ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1997, p. 557–562 Vol. 41, No. 3
0066-4804/97/$04.00+0

Association of Nitric Oxide Production by Kidney Proximal Tubular Cells in Response to Lipopolysaccharide and Cytokines with Cellular Damage

ALBERT F. KABORÉ,[1] MICHEL DENIS,[2] AND MICHEL G. BERGERON[1]*

Laboratoire et Service d'Infectiologie, Centre de Recherche du Centre Hospitalier de l'Université Laval, Département de Microbiologie, Faculté de Médecine, Université Laval, Québec, Québec, Canada G1V 4G2,[1] and Unité de Recherche Pulmonaire, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada J1N 5N4[2]

Received 2 November 1994/Returned for modification 6 October 1996/Accepted 30 December 1996

Recent findings suggest that nitric oxide (NO) is an important biologic mediator which exerts a wide variety of effects on numerous physiological and pathophysiological processes. L-Arginine is oxidized to L-citrulline with concomitant NO production; as a result, nitrate and nitrite accumulates. This study was conducted to determine the potential NO production by proximal tubular cells (PTC) in response to bacterial lipopolysaccharides (LPS) and cytokines and to evaluate the cytotoxic effect associated with NO release. After a 7-day stimulation with LPS (100 μg/ml), interleukin-1β (IL-1β) (10 ng/ml), and tumor necrosis factor alpha (TNF-α) (10 ng/ml), the nitrate and nitrite levels were determined by a spectrophotometric method based on the Griess reaction. Moreover, α-methylglucopyranoside phosphate and lactate dehydrogenase release and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay served as indicators of sodium-dependent hexose transport integrity and cell death, respectively. IL-1β and TNF-α used alone or together or combined with LPS led to a significant generation of NO by PTC. Our results also demonstrate that NO induced by LPS and cytokines could inhibit sodium-dependent transport and could induce PTC damage.

Nitric oxide (NO), also referred to as endothelium-derived relaxing factor (17), is a highly versatile molecule (30 Da) that regulates a large number of cellular functions with a very short biologic half-life (24). A role for inducible NO production in renal pathophysiology has been assumed from studies measuring NO production in glomeruli from nephritic kidneys (12). Moreover, recent studies confirmed that tubular epithelial cells are capable of high-output NO synthesis (29, 54). Consequently, intensive research activities have focused on the production and effects of NO in the kidney, since it is known that NO affects renal function and hemodynamics (43). It has been demonstrated that both glomerular microcirculation and medullary microcirculation in the kidneys are regulated by endogenous NO (3, 4, 28, 39, 40), which also influences tubular reabsorbtion, which may be relevant to hypertension (43). Thus, we have conducted the current study to determine to what extent endotoxin (lipopolysaccharide [LPS])- and cytokine-induced production of NO is associated with tubular cell dysfunction. Our results demonstrate that significant NO production may be induced by the single cytokines interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) or their combination in the presence or absence of LPS and that this NO production is associated with damage of proximal tubular cells (PTC).

(This work was presented in part at the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy, Orlando, Fla., 4 to 7 October 1994, session 20, paper G22.)

MATERIALS AND METHODS

Cell culture. Primary confluent monolayers were grown from proximal tubule fragments as reported by Bello-Reuss and Weber (5) with minor modifications (26). Briefly, kidneys were obtained aseptically from New Zealand male rabbits (1.5 to 2 kg), decapsulated, and sliced in 1-mm-thick sections which were kept at 4°C in Hanks balanced salt solution combined with 10 mM HEPES and 5 mM D-glucose, pH 7.4 (HBS-HEPES). The cortical slices were rinsed three times in HBS-HEPES and placed in a mixture of 4.25 ml of HBS-HEPES, 4 ml of culture medium, 25 mg of bovine serum albumin, and collagenase (final concentration, 0.75 mg/ml). The suspension was transferred to a trypsinizing flask and incubated for 35 min at 37°C in an atmosphere of 5% CO_2–95% air. The suspension was strained to remove large debris, and the strained effluent was washed by centrifugation in cold HBS-HEPES at 700 × *g* at 4°C for 5 min. The pellet was resuspended in the same solution, and the operation was repeated three times. The pellet was resuspended in 5% albumin–HBS-HEPES solution, kept on ice for 5 min, and centrifuged as described above. Homogeneous populations of nephron segments were separated as follows by Percoll centrifugation adapted from the method of Vinay et al. (53): the pellet was resuspended in 50% Percoll in a concentrated HBS solution (iso-osmotic) and centrifuged at 24,000 × *g* at 4°C for 35 min. There were four distinct bands in the gradient. The proximal fragments (>98% pure [53]) were found in the F4 layer obtained in the gradient. They were removed and washed twice in HBS-HEPES (700 × *g*, 4°C, 5 min). The final pellet was resuspended in culture medium, and tubules were seeded in 24-well plastic trays (2×10^5 to 5×10^5 fragments per well) which had been coated with calf skin collagen, type 1. Serum-free culture medium consisted of a 1:1 (vol/vol) mixture of Ham F-12 medium and Dulbecco modified Eagle medium supplemented with 25 mM HEPES, 8.9 mM $NaHCO_3$, 1 mM sodium pyruvate, 4 mM L-glutamine, 10 ml of a 100× nonessential amino acid mixture per liter, 5,000 IU of penicillin per ml, 5,000 mg of streptomycin per ml, 100 nM sodium selenite, 1.22 mg of ethanolamine per liter, 35 mg of transferrin per liter, 5 mg of insulin per liter, and 100 nM hydrocortisone. The medium was changed every 2 days. The cultures became confluent in 4 to 6 days. All studies were performed with cells at confluence, 4 to 6 days after incubation at 37°C in a 5% CO_2 environment.

