Transcriptional Activation following Cerebral Ischemia in Mice of a Promoter-deleted Nitric Oxide Synthase-2 Gene*

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Nitric oxide synthase (NOS)-2 is transcriptionally activated in a wide variety of injurious conditions, including cerebral ischemia, and the resulting nitric oxide is implicated both in tissue damage and recovery. Studies in vitro suggest that the proximal region of the NOS-2 promoter is obligatory for gene activation by proinflammatory cytokines. However, following cerebral ischemia in a NOS-2 gene-deficient mouse in which this region and exons 1–4 have been deleted, we find temporal and spatial expression, identical to wild-type, from a previously unidentified promoter region. The resulting protein is predicted to lack the first 113 amino acids and is NOS-2-incompetent. Fortuitously, this gene-deficient mouse presents a unique opportunity to determine more about the mechanisms of NOS-2 gene regulation in vivo.

Cloning and sequencing of CDNAs for the three isoforms of nitric oxide synthase (NOS)† has revealed products of distinct genes that share 50–60% homology at the nucleotide and amino acid levels (1). Two of these products, NOS-1 and NOS-3, are constitutively expressed enzymes that are calcium-calmodulin-dependent and produce small amounts of nitric oxide (NO) in response to transient elevations in intracellular calcium (2, 3). The third isoform, NOS-2, is not normally expressed but can be transcriptionally activated in a variety of cell types in response to proinflammatory cytokines and bacterial endotoxins (4, 5). Unlike the constitutive isoforms, NOS-2 has calmodulin bound tightly at all times, maintaining the enzyme in a tonically active state and making it capable of producing a large, continuous flux of NO. The relatively large amount of NO that can be synthesized by NOS-2 over a sustained period has been implicated in diverse functions associated with inflammation and injury (6). Expression of NOS-2 in the central nervous system has been associated with viral, parasitic, and bacterial infections; in multiple sclerosis and neurodegenerative diseases; and in trauma and ischemia (7). Given the potential involvement of NOS-2 in such pathologies, a clear understanding of the mechanisms of NOS-2 gene regulation in vivo is important.

The role of elements in the 5’ untranslated region of the NOS-2 gene and the mechanisms by which NOS-2 is induced and this induction is suppressed have all been well characterized in vitro. A 1749-bp fragment of the mouse NOS-2 promoter has been cloned and sequenced (8), and the transcription initiation site and 22 consensus sequences for the potential binding of transcription factors have been identified. Given this diversity and multiplicity, it is not surprising that maximal induction of the gene requires the synergistic effect of combinations of stimuli. The nuclear factor xB binding site in the proximal portion of the promoter is considered obligatory for NOS-2 induction by endotoxin in vitro, whereas the more distal region of the promoter is important for the synergistic effect of IFN-γ (9).

Recently, a NOS-2 gene-deficient mouse was generated (10) with a targeted deletion of the proximal region of the promoter (585 bp), as well as exons 1–4 (including the translational start site). This manipulation was designed to produce a NOS-2 null allele by interfering with both transcriptional and translational expression of the gene. In the descriptions and outcomes of studies in a variety of disease models using this genetic variant, and in a range of different cell types, the loss of expression appears to hold true (6). However, here we report that the generation of a cerebral infarct in such mice initiates transcriptional expression of the disrupted NOS-2 gene. Although there is translation of a product, this does not function as a NOS. As such, these mice provide a unique opportunity to explore further the regulation of the NOS-2 gene in vivo through the analysis of a promoter deletion introduced by genetic mutagenesis and should prove useful in the identification of previously undetected activators of this gene.

EXPERIMENTAL PROCEDURES

Mice and Genotyping—NOS-2−/− mice, generated from a mixed background of C57BL6 × 129/SvEv (10), were outcrossed to C57BL6 wild-type mice (+/+ ) to generate NOS-2 heterozygous (+/−) mice for breeding. A NOS-2 colony was established at the University of Iowa using heterozygote breeding pairs in order to generate littersmates of all three genotypes (+/+ , +/−, and −/−). Genomic screening was performed on DNA extracted from mouse tail snips. To detect the presence of the wild-type NOS-2 allele, a pair of PCR primers were designed that amplified a 328-bp region within exon 1 of the NOS-2 allele and absent in −/− mice (Fig. 1). The sequences of the NOS-2 primers are as follows: forward, 5’TGA AGT GAC TAC GTG CTG CC-3; reverse, 5’AGT CCC TTC ACC AAG GTG G-3’. To identify the presence of the altered NOS-2 allele, another primer pair was constructed that amplifies a 550-bp region of the neo insert specific to the disrupted NOS-2 allele. The sequences of the neo primers are as follows: forward, 5’TGC AGA GGC AAT CGT GCT ATG AC-3; reverse, 5’CAC CAT GAT ATT CGG CAA GCA G-3’.

