, Smalstig EB Clemens J, Paul SM (1998) Transient global forebrain ischemia induces a prolonged expression of the caspase-3 mRNA in rat hippocampal CA1 pyramidal neurons. *J Cereb Blood Flow Metab* 18: 248-256

101. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin T-T, Yu VL, Miller DK (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376: 37-43

102. Nicotera P, Ankarcrona M, Bonfoco E, Orrenius S, Lipton SA (1997) Neuronal necrosis and apoptosis: two distinct events induced by exposure to glutamate or oxidative stress. *Adv Neurol* 72: 95-101

103. Nishino T, Tamura I (1991) The mechanism of conversion of xanthine dehydrogenase to oxidase and the role of the enzyme in reperfusion injury. *Adv Exp Med Biol* 309A: 327-333

104. Nosseri C, Coppola S, Ghibelli L (1994) Possible involvement of poly(ADP-ribosyl) polymerase in triggering stress-induced apoptosis. *Exp Cell Res* 212: 367-373

105. Obrenovitch TP, Richards DA (1995) Extracellular neurotransmitter changes in cerebral ischaemia. *Cerebrovasc Brain Metab Rev* 7: 1-54

106. Oei SL, Griesenbeck J, Ziegler M, Schweiger M. A novel function of poly(ADP-ribosyl)ation: silencing of RNA polymerase II-dependent transcription (1998) *Biochemistry* 37: 1465-1469

107. Palmer RMJ, Ferrige AG, Moncada S (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524-526

108. Piantadosi CA, Zhang J, Forman HJ (1996) Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. *Stroke* 27: 327-332

109. Poskitt L, Wojnarowska F (1995) Treatment of cicatricial pemphigoid with tetracycline and nicotinamide. *Clin Exp Dermatol* 20: 258-259

110. Pourcyrous M, Leffler CW, Bada HS, Korones SB, Busija, DW (1993) Brain superoxide anion generation in asphyxiated piglets and the effect of indomethacin at therapeutic dose. *Pediatr Res* 34: 366-369

111. Pozzilli P, Browne PD, Kolb H (1996) Meta-analysis of nicotinamide treatment in patients with recent-onset IDDM. The Nicotinamide Trialists. *Diabetes Care* 19: 1357-1363

112. Pozzilli P, Visalli N, Cavallo MG, Signore A, Baroni MG, Buzzetti R, Fioriti E, Mesturino C, Fiori R, Romiti A, Giovannini C, Lucentini L, Matteoli MC, Crino A, Teodonio C, Paci F, Amoretti R, Pisano L, Suraci MT, Multari G, Suppa M, Sulli N, De Mattia G, Faldetta MR, Ghirlanda G, et al (1997) Vitamin E and nicotinamide have similar effects in maintaining residual β-cell function in recent onset insulin-dependent diabetes (the IMDIAB IV study). *Eur J Endocrinol* 137: 234-239

113. Pozzilli P, Visalli N, Signore A, Baroni MG, Buzzetti R, Cavallo MG, Boccuni ML, Fava D, Gragnoli C, Andreani D, et al (1995) Double blind trial of nicotinamide in recent-onset IDDM (the IMDIAB III study). *Diabetologia* 38: 848-852

114. Radi R, Rodriguez M, Castro L, Telleri R (1994) Inhibition of mitochondrial electron transport by peroxynitrite. *Arch Biochem Biophys* 308: 89-95

115. Radomsky MW, Palmer RMJ, Moncada S (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci USA* 87: 5193-5197

116. Robinson LJ, Weremowicz S, Morton CC, Michel T (1994) Isolation and chromosomal localization of the human endothelial nitric oxide synthase (NOS3) gene. *Genomics* 19: 350-357

117. Rosenthal DS, Ding R, Simbulan-Rosenthal CM, Vaillancourt JP, Nicholson DW, Smulson M (1997) Intact cell evidence for the early synthesis, and subsequent late apopain-mediated suppression, of poly(ADP-ribose) during apoptosis. *Exp Cell Res* 232: 313-321