Experimental protocol. (i) Immunohistochemistry. Confluent PTC were examined for their epithelial lineage by actin-vimentin staining: briefly, monolayers were fixed in ice-cold acetone, washed in phosphate-buffered saline (PBS), and incubated for 30 min at 37°C with mouse antiactin-antivimentin monoclonal antibody. Cells were rinsed in PBS and incubated for 30 min at 37°C with goat anti-mouse antibody. After a final wash in PBS for 10 min at room temperature, monolayers were observed directly in the well bottoms with an optical microscope.

(ii) Assay for nitrate and nitrite levels. At 37°C in a CO_2 environment, PTC were treated over a period of 7 days as follows: LPS, 100 μg/ml; IL-1β, 10 ng/ml; TNF-α, 10 ng/ml; IL-1β plus TNF-α; and LPS plus IL-1β plus TNF-α. In another series of experiments, LPS–IL-1β–TNF-α was used in the presence or absence of 1 mM N^G-monomethyl-L-arginine monoacetate (L-NMMA) supplemented or

* Corresponding author. Mailing address: Laboratoire et Service d'Infectiologie, 2705, Boul. Laurier, Sainte-Foy, Québec, Canada G1V 4G2. Phone: (418) 654-2705. Fax: (418) 654-2715.

not supplemented with 5 mM L-Arg. The LPS–IL-1β–TNF-α treatment was also carried out with or without 1 mM EGTA. Throughout these series of experiments, treatments were repeated every 48 h. At the end of the incubation time, the supernatants from the above-described conditions were harvested, aliquoted, and stored at −70°C until assayed. The level of nitrate and nitrite, the stable end products of nitric oxide, was determined by the Griess reaction (15, 20). Samples were first incubated for 24 h at room temperature with 100 μM NADPH and 0.1 U of *Aspergillus* nitrate reductase to convert nitrate to nitrite. The nitrite concentration of the resulting supernatants was taken to reflect total nitrate and nitrite in the medium. To this, 200 μl of Griess reagent [1% sulfanilamide in 30% acetic acid and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 60% acetic acid in a 1:1 mixture] was added. The Griess reagent reacted with nitrite to form a chromophore absorbing at 550 nm, which was quantified spectrophotometrically. Known concentrations of $NaNO_2$ were used as the standard. Nitrate and nitrite levels were normalized with respect to protein quantities determined by the method of Bradford (10).

(iii) Uptake studies. α-Methyl glucopyranoside (MGP) uptake, a marker of cellular integrity of PTC, was determined at 37°C in a buffered solution with the following composition per liter: NaCl, 137 mmol; KCl, 5.4 mmol; $CaCl_2$, 1 mmol; $MgSO_4$, 1.2 mmol; and HEPES, 1.5 mmol (pH 7.4). After removal of culture medium, cells were washed twice with a solution of 137 mM NaCl and 15 mM HEPES (pH 7.4) and incubated in the presence of methyl-(α-D-U-[^{14}C]gluco-)pyranoside ([^{14}C]MGP) (0.5 mCi/ml) and appropriate concentrations of unlabeled MGP. MGP was used instead of D-glucose because the latter is metabolized by kidney cells while the former is not. At the end of the incubation time (30 min), the uptake was stopped by washing the cells twice with 1 ml of the buffer solution. Cells were then solubilized in 0.5% Triton X-100 (250 μl per well), and aliquots were counted with a scintillation counter. Radioactive counts in each sample were normalized with respect to protein quantities determined by the method of Bradford (10).

