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Urea-induced inducible nitric oxide synthase inhibition and macrophage proliferation

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Background. Atherosclerosis is a major cause of morbidity and mortality in chronic renal failure and is associated with the proliferation of macrophages within atherosclerotic lesions.

Methods. Because the progression of atherosclerosis as a consequence of decreased nitric oxide (NO) synthesis has been described, we investigated the correlation between the inhibition of inducible NO synthase (iNOS) by urea, macrophage proliferation as assayed by cell counting, tritiated-thymidine incorporation and measurement of cell protein, and macrophage apoptosis.

Results. Urea induces a dose-dependent inhibition of inducible NO synthesis in lipopolysaccharide-stimulated mouse macrophages (RAW 264.7) with concomitant macrophage proliferation. Macrophage proliferation as determined by cell counting became statistically significant at 60 mmol/L urea corresponding to a blood urea nitrogen level of 180 mg/100 mL, concentrations seen in uremic patients. iNOS protein expression showed a dose-dependent reduction, as revealed by immunoblotting when cells were incubated with increasing amounts of urea. The decrease of cytosolic DNA fragments in stimulated macrophages incubated with urea shows that the proliferative actions of urea are associated with a decrease of diminished NO-mediated apoptosis.

Conclusions. These data demonstrate that inhibition of iNOS-dependent NO production caused by urea enhances macrophage proliferation as a consequence of diminished NO-mediated apoptosis. This fact may be important for the development of atherosclerotic lesions during chronic renal failure and is in accordance with recently published studies showing that under conditions with decreased constitutive NOS activity, iNOS might substitute the synthesis of NO. iNOS expression in vascular smooth muscle cells and macrophages is supposed to prevent restenosis following angioplasty or heart transplant vasculopathy. This is supported by the fact that specific inhibition of endogenous iNOS activity with L-N^6-(1-iminoethly)-lysine accelerates the progression of vasculopathy in transplantation atherosclerosis.

Atherosclerosis is a major cause of morbidity and mortality in chronic renal failure (CRF) [1, 2] and is associated with the proliferation of macrophages within atherosclerotic lesions. Vascular disease in uremic patients cannot be entirely explained by the prevalence of established risk factors for atherosclerosis such as hypertension or an increase in the serum levels of triglycerides, low-density lipoproteins, or lipoprotein(a) [3]. Because the progression of atherosclerosis as a consequence of decreased nitric oxide (NO) synthesis has been described, we investigated the correlation between the inhibition of inducible NO synthase (iNOS) by urea, macrophage proliferation as assayed by cell counting, tritiated-thymidine incorporation and measurement of cell protein, and macrophage apoptosis.

Key words: apoptosis, atherosclerosis, chronic renal failure, vascular disease, thymidine incorporation, transplantation.

METHODS

Materials

[^{3}H]thymidine (20 Ci/mmol) was obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA). 2,2′-(Hydroxynitrosohydrazino) bis-etanamine (NOC 18) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) were obtained from Dojindo Laboratories (Tokyo, Japan). Rabbit anti-iNOS polyclonal antibody and purified iNOS protein was supplied by Calbiochem (San Diego, CA, USA). Cell culture materials, *Escherichia coli* lipopolysaccharide serotype 055:B5 (LPS), and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 (ATCC TIB 67) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 25 mmol/L HEPES, 2 mmol/L glutamine, 100 U penicillin/mL, and 100 μg streptomycin/mL at 37°C, 5% CO_2, and 95% humidity. Cells were studied between passages 7 to 20. Cells were seeded in 24-well dishes at a density of 2×10^5 cells/well, stimulated by LPS (1 μg/mL) and incubated either with or without the indicated amount of urea for 48 hours.

Nitrite analysis

Nitrite was determined spectrophotometrically by using the Griess reagent (0.5% sulfanilic acid, 0.002% N-1-naphtyl-ethylenediamine dihydrochloride, 14% glacial acetic acid) in supernatants. Absorbance was measured at 550 nm with baseline correction at 650 nm, and nitrite concentration was determined using sodium nitrite as a standard [4].