Induction of Focal Cerebral Ischemia—Adult mice (19–30 g) were anesthetized with 2% halothane for induction and were maintained on 1% halothane. A midline incision was made on the ventral surface of the
Expression of an Altered NOS-2 Gene

**WILD-TYPE NOS2 ALLELE**

**DISRUPTED NOS2 ALLELE**

FIG. 1. Schematic comparison of the wild-type and disrupted NOS-2 alleles. Shown are the deletions in the exons; dark shaded boxes, promoters; light shaded boxes, promoter response elements; arrows, PCR primers.

The neck, and the right external carotid and common carotid arteries were isolated and ligated. The internal carotid artery and the pterygopallinal artery were temporarily occluded. An 8-0 monofilament coated with silicone was introduced into the extracranial internal carotid artery through an incision in the common carotid arteries and carefully advanced approximately 10 mm distal to the carotid bifurcation, beyond the origin of the middle cerebral artery. After suturing the incision, anesthesia was withdrawn and the animals allowed to recover for up to 72 h.

**Immunohistochemistry**—NOS-2 protein was detected in paraformaldehyde-fixed tissue using a rabbit anti-mouse NOS-2 polyclonal antibody (1:100, Transduction Laboratories) that recognizes the C-terminal region of the NOS-2 isoform. Immunohistochemistry was performed with only slight modifications of standard immunoperoxidase techniques suggested by Oncogene Science Inc. Immunoreactivity was detected using an avidin-biotin horseradish peroxidase complex kit (Vectorstain ABC kit, Vector Laboratories, Inc.) along with a peroxidase substrate kit containing 3,3′ diaminobenzidine as substrate following the manufacturer’s recommendations. All sections were additionally counterstained with toluidine blue (0.5% aqueous, acidified with 0.2% glacial acetic acid) and eosin (0.5% aqueous) and covered-slipped. To assist in the determination of cell types expressing NOS-2 protein, adjacent sections were labeled with cell-specific markers; a mouse monoclonal anti-glial fibrillary acidic protein antibody (1:200, Serotec Ltd.) was used to recognize monocytes and macrophages.

**RNA Isolation**—Total cellular RNA was isolated from approximately 100–150 mg (wet weight) of tissue with Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. Precipitated RNA was pelleted by centrifugation, washed with 0.6 volumes of 2M sodium acetate and 3 volumes of 100% ethanol to air dry. RNA was redissolved in RNase-free water and precipitated with 0.2% glacial acetic acid and ethanol (0.5% aqueous) and covered-slipped. To assist in the determination of cell types expressing NOS-2 protein, adjacent sections were labeled with cell-specific markers; a mouse monoclonal anti-glial fibrillary acidic protein antibody (1:200, Sigma) was used to identify astrocytes, and a rat anti-mouse F4/80 polyclonal antibody (1:100, Serotec Ltd.) was used to recognize monocytes and macrophages.

**RT-PCR**—First strand cDNA was synthesized from total RNA using Superscript™ II RNase H reverse transcriptase (Life Technologies, Inc.) according to the manufacturer’s protocol. Precipitated RNA was pelleted by centrifugation, washed with 75% ethanol, and allowed to air dry. RNA was redissolved in RNase-free water and precipitated with 0.5 volumes of 2 M sodium acetate and 3 volumes of 100% ethanol for storage at −70 °C. Prior to reverse transcription, RNA precipitates were recovered by centrifugation, washed with 75% ethanol, redissolved in RNase-free water, and quantitated.

**RESULTS**

**Expression of a Protein in NOS-2 Gene-deficient Mice Following Cerebral Ischemia**—In wild-type (+/+), mouse, immunohistochemistry performed with a specific C-terminal antibody revealed NOS-2 expression in both infiltrating cells and also in resident astrocytes after permanent middle cerebral artery occlusion (MCAO). In gene-deficient (−/−) mouse, immunoreactivity was detected 24 h postocclusion in the infarcted hemisphere in infiltrating inflammatory cells (Fig. 2A). Later, at 72 h after MCAO, immunostaining was still evident, but was additionally seen in cells surrounding the infarct that morphologically resemble astrocytes (Fig. 2B). This pattern of expression was identical, spatially and temporally, to that seen in wild-type mice following MCAO. As no immunolabeling was observed in the contralateral cerebral hemisphere at any time point, expression of the altered gene product appeared to be restricted to the ischemic hemisphere. No immunoreactivity was detected in tissue from normal −/− mouse brain (data not shown), suggesting that the altered NOS-2 gene was not constitutively activated.