(iv) LDH release. Lactate dehydrogenase (LDH) was assessed by using the Sigma diagnostics LDH reagents that measure the enzyme activity based on the optimized standard method. Each determination was standardized with respect to the total protein of the corresponding well.

(v) Colorimetric MTT assay. PTC were incubated for 72 h at 37°C in a 5% CO_2 environment under the following conditions: LPS, 100 μg/ml; IL-1β, 4 ng/ml; TNF-α, 20 ng/ml, IL-1β plus TNF-α; and LPS plus IL-1β plus TNF-α. These treatments were repeated every 12 h. At intervals cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay as follows: MTT was dissolved in PBS at 5 mg/ml and filter sterilized to remove the small amount of insoluble residue present. At intervals, stock MTT solution (10 μl/100 μl of medium) was added to all wells of an assay, and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The plates were read on a micro-enzyme-linked immunosorbent assay reader, using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99. Plates were normally read within 1 h of adding the isopropanol. For each series of experiments mentioned above, control groups which received no treatment were included.

Statistical analysis. Unless otherwise stated, all values are expressed as mean ± standard error of the mean. A one-way analysis of variance (ANOVA) was used to assess the statistical significance of results (P of <0.05 was considered significant).

Materials. Ethanolamine, insulin, transferrin, hydrocortisone, MGP, sodium selenite, endotoxin (lipopolysaccharide O127:B8 from *Escherichia coli*), Percoll, *N*-acetylglucosaminide, L-glutamyl-anilide, type 1 collagenase, bovine serum albumin, collagen from calf skin type 1, EGTA, *Aspergillus* nitrate reductase, LDH reagents, MTT, NADPH, and L-arginine were purchased from Sigma Chemical Co. St. Louis, Mo. [^{14}C]MGP was from Amersham, Oakville, Ontario, Canada. L-NMMA was from Calbiochem, La Jolla, Calif. Recombinant human IL-1β and recombinant human TNF-α were from Genzyme, Cambridge, Mass. Culture media were from Flow Laboratories, McLean, Va., and Sigma Chemical Co. Endotoxin-free plasticware was from Costar, Ottawa, Ontario, Canada.

RESULTS

PTC culture. PTC monolayers were 99% actin positive and vimentin negative, confirming the epithelial lineage of our cells. Trypan blue dye was excluded by more than 95% of untreated PTC.

Nitrate and nitrite level. Only LPS at 100 μg/ml failed to increase significantly the total nitrite level in the supernatants when used alone (129% ± 4% of the control level; $P < 0.05$), whereas IL-1β (10 ng/ml) and TNF-α (10 ng/ml) used separately induced significant total nitrite elevation (138% ± 4% and 142% ± 25% of the control level, respectively; $P < 0.05$) (Fig. 1). Both IL-1β–TNF-α and LPS–IL-1β–TNF-α combinations led to a further increase in total nitrite accumulation in the supernatants (175% ± 9% and 151% ± 14% of the control level, respectively; $P < 0.01$). The addition of 1 mM L-NMMA to LPS–IL-1β–TNF-α-treated cells prevented the total nitrite elevation in the supernatants (100% ± 3% of the control level; $P < 0.01$) (Table 1). The addition of 5 mM L-Arg reversed partially but not significantly the inhibitory effect of L-NMMA, leading to a weak increase of the total nitrite level (117% ± 4% of the control level; $P < 0.01$). Finally, no significant difference in the total nitrite was observed between the LPS–IL-1β–TNF-α-treated groups which did and did not receive 1 mM EGTA (data not shown).

TABLE 1. Nitrate and nitrite levels of kidney PTC following exposure to LPS, IL-1β, and TNF-α in the presence of L-NMMA with or without L-Arg

Treatment[a]	Nitrate and nitrite level (% of control)[b]
LPS + IL-1β + TNF-α	151 ± 14[c]
LPS + IL-1β + TNF-α + L-NMMA	100 ± 3
LPS + IL-1β + TNF-α + L-NMMA + L-Arg	117 ± 4

[a] Over a period of 7 days, cells were treated with LPS (100 μg/ml), IL-1β (10 ng/ml), and TNF-α (10 ng/ml). L-NMMA and L-Arg were used at 1 and 5 mM, respectively. The treatments were repeated every 48 h. Supernatants were harvested at the end of incubation time, and the total nitrite level was determined by using the Griess reagent as described in Materials and Methods.