Oxyhemoglobin preparation

Oxyhemoglobin was prepared from methemoglobin by reduction with excess sodium dithionite. Oxyhemoglobin was desalted and purified by passage through an Econopac 10-DG column (Bio-Rad Laboratories, Richmond, CA, USA) and identified spectrophotometrically [5].

Tritiated thymidine incorporation

Macrophage growth was assayed by the incorporation of [^{3}H]thymidine (20 Ci/mmol) into cellular DNA. [^{3}H]thymidine (1 μCi/well) was added directly to the culture medium for the last two hours of incubation. The medium was aspirated, and the cells were washed with 500 μL phosphate-buffered saline (PBS) on ice before the addition of 500 μL methanol. Cell DNA was precipitated by adding 500 μL of 10% trichloroacetic acid. Precipitates were lyzed with 200 μL of 300 mmol/L sodium hydroxide containing 1% sodium dodecyl sulfate (SDS). Samples were aspirated and subjected to liquid scintillation counting.

Cell number determination

To determine the number of RAW 264.7 cells during culture, the number of adherent cells was counted within standard-sized areas (0.25 mm^2) in each of three wells by inverted, phase-contrast microscopy.

Cell protein

Protein was determined according to the method of Bradford using bovine serum albumin (BSA) as the standard [6].

DNA fragmentation assay

Cytosolic DNA fragments were quantitated by a cell death detection enzyme-linked immunosorbent assay (ELISA; Boehringer Mannheim, Mannheim, Germany). The assay is based on the sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against histone-associated DNA fragments. Cells were washed with PBS and lyzed with ELISA buffer. The lysates were centrifuged at 200 × *g* for 10 minutes. The histone-associated DNA fragments of the supernatant were linked to the biotinylated antihistone antibody bound to the streptavidin-coated microtiter plate as described by the manufacturer. The DNA part of the nucleosomes was detected by peroxidase-labeled anti-DNA antibody. The amount of histone-associated DNA fragments was quantitated spectrophotometrically with 2.2′-azino-di(3-ethylbenzthiazolin-sulfonate) as the substrate. Samples were read at 405 and 492 nm on a Perkin-Elmer Lambda 2 Spectrophotometer.

Western blotting for inducible nitric oxide synthase

Cells were lyzed in ice-cold buffer containing 25 mmol/L monosodium phosphate (pH 7.4), 75 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 100 μg/mL phenylmethylsulfonyl fluoride, 10 μg/mL antipain, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 20 μg/mL aprotinin, and 10 μg/mL trypsin inhibitor and centrifuged at 50,000 *g* for 20 minutes at 4°C. The cytosolic proteins (20 μg per lane) were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose filters and then immunoblotted with a rabbit anti-iNOS polyclonal antibody at a 1:1000 dilution. Anti-rabbit horseradish peroxidase-conjugated antibody was used as a secondary antibody at a dilution of 1:2500. The blots were detected with the enhanced chemiluminescence method and exposed to photographic film.

Semiquantitative reverse transcription-polymerase chain reaction

Total RNA was isolated using the guanidinium thiocyanate method [7]. To determine the RNA concentration, the absorption at 260, 280, and 320 nm was measured photometrically (UV/VIS Spectrophotometer Lambda 2; Perkin-Elmer, Norwalk, CT, USA). Single-stranded cDNA synthesis was carried out on 2 μg of total RNA primed with oligo(dT)$_{12-18}$ (Pharmacia, Freiburg, Germany) using murine leukemia virus reverse transcriptase (MMLV-RT; MBI Fermentas, Vilnius, Lithuania) at 37°C for 60 minutes. Reactions were stopped by heating for five minutes at 70°C. iNOS cDNA was subjected to DNA amplification by polymerase chain reaction (PCR) using 0.5 units of Taq DNA polymerase (MBI) with oligonucleotide primers complementary to murine iNOS cDNA (MWG-Biotec, Ebersberg, Germany) at a final concentration of 0.25 μmol/L. Reaction mixtures were subjected to the following conditions in a PE 2400 DNA thermal cycler (Perkin-Elmer): denaturing at 94°C for 30 seconds, annealing at 55°C for 35 seconds, and extension at 72°C for 35 seconds. After 35 cycles, the reaction mixture was cooled down to 4°C. The primers for iNOS were 5′-CTA AGA GTC ACC AAA ATG GCT CCC-3′ (sense) and 5′-ACC AGA GGC AGC ACA TCA AAG C-3′ (antisense). The expected product length was 775 bp. As a control, we used the following primers for the "housekeeping gene" β-actin: 5′-ATG GTG GGA ATG GGT CAG AAG GAC-3′(sense) and 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′ (anti-

