Alternative Splicing of Human Inducible Nitric-Oxide Synthase mRNA

TISSUE-SPECIFIC REGULATION AND INDUCTION BY CYTOKINES

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N. Tony Eissa, Adam J. Strauss, Cynthia M. Haggerty, Esther K. Choo, Shan C. Chu, and Joel Moss

From the Pulmonary-Critical Care Medicine Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892-1590

Human inducible nitric-oxide synthase (iNOS) is responsible for nitric oxide synthesis in response to inflammatory mediators. The human iNOS gene, containing 26 exons, encodes a protein of 131 kDa. This study was aimed at investigating the presence of alternative splicing of human iNOS mRNA. Total RNA from human alveolar macrophages, nasal and bronchial epithelial cells, and several human tissues was transcribed to cDNA and analyzed using polymerase chain reaction with specific primers for segmental analysis of the iNOS gene. Four sites of alternative splicing were identified by sequence analysis; these included deletion of: (i) exon 5; (ii) exons 8 and 9; (iii) exons 9, 10, and 11; and (iv) exons 15 and 16. The deduced amino acid sequences of the novel iNOS cDNAs predict one truncated protein (resulting from exon 5 deletion) and three iNOS proteins with in-frame deletions. Southern analyses of polypeptide chain reaction products were consistent with tissue-specific regulation of alternative splicing. In cultured cells, iNOS induction by cytokines and lipopolysaccharide was associated with an increase in alternatively spliced mRNA transcripts. Because iNOS is active as a dimer, the novel forms of alternatively spliced iNOS may be involved in regulation of nitric oxide synthesis.

Nitric oxide (NO), an important mediator of physiological and inflammatory processes, is synthesized from L-arginine by isoforms of nitric-oxide synthase (NOS): the constitutive endothelial and neuronal subtypes and the high-output inducible enzyme (1–4). The human iNOS gene encodes a protein with a calculated molecular mass of 131 kDa. It contains 26 exons with a translational (ATG) start site in exon 2 and a stop codon (TGA) in exon 26 (9). Human iNOS cDNAs have been independently cloned from several tissues with only a few differences reported in deduced amino acid sequences (2, 10).

Alternative mRNA splicing can lead to tissue-specific patterns of gene expression by generating multiple forms of mRNA that can be translated into different protein products with distinct functions and regulatory properties (11). Regulated alternative splicing can also function as an on/off switch for gene expression. In the latter case, one mode of splicing generates an mRNA that yields a functional protein, whereas an alternative mode generates an mRNA that lacks an open reading frame (11, 12). This study was aimed at investigating the presence of alternative splicing of human iNOS mRNA. cDNAs obtained from human tissues and cultured cells were subjected to PCR-based segmental analysis of the iNOS gene. We demonstrate that the human iNOS gene has a previously unsuspected capacity to generate multiple mRNA isoforms, predicting novel forms of iNOS protein. Four distinct cDNAs were found, which included deletions of: (i) exon 5; (ii) exons 8 and 9; (iii) exons 9, 10, and 11; and (iv) exons 15 and 16 (see Fig. 1B). The alternatively spliced mRNA transcripts were regulated in a tissue-specific manner and induced by cytokines.

EXPERIMENTAL PROCEDURES

Isolation of Normal Human Nasal and Bronchial Epithelial Cells and Alveolar Macrophages—Informed consents approved by the Institutional Review Board were obtained from normal nonsmoking volunteers. Nasal and bronchial epithelial cells were obtained using a standard cytology brush and fiberoptic bronchoscopy. Alveolar macrophages were recovered by bronchoalveolar lavage and purified by adherence to plastic (5, 13, 14).

iNOS Induction—DLD-1 cells, a human colorectal adenocarcinoma cell line, and A549 cells, a human alveolar type II epithelium-like lung carcinoma cell line, were maintained as described previously (5). DLD-1 cells were incubated for 24 h with or without a mixture of interferon-γ (100 units/ml), interleukin-1β (IL-1β, 0.5 ng/ml), tumor necrosis factor-α (10 ng/ml), and interleukin-6 (IL-6, 200 units/ml). A549 cells were incubated for 24 h with or without a mixture of interferon-γ, IL-1β, tumor necrosis factor-α (same concentrations used with DLD-1 cells), and lipopolysaccharide (Escherichia coli serotype 0128:B12, 10 μg/ml; Sigma). Confluent cells were used for all experiments. Interferon-γ, tumor necrosis factor-α, and IL-6 were obtained from Boehringer Mannheim Corp. IL-1β was obtained from Genzyme Corp.

Identification of Alternatively Spliced Human iNOS mRNA Transcripts by Sequence Analysis—Total RNA was extracted from nasal and bronchial epithelial cells and from alveolar macrophages by the guanidinium thiocyanate-CsCl gradient method. Total RNA from human tissues was purchased from Clontech Laboratories, Inc. cDNA was transcribed from RNA using Molony murine leukemia virus RNase H reverse transcriptase and oligo (dT) 12–18 primer (Life Technologies, Inc.). cDNA was amplified by PCR using human iNOS-specific primers (see Fig. 1B): (i) INH1 in exon 4 (5′-TGAGGATCAAAACTGGG-3′)
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the binding site for FMN (Fig. 1) (2, 9).

