Transfection of Inducible Nitric Oxide Synthase Gene Causes Apoptosis in Vascular Smooth Muscle Cells

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Background—Excess production of nitric oxide (NO) by inducible NO synthase (iNOS) has been implicated in a variety of physiological processes including vascular remodeling. To elucidate whether endogenous NO generated by iNOS is involved in the programmed cell death (apoptosis) of the vasculature, iNOS cDNA– expressing construct was transfected into rat and human vascular smooth muscle cells (VSMCs) by lipofection.

Methods and Results—VSMCs transiently transfected with iNOS cDNA functionally expressed 130 kd iNOS protein with full catalytic activity to generate massive NO in proportion to the doses of cDNA used; its enzymatic activity as well as NO production was completely blocked by an NOS inhibitor, N\textsubscript{G\textsuperscript{-}}-monomethyl-L-arginine (LNMMA). Overexpression of iNOS led to a marked inhibition of DNA synthesis as well as induction of apoptosis in VSMCs. Evidence for apoptotic cell death was provided by internucleosomal DNA fragmentation by agarose gel electrophoresis, positive staining for TdT-mediated dUTP biotin nick end-labeling, and appearance of hypodiploid cells by flow cytometry analysis. Apoptosis after transfection with iNOS cDNA was abrogated by LNMMA. Transfection of iNOS cDNA caused accumulation of the tumor suppressor gene p53 but not of bcl-2, which was also blocked by LNMMA.

Conclusions—These results demonstrate that massive generation of endogenous NO derived from iNOS overexpression leads to a marked apoptosis in VSMCs, thus suggesting an important role of NO as a proapoptotic factor for VSMCs in the process of vascular remodeling. (Circulation. 1998;98:1212-1218.)

Key Words: vasculature ■ apoptosis ■ endothelium-derived factors ■ atherosclerosis ■ genes

Nitric oxide (NO), generated from L-arginine by nitric oxide synthases (NOS),\textsuperscript{1} plays diverse physiological functions, such as vascular tonus regulation, neurotransmission, and cytotoxicity.\textsuperscript{2} Two distinct isoenzymes of NOS are known to exist: Ca\textsuperscript{2+}/calmodulin-dependent and constitutive (c)NOS and Ca\textsuperscript{2+}/calmodulin-independent and cytokine-inducible (i)NOS. cNOS, dominantly expressed in brain (NOS1) and endothelium (NOS3), is rapidly activated by Ca\textsuperscript{2+}-mobilizing agonists, whereas iNOS (NOS2), widely distributed in a variety of cell types including vascular smooth muscle cells (VSMCs), can produce high output of NO on induction with bacterial lipopolysaccharides (LPS) and several cytokines.\textsuperscript{1,2} Thus excess NO generation resulting from iNOS induction is important not only for nonspecific host defense by its cytotoxic and bactericidal effect\textsuperscript{1} but also for regulation of cell growth and programmed cell death (apoptosis).\textsuperscript{1}

Apoptosis, a genetically programmed biological strategy to eliminate unwanted cells, is associated with characteristic morphologic features such as cellular shrinkage, nuclear condensation, and chromatin fragmentation and may function as a major determinant of cell number in several physiological and pathological conditions.\textsuperscript{4} Involvement of apoptosis has been extensively studied in the regulation of tumor growth, cell-mediated immunity, embryogenesis, and meta-morphosis.\textsuperscript{5} Genetic program of apoptosis in hematopoietic cells, fibroblasts, and thymocytes is under the control of environmental signals, such as growth factors and cytokines.\textsuperscript{4} Whether they induce or suppress apoptosis appears to depend on species or cell specificity, or alternatively on experimental designs used. NO functions either as a proapoptotic or antiapoptotic factor for different cell types.\textsuperscript{6} In the blood vessels, it has been reported that NO induces apoptosis in vascular endothelial\textsuperscript{7} and smooth muscle cells.\textsuperscript{8,9} Recently, it has been shown that apoptosis occurs during the process of vascular remodeling and lesion formation.\textsuperscript{10} These observations suggest that NO-induced apoptosis in VSMCs and endothelial cells may be an important determinant to regulate cell number of normal arterial wall and may contribute to the pathogenesis of atherosclerosis.