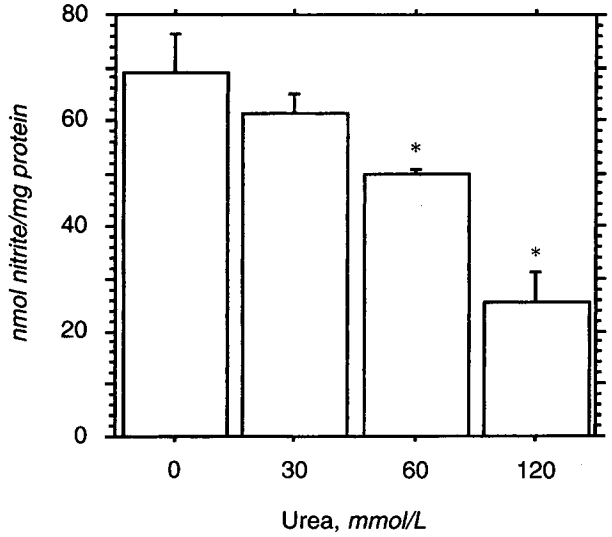

Fig. 1. Dose-dependent inhibition of inducible nitric oxide (NO) production by urea in RAW 264.7 cells. Cells were activated with 1 μg/mL lipopolysaccharide (LPS) and were incubated with increasing amounts of urea as indicated. NO production was determined as nitrite accumulation in the medium after 48 hours as described in the **Methods** section. Each bar shows the mean of triplicate measurements. Error bars show the standard deviation. $*P < 0.05$ when compared with controls without urea.

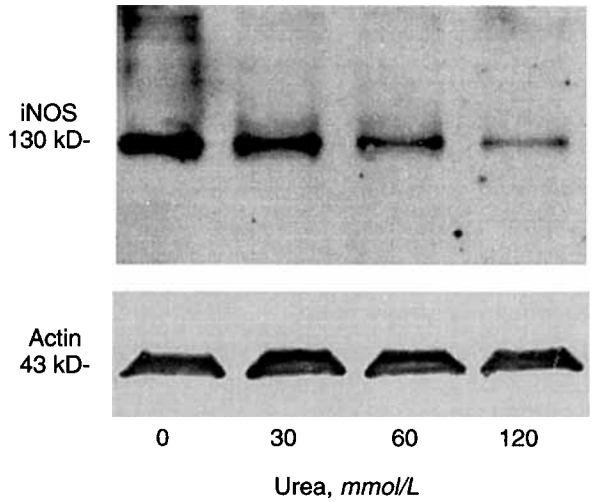

Fig. 2. Immunoblotting against inducible NO synthase (iNOS). Lipopolysaccharide (LPS)-stimulated RAW 264.7 mouse macrophages were cultured with increasing amounts of urea (0 to 120 mmol/L) for 48 hours. Western blotting was performed as described in the **Methods** section. Lane 1, control incubations (stimulated RAW 264.7 mouse macrophages without urea); lanes 2 through 4, incubations containing 30 to 120 mmol/L urea. Immunoblotting identified a band with an estimated molecular mass of 130 kD in stimulated RAW 264.7 mouse macrophages. iNOS protein was markedly reduced in cells incubated with urea (lanes 2 through 4). In contrast, actin (43 kD) levels remained unchanged during incubations of LPS-stimulated RAW 264.7 mouse macrophages with increasing amounts of urea.

sense). PCR conditions were as mentioned previously in this article with the exception that only 30 cycles were run. The expected product length was 513 bp. All PCR reactions were in linear range. Final PCR products were separated on a 1.2% agarose gel and detected by ethidium bromide staining. Semiquantitative estimation was done by comparing mRNA expression of iNOS to β-actin represented by the amount of the PCR product formed.