118. Rudat V, Kupper JH, Weber KJ (1998) Trans-dominant inhibition of poly(ADP-ribosyl)ation leads to decreased recovery from ionizing radiation-induced cell killing. *Int J Radiat Biol* 73: 325-330

119. Saito I, Asano T, Sano K, Takakura K, Abe H, Yoshimoto T, Kikuchi H, Ohta T, Ishibashi S (1998) Neuroprotective effect of an antioxidant, ebselen, in patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage. *Neurosurgery* 42: 269-277

120. Salgo MG, Bermudez E, Squadrito G, Pryor W (1995a) DNA damage and oxidation of thiols peroxynitrite causes in rat thymocytes. *Arch Biochem Biophys* 322: 500-505

121. Salgo MG, Stone K, Squadrito GL, Battista JR, Pryor WA (1995b) Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochem Biophys Res Comm* 210: 1025-1030

122. Samdani AF, Dawson TM, Dawson VL (1997) Nitric oxide synthase in models of focal ischemia. *Stroke* 28: 1283-1288

123. Satoh MS, Lindahl T (1992) Role of poly(ADP-ribose) formation in DNA repair. *Nature* 356: 356-358

124. Schmidt HHHW, Walter U (1994) NO at work. *Cell* 78: 919-925

125. Schoepp DD, Conn PJ (1993). Metabotropic glutamate receptors in brain function and pathology. *Trend Pharmacol Sci* 14: 13-20

126. Shall S (1995) ADP-ribosylation reactions. *Biochimie* 77: 313-318

127. Silver IA, Erecinska M (1990) Intracellular and extracellular changes of [Ca^{2+}] in hypoxia and ischemia in rat brain in vivo. *J Gen Physiol* 95: 837-866

128. Silver IA, Erecinska M (1992) Ion homeostasis in rat brain *in vivo*: intra- and extracellular [Ca^{2+}] and [H^{+}] in the hippocampus during recovery from short-term, transient ischemia. *J Cereb Blood Flow Metab* 12: 759-772

129. Simbulan-Rosenthal CM, Rosenthal DS, Hilz H, Hickey R, Malkas L, Applegren N, Wu Y, Bers G, Smulson ME (1996) The expression of poly(ADP-ribose) polymerase during differentiation-linked DNA replication reveals that it is a component of the multiprotein DNA replication complex. *Biochemistry* 35: 11622-11633

130. Sims NR, Zaidan E (1995) Biochemical changes associated with selective neuronal death following short-term cerebral ischaemia. *Int J Biochem Cell Biol* 27: 531-550

131. Spencer JP, Jenner A, Chimel K, Aruoma OI, Cross CE, Wu R, Halliwell B (1995) DNA strand breakage and base modification induced by hydrogen peroxide treatment of human respiratory tract epithelial cells. *FEBS Lett* 374: 233-236

132. Spencer JP, Wong J, Jenner A, Aruoma OI, Cross CE, Halliwell B (1996) Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin epidermal keratinocytes exposed to peroxynitrite or 3-morpholinosydnonimine. *Chem Res Toxicol* 9: 1152-1158

133. Szabó C, Ohshima H (1997) DNA damage induced by peroxynitrite: subsequent biological effects. *Nitric Oxide* 1: 373-385

134. Szabó C, Zingarelli B, O'Connor M, Salzman AL (1996) DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. *Proc Natl Acad Sci USA* 93: 1753-1758

135. Szatkowski M, Attwell D (1994) Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. *Trend Neurosci* 17: 359-365

136. Takahashi M, Billups B, Rossi D, Sarantis M, Hamann M, Attwell D (1997) The role of glutamate transporters in glutamate homeostasis in the brain. *J Exp Biol* 200: 401-409

137. Takahashi K, Greenberg JH, Jackson P, Maclin K, Zhang J (1997) Neuroprotective effects of inhibiting poly(ADP-ribose) synthetase on focal cerebral ischemia in rats. *J Cereb Blood Flow Metab* 17: 1137-1142