**A Truncated NOS-2 Transcript Is Expressed in Gene-deficient Mice**—We used RT-PCR both to verify expression of the disrupted NOS-2 gene following MCAO, and in an attempt...
Expression of an Altered NOS-2 Gene

The Disrupted NOS-2 Gene Can Be Activated in Cultured Glial Cells—A period of cerebral ischemia is immediately followed by the induction of genes encoding for proinflammatory cytokines, such as IL-1β and TNFα. Assuming that the +/+ and −/− NOS-2 genes are induced by similar mechanisms, we tested the ability of various combinations of proinflammatory cytokines, as well as hypoxia, to induce the altered gene in mixed glial cultures. Although the known hypoxia response element in the NOS-2 promoter is deleted in the genetically altered mice, we thought that another site might exist more distally. With this in mind, we tested the responsiveness of the altered gene to hypoxic conditions and to stimuli (IFN-γ and the iron chelator desferrioxamine) shown to induce the NOS-2 gene in macrophages through a hypoxia response element (14).

FIG. 3. Detection of NOS-2 transcript in ischemic +/+ and −/− mouse brain. A, using a forward PCR primer (436F) located in exon 4, transcript was detected in +/+ but not −/− mice. NOS-2 mRNA was detected in +/+ and −/− animals using primers (489F–1181R) that amplified regions downstream of the exon 4/5 boundary. The predicted product sizes (692 and 745 bp) are indicated by arrows. B, the NOS-2 transcript was also detected in +/+ and −/− mice using primers (2721F–3579R) located in the 3′ region. The predicted 858-bp product is indicated by an arrow.

FIG. 4. Activation of the NOS-2 gene in tissue and cells derived from −/− mouse brain. A, RNA from infarcted hemispheres was probed for NOS-2 and the transcript for ribosomal protein L32 by RPA at various times after MCAO. Densitometric analysis of autoradiograms was performed using Molecular Analysis imaging software. Individual data points (2–4 animals per group) are indicated as the ratio of NOS-2:L32. B, RNA isolated from cultured glial cells was probed for NOS-2 by RPA. Lanes 1 and 3, unstimulated cells; lanes 2 and 5, IFN-γ (100 units/ml) and desferrioxamine (0.8 mM); lane 4, IFN-γ and IL-1β (1 ng/ml); lanes 6 and 11, mixture of IFN-γ, IL-1β, TNFα (50 ng/ml), and lipopolysaccharide (1 μg/ml); lanes 7 and 9, TNFα; lanes 8 and 10, hypoxia; lane 12, 8-bromo-cyclic GMP (1 mM). L32 indicates relative loading. C, glial cultures were exposed to cytokines for 30 h, lysed, immunoprecipitated with anti-NOS-2, and then immunoblotted. Lane 1, +/+ cells stimulated with cytokine mixture; lane 2, unstimulated −/− cells; lane 3, cytokine mixture stimulated −/− cells. The position of molecular mass markers (kDa) is indicated.
Expression of an Altered NOS-2 Gene

The disrupted NOS-2 gene transcript was detected, using RPA, in glial cells that had been exposed to a mixture of cytokines, but not in response to other stimuli (Fig. 4B). A protein product could also be detected in immunoprecipitates from lysates of cytokine-activated cells (Fig. 4C). This protein was smaller than that of NOS-2 in wild-type cells (131 kDa) and displayed a molecular mass (approximately 112 kDa) similar to that predicted if the first 113 amino acids of NOS-2 were absent (116 kDa). In similar cell cultures exposed to the cytokine mixture, we determined nitrite accumulation in the medium, using the Griess reaction. Detectable NO production was only found in cells derived from wild-type animals, indicating a loss of NOS-2 function in −/− mice (data not shown).

As neo has its own promoter (PGK), we predicted that it should be expressed in −/− mice, even though it was inserted into the NOS-2 construct in the reverse orientation. With the same primer pair used for genotyping mice, and using RT-PCR, we were able to amplify a product from DNase-treated RNA derived from −/− mice (Fig. 5). In cells from −/− mice, a mixture of cytokines also induced expression of neo, as determined by northern hybridization using a specific probe. Only one transcript was detected, and it was of a size corresponding to neo mRNA (data not shown).

