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Inducible nitric oxide synthase expression in human cerebral infarcts

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Abstract The inducible or "immunological" isoform of nitric oxide synthase (iNOS) is induced in many cell types by inflammatory stimuli and synthesizes toxic amounts of NO. In rodent models of focal cerebral ischemia, iNOS is expressed in neutrophils invading the injured brain and in local blood vessels. Studies with iNOS inhibitors and iNOS null mice indicate that NO produced by iNOS contributes to ischemic brain injury. In the present study, we sought to determine whether iNOS is also expressed in the human brain after ischemic stroke. Studies were conducted using immunohistochemistry on autopsy brains with neuropathological evidence of acute cerebral infarction. iNOS immunoreactivity was observed in neutrophils infiltrating the ischemic brain and in blood vessels within the ischemic territory. iNOS-positive cells also were immunoreactive for nitrotyrosine, reflecting protein nitration by NO-derived peroxynitrite and nitrites. iNOS or nitrotyrosine immunoreactivity was not detected outside the region of the infarct. These observations provide evidence that iNOS is expressed in the human brain after ischemic infarction and support the hypothesis that iNOS inhibitors may be useful in the treatment of ischemic stroke in humans.

Key words Cerebral ischemia · Inflammation · Immunohistochemistry · Nitrotyrosine

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Introduction

Cerebral ischemia is associated with an inflammatory reaction involving the injured brain. A few hours after induction of ischemia, intravascular leukocytes adhere to the cerebral endothelium and infiltrate that ischemic brain [18, 34]. At later times, microglial cells become activated and astrocytes become reactive [17, 19]. Experimental evidence suggests that this inflammatory reaction is deleterious to the ischemic brain [15]. Thus, if the neutrophilic infiltration is prevented, for example, by antibodies against adhesion molecules or by depletion of neutrophils prior to induction of ischemia, ischemic damage is reduced [8, 9]. Therefore, post-ischemic inflammation is thought to contribute to the evolution of the tissue damage that occurs after cerebral ischemia [15].

Recent evidence suggests that expression of inducible nitric oxide synthase (iNOS) in neutrophils is one of the mechanisms by which post-ischemic inflammation exerts its deleterious effects. iNOS is transcriptionally induced in many cell types during inflammation and generates toxic levels of NO continuously [33]. NO produced by iNOS is a major mechanism of cytotoxicity in models of inflammation [30]. In rats, iNOS is expressed in infiltrating neutrophils and cerebral blood vessels 6–96 h after occlusion of the middle cerebral artery (MCA) [25, 26]. Inhibition of iNOS by the relatively selective inhibitor aminoguanidine, administered starting 6–24 h after ischemia, reduces the ensuing tissue damage and neurological deficits [24, 26, 32]. In addition, mice with a null mutation of iNOS do not express the enzyme following MCA occlusion and have smaller infarcts and a better neurological outcome than wild-type controls [27]. These data indicate that iNOS expression is deleterious to the post-ischemic brain, and raise the possibility that iNOS inhibitors could be used in the treatment of cerebral ischemia in patients.

It is unknown, however, whether iNOS is also expressed in the human brain following cerebral ischemia. Therefore, in this study we used immunohistochemical techniques to study iNOS expression in human cerebral infarction. We found that iNOS immunoreactivity is present in neutrophils and cerebral blood vessels in the infarcted brain. In addition, nitrotyrosine immunoreactivity, reflecting protein nitration by NO-derived peroxynitrite and nitrites, is also found in iNOS-positive neutrophils. These data provide evidence of iNOS expression and NO production in the human brain following acute infarction and strengthen the rationale for using iNOS inhibitors in the treatment of human stroke.

Materials and methods

Patients

Brains from four patients with a pathological diagnosis of acute cerebral infarcts were studied. A brief description of the patients studied is presented in Table 1. All infarcts were secondary to thrombosis or embolism. In all cases, the infarct was in the territory of the MCA. In one case, there also were bilateral thalamic infarcts.