Data analysis

Each experimental result as shown in the figures is the mean ± SD for at least three measurements. When the SD is not displayed, it is smaller than the size of the symbol. Statistical analyses were performed using analysis of variance followed by Student's *t*-tests for unpaired data. Statistical significance was defined as $P < 0.05$.

RESULTS

Urea induces a dose-dependent inhibition of inducible NO synthesis in lipopolysaccharide-stimulated mouse macrophages (Fig. 1). Monolayers of RAW 264.7 macrophages were activated with 1 μg/mL LPS and incubated with increasing amounts (0 to 120 mmol/L) of urea as indicated. After 48 hours, the culture media were collected and assayed for nitrite. Activated RAW 264.7 cells released large amounts of nitrite into the culture medium (69 ± 7.3 nmol nitrite per mg protein within 48 hours). Incubation of activated RAW 264.7 cells with increasing amounts of urea was associated with a dose-dependent reduction in NO production. Measurements of cytotoxicity were performed to exclude toxic effects of urea towards RAW 264.7 cells. Up to a concentration of 150 mmol/L urea, cell viabilities were more than 95%, as measured by trypan blue exclusion. To exclude an interference of urea with the detection of nitrite by the Griess reaction, we incubated the NO donor NOC 18 (30 μg/mL) with increasing amounts urea. Urea (0 to 150 mmol/L) failed to affect nitrite measured from the NO donor NOC 18 for up to 48 hours (data not shown).

Figure 2 shows the Western blot analysis of iNOS in RAW 264.7 cells. LPS-stimulated macrophages were cultured with increasing amounts of urea (0 to 120 mmol/L) for 48 hours. Immunoblotting shows a band with an estimated molecular mass of 130 kD (the known molecular mass of iNOS) in stimulated RAW 264.7 mouse macrophages. An identical molecular mass was determined by blotting against purified iNOS protein. iNOS protein showed a dose-dependent reduction when cells were incubated with 0 to 120 mmol/L urea. In contrast, actin (43 kD) levels remained unchanged during incubations of LPS-stimulated RAW 264.7 mouse macrophages with increasing amounts of urea. This shows that high concentrations of urea do not cause a generalized decrease in protein expression. We performed additional experiments by the use of RT-PCR to determine iNOS mRNA levels. iNOS mRNA levels

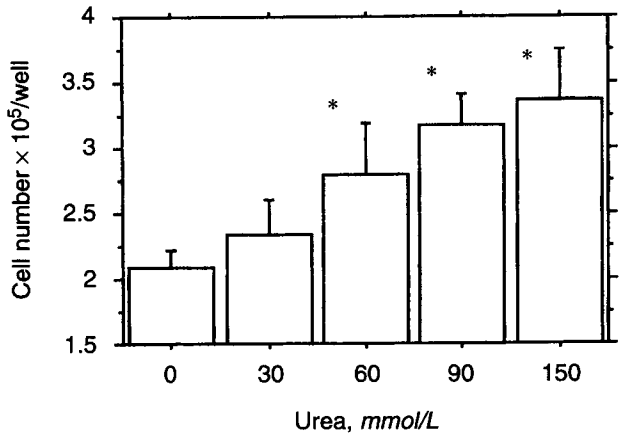

Fig. 3. Urea-induced macrophage proliferation. RAW 264.7 macrophages were stimulated with LPS and were incubated with increasing amounts (0 to 150 mol/L final concentration) of urea for 48 hours. Cell counting was performed by phase-contrast microscopy as described in the **Methods** section and is shown as cell number × 10^5 per well. Data show the mean of triplicate measurements ± SD and are representative of three independent experiments. *$P < 0.05$ compared with control incubations without urea.

