Urea inhibits inducible nitric oxide synthase in macrophage cell line

SHARMA S. PRABHAKAR,[1,2] GUILLERMO A. ZEBALLOS,[1]
MARTIN MONTOYA-ZAVALA,[3] AND CLAIRE LEONARD[3]
[1]*Renal Section, Veterans Affairs Medical Center, Bronx 10468;* [2]*Department of Medicine,*
Mount Sinai School of Medicine, New York, New York 10029; and [3]*Department of*
Molecular Biology, Alteon Inc., Ramsey, New Jersey 07446

Prabhakar, Sharma S., Guillermo A. Zeballos, Martin Montoya-Zavala, and Claire Leonard. Urea inhibits inducible nitric oxide synthase in macrophage cell line. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C1882–C1888, 1997.— Macrophage dysfunction is considered an important contributory factor for increased propensity of infections in uremia. Because nitric oxide (NO) is believed to be an effector molecule of macrophage cytotoxicity, we propose that the dysfunction may be related to impaired NO synthesis. To verify this hypothesis, we evaluated macrophage NO synthesis in the presence of urea, a compound that accumulates in renal failure and is believed by some to be a uremic toxin. Macrophages (RAW 264.7 cells) were incubated with bacterial lipopolysaccharide to induce NO synthesis, whereas the test groups had various concentrations of urea in addition. NO synthesis was measured by assaying the supernatant for nitrites and nitrates by chemiluminescence. We observed that urea consistently produced a dose-dependent reversible inhibition of inducible NO production in macrophages, whereas parathormone, another toxin retained in uremia, had no such inhibitory effects. Further studies revealed that mRNA for inducible NO synthase was not inhibited by urea. We thus conclude that urea inhibits inducible NO synthesis in macrophages by a posttranscriptional mechanism and that this may be important in macrophage dysfunction of uremia.

inducible nitric oxide synthesis; macrophage dysfunction; uremia

INFECTIONS CONTINUE TO BE an important cause of morbidity and mortality in patients with end stage renal disease. Although several immunologic abnormalities have been described in uremia, the precise mechanisms responsible for the impaired host defenses remain unclear. Macrophages constitute the first line of host defenses in conferring immunity against infections in humans. Macrophages have been shown to be dysfunctional in uremia, and impaired function of macrophage Fc$_\gamma$ receptors was recently described in end stage renal disease (18). However, the impaired cytotoxicity of macrophages in uremia remains to be explained. Macrophages are now well known to produce nitric oxide (NO), and there is evidence to believe that NO may be an effector molecule of several functions of macrophages, especially cytotoxicity (6a, 7). It is thus possible that macrophage dysfunction in uremia may be related to defective NO production. We, therefore, studied the NO production from macrophages and the effect of some of the known uremic toxins such as urea. The results indicated that the urea inhibited synthesis of inducible NO synthase (iNOS) in activated macrophages. We further attempted to define the mechanisms of such an inhibition.

MATERIALS AND METHODS

Cell cultures. Murine peritoneum-derived macrophages (RAW 264.7 cell line) were obtained from the American Type Culture Collection, and *passages 5–10* were grown in RPMI 1640 (GIBCO BRL, Life Technologies, Grand Island, NY) enriched with fetal calf serum (GIBCO BRL). Medium was customized to contain L-glutamine but no nitrate to avoid interference with the nitrate assay. Cells were incubated at 1 $\times$ 10^5 cells/well in 96-well plates in a 5% CO$_2$ incubator at 37°C. NO synthesis was induced by bacterial lipopolysaccharide (LPS) derived from *Escherichia coli* serotype 026:B6 (Sigma Chemical, St. Louis, MO) at a concentration of 1 µg/ml in each well.

Chemicals. L-Arginine, murine parathyroid hormone (PTH), and urea were purchased from Sigma. Stock solutions of *E. coli* LPS serotype 026:B6 were stored at 4°C until needed. Endotoxin-tested fetal calf serum purchased from GIBCO BRL was used in the preparation of culture medium. Reconstituted culture medium was stored at 4°C until needed.

NO measurement. The supernatant of medium from each well was analyzed for NO production by chemiluminescence, using a Sievers Instruments NO analyzer (Boulder, CO). The NO produced by the cells is quickly converted to its stable end products, namely nitrite and nitrate. Nitrite-nitrate content is reduced to NO with a vanadium reducing mixture in a reaction tube on-line with the analyzer. The NO is propelled with nitrogen gas into another reaction chamber in the NO analyzer, where NO reacts with ozone to form NO$_2$. The luminescence associated with this reaction is read in millivolts and recorded as a deflection on a data recorder. The area under the curve reflects the NO content in the sample. This is read initially as a value in luminescence units, which when divided by the slope of a standard curve gives the precise NO content. The standard curve was made in culture medium spiked with nitrate. The NO activity of the untreated medium was subtracted from all the measurements to eliminate the baseline NO content.