Immunocytochemistry

All brain specimens were fixed in formalin for 2–4 weeks, blocked and embedded in paraffin. Sections from the blocks including the ischemic tissue were cut at 4 µm. Immunocytochemical procedures were identical to those previously described [25–27]. After removing paraffin, sections were treated with 0.01% Triton-X and incubated with 10% normal goat serum for 30 min. Adjacent sections were incubated overnight (4° C) with monoclonal antibodies to iNOS (Research and Diagnostic Antibodies, Berkley, Calif.; clone 2D2-B2 or 21E82-D4; dilution 1:20–40) or polyclonal antibodies to nitrotyrosine (Upstate Biotechnology, Lake Placid, NY; lot 1642; dilution 1:1000). Nitrotyrosine is formed from nitration of tyrosine residues by peroxynitrite, the reaction product of NO with superoxide, or by nitrite, the end-product of NO [13]. Nitrotyrosine immunohistochemistry has been used extensively as a marker of NO in brain [2, 3, 35]. Following incubation with the appropriate secondary antibody and quenching with 0.3% H_2O_2, the immunocomplex was visualized using diaminobenzidine as a chromogen in a peroxidase reaction (Kirkegard and Perry Laboratories, Gaithersburg, Md.). To assist in the determination of the cellular localization of the label, adjacent sections were stained with hematoxylin and eosin (H&E) using conventional methods. Slides were viewed and photographed using a Nikon Optiphot microscope.

Controls

Three types of controls were performed. First, sections were incubated overnight without the primary antibody and processed as above. Second, to demonstrate specificity of nitrotyrosine immunoreactivity, nitrotyrosine antibody prepared at working dilution was preabsorbed with 1 mM nitrotyrosine (Aldrich Chemical, Milwaukee, Wis.; lot 07124JR) prior to incubation with the sections and further processing. Third, to demonstrate the specificity of the iNOS stain, the iNOS antibody (dilution 1:20) was preabsorbed with a tenfold excess (w/w) of the specific peptide used to generate the antibody (PS-5166; lot 7792; Research and Diagnostic Antibodies). Brain sections were then incubated with this solution and processed further as described in the previous section.

Results

In H&E-stained sections, the infarcts were characterized by widespread eosinophilic neuronal necrosis and prominent neutrophilic infiltration (Fig. 1). In some instances, areas of hemorrhagic transformation were observed. In all brains, iNOS immunohistochemistry revealed abundant iNOS-immunoreactive cells with the morphological characteristics of neutrophils (Fig. 1). The identity of these cells was confirmed by examination of adjacent sections stained with H&E (Fig. 1). iNOS-positive neutrophils were observed in perivascular spaces and in the lumen of blood vessels (Figs. 1, 2). iNOS immunoreactivity also was observed in the wall of blood vessels (Fig. 2). In some cases, the iNOS reaction product involved the full thickness of the vessel (Fig. 2). Both small and large vessels expressed iNOS immunoreactivity. iNOS immunoreactivity was not observed in neurons or vascular cells in non-infarcted areas. This observation attests to the lack of cross-reactivity of the iNOS antibody with neuronal or endothelial NOS. Immunoreactivity in the areas of pathology was not observed if the primary antibody was omitted or if the primary antibody was preabsorbed with an excess of the antigen (Fig. 1). An identical pattern of immunostain was obtained with two different iNOS antibodies (Research and Diagnostic Antibodies, clones 21E82-D4 or 2D2-B2).

To provide evidence of NO production in iNOS-positive cells, we performed nitrotyrosine immunohistochemistry (Fig. 1). In all brains, nitrotyrosine immunoreactivity was observed in iNOS-positive neutrophils (Fig. 1). In general, nitrotyrosine-positive cells were less numerous than iNOS-positive cells (Fig. 1). Furthermore, immunoreactivity was not observed in the blood vessel wall (Fig. 1). Nitrotyrosine immunoreactivity was abolished by

Table 1 Summary of patients studied (*F* female, *M* male, *MCA* middle cerebral artery)

Case	Age/sex	Infarct territory	Interval between stroke and death	Cause of death
1	75/F	Right MCA	1 day	Cardio-respiratory insufficiency
2	53/M	Right MCA Bilateral thalamic infarcts	1–2 days	Left intracerebral hematoma, uncal herniation
3	60/F	Right MCA	1–2 days	Sepsis
4	80/M	Left MCA	1–2 days	Right intracerebral hematoma, uncal herniation