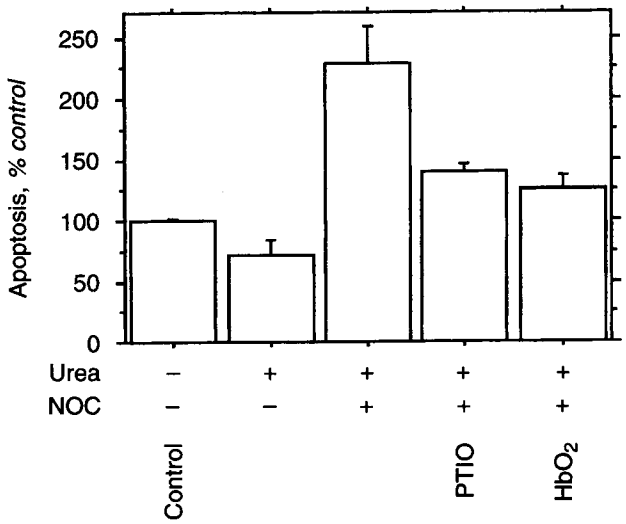

Fig. 4. Inhibition of inducible NO synthesis by urea prevents apoptosis in RAW 264.7 macrophages. Apoptosis was quantitated by detection of histone-associated DNA fragments. Stimulated RAW 264.7 mouse macrophages were cultured with or without 120 mmol/L urea for 12 hours. Urea inhibits apoptosis of macrophages expressing iNOS activity ($P < 0.02$). Decreased DNA fragmentation by urea was increased by the NO donor NOC 18 (30 μg/mL, $P < 0.005$). The NO-mediated increase in apoptosis could be anti-agonized by adding the NO scavengers carboxy-PTIO (10 μg/mL, $P < 0.01$) and oxygenated hemoglobin (100 μmol/L HbO_2, $P < 0.01$). Error bars represent SD.

were not reduced by urea. This confirms previous results [8] and hints at a post-transcriptional mechanism of urea-induced iNOS inhibition.

Urea-induced iNOS inhibition was associated with macrophage proliferation (Fig. 3). The number of macrophages per well was determined by inverted phase-contrast microscopy. Cells were seeded at a density of 2 × 10^5 per well in 24-well plates. Stimulation by LPS reduced the proliferation of RAW 264.7 cells within 48 hours from 183 ± 17% (increase in cell number) for unstimulated macrophages to 4.7 ± 6.1% for stimulated cells. Macrophage proliferation was enhanced because of the addition of urea to stimulated cells. Cell number increased from 2.09 ± 0.12 × 10^5 cells/well for control incubations without urea to 3.18 ± 0.22 × 10^5 cells/well for 90 mmol/L urea and 3.36 ± 0.38 × 10^5 cells/well for 150 mmol/L urea ($P < 0.05$). Macrophage proliferation as determined by cell counting became statistically significant within 48 hours at 60 mmol/L urea corresponding to a blood urea nitrogen level of 180 mg/100 mL, concentrations seen in uremic patients. Urea-induced proliferation was inhibited by the NO donor NOC 18 (data not shown). The inhibition of cell proliferation by NOC 18 could be antagonized by adding the NO scavengers carboxy-PTIO and oxygenated hemoglobin. The addition of urea to unstimulated cells resulted in a decrease of cell number, which was not statistically significant. The addition of the NOS inhibitor L-NAME (300 μmol/L final concentration) to LPS-stimulated macrophages increased the cell number after a 48-hour incubation. This was accompanied by an almost complete inhibition of NO synthesis. The addition of urea to L-NAME containing incubations showed no further statistically significant increase in cell number. This hints to the fact that urea-induced proliferation of stimulated macrophages is a consequence of iNOS inhibition. The estimation of cell proliferation by measuring cell protein or thymidine incorporation gave comparable results.

