Nitric oxide, ischaemia and brain inflammation

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Abstract
Cerebral ischaemia results in the activation of three isoforms of NOS (nitric oxide synthase) that contribute to the development of and recovery from stroke pathology. This review discusses, in particular, the role of the transcriptionally activated NOS-2 (inducible NOS) isoform and summarizes the outcomes of experimental stroke studies with regard to the therapeutic utility of nitric oxide donors and NOS inhibitors.

Introduction
In an aging population, one in four of us may expect to experience a stroke by the age of 85. At this time, nearly 300 clinical trials concerns with acute ischaemic stroke have been registered (http://www.strokecenter.org/trials/) and none of the completed trials show evidence of patient benefit, apart from modest (but clinically relevant) results with tPA (tissue plasminogen activator). Stroke causes excitotoxicity, inflammation, cell death and compensatory neurogenesis. Resident and infiltrating cells generate NO (nitric oxide) as a result of the activation of NOSs (nitric oxide synthases), and NO and the products of its further oxidation are generally implicated in stroke pathology. In this review, we focus on the NOS-2 isoform and examine experimental stroke studies for evidence suggesting that manipulation of NO production could have therapeutic benefit.

NO and the NOS isoforms
Through a reaction with molecular oxygen, NO can form an intermediate species \( \text{NO}_x \) that efficiently nitrosates thiol and amine, and NO out-competes the superoxide dismutase reaction with \( \text{O}_2^- \) (superoxide) to produce \( \text{ONOO}^- \) (peroxynitrite). The latter reacts rapidly with \( \text{CO}_2 \) to produce short-lived reaction intermediates that are probably responsible for many of the reported cytotoxic effects of NO. NO can potentially damage DNA, and also nitrosate and nitrate key amino acid residues in transcriptional regulators, enzymes and receptors (for reviews, see [1,2]). In so doing, NO can turn off constitutively expressed genes, activate transcriptionally regulated genes or prevent their activation. Critical signalling proteins can be influenced by reactive nitrogen species functioning at the transcriptional and/or post-transcriptional level, either as activators or as inhibitors. The mitochondrial respiratory chain is also susceptible: NO inhibits cytochrome c oxidase (complex IV) in a reaction that is reversible and competitive with oxygen, and \( \text{ONOO}^- \) irreversibly inhibits respiratory complexes I–III.

NO can have diverse effects on cell fate, initiating or protecting against apoptosis depending on the cell type, NO concentration and redox environment. The caspases are also targets for NO, which inhibits their activity in a reversible manner.

There are three isoforms of the classical mammalian NOS, namely NOS-1 (nNOS, neuronal NOS), NOS-2 (iNOS, inducible NOS) and NOS-3 (eNOS, endothelial NOS), characterized by N-terminal oxygenase and C-terminal reductase domains, which generate NO via the combination of L-arginine and molecular oxygen [2,3]. The three isoforms are products of different single-copy genes and share 50% amino acid homology. Common features include binding domains for the substrates arginine and NADPH and also for cofactors. There is an N-terminal PDZ domain in NOS-1, a C-terminal PDZ-binding ligand motif in NOS-2, and myristoylation and palmitoylation sites on NOS-3, all of which are involved in protein targeting.

The NOS-1 isoform displays multiple promoter sites, RNA splice variants and alternative translation products. The major transcript (NOS-1α) generates a protein with a single interactive PDZ domain in the N-terminal region. In neurons, this targets the enzyme to the synaptic assembly. In endothelium, the NOS-3 protein is targeted to caveolae through dual acylation by myristate and palmitate. Caveolin inhibits NOS-3 activity, which is reversed by increases in cell \( \text{Ca}^{2+} \) [4]. Unlike NOS-2, both NOS-1 and NOS-3 are constitutively expressed enzymes but it is clear that their expression can be dynamically controlled during development and in response to injury. The promoter regions of the genes display binding sites for a wide variety of transcription factors, such as activator protein 2, acute-phase reactants, NF-1 (nuclear factor-1) and NF-κB (nuclear factor-κB). The many routes that lead to the transcriptional activation of NOS-2 have been clearly described in vitro [5], but almost nothing is known in vivo.