138. Tanaka Y, Yoshihara K, Tohno Y, Kojima K, Kameoka M, Kamiya T (1995) Inhibition and down-regulation of poly(ADP-ribose) polymerase results in a marked resistance of HL-60 cells to various apoptosis-inducers. *Cell Molec Biol* 41: 771-781

139. van der Vliet A, Eiserich JP, Halliwell B, Cross CE (1997) Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J Biol Chem* 272: 7617-7625

140. Walder CE, Green SP, Darbonne WC, Mathias J, Rae J, Dinauer MC, Curnutte JT, Thomas GR, Wolin MS (1997) Ischemic stroke injury is reduced in mice lacking a functional NADPH oxidase. *Stroke* 28: 2252-2258

141. Wang ZQ, Auer B, Stingl L, Berghammer H, Haidacher D, Schweiger M, Wagner EF (1995) Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. *Genes Devel* 9: 509-520

142. Wang ZQ, Stingl L, Morrison C, Jantsch M, Los M, Schulze-Osthoff K, Wagner EF (1997) PARP is important for genomic stability but dispensable in apoptosis. *Genes Dev* 11: 2347-2358

143. Weinfeld M, Chaudhry MA, D'Amours D, Pelletier JD, Poirier GG, Povirk LF, Lees-Miller SP (1997) Interaction of DNA-dependent protein kinase and poly(ADP-ribose) polymerase with radiation-induced DNA strand breaks. *Radiat Res* 148: 22-28

144. Wink DA, Kasprzak KS, Maragos CM, Elespuru RK, Misra M, Dunams TM, Cebula TA, Kock WH, Andrews AW, Allen JS, Keefer LK (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science* 254: 1001-1003

145. Wisden W, Seeburg P. (1993). Mammalian ionotropic glutamate receptors. *Current Opinion Neurobiol* 3: 291-298

146. Xu W, Gorman P, Sheer D, Bates G, Kishimoto J, Lizhi L, Emson P (1993) Regional localization of the gene coding for human brain nitric oxide synthase (NOS1) to 12q24.2-24.31 by fluorescent in situ hybridization. *Cytogenet Cell Genet* 64: 62-63

147. Yamaguchi T, Sano K, Takakura K, Saito I, Shinohara Y, Asano T, Yasuhara H (1998) Ebselen in acute ischemic stroke: a placebo-controlled, double-blind clinical trial. Ebselen Study Group. *Stroke* 29: 12-7

148. Yoon YS, Kim JW, Kang KW, Kim YS, Choi KH, Joe CO (1996) Poly(ADP-ribosyl)ation of histone H1 correlates with internucleosomal DNA fragmentation during apoptosis. *J Biol Chem* 271: 9129-9134

149. Yoshida T, Limmroth V, Irikura K, Moskowitz MA (1994) The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. *J Cereb Blood Flow Metab* 14: 924-929

150. Zaharchuk G, Hara H, Huang PL, Fishman MC, Moskowitz MA, Jenkins BG, Rosen BR (1997) Neuronal nitric oxide synthase mutant mice show smaller infarcts and attenuated apparent diffusion coefficient changes in the peri-infarct zone during focal cerebral ischemia. *Magnetic Resonance Med* 37: 170-175

151. Zhang F, Casey RM, Ross ME, Iadecola C (1996) Aminoguanidine ameliorates and L-arginine worsens brain damage from intraluminal middle cerebral artery occlusion. *Stroke* 27: 317-323

152. Zhang ZG, Chopp M, Gautam S, Zaloga C, Chang RL, Schmidt HHHHW, Pollock JS, Förstermann U (1994) Upregulation of neuronal nitric oxide synthase and mRNA, and selective sparing of nitric oxide synthase-containing neurones after focal cerebral ischemia in rat. *Brain Res* 654: 85-95

153. Zhang ZG, Chopp M, Zaloga C, Pollock JS, Forstermann U (1993) Cerebral endothelial nitric oxide synthase expression after focal cerebral ischemia in rats. *Stroke* 24: 2016-2021