However, our understanding of the effects of NO on apoptosis in VSMCs thus far reported is based on studies that used either several NO donors or certain cytokines that induce iNOS gene expression. Given the short half-life of NO, the different kinetics of NO release from these NO donors, and the accumulation of their metabolites, which may affect cellular functions, it is difficult to determine the exact role of NO involved in VSMC apoptosis. Likewise, cytokines cause a variety of signals and cell responses other than induction of iNOS.\textsuperscript{11} To elucidate the physiological conse-
quences of increased NO production in VSMCs, the present study was undertaken to determine whether endogenous NO regulates VSMC apoptosis by transfecting rat iNOS cDNA construct into cultured rat and human VSMCs.

Methods

Materials

N^6-monomethyl-L-arginine (LNMMMA) was purchased from Calbiochem-Novabiochem, Dulbecco’s modified Eagle’s medium (DMEM) from Flow Laboratories, fetal bovine serum (FBS) from Cell Culture Laboratories, deoxyctydine 5'-[^32]P]trisphosphate ([^32]P)dCTP) from DuPont NEN Research Products, Lipofectin from Transduction Laboratories, and rabbit polyclonal antibody for bcl-2 from PharMingen. Other materials were molecular biology grade.

Cell Culture

Rat aortic VSMCs were prepared by the explant method as reported12 and cultured in DMEM supplemented with 10% FBS in a 5% CO_2 atmosphere at 37°C. Human aortic VSMCs were purchased from Kurabo Biomedical (Osaka, Japan) and were cultured in HuMedia-SB2 supplemented with 5% FBS, recombinant EGF (0.5 ng/mL), recombinant FGF-B (2 ng/mL), and insulin (5 μg/mL). After reaching confluency, medium was replaced with serum-free DMEM and the cells (rat 15th to 20th passages, human 3rd to 5th passages) were used in the experiments.

Expression of iNOS cDNA in Rat VSMCs

Rat iNOS cDNA/SRα296 construct was prepared by ligating iNOS cDNA (4117 bp) recently cloned from rat endothelial cells13 to the expression plasmid vector (pcDLSR296) driven by SV40 promoter.14 Transfection of cultured rat VSMCs with iNOS cDNA/SRα296 was carried out by lipofection as recommended by the manufacturer’s instructions. iNOS cDNA/SRα296 or the empty vector in indicated doses dissolved in 500 μL serum-free DMEM was mixed with 25 μL Lipofectin dissolved in 500 μL serum-free DMEM, followed by incubation for 20 minutes at room temperature. Liposome-DNA complexes were overlaid on confluent VSMCs and followed by incubation for four hours under serum-free conditions. To determine the transfection efficiency, pSV/β GAL plasmid DNA (20 μg; Promega, Inc) was transfected as indicated above; the approximate transfection efficiency, pSV/β GAL, was determined with a Sysmex CDA-500 Autoanalyzer (Toa Medical Electronics).

DNA Synthesis

DNA synthesis was determined by incorporation of [3H]-thymidine as reported.17 After transfection, quiescent cells (10^5 cells per well) were washed with serum-free DMEM, incubated with DMEM containing 1% FBS, and further incubated for 4 hours with 0.5 mCi [3H]-thymidine (Amersham International); the radioactivity incorporated was determined with a liquid scintillation counter.

