Urea induces macrophage proliferation by inhibition of inducible nitric oxide synthesis

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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 synthesis has been described, we investigated the correlation between the inhibition of inducible nitric oxide 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 nitric oxide 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 mM 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 NO-induced apoptosis.

Conclusions. Our data demonstrate that the inhibition of iNOS-dependent NO production caused by urea enhances macrophage proliferation as a consequence of diminished NO-mediated apoptosis.

Atherosclerosis is a major cause of morbidity and mortality in chronic renal failure (CRF) [1, 2]. 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]. The dose-dependent inhibition of inducible nitric oxide synthesis for lipopolysaccharide (LPS)-activated macrophages incubated with urea in vitro has been recently described [4].

The development of atherosclerotic lesions as a consequence of decreased nitric oxide (NO) synthesis has been reported [5, 6]. Anti-atherogenic properties of NO include antiproliferative actions, such as the inhibition of smooth muscle cell and T-cell proliferation [7], reduced neutrophil adhesion [8, 9], inhibition of platelet activation [10], and the reduction of endothelial hyperpermeability [11]. The low release of NO, both basal and stimulated, has been reported for atherosclerotic vessels [12]. Atherosclerosis-associated factors with the ability to reduce NO synthesis include oxidatively modified low-density lipoproteins [13] and heat-shock proteins [14].

Nitric oxide is synthesized from L-arginine by the L-arginine NO pathway [15] and is converted to nitrite and nitrate in oxygenated solutions [16]. A family of enzymes, termed the NO synthases (NOS), catalyze the formation of NO and citrulline from L-arginine, O2, and nicotinamide adenine dinucleotide phosphate (NADPH) [17]. The constitutive NOS isoforms (NOS-1 and NOS-3) produce low levels of NO as a consequence of increased intracellular Ca2+ [18]. By contrast, the inducible isoform of NOS (NOS-2 or iNOS) generates large amounts of NO over a prolonged period of time through a Ca2+-independent pathway [19]. iNOS expression has been observed in many cells, including murine macrophages [20], endothelial cells [21], smooth muscle cells [22], and cardiac myocytes [23]. NO inhibits the proliferation of vascular smooth muscle cells, mesangial cells, and fibroblasts [7, 24, 25]. High levels of NO induce changes suggestive of apoptosis in mouse peritoneal macrophages and in the RAW 264.7 mouse macrophage cell line [26–28].

Because the proliferation of macrophages within atherosclerotic lesions has been described [29, 30], we investigated the correlation between the inhibition of inducible NO synthesis by urea, macrophage apoptosis, and...
macrophage proliferation. Using LPS-stimulated macrophages, we examined the influence of iNOS antagonists (N-NAME), NO donors (NOC 18), and NO scavengers [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), hemoglobin] on urea-induced macrophage proliferation. The inhibition of inducible NO synthesis by urea might initiate macrophage proliferation because NO decreases cell proliferation by increasing the rate of apoptosis. This might be a key event for the development of atherosclerotic lesions in uremia.

**METHODS**

**Materials**

[3H]Thymidine (20 Ci/mmol) was obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA). 2,2'-[Hydroxy-nitrosohydrazino] bis-etanamine (NOC 18) and carboxy-PTIO were obtained from Dojindo Laboratories (Tokyo, Japan). Rabbit anti-iNOS polyclonal antibody and purified iNOS protein were 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 (FBS), 25 mM HEPES, 2 mM glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml at 37°C, 5% CO2, and 95% humidity. Cells were studied between passages 7 to 20. Cells were seeded in 24-well dishes at a density of 2 × 10⁵ cells per 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-naphthylethylenediamine dihydrochloride, 14% glacial acetic acid) in supernatants. Absorbance was measured at 550 nm, with baseline correction at 650 nm, and the nitrite concentration was determined using sodium nitrite as a standard [31].