The decrease of cytosolic DNA fragments in stimulated macrophages incubated with urea shows that the proliferative actions of urea are associated with a decrease of NO-induced apoptosis. NO-induced apoptosis has been described for various cell lines, including monocyte/macrophages [9–11]. To test whether urea-induced inhibition of inducible NO synthesis prevents macrophage apoptosis, we assessed cytosolic DNA fragments. Urea (120 mmol/L) inhibits the apoptosis of macrophages expressing iNOS activity ($P < 0.02$; Fig. 4). The reduced DNA fragmentation by urea was increased by the NO donor NOC 18 (30 μg/mL, $P < 0.005$). The NO-mediated increase in apoptosis could be antagonized by adding NO scavengers carboxy-PTIO (10 μg/mL, $P < 0.01$) and oxygenated hemoglobin (100 μmol/L HbO_2, $P < 0.01$). The decrease of cytosolic DNA fragments in stimulated macrophages incubated with urea shows that the proliferative actions of urea are associated with a decrease of NO-induced apoptosis.

DISCUSSION

Macrophage proliferation is a hallmark for the development of atherosclerotic lesions [12]. Decreased NO

synthesis is supposed to contribute to the development of atherosclerotic lesions [13, 14]. Morphometric analyses revealed a marked enlargement of intimal atherosclerotic areas in aortas from L-NAME–treated animals. L-NAME inhibits both inducible and constitutive NOS. Anti-atherogenic properties of NO include antiproliferative actions such as the inhibition of smooth muscle and T-cell proliferation [15], reduced neutrophil adhesion [16, 17], inhibition of platelet activation [18], and the reduction of endothelial hyperpermeability [19]. The low release of NO both basal and stimulated has been reported for atherosclerotic vessels [20].

Inducible nitric oxide synthesis-dependent NO release may play protective roles in oxidative modification of LDL during the atherosclerosis process [21]. Therefore, urea-induced inhibition of inducible NO synthesis could result in an increased lipoprotein oxidation. Because the inhibition of iNOS by ox-LDL has been described, this could result in a vicious circle by a further lipoprotein oxidation with concomitant iNOS inhibition.

Nitric oxide is synthesized from L-arginine by the L-arginine-NO pathway [22] and is converted to nitrite and nitrate in oxygenated solutions [23]. A family of enzymes, termed the nitric oxid synthases (NOSs), catalyze the formation of NO and citrulline from L-arginine, molecular O_2, and NADPH [24]. The constitutes NOS isoforms (NOS-1 and NOS-3) produce low levels of NO as a consequence of an increased intracellular Ca^{2+} concentration [25]. In contrast, the inducible isoform of NOS (NOS-2 or iNOS) generates large amounts of NO over a prolonged period of time through a Ca^{2+}-independent pathway [26]. Inducible NOS expression has been observed in many cells, including murine macrophages [27], endothelial cells [28], smooth muscle cells [29], and cardiac myocytes [30]. NO inhibits the proliferation of vascular smooth muscle cells, mesangial cells, and fibroblasts [15, 31, 32].

Atherosclerosis-associated factors with the ability to reduce NO synthesis have been described. These factors include oxidatively modified low density lipoproteins (LDLs) [33], heat shock proteins (HSPs) [34], platelet-derived growth factor (PDGF) [35], and transforming growth factor-β (TGF-β) [36].

Inducibile nitric oxide synthase is expressed within macrophages of atherosclerotic lesions [37, 38]. iNOS was not detected in normal vessels, but widespread iNOS protein staining was found in macrophages, foam cells, and vascular smooth muscle cells of early and advanced atherosclerotic lesions. Inhibition of iNOS by urea might contribute to the development of atherosclerotic plaques by an increased proliferation of macrophages because of the diminished release of antiproliferative NO in these lesions.

As shown by our data, macrophage proliferation (increase in cell number) as a consequence of urea-induced inhibition of inducible NO synthesis became statistically significant at concentrations of 60 mmol/L urea corresponding to a blood urea nitrogen concentration of approximately 180 mg/100 mL. These concentrations are seen in uremic patients, implicating a pathophysiological relevance of urea-induced proliferation of macrophages expressing iNOS activity for the development of atherosclerotic lesions in chronic renal failure (CRF). Urea concentrations of up to 60 mmol/L are seen in patients suffering from CRF without renal replacement therapy [8, 39]. Thus, dialysis promises to prevent the urea associated proliferative effects by preventing urea-induced inhibition of iNOS.