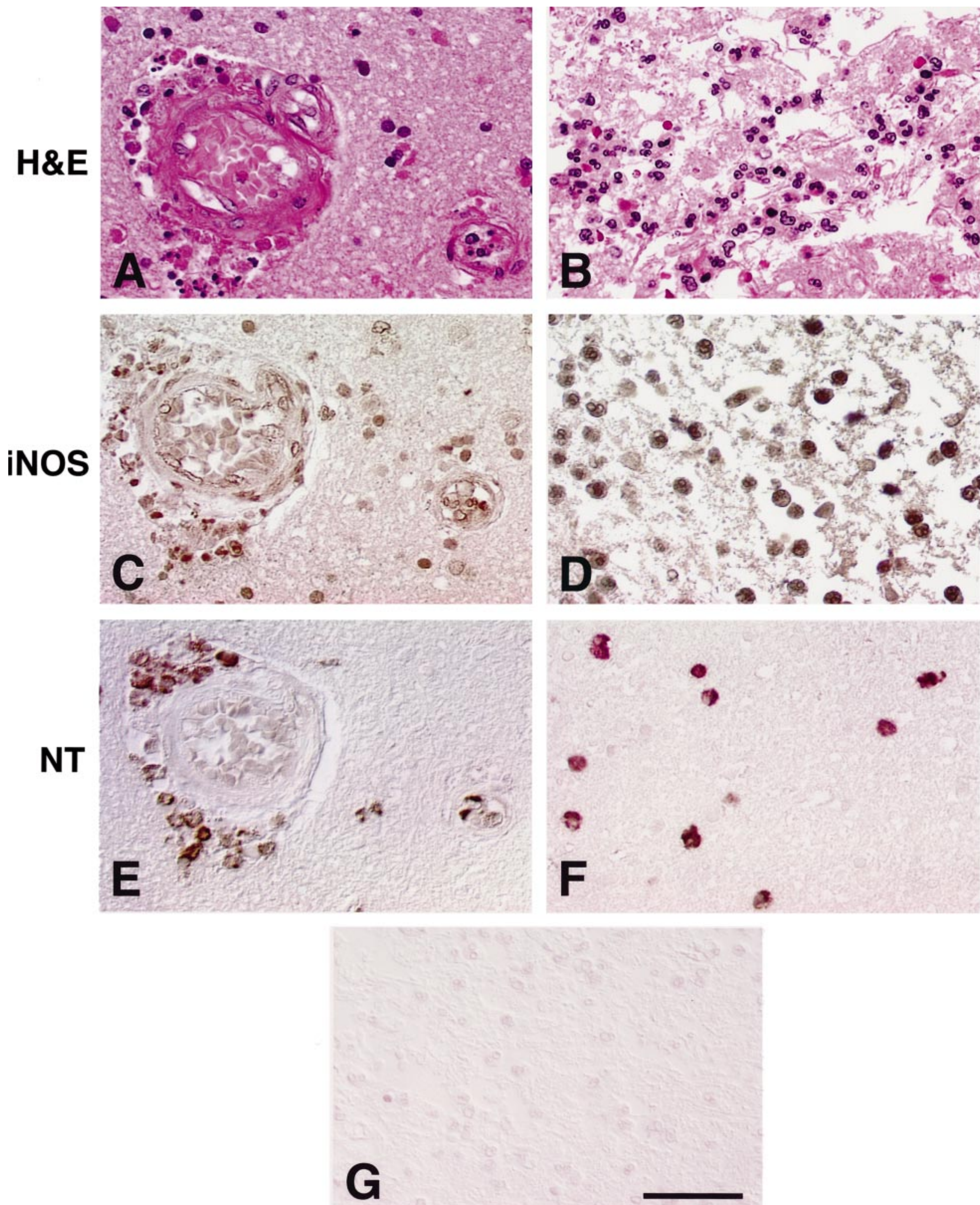

Fig. 1 A–G iNOS and nitrotyrosine immunoreactivity in human stroke. Studies were conducted in paraffin-embedded sections (4 μm thick) of autopsy brains. The infarct was characterized by parenchymal and perivascular neutrophilic infiltration (**A, B**). iNOS immunoreactivity was observed in small cells with multilobular nuclei (**C, D**). These cells have the morphological characteristics of neutrophils in H&E-stained sections (**A, B**). iNOS-positive neutrophils also are endowed with nitrotyrosine immunoreactivity (**E, F**). Nitrotyrosine immunoreactivity is not observed in the wall of cerebral blood vessels (**E**). Nitrotyrosine reflects protein nitration by NO-derived peroxynitrite and nitrite and is used as a "footprint" of NO. The iNOS stain is abolished by preabsorption of the iNOS antibody with the specific peptide used to generate the antibody (**G**). *Bar* in **G** 50 μm in **A–C, E, G** and 25 μm in **D** and **F** (*H&E* hematoxylin and eosin, *iNOS* inducible nitric oxide synthase, *NT* nitrotyrosine)