mRNA extraction and reverse transcription-polymerase chain reaction. Total cellular RNA was extracted using the guanidinium thiocyanate method (3). After aspiration of the supernatant, the pellet of cells was washed with phosphate-buffered saline and resuspended at a concentration of 1 ml 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) buffer/10^7 cells. The lysate was passed through a pipette several times before addition of 0.1 ml of 2 M sodium acetate at pH 4.0, 1 ml of water-saturated phenol, and 0.2 ml 49:1 chloroform-isoamyl alcohol. The lysate was extracted by gentle mixing before incubation at 4°C for 15 min. The lysate was centrifuged at 10,000 *g* for 20 min. The aqueous phase was precipitated in an equal volume of 100% isopropyl alcohol at −20°C for 30 min. The RNA was further centrifuged at 10,000 *g* for 10 min. Once dissolved in 1/3 volume of GITC

buffer, the RNA was precipitated with 1 volume of 100% isopropyl alcohol for 30 min at −20°C. The RNA pellet was cleaned using 75% ethanol in diethyl pyrocarbonate (DEPC)-treated water. The RNA pellet was resuspended in DEPC-treated water. RNA was quantitated by spectrophotometry at 260 nm. The purity was assessed by measuring the 260 nm-to-280 nm ratio. For reverse transcription-polymerase chain reaction (RT-PCR), the primers were designed from the highly homologous region of the published mouse cDNA sequences and chemically synthesized by Biosynthesis (Lewisville, TX). The cDNA was then amplified with specific primers for 30 cycles with 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The PCR products were size fractionated on 1.2 agarose gel and detected by ethidium bromide staining.

Northern blot analysis. RAW 264.7 cells were grown in large 150 cm² flasks to the point of confluence and further incubated with fresh medium. The test groups had either LPS alone or LPS and urea, whereas the control group had neither. The incubations were carried out for 18 h at 37°C in a CO_2 incubator. At the end of the incubation, the supernatant was collected separately for NO analysis and the cells were centrifuged into a pellet for mRNA extraction. Total cellular RNA was extracted from RAW 264.7 cells in guanidine thiocyanate. Proteins were denatured and eliminated by phenol-chloroform-isoamyl alcohol extraction. The total RNA was precipitated using isopropanol. Contaminating DNA was removed by digestion with ribonuclease-free deoxyribonuclease (Promega, Madison, WI). Approximately 50 µg of total RNA was electrophoresed through a 1% agarose-formaldehyde gel, transferred to GeneScreen nylon membrane (DuPont-NEN, Boston, MA), and vacuum dried at 80°C for 2 h, followed by ultraviolet cross-linking (under conditions recommended by manufacturer). iNOS mouse macrophage cDNA probe (Cayman Chemical, Ann Arbor, MI) was labeled with ^{32}P, using the random prime kit (Boehringer Mannheim) to a specific activity of ~2×10^8 counts·min^{-1}·µg^{-1}. The labeled probe was then hybridized to the membrane in 50% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), and 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M NaHPO$_4$, and 0.001 M EDTA, pH 7.4). The hybridization was done at 42°C for 24 h. The membranes were washed once at room temperature for 15 min with 2× SSPE. This was followed by a wash with 1× SSPE and 2% SDS at 65°C for 30 min, followed by a wash in 0.1× SSPE at room temperature for 15 min. Autoradiograms were prepared using Kodak XAR-5 film with intensifying screens at −80°C for 24 h.

Data analysis. All data are expressed as means ± SE. The data were analyzed using commercial computer software (SlideWrite Plus 2.0 for Windows, Advanced Graphics Software) for statistical significance, means, and SE. Comparisons between groups of samples were performed by independent *t*-test or repeated measures analysis of variance as appropriate. Specific comparisons between the means were done with Student-Newman-Keuls multiple range test. A value of $P < 0.05$ was considered statistically significant. The slope of the standard curves, the intercept, and the *r* values for nitrite-nitrate measurements were calculated by linear regression analysis, and all illustrations were made with SlideWrite Plus 2.0 for Windows. In some cases, the means of several experiments were used to illustrate the observations.

RESULTS

Previous work demonstrated that NO produced by activated macrophages may be an effector molecule in mediating the cytotoxicity (5, 6a). Macrophages are believed to be functionally defective in renal failure

(18). The nature of this functional alteration is not completely clear, and thus it is reasonable to hypothesize that the defect may relate to the ability of macrophages to produce NO in the uremic environment. To further explore this hypothesis, we evaluated the effects of urea, a metabolite that accumulates in renal failure, on the NO production in RAW 264.7 cells.

NO production in activated macrophages. Initially, we evaluated the ability of RAW 264.7 cells to synthesize NO by induction with bacterial endotoxin. RAW 264.7 cells were incubated at 1×10^5 cells/well in 96-well plates. After a 2-h incubation, the nonadherent cells were removed by aspiration of the supernatant and fresh medium was added. Bacterial LPS derived from the *E. coli* of the serotype 026:B6 was added to the test group to yield a final concentration of 1 µg/ml. The incubation was continued at 37°C for 18 h, after which the supernatants were collected for measurement of NO production. The NO activity of the untreated medium was subtracted from all the measurements to eliminate the baseline NO content. The NO content (as reflected by the nitrite and nitrate content in the medium) in the control group ($n = 8$) was 21.8 ± 2.1 µM, whereas the activated cells produced 93.2 ± 3.6 µM ($P < 0.001$). The magnitude of LPS induction of NO synthesis by macrophages was dose dependent. Although we found significant and progressively greater induction of NO synthesis with concentrations of 0.1, 1, and 10 µg/ml (Fig. 1), the cytopathic effects of LPS were distinctly prominent at a concentration of 10 µg/ml. We thus used an LPS concentration of 1 µg/ml in all our experiments. We also noted that the induction of NO synthesis was noticeable as early as after 6 h of incubation, with maximal activity seen after 18 h.