Ischaemia and NOS expression
Experimental cerebral ischaemia leads to the up-regulation of all three isoforms of NOS, although their patterns of expression differ both temporally and spatially post-injury. After transient ischaemia in rats, the number of NOS-1 immunoreactive neurons in the cortex increased markedly as early as 15 min, and expression persisted for 24 h [6]. During
permanent ischaemia, NOS-3 expression in cerebral vessels of the ischaemic core increased to reach a peak at 24 h.

The expression of NOS-2 is induced in both resident and infiltrating cells in response to experimental cerebral ischaemia [7] and also in human stroke [8]. This induction occurs later than NOS-1 or NOS-3, suggesting that NOS-2 does not contribute to early injury. Indeed, following permanent ischaemia, mice lacking a functional NOS-2 gene show no alterations in infarct volume compared with wild-type mice [9,10]. However, at later times, NOS-2-deficient male mice show a significant reduction in infarct volume (Figure 1) and attendant behavioural changes (Figure 2). Furthermore, the infusion of arginine at later times following ischaemia increases injury in wild-type but not in NOS-2-deficient mice [11]. The activity of NOS-2 following transient ischaemia increases progressively over time, with maximal levels after 24 h in the striatum and 48 h in the cortex [12,13]. The induction of NOS-2 mRNA expression is clearly related to the duration of ischaemia. For example, infarcts of increasing size can be produced in mice by lengthening the duration of MCAO (middle cerebral artery occlusion). We have found that the level of NOS-2 mRNA expression surpasses that found in shams only after 60 min of ischaemia and that the expression of NOS-2 protein following transient ischaemia is restricted to infiltrating cells and/or microglia and macrophages [14].

There are significant increases in IL-1β (interleukin-1β) and TNFα (tumour necrosis factor α) mRNA expression within a few hours of ischaemia [15]. On the basis of in vitro observations, it is assumed that these cytokines trigger transcriptional activation of the NOS-2 gene, and direct injection of IL-1β into the cerebral ventricles in the absence of injury does indeed up-regulate NOS-2 expression along the injection tract [16]. The NOS-2 promoter contains a hypoxia response element, and Matrone et al. [17] provide compelling evidence that HIF-1α (hypoxia-inducible factor-1α) can also activate the gene following ischaemia. However, transcriptional activation following ischaemia may not account for the very rapid appearance of NOS-2-positive cells infiltrating the infarct, and one possibility is that these cells express NOS-2 mRNA constitutively. Lindemann

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**Figure 1** | Anatomic outcomes in NOS-2+/+ and NOS-2−/− mice following permanent focal ischaemia

Brain slices stained with 2,3,5-triphenyltetrazolium chloride reveal the location of the infarct 48 h after MCAO in striatal and cortical areas. Measurements of lesion volume indicate a significant reduction (two-way ANOVA) in total lesion volume in NOS-2−/− compared with wild-type mice (n=8). Post hoc analysis (*) reveals a significant reduction in cortical lesion volume. Scale bar, 1 mm.

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**Figure 2** | Functional outcomes in NOS-2+/+ and NOS-2−/− mice following transient focal ischaemia

(A) Motor performance (Rotarod) is deficient following cerebral ischaemia in NOS-2+/+ mice. However, NOS-2−/− mice are less impaired than NOS-2+/+ and are able to remain on the rotarod for a significantly longer period (*; n=7). (B) Unilateral deficits (contralateral footfaults on the grid test) are the same in NOS-2−/− and NOS-2+/+ mice (*; n=7).
et al. [18] found that constitutive mRNAs in neutrophils are translated rapidly in response to external signalling molecules, involving an intracellular kinase [mTOR (mammalian target of rapamycin)] that regulates initiation of translation of a subset of mRNAs. The NOS-2 transcript may well be one and, if so, it will be important to identify the chemical triggers that activate extravasating cells. However, it is not at all clear whether neutrophils contribute to cerebral infarction. In their review, Emerich et al. [19] report the lack of evidence for neutrophil invasion prior to terminal stage infarction, as well as the lack of correlation between numbers of invading neutrophils and subsequent pathology. Beray-Berthat et al. [20] induced neutropenia, which reduced infiltration by >60%, but had no effect on infarct volume. Interestingly, NOS-2 activity was also unaffected, suggesting that macrophages/microglia and astrocytes are the cells most likely to be responsible for enzyme expression.