Our data show that inhibition of iNOS-dependent NO production by urea enhances macrophage proliferation and is associated with diminished NO-mediated apoptosis. NO has previously been shown to inhibit DNA synthesis, cell growth, and division [15, 40]. In addition, induction of iNOS was shown to be associated with an increased apoptosis rate of murine peritoneal macrophages [9]. This was shown by microscopic examination of the cells, which revealed the presence of nuclear and cytoplasmic alterations characteristic of apoptosis, and by the specific pattern of internucleosomal DNA fragmentation [9]. NO-induced apoptosis is presumed to be due to cGMP-dependent protein kinase G activation [41]. As a consequence, NO synthesis by iNOS correlates inversely with macrophage life span in vitro. It has been proposed that NO-dependent death of murine peritoneal macrophages activated in vitro with interferon-γ and LPS is mediated through apoptosis. Inhibition of NO formation by N-monomethyl-L-arginine (L-NMA) diminishes apoptosis of LPS activated RAW 264.7 macrophages [42].

In addition to a local proliferation, the appearance of macrophages within atherosclerotic lesions has been suggested to be the consequence of an increased recruitment of monocytes via a nuclear factor-κB (NF-κB)–dependent expression of vascular endothelial cell adhesion molecule-1 (VCAM-1), macrophage colony-stimulating factor (M-CSF) and monocyte chemoattractant protein 1 (MCP-1) by human vascular endothelial cells. NO has been shown to inhibit VCAM-1 [16], M-CSF [43], and MCP-1 expression [17] by inhibiting NF-κB activation. The inhibition of NO synthesis by L-NMA was shown to activate NF-κB with concomitant VCAM-1, M-CSF, and MCP-1 expression [16, 17, 43]. Inhibition of inducible NO synthesis by urea might influence macrophage accumulation within atherosclerotic lesions in addition to a decreased apoptosis rate by enhancing NF-κB–dependent VCAM-1, M-CSF, and MCP-1 expression.

Formation of NO by the constitutive NOS expressed in endothelial cells is believed to protect against the development of atherosclerotic lesions. Under conditions with decreased constitutive NOS activity, iNOS might substi-

tute the synthesis of NO [44]. iNOS expression in vascular smooth muscle cells and macrophages may be beneficial as a compensatory mechanism for the lack of endothelial NO synthesis, thereby preventing restenosis following angioplasty or heart transplant vasculopathy [45]. This idea is supported by a recent article showing that the administration of an iNOS selective inhibitor L-N^6-(1-iminoethyl)-lysine accelerated the progression of vasculopathy in transplantation atherosclerosis, and transduction with iNOS using an adenoviral vector has been shown to completely suppress the development of allograft arteriosclerosis [46]. Induction of iNOS leads to a reduced proliferation in both medial and intimal smooth muscle cells [47], and iNOS was shown to restore blood flow in the injured artery after denudation of the rat carotid artery as a model for arterial injury and restenosis [44]. Thus, expression of iNOS in lesions may represent a protective mechanism that compensates for the loss of endothelium.

Inducible nitric oxide synthase gene transfer to rats and pigs has been shown to inhibit intimal hyperplasia in response to vascular injury in vivo. An adenoviral vector carrying human iNOS cDNA was used to express iNOS protein at sites of arterial injury. In vivo iNOS gene transfer to injured rat arteries resulted in a near complete (>95%) reduction in neointima formation even when followed long term to six-weeks postinjury. This protective effect was reversed by selective iNOS inhibition [48]. Vein graft failure as the result of intimal hyperplasia following coronary artery bypass is a significant clinical problem. Gene transfer of the *iNOS* gene to vein grafts has been shown to reduce intimal hyperplasia effectively [49]. According to these results, inducible NO synthesis can be supposed to be a compensatory mechanism for the lack of endothelial NO formation, thereby maintaining vascular homeostasis.

Numerous anti-atherogenic properties of NO have been described. NO inhibits smooth muscle cell proliferation [15], platelet activation [18], and neutrophil adhesion [50], and it inhibits NF-κB activation [17]. Our results support the hypothesis that inducible NO release protects against atherogenesis by preventing macrophage proliferation.

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