Effects of urea on NO synthesis. We found that urea inhibited the NO production by activated RAW 264.7
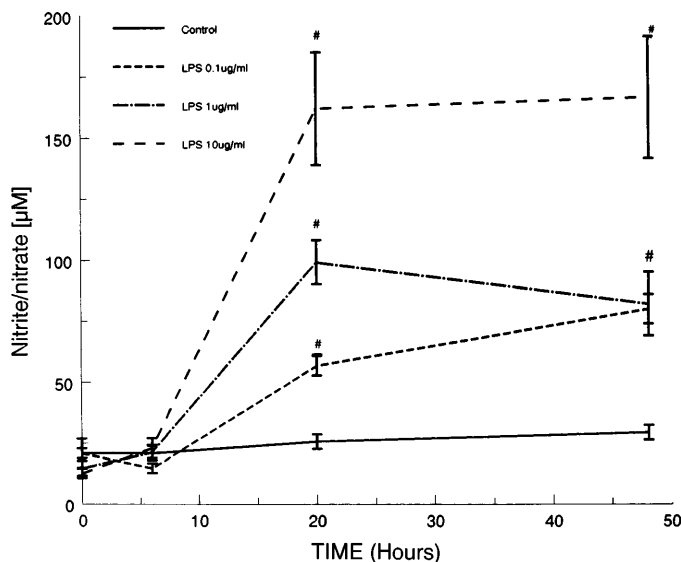

Fig. 1. Effects of bacterial lipopolysaccharide (LPS) on nitric oxide (NO) synthesis by macrophages expressed as nitrite-nitrate content in medium. Time- and dose-response relationship ($n = 8$ in control and all test groups). Values are means ± SE of 4 experiments. #$P < 0.05$ vs. control.
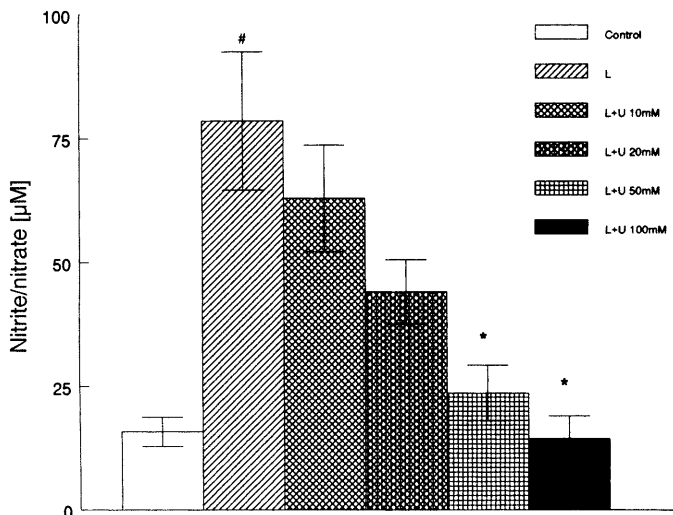
Fig. 2. Effects of urea (U) on LPS (L)-induced NO synthesis by macrophages: dose dependence of response. [#]$P < 0.05$ vs. all other groups;. [*]$P < 0.05$ vs. LPS alone; $n = 8$ in control and all test groups. Values are means ± SE in μM nitrite-nitrate content. Results are from 1 experiment representative of 6 similar experiments.

macrophages. The inhibition was concentration dependent (Fig. 2) and was seen at concentrations as low as 10 mM, and maximal inhibition was seen at 100 mM concentration. Because a concentration of 50 mM corresponds to a urea nitrogen of ~150 mg/100 ml, concentrations seen in uremic humans, we continued further experiments at a urea concentration of 50 mM in the culture medium.

When RAW 264.7 cells were incubated with LPS, urea inhibited the inducible NO synthesis significantly ($n = 8$). The LPS group had an NO activity of 93.2 ± 3.6 μM, whereas the control group had an activity of 21.8 ± 2.1 μM ($P < 0.001$). In the presence of urea, LPS failed to increase NO synthesis, generating only 22.7 ± 2.7 μM of nitrite-nitrate (vs. 28.6 ± 2.1 μM in cells incubated in urea without LPS), significantly lower than cells activated with LPS in the absence of urea ($P < 0.001$). This inhibition was not seen earlier than at 6 h and was best seen at 18 h of incubation.