Measurements of Apoptosis

For assessment of nucleosomal laddering, apoptotic DNA fragments of the floating and the adherent cells 24 hours after transfection were extracted with the NP-40 lysis method and fractionated on 2% agarose gels as described.18

For flow cytometric analysis, both floating and trypsinized adherent cells were collected, washed with PBS, and stained with 0.1% Triton X-100, 0.1% sodium citrate, pH 7.0, containing 50 μg/mL propidium iodide by incubation under subdued light (30 minutes, 4°C). Stained cells were then analyzed with a FACS Calibur flow cytometer (Becton Dickinson), as described.19

Apopotic cells were also detected in situ by TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method with an in situ cell death detection kit (Takara Biomedicals) according to the manufacturer’s instructions. Cells grown on LAB-TEK Chamber Slide (Nalge Nunc Int. IL) were fixed for 15 minutes in 4% paraformaldehyde in PBS, blocked for 15 minutes with 0.3% H_2O_2 in methanol, washed and permeabilized for 2 minutes with 0.1% sodium citrate in PBS, followed by sequential exposure to the enzymatic reaction mixture for 60 minutes at 37°C, anti-FTC HRP conjugate for 30 minutes at 37°C, and 0.05% diaminobenzidine in 1% nickel sulfate and 0.01% H_2O_2. To quantify an apoptotic event, the percentage of TUNEL-positive cells to total cell population was calculated by counting all cells from 5 random microscopic fields at the magnification of ×100.

Floating and trypsinized adherent cell numbers were determined with a Sysmex CDA-500 Autoanalyzer (Toa Medical Electronics).

Western Blot Analysis

Western blot analysis with the use of mouse monoclonal antibodies against iNOS, p53, and bcl-2 were performed as reported.20 Twenty-four hours after transfection, cells were homogenized in lysis buffer and centrifuged. The supernatant was subjected to SDS-PAGE and then incubated for 24 hours under serum-free conditions. To determine the transfection efficiency, pSV/β GAL, plasmid DNA (20 μg; Promega, Inc) was transfected as indicated above; the approximate transfection efficiency by lipofection, as monitored by β-galactosidase activity, was >70%.

Determination of NOx (NO_2^-/NO_3^-)

Concentrations of NOx in culture media were determined essentially in the same manner as reported.19 In brief, samples were applied to a copperized cadmium reduction column and NOx were determined by the Griess method with the use of an autoanalyzer (TCI-NOX 1000, Tokyo Kasei Kogyo); the absorbance of 540 nm was measured.

NOS Enzymatic Assay

NOS activity was determined essentially in the same method as citrulline assay.16 Cells were homogenized in 50 mmol/L Tris-HCl, pH 7.4, containing 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 1 μg/mL pepstatin, 2 μg/mL leupeptin, and 0.1 mmol/L phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 30 minutes at 10,000g and the supernatant was used. Enzymatic reaction was performed at 37°C for 1 hour in a final volume of 100 μL assay buffer containing cytosolic fraction (200 μg protein). Assay buffer consists of 12.5 mmol/L Tris-HCl, pH 7.4, containing 1 mmol/L l-arginine, 1 mmol/L NaDPH, 10 μg/mL calmodulin, 9 μmol/L tetrahydrobiopterin, 9 μmol/L FAD, and 5 mmol/L CaCl_2. Enzymatic reaction was terminated by adding 0.2 mL 20 mmol/L HEPEs buffer, pH 5.5, containing 2 mmol/L EDTA. Reaction mixtures were centrifuged for 5 minutes at 10,000g; NOx concentration of supernatant was measured by the Griess method.

Statistical Analysis

Data are expressed as mean±SE. Statistical analysis was performed by using ANOVA for repeated measures. A value of P<0.05 was considered statistically significant.

Results

Functional Expression of iNOS cDNA in Rat VSMCs

To confirm that rat iNOS cDNA encodes functional iNOS activity, iNOS cDNA/SRα296 construct was transiently expressed in cultured rat VSMCs by lipofection. Transfection of iNOS cDNA/SRα296 into rat VSMCs resulted in a marked and dose-dependent increase in NOx production (Figure 1A); a significant increase (3.7-fold) was induced with 10 μg DNA, and further increases (7.1 to 7.5-fold) with 20 to 40 μg DNA. Addition of 1 mmol/L LNMMMA to the cells transfected with iNOS cDNA/SRα296 (20 μg) completely blocked NOx production, whereas NOx production by VSMCs transfected with empty vector (20 μg) was comparable to that of control
Western blot analysis revealed that transfection of iNOS cDNA/SRα296 (20 μg) but not of control empty vector resulted in a distinct expression of 130 kd protein, whose molecular size was identical to that induced by IL-1β (Figure 2). Enzymatic activity was measured in cytosolic fraction prepared from VSMCs transfected with either iNOS cDNA/SRα296 or control empty vector (Figure 3). VSMCs transfected with iNOS cDNA/SRα296 (20 μg) showed a far greater enzymatic activity (12-fold) than those with empty vector, whose effect was completely blocked by coincubation with 1 mmol/L LNMMA.