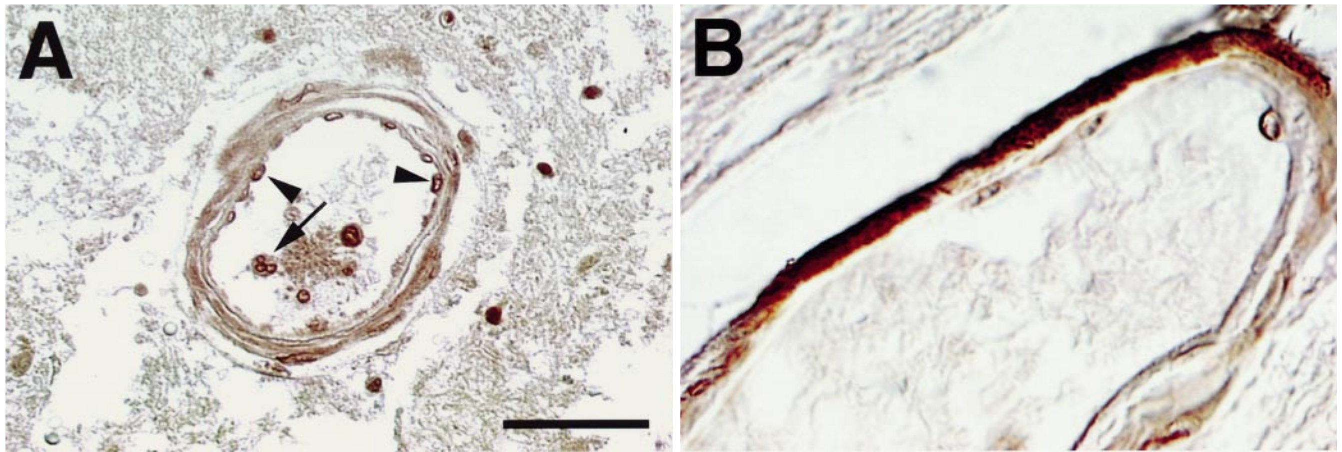

Fig. 2 iNOS immunoreactivity was also observed in the wall of cerebral blood vessels (**A, B**) and in intravascular neutrophils (*arrow* in **A**). *Arrowheads* in **A** indicate iNOS-positive endothelial cells. *Bar* in **A** 50 μm in **A** and 25 μm in **B**

omission of the primary antibody or by pre-incubation of the antibody with 1 mM nitrotyrosine.

Discussion

Studies in rodents have demonstrated that iNOS expression in inflammatory cells is involved in the mechanisms by which post-ischemic inflammation contributes to progression of cerebral ischemic damage [23]. However, the relevance of iNOS expression to human cerebral ischemia remained unclear. While iNOS is easily induced in rodent cells by treatment with endotoxins and cytokines, it is more difficult to elicit iNOS expression in human leukocytes [12]. In this study, therefore, we investigated whether iNOS is expressed in the human brain following ischemic infarction. Using immunohistochemical techniques, we found that iNOS immunoreactivity is present in neutrophils infiltrating the infarcted brain and in cerebral blood vessels within the infarct. The present data clearly demonstrate that in the human brain, as in the brain of rodents, iNOS is expressed in inflammatory cells following ischemic infarction.

iNOS-immunoreactive cells also exhibited nitrotyrosine immunoreactivity. Nitrotyrosine is formed by nitration of tyrosine residues by peroxynitrite, the product of the reaction of NO with superoxide [4]. Nitrotyrosine can also be generated by reaction of nitrite, the autoxidation product of NO, with either myeloperoxidase, present in neutrophils, or hypochlorous acid [13, 14]. Although the mechanisms of protein nitration in human cerebral ischemia have not been elucidated, the presence of both iNOS and nitrotyrosine immunoreactivity suggests strongly that NO is produced by iNOS-positive neutrophils. However, not all iNOS-positive neutrophils were found to have nitrotyrosine immunoreactivity. This observation suggests that either iNOS is not active in all positive cells or that NO is synthesized at a level insufficient to induce detectable protein nitration. It is also of interest that nitrotyrosine immunoreactivity was not observed in the wall of cerebral vessels in which iNOS immunoreactivity was present. One possible explanation for this observation is that NO produced at the vascular level is rapidly captured by intravascular hemoglobin, a molecule whose heme group binds NO avidly [20], thereby reducing the NO available for reaction with superoxide. The recent demonstration that nitrotyrosine immunoreactivity is also present in the ischemic human spinal cord [3] is consistent with the hypothesis that iNOS is also induced following spinal cord ischemia. However, at variance with the present study, in the ischemic spinal cord nitrotyrosine immunoreactivity was observed in the neuropil [3]