Experiments were conducted to evaluate the effects of urea alone on NO production in the absence of LPS. We found that urea had no independent effects on NO synthesis at the end of 18 h of incubation [28.6 ± 2.1 vs. 23.8 ± 2.4 μM in the absence of urea; not significant (NS); $n = 8$]. Additionally, we performed experiments to assess whether the observed inhibition is a reversible phenomenon. Cells preincubated with urea at 50 mM for 20 h retained the ability to be stimulated by LPS when reincubated in LPS after urea washout. After 18 h of such reincubation, NO synthesis increased from 18.8 ± 2.7 to 57.7 ± 3.4 μM, ($n = 6$; $P < 0.01$). This is statistically not different from the control cell population, in which the NO synthesis increased from 17.5 ± 2.4 to 49.7 ± 3.1 μM with LPS induction ($n = 6$). These results further strengthen the observations that urea did not interfere with viability and that the inhibition is reversible. In addition, the cell groups initially incubated in urea with LPS were washed free of urea

and reincubated with LPS. The NO synthesis increased from 21.4 ± 3.0 μM (urea + LPS) to 54.9 ± 2.9 μM (reincubation with LPS) ($n = 6$; $P < 0.01$; Fig. 3). These observations confirm that the urea-induced inhibition of NO synthesis is reversible if urea is eliminated from the medium.

Effects of urea on cell viability. To evaluate the effects of urea on cell viability, we incubated the macrophages in the presence of urea (50 mM), and we found that under such conditions these cells exhibited growth and proliferation comparable to control conditions (i.e., in the absence of urea in the medium). When cells were incubated in 75-cm[2] flasks ($n = 4$) at a concentration of 10[6] cells/ml, the cell growth and proliferation at the end of 48 h of incubation were not significantly different between control conditions and in the presence of urea. The mean density of cell population as measured by a cell counter was 4.86×10^6 cells/ml in the control flask vs. 5.18×10^6 cells/ml in the flask containing urea in the medium. In addition, the time needed to attain confluence in the flask was similar under both conditions (4 days in both). Furthermore, the cell viability as measured with trypan blue staining was 97.5 vs. 98.1%, respectively, in the control and urea groups. These findings indicated clearly that urea did not affect the viability of the macrophages in culture.

Effects of PTH. To evaluate whether other known uremic toxins have an effect on NO production by macrophages, we examined the effects of PTH on NO production. PTH is incriminated in defective phagocytosis in uremia, but its effects on iNOS are unknown. We incubated RAW 264.7 cells with a rat PTH preparation at a concentration of 400 pg/ml, a concentration of PTH seen in rodent models of advanced renal failure. The control group had an NO content of 22.9 ± 2.3 μM,
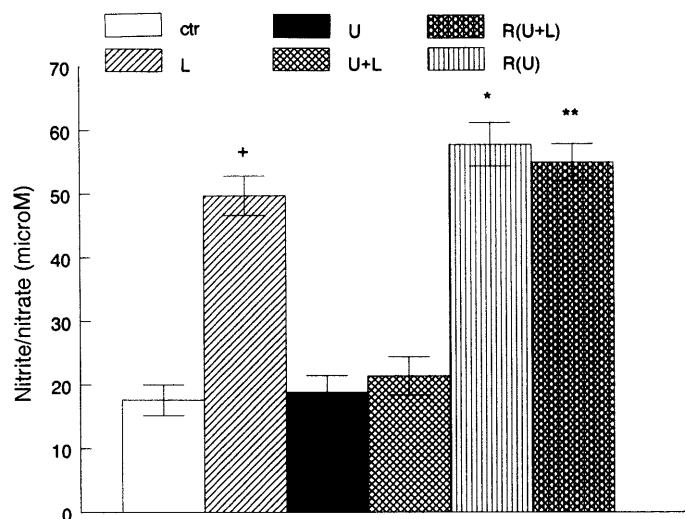

Fig. 3. LPS-induced NO synthesis in macrophages: effects of preincubation with urea. ctr, Control; R(U), cells grown in presence of urea, washed, and reincubated in presence of LPS in a urea-free medium; and R(U+L), cells previously grown in presence of urea and LPS, washed free of urea, and reincubated in fresh medium with LPS. Values are means ± SE in μM nitrite-nitrate content. Results are from 1 experiment typical of 6 others. [*]$P < 0.01$, R(U) vs. U; [**]$P < 0.01$, R(U+L) vs. U+L group. [+]$P < 0.01$ L vs. control; R(U+L) vs. L and R(U) vs. L were not significant.
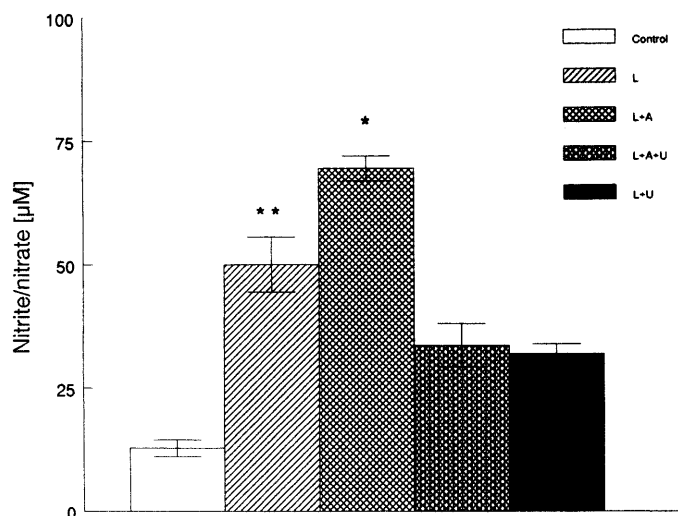
Fig. 4. Effects of L-arginine supplementation on urea-induced inhibition of NO synthesis in RAW 264.7 macrophage cell line. A, L-arginine. Values are means ± SE in µM nitrite-nitrate content. Results are from 1 experiment typical of 3 others. ** $P < 0.001$, L vs. control; * $P < 0.05$, L+A vs. L; L+A+U vs. L was not significant.