**Supression of DNA Synthesis After Transfection With iNOS cDNA**

To determine whether excess NO generation resulting from overexpression of iNOS gene affects DNA synthesis in rat VSMCs, [³H]-thymidine incorporation was measured in rat VSMCs transfected with either iNOS cDNA/SRα296 or control empty vector (Figure 4). Compared with the control cells transfected with the empty vector, significant reduction of DNA synthesis occurred after transfection with iNOS cDNA/SRα296 in proportion to the doses used: 10% decrease (10 μg), 34% (20 μg), and 40% (40 μg), respectively (Figure 4A). Reduction of DNA synthesis in cells transfected with...
iNOS cDNA/SRα296 (20 μg) was reversed by treatment with 1 mmol/L LNMA (Figure 4B).

Apoptosis After Transfection With iNOS cDNA
Transient transfection of rat VSMCs with iNOS cDNA/SRα296 resulted in the appearance of many floating cells after 24 hours; the number of floating cells significantly increased, whereas the number of adherent cells conversely decreased in proportion to the doses (10 to 20 μg) of DNA used (Figure 5). These floating cells neither reattached to the plate nor regrew when replated in fresh DMEM containing 10% FBS, suggestive of apoptotic cell death. To determine whether nucleosomal laddering takes place, fragmented DNA extracted from both floating and adherent rat VSMC culture after cell lysis with NP-40 was subjected to agarose gel electrophoresis (Figure 6A). Transfection with iNOS cDNA/SRα296 caused nucleosomal laddering in a dose-dependent fashion (10 to 40 μg), which was blocked by addition of 1 mmol/L LNMA, whereas cells transfected with control empty vector exhibited a minimal nucleosomal laddering (data not shown). Transfection of human VSMCs with rat iNOS cDNA/SRα296 (20 μg) also caused nucleosomal laddering (Figure 6B).

Rat and human VSMCs transfected with either iNOS cDNA/SRα296 (20 μg) or control empty vector were stained in situ by the TUNEL method (Figure 7). Very few cells transfected with empty vector were TUNEL positive (Figure 7, A and D), whereas many adherent cells transfected with iNOS cDNA/SRα296 showed positive TUNEL staining (Figure 7, B and E); treatment with 1 mmol/L LNMA reduced the number of TUNEL-positive cells (Figure 7, C and F). Quantitation of TUNEL-positive cells revealed significant (P<0.005) increases in the transfected rat VSMCs (33.1 ± 1.3%) and human VSMCs (22.1 ± 0.9%) compared with control rat VSMCs (1.6 ± 0.2%) and human VSMCs (0.2 ± 0.1%), respectively (Figure 8). After treatment with 1 mmol/L LNMA, TUNEL-positive cells significantly (P<0.01) decreased in rat VSMCs (7.1 ± 1.0%) and human VSMCs (2.9 ± 2.0%) (Figure 8).

For quantitation of total apoptotic events, flow cytometric analysis with FACS Calibur was performed. Only 6% of total rat VSMCs was hypodiploid when transfected with control empty vector (Figure 9A). Frequency of apoptotic event as
determined by hypodiploidity increased in proportion to the doses of iNOS cDNA/SRα296 used for transfection. After transfecting 80 μg iNOS cDNA/SRα296, almost entire cells (97%) became hypodiploid (Figure 9B), which was completely abrogated by 1 mmol/L LNMMA (Figure 9C). Flow cytometry analysis of human VSMCs transfected with rat iNOS cDNA/SRα296 revealed the appearance of a greater frequency of hypodiploid cells in the same fashion as in rat VSMCs (data not shown).