**Oxyhemoglobin preparation**

Oxyhemoglobin was prepared from methemoglobin by reduction with excess sodium dithionite. Oxyhemoglobin was desalted and purified by passage through a Econopac 10-DG column (Bio-Rad Laboratories, Richmond, CA, USA) and was identified spectrophotometrically [32].

**Tritiated thymidine incorporation**

Macrophage growth was assayed by the incorporation of [3H]thymidine (20 Ci/mmol) into cellular DNA. [3H]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 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 mM sodium hydroxide containing 1% sodium dodecyl sulfate. 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 mm²) 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 as standard [33].

**DNA fragmentation assay**

Cytosolic DNA fragments were quantitated by a cell death detection enzyme-linked immunosorbent assay (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 phosphate-buffered saline and lyzed with enzyme-linked immunosorbent assay buffer. The lysates were centrifuged at 200 g for 10 minutes. The histone-associated DNA fragments of the supernatant were linked to the biotinylated anti-histone 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 nm 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 mM monosodium phosphate (pH 7.4), 75 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 100 μg/ml phenylmethylsulfonylfluoride, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 20 μg/ml aprotenin, and 10 μg/ml trypsin inhibitor and were 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 electrophore-
Measurements of cytotoxicity were performed to exclude toxic effects of urea toward RAW 264.7 cells. Up to a concentration of 150 mM 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 mM) failed to affect nitrite measured from the NO donor NOC 18 for up to 48 hours (data not shown).

**Effects of urea on inducible nitric oxide synthase protein expression**

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 mM) for 48 hours. Immunoblotting shows a band with an estimated molecular mass of 130 kDa (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 mM urea. In contrast, actin (43 kDa) 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.

**Effects of urea on macrophage growth**

Tritiated thymidine incorporation was determined in LPS-stimulated and -unstimulated cells to examine whether inhibition of inducible NO synthesis by urea stimulates the proliferation of RAW 264.7 macrophages. iNOS induction by LPS reduced basal tritiated thymidine incorporation of RAW 264.7 cells by more than 90%. The inhibition of basal tritiated thymidine incorporation was antagonized by urea. As shown in Figure 3, the tritiated thymidine uptake increased 9.8-fold after a 48-hour incubation with 90 mM urea for stimulated cells in contrast to a slight decrease for unstimulated cells when compared with controls without urea. Tritiated thymidine incorporation increased in a dose-dependent manner up to 120 mM urea for LPS-stimulated macrophages. Higher urea concentrations decreased the incorporation of tritiated thymidine into cellular DNA (data not shown). These results suggest that urea has the capability to augment growth (that is, increase in cell number) of LPS-stimulated RAW 264.7 macrophages.

In addition, the number of macrophages per well was determined by inverted phase-contrast microscopy for LPS-stimulated cells to prove that urea stimulates the proliferation of RAW 264.7 macrophages (Fig. 4). Cells were seeded at a density of 2 × 10^6 per well in 24-well plates. Stimulation by LPS reduced the proliferation of RAW 264.7 cells within 48 hours from 183 ± 17% (in-
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Fig. 2. Immunoblotting against inducible nitric oxide synthase (iNOS). Lipopolysaccharide (LPS)-stimulated RAW 264.7 mouse macrophages were cultured with increasing amounts of urea (0 to 120 mM) 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 mM urea. Immunoblotting identified a band with an estimated molecular mass of 130 kDa in stimulated RAW 264.7 mouse macrophages. iNOS was markedly reduced in cells incubated with urea (lanes 2 through 4). In contrast, actin (43 kDa) levels remained unchanged during incubations of LPS-stimulated RAW 264.7 mouse macrophages with increasing amounts of urea.

Fig. 3. Effects of urea on macrophage proliferation measured as tritiated thymidine incorporation. Monolayers of RAW 264.7 macrophages were activated with 1 μg/ml LPS (○) or not activated (●) and incubated with increasing amounts (0 to 120 mM f.c.) of urea as indicated for 48 hours. [3H]Thymidine was added directly to the incubation medium, and thymidine incorporation into DNA was analyzed as described in the Methods section. Data show the mean of triplicate measurements ± sd and are representative of three independent experiments.