Inflammation-related Gene Expression following Ischemia is Similar in +/+ and −/− Mice—The expression of cytokine and other inflammation-related genes was examined to evaluate possible variations in the inflammatory reaction in −/− mice. Multiprobe RPA analysis was performed on RNA isolated from infarcted and contralateral hemispheres in order to detect transcripts for the cellular response genes ICAM-1, A20, Mac-1, EB22, and glial fibrillary acidic protein and for the cytokines TNF-α, IL-4, IL-5, IL-1α, IFN-γ, IL-2, IL-6, IL-1β, and IL-3. Increased expression of Mac-1 and glial fibrillary acidic protein genes is indicative of activation of macrophages/microglia and astrocytes, respectively. Expression of EB22, an acute-phase response gene, was detected as early as 6 h and was increased at 24 and 48 h postocclusion. Induction of the gene for the cellular adhesion molecule ICAM-1 was also apparent in NOS-2 −/− mice. Quantification revealed similar ischemic induction of ICAM-1 and Mac-1 in both genotypes at all times following permanent MCAO (Fig. 6). Transcript for IL-1β, a potent inducer of the NOS-2 gene, was markedly elevated in ischemic hemispheres of wild-type and NOS-2-deficient mice, whereas expression of IL-6 and TNF-α was increased to a lesser extent (Fig. 7). Minimal or no induction was seen for the other cytokine genes.

DISCUSSION

We have found that mice with a disruption of the NOS-2 gene can undergo tissue-specific transcription, and translation of a NOS-incompetent product, despite the absence of exons 1–4 and proximal regions of the promoter considered to be essential for activation. In vitro investigation into the responsiveness of this gene to endotoxin, proinflammatory cytokines, and hypoxia have identified the promoter sequences required for induction, many of which are absent in the gene-deficient mouse. However, the 5’ flanking region of the mouse NOS-2 gene contains sequences of DNA of which the function is not yet known, and studies using reporter constructs are limited by the amount upstream that has been sequenced to date. Therefore, the regulatory mechanisms that occur in vivo might be complex and may be oversimplified by in vitro representation. Because they exhibit a truncated but active promoter, these −/− mice provide a unique opportunity to study NOS-2 gene regulation in vivo.

Mice with targeted deletions in the NOS-1 (15), NOS-2 (10, 16, 17), and NOS-3 (18) genes have been produced. Although gene disruption is designed to result in a null allele, this is not always the case, and the resulting mice may produce a non-functional gene product, a truncated product, or alternatively...
Expression of an Altered NOS-2 Gene

A

IL-1β/L32

Left
Right

+/+ -/-
+/+ -/-
+/+ -/-
+/+ -/-

B

IL-6/L32

+/+ -/-
+/+ -/-
+/+ -/-
+/+ -/-

C

TNFα/L32

+/+ -/-
+/+ -/-
+/+ -/-
+/+ -/-

Fig. 7. Expression of proinflammatory cytokine genes after MCAO in NOS-2 +/+ and -/- mice. RNA from both infarcted (right) and noninfarcted (left) hemispheres was probed by RPA for IL-1β (A), IL-6 (B), and TNFα (C) at the indicated times following MCAO. Densitometric analysis of autoradiograms was performed using Molecular Analysis imaging software. Mean values (as a ratio to L32) for 2–4 mice are indicated.

In the mice used here, a gene replacement vector was designed to delete the proximal 585 bases of the promoter, as well as exons 1–4. Because the proximal portion of the promoter was previously shown in vitro to be required for NOS-2 induction in macrophages in response to lipopolysaccharide, and because exon 2 contains the translational start site, homologous recombination of the targeting vector with the NOS-2 gene was predicted to interfere with both transcription and translation. Screening of NOS-2-deficient mice for mRNA, protein, and NOS activity indicated that the gene had been inactivated (10). However, this screening was performed on organ blots following lipopolysaccharide injection, or on isolated macrophages stimulated with various combinations of lipopolysaccharide, TNFα, IFN-α/β, and IFN-γ. As activation of the NOS-2 gene via these stimuli has been shown to be dependent upon the proximal portion of the NOS-2 promoter (8), induction of the disrupted gene would not be predicted. We have shown here that glial cells from gene-deficient mice did not express the aberrant transcript under such conditions. However, both a transcript and a protein product could be detected when a cytokine mixture was used, although there was no evidence for a NOS function.