**Inflammation and extravasation**

The glial expression of NOS-2* in vitro* has been described in response to various stimuli, including pro-inflammatory cytokines, the products of infectious agents, various signalling molecules (glutamate and purines) and amyloid. Suppressors of NOS-2 up-regulation include anti-inflammatory cytokines, angiotensin II, noradrenaline (norepinephrine) and dopamine [21], HSP70 (heat-shock protein 70) and NO itself [22].

The production of pro-inflammatory cytokines and chemokines following ischaemia up-regulates adhesion molecules on the endothelium and directs infiltrating cells to the infarcted area. NO is known to down-regulate the expression of adhesion molecules, and it might be predicted that one function of the later production of NO from NOS-2 would be to terminate extravasation. However, NO from NOS-2 does not appear to alter neutrophil recruitment *in vivo* [23]. Observations in NOS-1- and NOS-3-knockout mice suggest that there is enhanced leucocyte adhesion to vascular endothelium [24], indicating that these two isoforms may be more important than NOS-2. Once cells such as neutrophils are in the infarct, NO could modulate the catalytic activity of MPO (myeloperoxidase), an enzyme that generates cytotoxic oxidants and diffusible radicals, because low levels of NO stimulate whereas high levels block MPO activity [25]. However, as indicated earlier, it is not at all clear whether neutrophils contribute to ischaemic damage [19,20]. The NO generated could also protect susceptible cells by inhibiting caspase activity [26], although there is recent evidence that neurons may then be driven towards a caspase-independent form of cell death [27]. NO, through the process of preconditioning, can protect neurons *in vitro* [28], an effect that is absent from NOS-2*−/−* mice [29].

**Inhibition of NOS and ischaemic outcome**

Gene knockout studies suggest that the NO from NOS-1 and NOS-2 is detrimental, and that NO derived from NOS-3 is beneficial [30]. However, studies of various NOS inhibitors have given conflicting results for effects on lesion size and cerebral blood flow. The early NOS inhibitors (guanidino amino acids) competed with substrate at the active site but none discriminated sufficiently to enable them to target specific NOS isoforms. In contrast, some inhibitors possess higher affinity against one isoform and are commonly referred to as selective, although this term is used indiscriminately [2]. We examined, systematically, the effects of NOS inhibitors on infarct size in animal studies [31]. The application of NOS inhibitors appears to cause a significant reduction in total, cortical and subcortical infarct volume. Treatment before stroke onset was effective at reducing infarct volume in transient models, whereas early administration of NOS inhibitors (<1 h of onset) was effective in permanent stroke. Later treatment (>1 h of onset) had a beneficial effect on infarct volume. Non-selective inhibitors did not alter infarct volume in permanent ischaemia, whereas ‘selective’ NOS-1 and NOS-2 inhibitors reduced lesion size regardless of experimental model. It is likely that the beneficial effects of non-selective inhibitors were limited because they inhibit NOS-3 to a similar degree. Consequently, they may aggravate brain ischaemia by increasing platelet aggregation and leucocyte activity, raising blood pressure and by restricting penumbral blood supply. Evidence of reduced cerebral blood flow after administration of non-selective inhibitors to permanent stroke models is consistent with this hypothesis. Hence, non-selective inhibitors are not agents of first choice for clinical stroke.

**NO donors and ischaemic outcome**

We have also examined the effects of NO donors on infarct size in experimental stroke [32]. Sources of NO available for experimental study originate either from the substrate l-arginine or from pharmacological donors. Exogenously applied l-arginine appears to increase levels of NO partly by the NOS pathway, but also via the release of other vasoactive and arginase enzymes [33]. However, experimental results have provided conflicting evidence. Similar conflicting results arise when investigating the effect of l-arginine on cerebral blood flow, which, if enhanced, may rescue salvageable tissue. Thus l-arginine would not be recommended for clinical trial following human stroke. This conflicting evidence from l-arginine may be due to the ability to enhance NO via detrimental routes as well as via beneficial routes, as it potentially enhances NO via all three isoforms of NOS. Interestingly, patients with acute ischaemic stroke have lower levels of l-arginine in plasma and CSF (cerebrospinal fluid), and low concentrations appear to associate with greater cerebral damage [34].