whereas the group incubated in the presence of PTH produced 24.3 ± 1.8 µM ($n = 8$; NS). Thus PTH had no independent effects on NO synthesis in RAW 264.7 cells. Furthermore, when RAW 264.7 cells were activated with LPS in the presence of PTH, they produced NO measuring 79.2 ± 3.36 µM ($n = 8$), indicating that PTH did not interfere with LPS-induced NO synthesis.

Effects of L-arginine supplementation. To understand the mechanism of urea-induced inhibition of NO synthesis in macrophages, we first evaluated the effects of L-arginine supplementation to assess whether this inhibition was at the level of substrate utilization. Because L-arginine is a precursor of NO, enrichment of the culture medium with L-arginine would overcome the inhibition of NO by urea. RAW 264.7 cells were incubated in RPMI 1640 medium saturated with additional L-arginine to yield a final concentration of 1 mM. LPS-induced NO synthesis was 51.2 ± 5.58 µM vs. a control value of 12.5 ± 2.02 µM ($P < 0.01$; $n = 12$).

L-Arginine supplementation enhanced the NO synthesis by LPS to 69.8 ± 2.6 µM ($P < 0.05$). Addition of urea significantly inhibited LPS-induced NO synthesis ($n = 12$; 31.2 ± 2.27 vs. 51.2 ± 5.58 µM; $P < 0.01$). Supplementation of L-arginine did not prevent urea-induced inhibition of NO synthesis (33.24 ± 2.94 µM compared with 32.16 ± 2.27 µM without additional L-arginine; NS). Thus L-arginine supplementation did not overcome the urea-induced suppression of NO synthesis from activated macrophages (Fig. 4), suggesting that inhibition is not due to interference with substrate utilization.

mRNA analysis and northern blot studies. Our studies evaluating the iNOS mRNA expression in macrophages using RT-PCR and Northern blot techniques indicate that urea did not interfere with the iNOS gene expression. Figure 5 shows a representative Northern blot analysis of RNA ($n = 3$) hybridized to species-specific ^{32}P-labeled iNOS. The untreated cells contain no discernible iNOS mRNA; however, LPS treatment induced iNOS mRNA synthesis. This induction of mRNA was not diminished to any appreciable level by the addition of urea to LPS, whereas the NO production was inhibited by >95%. This indicates that the inhibitory effects of urea may involve a posttranscriptional step at or beyond translation. However, more studies are required to define the precise mechanism of this inhibition.

DISCUSSION

The defects of host defense mechanisms in renal failure are multiple and involve alterations at various levels. Macrophage dysfunction appears to be a major site of impairment of host defenses. First, Hibbs et al. (6a, 7) showed that NO may be a mediator of macrophage-mediated cytotoxicity. More recently, Vallance et al. (22) described and Brenner and Yu (2) discussed the inhibition of NO synthesis due to retained endogenous factors in uremia. It is therefore conceivable that the dysfunction of macrophages in uremia may indeed be
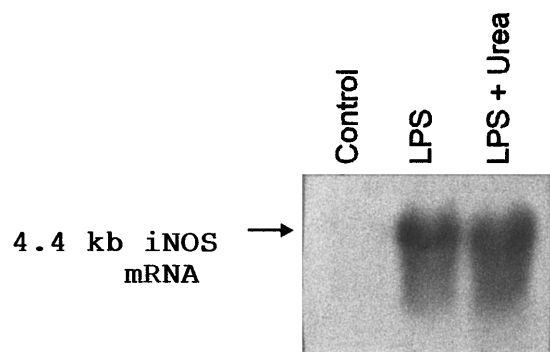

Fig. 5. Results of Northern blot analysis of inducible NO synthase (iNOS) mRNA expression in RAW 264.7 macrophage cell line. Results are from 1 experiment representative of 2 others. Difference in signal between LPS and LPS + urea (50 mM) groups was <10%, whereas effects of these 2 groups on NO synthesis were different by ~400%. Results of RT-PCR studies were qualitatively very similar. kb, Kilobases.

NORTHERN BLOT ANALYSIS OF iNOS mRNA

EXPRESSION IN RAW 264.7 CELLS

due to inhibition of NO synthesis. Our experiments were designed to examine this hypothesis.