Expression of p53 and bcl-2
To determine whether NO-induced VSMC apoptosis involves a specific set of apoptosis-related genes, the expression of tumor suppressor gene product (p53) and B-cell leukemia/lymphoma gene product (bcl-2) was examined by Western blot analysis with specific antibodies for p53 and bcl-2, respectively (Figure 10). Transfection of rat VSMCs with iNOS cDNA/SRα296 (20 μg), but not the empty vector resulted in a marked accumulation of nuclear phosphoprotein, p53, which was abrogated by addition of 1 mmol/L LNMMA (Fig 10, top blot), whereas expression of bcl-2 protein was unaffected either by iNOS/SRα296 or empty vector transfection (Figure 10, lower blot).

Discussion
In this study, cultured rat and human VSMCs were transfected with rat iNOS cDNA expressing plasmid by the lipofection method. Transient transfection of iNOS cDNA/SRα296 but not of control empty vector resulted in marked increases in NOX production in proportion to the doses of DNA used. Rat VSMCs transfected with iNOS cDNA/SRα296 expressed a distinct protein band corresponding to the position (130 kd) of that induced by IL-1β as evaluated by Western blot analysis with a specific iNOS antibody. Furthermore, transfection with iNOS cDNA/SRα296 but not with control empty vector also led to a marked increase in enzymatic activity. The augmented NOX production and its enzymatic activity in VSMCs transfected with iNOS cDNA/SRα296 were completely blocked by a nonselective NOS inhibitor, LNMMA. These results demonstrate an efficient and functional expression of iNOS in cultured rat VSMCs by transfection with rat iNOS cDNA/SRα296 plasmid.

Several investigators have reported that NO donors, such as nitroglycerin and sodium nitroprusside (SNP), inhibit...
DNA synthesis and mitogenesis of cultured VSMCs. In our study, DNA synthesis in rat VSMCs induced by 1% FBS was reduced by transfecting iNOS cDNA/SRα296 in proportion to the doses of DNA used, whose effect was blocked by a nonselective NOS inhibitor, LNMA. These results corroborate the notion that NO potently inhibits mitogenesis of rat VSMCs. However, it is also possible that NO-induced inhibition of DNA synthesis may be primarily due to cell death rather than cell cycle arrest. Notably, after transient transfection with iNOS cDNA/SRα296 but not with control empty vector into rat and human VSMCs, significant numbers of rounded, floating cells began to accumulate, which did not reattach when harvested and replated in new culture dishes even supplemented with 10% FBS. Gel electrophoresis of DNA samples extracted from the floating and the adherent VSMCs after transfection with iNOS cDNA/SRα296 displayed a nucleosomal ladder characteristic of apoptotic cells. These observations were further strengthened by the appearance of many TUNEL-positive cells in the adherent VSMCs transfected with iNOS cDNA/SRα296, a hallmark of apoptotic events by specific immunolabeling of nuclear DNA fragmentation. Flow cytometry analysis also revealed that after transfection with iNOS cDNA/SRα296 but not with control empty vector, both floating and adherent cells underwent hypodiploidy. For example, after transfection with a higher dose of iNOS cDNA/SRα296 (80 μg), almost entire cells became hypodiploid by flow cytometry analysis. Furthermore, LNMA, a nonselective NOS inhibitor, markedly suppressed the fraction of apoptotic cells after transfection with iNOS cDNA/SRα296 as determined by nucleosomal laddering, TUNEL staining, and flow cytometric analysis. These data demonstrate for the first time that excess endogenous NO production by transient and functional expression of iNOS gene induced massive apoptosis of both rat and human VSMCs.