When gene targeting is designed to modify or delete regulatory DNA sequences, the activity of the marker gene should be considered as it brings its own promoter and enhancer (21). Although we have shown that neo is expressed in NOS-2 -/- mice following cerebral ischemia, and in cytokine-activated glial cells, we believe it is activation of the remaining (distal) NOS-2 promoter region that is responsible. The temporal and spatial expression of the disrupted gene product following cerebral ischemia is identical to that in the wild-type, suggesting a similar mechanism of induction. Less than 2 kilobase pairs of the 5' flanking DNA of the murine NOS-2 gene has been sequenced. It is possible that unidentified promoter response elements could be involved in the activation of both the disrupted and the wild-type gene in response to focal ischemia. By comparison, the human NOS-2 gene has important regulatory elements extending 16 kilobase pairs upstream (22). It is also possible that the remaining transcription element consensus sequences mediate expression by some mechanism(s) previously undetected by in vitro study.

The NOS-2 protein is a dimeric, bidomain enzyme with iron protoporphyrin IX, flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin as bound prosthetic groups (23). The enzyme has an N-terminal oxygenase domain, containing binding sites for L-arginine and heme, and a C-terminal reductase domain that binds FMN, FAD, and NADPH. When NOS-2 is cleaved at the junction of the oxygenase and reductase domains, the resulting protein fragments remain catalytically active but unable to generate NO (24). Activity of NOS-2 is dependent upon dimerization, and recent evidence has identified amino acids 66–114 of the oxygenase domain as required for dimeric interaction (25). Exons 2–4 of the NOS-2 gene, deleted in the deficient mice, encode the first 100 amino acid residues of the NOS-2 protein. Although the native translational initiation site is located in exon 2, an ATG codon at bases 524–526 could potentially act as a translational start site in the gene-disrupted mice. If so, then the disrupted gene would be translated in the same open reading frame and, using the same termination codon as the wild-type gene, generate a trun-

spliced products (19). In some cases, generation of an inactive product is specifically designed, as this mimics the effects of pharmacological inhibition. Mice deficient for the NOS-1 gene showed residual NOS activity, indicating the presence of splice variants (20). In the NOS-2-deficient mice generated by Wei et al. (16), an attempt to delete the first five exons of the gene failed, and a genomic rearrangement occurred. From this genomic alteration there resulted an aberrant transcript larger than wild-type NOS-2 mRNA, but reportedly, this was not translated into a functional NOS. The targeting strategy for another NOS-2-deficient mouse involved replacement, with the neomycin resistance gene, of exons 12 and 13, which encode the calmodulin-binding domain (17). Low levels of two abnormal transcripts were detected, and both contained sequences of neo. Although immunoblotting revealed no detectable NOS-2 protein, a low level of NO-producing activity was observed.

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cated protein lacking the N-terminal 113 amino acid residues. We have found evidence, using an antibody against the C terminus, for the translation of a truncated protein both in ischemic NOS-2-deficient mouse brain, and in cytokine-activated glial cultures. Based on the work of Ghosh et al. (25) and the lack of NO production in glial cells activated with a cytokine mixture, we conclude that this truncated protein is NOS-incompetent because of its inability to dimerize.

Although translation of this altered gene product would not result in a functional NOS, alternate functions of a truncated protein cannot be ruled out. The reductase domain of NOS is capable of catalyzing the transfer of electrons from NADPH to a variety of exogenous acceptors, including cytochrome c and dioxygen. The NOS-1 protein can reduce molecular oxygen to superoxide (26), and the reductase domain is both necessary and sufficient for this reaction (27). Although NADPH oxidation by NOS-1 is not influenced by the substrate arginine, the rate of oxidation by NOS-2 increases in the presence of substrate (28). Recent evidence, though, suggests that the NOS reductase domains are actually poor superoxide generators, as they are slow to transfer electrons to dissolved O2 (23). It is therefore predicted that although the truncated protein might catalyze a superoxide dismutase-insensitive reduction of cytochrome c or similar artificial electron acceptors, NADPH oxidation leading to superoxide formation is unlikely (29).

The serendipitous finding that the disrupted NOS-2 gene can be expressed, despite the absence of four exons and important promoter response elements, has several implications. This NOS-2-deficient mouse can contribute important insights into the regulation of the gene and to structure-function aspects of the NOS enzymes. The residual expression of the disrupted gene challenges the idea that proximal promoter sequences are obligatory for induction. These mice provide a unique opportunity to study the molecular and physiological effects of both a truncated NOS-2 promoter and protein in intact animals following neurodegeneration, trauma, and viral infection, conditions in which the NO from NOS-2 is proposed to play a role (7).

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