It must be noted that the patients described here died 1–2 days after suffering a stroke. In all cases there were associated medical or neurological events that were responsible for the patients' death. The influence of these associated conditions on iNOS expression in the infarcts remains unclear. It is, however, of interest that the cellular localization and regional pattern of iNOS expression in the human infarcts were identical to those observed in rodents subjected to acute middle cerebral artery occlusion. It seems, therefore, unlikely that the associated conditions, rather than cerebral infarction, were responsible for iNOS expression in the areas of infarction.

In rodents, iNOS expression occurs 12–96 h after permanent focal ischemia and 6–48 h after transient ischemia [25, 26]. The time course of iNOS expression in the human brain has not yet been defined. The limitations imposed by studying autopsy material preclude a detailed analysis of the time course. However, our data suggest that iNOS is expressed during the first 1–2 days following stroke. The nature of our autopsy material also precluded a detailed topographical analysis of the distribution of iNOS-positive cells in the different regions of the infarct. Additional studies will be required to elucidate topography and temporal profile of iNOS expression. Irrespective of the spatial and temporal features of iNOS induction, however, the observation that the cellular site of the expression is the same in humans and rodents suggests that post-ischemic iNOS expression in inflammatory cells and blood vessels is a fundamental cellular reaction highly conserved across species.

The pathogenic significance of post-ischemic iNOS expression in the human brain has not been elucidated. The results of the present studies do not permit firm conclusions to be drawn about the pathogenic significance of the iNOS expression in neutrophils and cerebral blood vessels. However, as discussed above, experimental evidence in rodents suggests that NO produced by iNOS contributes to cerebral ischemic damage [23]. Different mechanisms are thought to be responsible for NO toxicity (see [21] for review). These include formation of peroxynitrite, a strong oxidant that is protonated to form the highly reactive peroxynitrous acid [4], induction of energy failure [31, 37] and DNA damage [38]. DNA damage can trigger apoptosis via p53-mediated mechanisms and can activate poly(ADP)ribose polymerase (PARP), a DNA-repairing enzyme that leads to energy depletion [7, 16, 39]. Therefore, large amounts of NO are likely to exacerbate the pathogenic consequences of cerebral ischemia. In support of this hypothesis, NO produced by iNOS aggravates the excitotoxicity resulting from glutamate or from oxygen-glucose deprivation in neuronal cultures [11, 22]. It is, therefore, likely that NO produced by iNOS is also detrimental to the human brain following cerebral ischemia. iNOS expression has also been implicated in the pathogenesis of Alzheimer's dementia, multiple sclerosis, AIDS dementia, progressive supranuclear palsy and brain tumors [1, 6, 10, 28, 36].

The observation that iNOS is also expressed in the human brain following stroke has important implications for the treatment of cerebral ischemia. A substantial proportion of stroke patients reach medical attention many hours after the onset of ischemia [5]. At this time, current therapeutic approaches, such as those based on thrombolysis or inhibition of glutamate receptors or calcium channels, are no longer effective [29]. iNOS inhibitors reduce ischemic damage even when they are administered several hours after induction of ischemia [24, 26, 32]. The finding that iNOS is expressed in the human brain after stroke, in concert with the protection observed in rodents with iNOS inhibition, supports the hypothesis that iNOS inhibitors may be a useful treatment modality in stroke patients.

In conclusion, we have demonstrated that following acute cerebral infarction there is immunohistochemically verified expression of iNOS in neutrophils invading the ischemic brain and in cerebral blood vessels within the area of injury. iNOS expression is associated with accumulation of nitrotyrosine immunoreactivity, which reflects protein nitration by NO-derived peroxynitrite and nitrite. The data provide strong evidence that, in rodents as in humans, iNOS is expressed following cerebral ischemia.

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