Accumulating lines of evidence implicate the pivotal roles of NO for cell death and proliferation. It has been reported that NO induces apoptosis in a variety of cells, including macrophage, pancreatic β-cells thymocytes, chondrocytes, and neuronal cells, whereas NO shows cell protective effects in hepatocytes, lymphocytes, and ovarian follicles. The discrepancy may be due not only to different cell types used but also to different experimental designs used, especially the compounds used for NO action. Most previous studies have used either bacterial LPS and cytokines (interleukin-1β, tumor necrosis factor-α, and interferon-γ) for iNOS induction or several NO donors such as organic nitrates, SNP, 3-morpholinosydnonimine (SIN-1), and 5-nitroso-N-acetylpenicillamine (SNAP). However, LPS and cytokines not only induce iNOS gene expression but stimulate many other signals and specific set of genes that modulate apoptosis. For example, tumor necrosis factor-α generates a sphingolipid hydrolysis product, ceramide, which stimulates apoptosis, whereas nuclear factor kappa B (NF-κB), activated by tumor necrosis factor receptor 1–associated protein death domain, suppresses apoptosis. These findings suggest that apoptosis/antiapoptosis elicited by these cytokines is largely due to the consequences of the net effects of diverse signaling pathways. Although NO donors are known to liberate NO exogenously, its labile and short-lived (5 to 20 seconds) nature as well as the different kinetics of its release by NO donors during long incubation has made it difficult to interpret the precise role of NO. Furthermore, various metabolites generated from these NO donors may make it difficult to determine whether NO itself is responsible. For example, various metabolites and byproducts such as cyanate (SNP), superoxide anion (SIN-1) and penicillamine (SNAP), may affect many cellular functions. Therefore this study with an efficient and functional expression of iNOS gene into cultured VSMCs should be a suitable cell model to study biological consequences of endogenous NO production by iNOS, distinct from other NO-unrelated effects by cytokines or NO donors.

Excess NO generation may lead to DNA damage through several mechanisms, including nitrosative deamination of deoxynucleotides, DNA strand break by NO2 and DNA modification by metabolically activated N-nitrosoamines. Because NO rapidly and readily reacts with superoxide anion to form peroxynitrite, a more potent reactive oxygen species, to cause DNA strand break through the activation of poly-ADP ribosyl synthetase, it is also likely that NO-induced apoptosis is mediated by peroxynitrite generation.

Tumor suppressor gene, p53, has been shown to induce apoptosis in many cell types. It has been shown that NO-induced apoptosis is accompanied by increased expression of p53 in macrophages and thymocytes. Recently, it has been suggested that inhibition of p53 by viral protein, such as cytomegalovirus, may block VSMC apoptosis, thereby contributing to vascular remodeling and lesion formation in atherosclerosis and restenosis after angioplasty. On the other hand, bcl-2 has been shown to prevent apoptosis induced by diverse stimuli. It has recently been reported that stable expression of bcl-2 protected VSMC apoptosis, although there was no difference in endogenous bcl-2 expression between normal and atherosclerotic vessels. In this study, we have demonstrated that transient transfection with iNOS cDNA caused a marked accumulation of p53 protein in...
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rat VSMCs that was inhibited by LNMMA, whereas bcI-2 expression was not affected. These results raise a possible intermediate role of p53 protein for NO-induced apoptosis in VSMCs. Alternatively, accumulation of p53 may play some role in the repair process to NO-induced DNA damage. 33

Recently, it has been reported that in vivo gene transfer of endothelial eNOS resulted in a marked reduction of neointimal formation after balloon injury in rats caused by constitutive generation of endogenous NO at the site of injured vessels. 34 Furthermore, it has recently been shown that expression of iNOS mRNA and protein is localized not only to macrophages and foam cells but also to VSMCs in atherosclerotic lesions and neointima after balloon angioplasty. 35,36 These data suggest that overexpression of iNOS in the vascular lesions may represent a compensatory mechanism to reduce proliferation and/or accelerate apoptosis of VSMCs through excess generation of endogenous NO.

In conclusion, we have demonstrated for the first time that excess NO production by iNOS cDNA transfection caused massive apoptosis in VSMCs accompanied by increased expression of p53. These findings have important implications for the role of NO as a proapoptotic factor for VSMCs in the process of vascular remodeling and lesion formation in atherosclerosis and restenosis after angioplasty.

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References

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