Macrophages are one of the earliest known sources of NO synthesis (4). NO is a labile free radical that acts as a paracrine and autocrine agonist formed exclusively from L-arginine by the action of the enzyme NO synthase (15), of which there are at least two subtypes: the constitutive subtype commonly expressed in endothelium and neurons, which is calcium dependent, and the inducible subtype, as seen in the macrophage and vascular smooth muscle, which is calcium independent (6) and is induced by cytokines and endotoxins and regulated by prostaglandins (9) and guanidines (14). Although NO was first discovered to be the endothelium-derived relaxing factor (8), subsequently many physiological roles have been ascribed to NO in various tissues and organ systems, such as control of systemic blood pressure, platelet aggregation, control of renal hemodynamics, regulation of renal tubular transport, immune defenses, and neuronal signaling in central and peripheral nervous systems (13, 20). In the area of immune defense mechanisms, NO, which is released by macrophages on induction with bacterial endotoxins, γ-interferon, or tumor necrosis factor, is believed to function as the effector molecule mediating the cytotoxicity (7). The NO inhibits the iron-containing enzymes in the target cells, including several enzymes involved in mitochondrial respiration (10). In addition, NO also inhibits DNA synthesis in the target cells. Thus NO may mediate the macrophage-dependent killing of bacteria, protozoa, and fungi. Thus, although the increased susceptibility to infections that occurs in uremic syndrome has been largely attributed to lymphocytopenia and leukocyte dysfunction, impairment of NO-mediated macrophage cytotoxicity may play an important role in the immune dysfunction in uremia.

Our preliminary experiments demonstrated that incubation of RAW 264.7 cells in uremic plasma inhibited iNOS production in these cells. However, these findings are similar to those of Arese et al. (1), who showed that incubation of J774 murine macrophage cell line in uremic human plasma caused inhibition of iNOS synthesis. We then proceeded to examine the specific factors in uremia that could influence iNOS production in macrophages.

Figure 1 shows that NO production can be induced in the activated macrophages by bacterial LPS derived from *E. coli*. Our results indicated that the induction caused a significant increase in NO content from a control value of 21.8 ± 2.1 to 93.2 ± 3.6 µM and that the maximal increase was seen at 18 h of incubation. It is also of interest that the basal nitrite-nitrate level is of considerable amount, and possibly there is some release of constitutive NO from macrophages, which is seen particularly with cells in late passages. This observation is consistent with the studies described earlier in RAW 264.7 cells (19). We also noticed that the baseline NO generated from the control cells was a function of the passage number, i.e., the higher the passage number the higher the NO production or constitutive NO synthesis. A similar observation was made by Schmidt et al. (19).

Our experiments showed that urea inhibited inducible NO production from macrophages in a dose-dependent fashion. The inhibition was observed in low concentrations of 10 mM but was maximal in concentrations of 50–100 mM (Fig. 2). It is interesting that significant inhibition was seen at a urea concentration of 50 mM, which corresponds to a blood urea nitrogen of ~150 mg/dl, which is a concentration that is seen in patients with advanced renal failure. Thus our observations are relevant to human or animal models of advanced renal failure, and the inhibition of NO synthesis by urea may have an important consequence. The fact that the inhibition of NO production was seen at 6 h and not earlier indicates that the mechanism may involve protein synthesis or gene expression. The maximal inhibition was seen at 18–24 h, which is again similar to the NO production from activated macrophages. These temporal correlations indicate that the inhibition of urea may be directly related to inhibition of NO synthase. We evaluated the effects of urea on NO production because there is renewed interest in urea as a potential cellular toxin. Urea is well known as a protein metabolite that accumulates in renal failure and is used conventionally to measure the severity of renal impairment. Because we wanted to examine the effects of renal failure, we thought that urea might be a reasonable candidate, since urea may have effects on cellular metabolism that have not been hitherto described. Notwithstanding the fact that urea was conventionally regarded as an innocuous metabolite that accumulated in renal failure, the recent evidence indicates that urea in fact may interfere with cellular metabolism and protein synthesis (17). In addition, recently several studies have demonstrated that urea may interfere with transport of electrolytes and also inhibit cellular and extracellular enzyme synthesis (12). So, we hypothesized that urea may also have an effect on NO synthesis in cells that are known to synthesize NO. In this context, it is interesting to note that Szabo et al. (21) showed recently that *S*-methyl-isothiourea was a potent and selective inhibitor of iNOS in a mouse macrophage cell line, and they further concluded that this compound may be beneficial in the rodent model of septic shock. It is possible that the urea part of this compound may play a role in this inhibition. We chose to examine the effects of urea on NO synthesis because urea has been shown to interfere with cellular transport mechanisms and synthesis of various intracellular enzymes (12, 17). However, we recognize the fact that many other factors retained in uremia may also potentially interfere with NO synthesis.