Fig. 4. Urea-induced macrophage proliferation. RAW 264.7 macrophages were stimulated with lipopolysaccharide (LPS) and were incubated with increasing amounts (0 to 150 mM f.c.) 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 \times 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.

cells/well for control incubations without urea to 3.18 ± 0.22 \times 10^5 cells/well for 90 mM urea and 3.36 ± 0.38 \times 10^5 cells/well for 150 mM urea (P < 0.05). The addition of urea to unstimulated cells resulted in a decrease of cell number from 5.68 ± 0.35 \times 10^5 cells/well for control
incubations without urea to $4.44 \pm 0.86 \times 10^5$ cells/well for 90 mM urea and $4.16 \pm 0.79 \times 10^5$ cells/well for 150 mM urea (differences not statistically significant). The estimation of cell proliferation by measuring cell protein, as described in the Methods section, gave comparable results. A correlation between the means of thymidine incorporation and cell count was linear with a correlation coefficient of 0.94. The correlation between the means of cell protein and cell count was linear with $r = 0.99$.

The addition of the NOS inhibitor L-NAME (300 µM final concentration) to LPS-stimulated RAW 264.7 macrophages increased the cell number after a 48-hour incubation from $2.09 \pm 0.12 \times 10^5$ (control incubations without L-NAME) to $2.94 \pm 0.25 \times 10^5$ cells/well. This was accompanied by an almost complete inhibition of NO synthesis. The addition of urea (0 to 150 mM) to L-NAME containing incubations showed no further statistically significant increase in cell number (data not shown). This hints to the fact that urea-induced proliferation of stimulated macrophages is a consequence of iNOS inhibition.

**Effect of nitric oxide on urea-induced macrophage proliferation**

We next examined whether the increased macrophage proliferation could be antagonized by the addition of a NO donor (NOC 18) and if the antiproliferative actions of NO could be reversed by NO scavengers (carboxy-PTIO, hemoglobin). Figure 5 shows the increased proliferation of LPS-stimulated macrophages by 120 mM urea in vitro, as quantitated by cell counting after a 48-hour incubation. Urea-induced proliferation was inhibited by the NO donor NOC 18 (30 µg/ml, $P < 0.005$). This inhibition of cell proliferation could be antagonized by adding the NO scavengers carboxy-PTIO (10 µg/ml, $P < 0.01$) and oxygenated hemoglobin (100 µM HbO2, $P < 0.01$). Error bars represent sd.

**Effects of urea and nitric oxide on macrophage apoptosis**

Nitric oxide-induced apoptosis has been described for various cell lines [26, 34, 35]. To test if urea-induced inhibition of inducible NO synthesis prevents apoptosis in RAW 264.7 macrophages, we assessed cytosolic DNA fragments as described in the Methods section. Urea (120 mM) inhibits the apoptosis of macrophages expressing iNOS activity ($P < 0.02$; Fig. 6). 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 the NO scavengers carboxy-PTIO (10 µg/ml, $P < 0.02$) and oxygenated hemoglobin (100 µM HbO2, $P < 0.01$). Error bars represent sd.
DISCUSSION

This article shows a correlation between the urea-induced inhibition of iNOS and macrophage growth. Recently, the inhibition of iNOS by urea has been demonstrated [4]. When activated cells were incubated with urea up to 100 mM, almost complete inhibition of inducible NO synthesis was observed. Northern blot analysis of iNOS mRNA showed a less than 10% difference in signal between LPS and LPS + urea incubations, whereas differences of NO synthesis between these groups were more than 400%. Our results show a distinct reduction of iNOS protein in cells incubated with increasing amounts of urea as revealed by immunoblotting. From these data it can be concluded that the inhibitory effects of urea toward inducible NO synthesis seem to act on a translational level.