All of the alternative splice junctions, except for the exon 8 and 9 deletion, follow a complete codon. In splicing leading to deletion of exons 8 and 9, exon 7 terminates after the first two nucleotides of the codon for Arg241. In this iNOS isoform, however, the first nucleotide in exon 10 (adenine) becomes the third nucleotide needed to encode Arg, providing an example of complementary split codons that characterize alternatively spliced exons (15).

The presence of alternatively spliced human iNOS mRNA transcripts was evaluated in pulmonary cells and in several human tissues. PCR products of a segmental analysis of the human iNOS gene were subjected to Southern analysis involving hybridization with a full-length human iNOS cDNA probe. With RNA from nasal epithelium, bronchial epithelium, and alveolar macrophages, products of the four identified sites of alternative splicing were detected, which constituted a minor fraction of total iNOS mRNA (Fig. 2). Overall, iNOS mRNA was more abundant in nasal and bronchial epithelium than in alveolar macrophages, taking into consideration the difference in the number of PCR cycles utilized in amplification (5, 10).

Applying the same analysis to cDNAs from other human tissues, the four patterns of alternatively spliced iNOS mRNA transcripts were observed (Fig. 3). The ratios of the alternatively spliced to constitutively spliced mRNA species differed, however, among the tissues. In particular, iNOS mRNA transcripts with deletion of exon 5 were notably abundant in human cerebellum, suggesting that the isoform may have a specific tissue-related function (11, 15).

To determine whether induction of iNOS is associated with concomitant alterations in amounts of the alternatively spliced mRNA transcripts, DLD-1 cells and A549 cells were incubated for 24 h with a mixture of cytokines and lipopolysaccharide. Upon induction of iNOS, a marked increase was identified in the levels of iNOS mRNA produced both by constitutive splicing and by alternative splicing involving the four sites (Fig. 4).

In the synthesis of NO, iNOS is active as a dimeric complex that contains two identical subunits in which electrons from NADPH reduction are transferred by FAD and FMN to the catalytic site at the amino terminus (7, 16). The transfer of electrons is facilitated by a tightly bound CaM (17, 18), and dimerization requires tetrahydrobiopterin (16). The requirement for such complex interactions between different domains of iNOS for NO production makes the finding of alternative splicing of human iNOS mRNA rather unexpected. Because the four sites of alternative splicing result in deletion of conserved sequences, it is unlikely that any of these splice variants by themselves would be capable of generating NO. It seems more likely that alternative splicing may be involved in modifying the kinetics of NO synthesis or conferring new functions to iNOS.

Although NO production by the constitutive NOS isoforms is critically controlled through regulation of intracellular Ca$^{2+}$ levels, iNOS, apparently, is regulated primarily at the transcriptional level (1, 2). Alternative splicing of mRNA may participate in the post-transcriptional regulation of NO production. Up-regulation of iNOS splice variants by cytokines may imply a role for these variants in maintaining or augmenting iNOS activity during infection or inflammatory response. Mod-
ulation of NO synthesis by alternatively spliced variants could be achieved through the formation of heterodimers. Lee et al. (19) demonstrated by co-expression that truncated mutants of endothelial NOS formed heterodimers with wild-type endothelial NOS and exerted a dominant negative effect on its activity. Recently, Skerjanc et al. described a splice variant of the ITF-2 transcript encoding a transcription factor that inhibits MyoD activity through the formation of inactive heterodimers (20).

Alternatively, in the case of exon 5 deletion, a function of the frameshift may be to down-regulate iNOS gene expression, merely by producing prematurely terminated open reading frames. In such a case, the function of alternative splicing would be to act as a simple on/off switch, in much the same way as transcription is used to control gene expression (11, 12). Down-regulation of iNOS, if involved in protection from the cytotoxic effects of excessive NO production, should be more important in organs where cells are terminally differentiated, such as the brain, which may explain the abundance of exon 5 deletion in mRNA transcripts in the cerebellum.

Because the cytochrome c reductase activity of the carboxyl terminus of iNOS is equivalent to that of full-length iNOS (7), the function of some of these variants could be similar to other cytochrome P-450 reductase proteins. Recently, alternative splicing of mRNA has been reported for neuronal NOS (21–23). One of these splice variants has an in-frame deletion that corresponds to exons 9 and 10 of human iNOS. The presence of a relatively conserved pattern of alternative splicing in NOS suggests a common function for the resulting isoforms. Future studies will be necessary to determine the functional consequences of alternative splicing of human iNOS mRNA. Gene expression studies and generation of transgenic mice expressing different forms of iNOS may help to resolve this issue.

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