Following experimental ischaemia, organic nitrates, sodium nitroprusside, sydnonimines, S-nitrosothiols, NONOates (diazoniumdiolates) and also hybrid NO donors have been investigated. Many have shown benefit, albeit within a relatively short therapeutic time window. Although NO donors have shown promising results following experimental ischaemia (nitroaspirin; [35]), their advancement into
clinical practice has not yet occurred. Further experimental studies are required in order to fully determine therapeutic potential, as most studies to date have concentrated on lesion volume and/or cerebral blood flow. A Phase III clinical trial [ENOS (Efficacy of Nitric Oxide in Stroke); 5000 patients, 100 centres worldwide] is under way to consider the benefits (at 3 months) of reducing hypertension through application of transdermal glyceryl trinitrate (5 mg/day) for 7 days following stroke (http://www.enos.ac.uk).

NO, neuroprotection and neurogenesis

A brief period of exposure to subtoxic injury induces protection against future insult. Hypoxic preconditioning in the newborn seems to involve NOS-3 [36]. Atochin et al. [37] induced transient MCAO and then permanently occluded the artery. Wild-type mice showed a 20% reduction in subsequent lesion volume, but NOS-1 and NOS-3 knockouts were not protected. In vitro studies (reviewed in [38]) reveal that NOS inhibitors can reverse preconditioning and NO donors can reproduce it. There is in vitro evidence to suggest that NO can precondition neurons to withstand the effects of oxygen–glucose deprivation [28]. In the heart, it is now well known that the late phase of ischaemic preconditioning is associated with the up-regulation of NOS-2, and knockout mice fail to show the infarct sparing effect of preconditioning [39].

The infarct volume in female mice after ischaemia is reported to be smaller than in males [40,41] and this may be due to steroidal regulation of NOS-2 expression. Park et al. [41] found that ovariectomy increased NOS-2 expression, which could be blocked by oestrogen replacement, and the oestradial effect is absent from knockout mice [42]. In addition, progesterone administration reduces lesion volume in male and female rodents following ischaemia and improves functional outcome as assessed by using neurological scoring, motor and cognitive tests [43]. Furthermore, the increase in NOS-2 mRNA expression following transient and permanent ischaemia is suppressed in mice treated with progesterone and also reduces aspects of the inflammatory response to injury [44].

There is an increase in the rate of neurogenesis following ischaemic brain injury (reviewed in [45]). In some cases, it is clear that these new neurons differentiate appropriately following ischaemia. The efficiency of the neurogenic response following stroke is limited, presumably because the concomitant inflammation is highly detrimental to the process of neurogenesis [46]. While NO has been reported to be cytostatic, or to promote terminal differentiation of neural stem cells in the uninjured brain, it appears to be anti-apoptotic in the ischaemic brain. Zhang et al. [47] have shown that administration of an NO donor increased neurogenesis after MCAO in rats, and recipients exhibited significant improvements in neurological outcome. Furthermore, administration of NO donors in combination with marrow stromal cells significantly enhanced angiogenesis and neurogenesis following cerebral ischaemia compared with either treatment alone, and significantly improved functional outcome. Interestingly, Zhu et al. [48] have observed that NOS-2−/− mice do not display the predicted neurogenic response in the dentate gyrus following ischaemia.

In conclusion, NOS activity clearly contributes to pathology following ischaemia and yet NO can also be beneficial. This apparent contradiction can be explained by the cell type responsible, the amount produced and whether the NO undergoes further oxidation. Important questions concerning the roles of NO following cerebral ischaemia remain, including the identity of the transcription-activating signals for NOS-2 expression in resident (microglia and astrocytes) and infiltrating cells (neutrophils and macrophages), and the effects of NO on the neurogenic response.

References


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