The development of atherosclerotic lesions as a consequence of chronic inhibition of NO production by L-NAME has been reported [5, 6]. 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. The expression of iNOS within macrophages of atherosclerotic lesions has been described [36, 37]. 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. The 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.

Macrophages are commonly detected in experimental and human atherosclerotic plaques. Autoradiographic and ultrastructural observations in human fibro-atheromatous plaques showed that most of the autoradiographic staining appeared on foam cells and monocyte-like cells, thus suggesting a local proliferation of these cells [29]. Simultaneous thymidine autoradiography and immunostaining with cell-type–specific monoclonal antibodies revealed that approximately 30% of the labeled cells were macrophages and 45% were smooth muscle cells in advanced atherosclerotic lesions [30]. Increased macrophage proliferation resulting from a urea-induced iNOS inhibition might enhance the development of atherosclerotic lesions.

As shown in Figure 4, macrophage proliferation (increase in cell number) as a consequence of urea-induced inhibition of inducible NO synthesis became statistically significant at concentrations of 60 mM 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 CRF. Urea concentrations of up to 60 mM are seen in patients suffering from CRF without renal replacement therapy [4, 38]. Nowadays, such values cannot be considered an adequate target of urea control because the widespread use of dialysis has become state-of-the-art therapy for patients suffering from CRF. 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 [7, 39]. In addition, induction of iNOS was shown to be associated with an increased apoptosis rate of murine peritoneal macrophages [26].

As a consequence, NO synthesis by iNOS correlates inversely with macrophage lifespan in vitro. It has been proposed that the NO-dependent death of murine peritoneal macrophages activated in vitro with interferon-γ and LPS is mediated through apoptosis. 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 detected by electrophoresis [26]. NO-induced apoptosis is presumed to be due to cGMP-dependent protein kinase G activation [40]. Inhibition of NO formation by N-monomethyl-L-arginine (L-NMA) diminishes apoptosis of LPS-activated RAW 264.7 macrophages [41].

According to our opinion, there is no need to reduce erythropoietin administration during the treatment of anemia found in patients suffering from CRF, although hemoglobin is known to antagonize the biological effects of NO and reduces NO-induced apoptosis, as shown in Figure 6. Erythropoietin administration raises hematocrit levels and increases hemoglobin, which is found predominantly within the erythrocyte and only to a minor extent in its free form in the plasma. On the other hand, macrophage proliferation representing a hallmark for the development of atherosclerotic lesions takes place within the vessel wall, where the cellular populations can be considered to be “out of reach” for almost any free hemoglobin.

The appearance of macrophages within atherosclerotic lesions has been supposed 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 chemotactic protein-1 (MCP-1) by human vascular endothelial cells. NO has been shown to inhibit VCAM-1 [8], M-CSF [42], and MCP-1 expression [9] by inhibiting NF-κB activation. The inhibition of NO synthesis by L-NMA was shown to activate NF-κB with concomitant

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VCAM-1, M-CSF, and MCP-1 expression [8, 9, 42]. 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. Although our data suggest a causative role of urea-induced inhibition of inducible NO synthesis with respect to inflammation and atherosclerosis, it is still unclear whether changes in cellular proliferation is a consequence of diminished NO synthesis.

Numerous anti-atherogenic properties of NO in vitro have been described. NO inhibits smooth muscle cell proliferation [7], platelet activation [10], neutrophil adhesion [43], and MCP-1 expression [9] and inhibits NF-κB activation [9, 42]. Our results support the hypothesis that inducible NO release protects against atherogenesis by preventing macrophage proliferation. Urea might contribute to the development of atherosclerotic lesions associated with CRF and elevated blood urea nitrogen levels by inducing NO synthesis with concomitant macrophage proliferation as a consequence of diminished macrophage apoptosis.

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