Our data suggest that urea had no effect on the basal or constitutive NO synthesis as reflected by our results (28.6 ± 2.1 µM vs. control 23.8 ± 2.4 µM after 18 h of incubation; $n = 8$; NS) These data lend further support to the conclusion that urea-induced effects on iNOS are quite specific. Our studies also demonstrate that urea-induced inhibition of NO synthesis is reversible as is evident from the observations that cells preincubated

in the presence of urea retained their ability to be induced by LPS to synthesize NO when reincubated in a urea-free medium (Fig. 3). Thus urea specifically and reversibly inhibited the inducible NO synthesis in macrophages, and these observations may have important implications in the macrophage dysfunction of uremia.

The impact of renal failure on NO production in vivo is not well understood (11). There are conflicting data about the significance of NO in the pathophysiology of renal failure (16). It appears that the plasma levels may be elevated in patients with renal failure, especially in the face of fluctuations of blood pressure. However, most of this NO is derived from endothelial cells, and hence it is not clear how uremia affects the production of iNOS from macrophages. Our data show that urea, a metabolite that accumulates in the presence of renal failure, inhibited iNOS in RAW 264.7 cells (Fig. 2). We also explored other compounds that accumulate in renal failure in terms of their effects on NO production. PTH is known to accumulate in the presence of renal failure in humans and also in rodent models of renal failure. Interestingly, there are very few data on the effect of PTH on the iNOS in macrophages. We used various concentrations of PTH and found that there was no effect on NO production at any concentration. At 400 pg/ml, a concentration seen in rodent models of advanced renal failure, we did not find any effect on iNOS production in the RAW 264.7 cells. Our data indicate that PTH did not interfere with LPS-induced NO synthesis. Although iNOS was not analyzed by Northern blots, it is extremely unlikely that LPS-induced NO synthesis in PTH-treated cells occurred without iNOS mRNA synthesis. It is known that certain agents such as cyclohexamide inhibit NO synthesis beyond the phase of iNOS mRNA synthesis, but there is no substance described that facilitates an LPS-induced NO synthesis in the absence of the iNOS mRNA synthesis. Hence we did not feel it necessary to perform Northern blots in PTH experiments.

We thus concluded that the NO inhibition from macrophages in renal failure could be predominantly from the effects of urea. In this context, it is interesting to review the results of Vallance et al. (22), who found that NO synthesis is impaired in the uremic syndrome. They proposed that this inhibition is mediated by asymmetric dimethyl arginine, a compound that accumulates markedly in patients with end stage renal failure. So it is possible that there may be several compounds that accumulate in renal failure that inhibit the NO synthesis in vivo.

The fact that L-arginine supplementation, which normally enhances NO synthesis, failed to prevent the urea-induced inhibition of NO synthesis indicates that urea-induced inhibition could not be overcome by enhanced substrate availability (Fig. 4). Despite saturation of the cell system with maximum concentrations of L-arginine (1 mM), urea continued to inhibit NO synthesis, implying that the inhibition is not at the level of substrate utilization. If significant, uncontrolled arginase were present in the medium, the resultant conver-

sion of arginine to urea would actually decrease and not increase NO synthesis with L-arginine supplementation. Our experimental data indeed showed that L-arginine significantly enhanced inducible NO synthesis (51.2 ± 5.58 µM with LPS vs. 69.8 ± 2.6 µM with supplemental arginine; $P < 0.05$), implying that, if any arginase was present, it was insufficient to affect NO synthesis. Additionally, because the same medium was used in all our experiments, arginase, if at all present in any amount, would affect all experimental groups equally, diminishing its significance. Furthermore, we found no evidence in the literature to postulate that urea stimulates arginase activity.

Our extensive studies examining the iNOS mRNA expression using the RT-PCR and Northern blot analysis indicated that the urea did not interfere with the synthesis of mRNA (Fig. 5), and hence we believe that the inhibition is at a posttranscriptional level. It is possible that urea inhibits translation of iNOS or inhibits a posttranslational step such as calmodulin binding of iNOS or binding with cofactors such as NADPH, tetrahydrobiopterin, or flavin mononucleotide. Further experiments would be necessary to clarify the details of this mechanism. It is interesting that, although urea inhibits many enzymes by impairing the cellular synthesis of these proteins, urea-induced inhibition of NO synthase may involve a step different from the other enzymes. The significance of this inhibition may be clinically important. If these results of in vitro studies are extrapolated into an in vivo situation, it is possible that, with increasing concentration of urea in advancing renal failure, there may be impairment of intrarenal production of NO. This effect could potentially influence the intrarenal hemodynamics in an unfavorable manner, leading to a progressive intrarenal vasoconstriction. With progressive loss of renal mass, elevation of blood urea may thus be an important factor contributing to decreased intrarenal NO synthesis in addition to renal depletion of arginine synthesis. The decreased NO synthesis may thus contribute to the progressive nature of renal disease. Thus one of the several adverse factors leading to relentless progression of renal failure may be impaired production or "dysregulation" of NO synthesis within the kidney, possibly resulting from retention of endogenous metabolites such as urea.

In conclusion, we believe that urea, a compound considered to be innocuous that accumulates in renal failure (23), may have potentially important implications in the pathogenesis of the uremic syndrome. Our experiments show that urea inhibits NO production by the macrophages by a posttranscriptional mechanism. This observation may have a clinical relevance in that urea may play a role in impaired macrophage function in renal failure by decreasing the NO production, since NO may be the effector molecule of a macrophage cytotoxicity (6a). In addition, these observations may have even more serious clinical implications in that urea may impair the production of NO from other known sources in the kidney, such as the mesangial

cells or glomerular endothelium, and thus may contribute to the progression of renal failure.

REFERENCES

1. **Arese, M., M. Strasly, C. Ruva, C. Costamagna, D. Ghigo, R. MacAllister, G. Verzetti, C. Tetta, A. Bosic, and F. Bussolino.** Regulation of nitric oxide synthesis in uremia. *Nephrol. Dial. Transplant.* 10: 1386–1397, 1995.

2. **Brenner, B. M., and A. S. L. Yu.** Uremic syndrome revisited: a pathogenic role for retained endogenous inhibitors of nitric oxide synthesis. *Curr. Opin. Nephrol. Hypertens.* 1: 3–7, 1992.

3. **Chomczynski, P., and N. Sacchi.** Single-step method for RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156–159, 1987.

4. **Ding, A. H., C. F. Nathan, and D. Stuehr.** Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. *J. Immunol.* 141: 2407–2412, 1988.

5. **Faruqi, R. M., and P. E. DiCorleto.** Mechanisms of monocyte recruitment and accumulation. *Br. Heart J.* 69, Suppl. 1: 519–529, 1993.

6. **Gross, R. W., A. E. Rudolph, J. Wang, C. D. Sommers, and M. J. Wolf.** Nitric oxide activates the glucose-dependent mobilization of arachidonic acid in a macrophage-like cell line (RAW 264.7) that is largely mediated by calcium-independent phospholipase A2. *J. Biol. Chem.* 270: 14855–14858, 1995.

6a. **Hibbs, J. B., Jr., R. R. Taintor, and Z. Vavrin.** Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* 235: 473–476, 1987.

7. **Hibbs, J. B., Jr., R. R. Taintor, Z. Vavrin, and E. M. Rachlin.** Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* 157: 87–94, 1988.

8. **Ignarro, J. J., G. M. Buga, K. S. Wood, and R. E. Byrns.** Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. USA* 84: 9265–9269, 1987.

9. **Imai, Y., H. Kolb, and V. Burkar.** Nitric oxide production from macrophages is regulated by arachidonic acid metabolites. *Biochem. Biophys. Res. Commun.* 197: 105–109, 1993.

10. **Iyengar, R., D. J. Stuehr, and M. A. Marletta.** Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. USA* 84: 6369–6373, 1987.

11. **Klahr, S., and J. Morrissey.** Renal disease: the two faces of nitric oxide. *Lab. Invest.* 72: 1–3, 1995.

12. **Lim, J., C. Gasson, and D. M. Kaji.** Urea inhibits NaK$_2$Cl cotransport in human erythrocytes. *J. Clin. Invest.* 96: 2126–2132, 1995.

13. **Lowenstein, C. J., J. L. Dinerman, and S. H. Snyder.** Nitric oxide: a physiologic messenger. *Ann. Intern. Med.* 120: 227–237, 1994.

14. **Misko, T. P., W. M. Moore, T. P. Kasten, G. A. Nickols, J. A. Corbett, R. G. Tilton, M. L. McDaniel, J. R. Williamson, and M. G. Currie.** Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur. J. Pharmacol.* 233: 119–125, 1993.

15. **Moncada, S., and A. Higgs.** The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* 329: 2002–2012, 1993.

16. **Narita, I., W. A. Border, M. Ketteler, and N. A. Noble.** Nitric oxide mediates immunologic injury to kidney mesangium in experimental glomerulonephritis. *Lab. Invest.* 72: 17–24, 1995.

17. **Rajagopalan, K. V., I. Fridovich, and P. Handler.** Competitive inhibition of enzymic activity by urea. *J. Biol. Chem.* 236: 1059–1065, 1963.

18. **Ruiz, P., F. Gomez, and A. D. Schreiber.** Impaired function of macrophage Fc$_\gamma$ receptors in end-stage renal disease. *N. Engl. J. Med.* 322: 717–722, 1990.

19. **Schmidt, H. H. H. W., T. D. Warner, M. Nakane, U. Forstermann, and F. Murad.** Regulation and subcellular location of nitrogen oxide synthases in RAW 264.7 macrophages. *Mol. Pharmacol.* 41: 615–624, 1992.

20. **Star, R. A.** Southwestern Internal Medicine Conference: nitric oxide. *Am. J. Med. Sci.* 306: 348–358, 1993.

21. **Szabo, C., G. J. Southan, and C. Thiemermann.** Beneficial effects and improved survival in rodent models of septic shock with S-methylisothiourea sulfate, a potent and selective inhibitor of inducible nitric oxide synthase. *Proc. Natl. Acad. Sci. USA* 91: 12472–12476, 1994.

22. **Vallance, P., A. Loene, A. Calver, J. Collier, and S. Moncada.** Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 339: 572–575, 1992.

23. **Walser, M.** Determinants of ureagenesis, with particular reference to renal failure. *Kidney Int.* 17: 709–721, 1980.