[b] Each value is the mean ± standard error of at least five determinations. Nitrate and nitrate levels were measured in nanomoles per milligram of protein.

[c] $P < 0.01$ versus control, $P < 0.01$ versus when L-NMMA was added, and $P < 0.05$ versus when L-NMMA and L-Arg were present (ANOVA).

MGP uptake. LPS (100 μg/ml), IL-1β (10 ng/ml), and TNF-α (10 ng/ml) alone led to a significant decrease of the

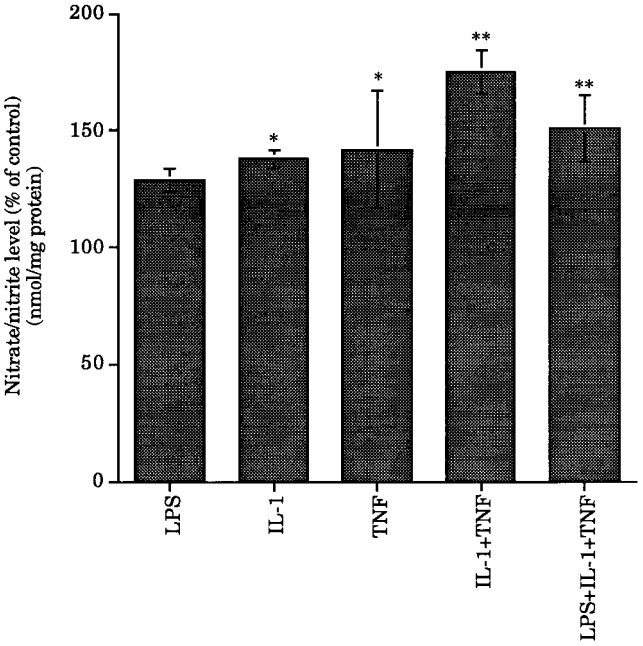

FIG. 1. Nitrate and nitrite levels in supernatants of cultured kidney PTC exposed to LPS, IL-1β, and TNF-α. Over a period of 7 days, cells were treated with LPS (100 μg/ml), IL-1β (10 ng/ml), and TNF-α (10 ng/ml) alone or combined together. The treatments were repeated every 48 h. Supernatants were harvested at the end of the incubation time, and the nitrite level was determined by using the Griess reagent as described in Materials and Methods. ** and *, $P < 0.01$ and $P < 0.05$, respectively, versus control (ANOVA). Each value is the mean ± standard error of four to six determinations from two different cultures.

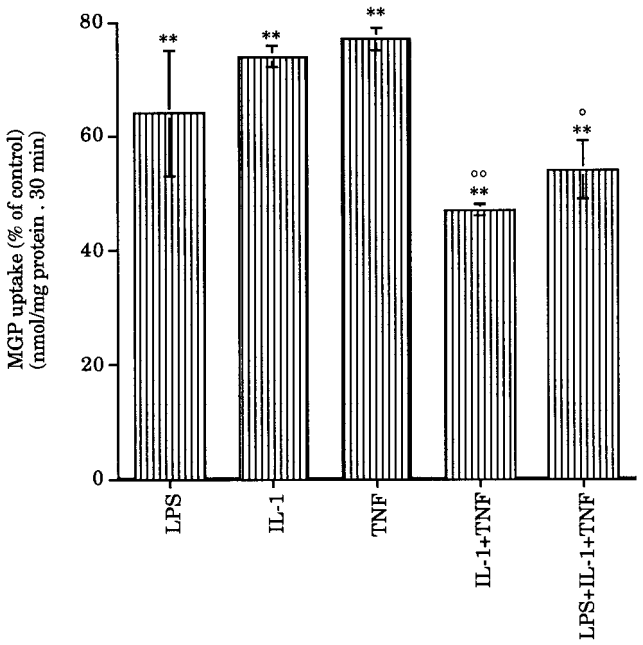

FIG. 2. Sodium-dependent uptake of MGP following 7 days of exposure of kidney PTC to LPS, IL-1β, and TNF-α. Cells were treated with LPS (100 μg/ml), IL-1β (10 ng/ml), and TNF-α (10 ng/ml) alone or combined together. The treatments were repeated every 48 h. At the end of incubation time, sodium-dependent uptake of MGP was measured in the cell homogenate as described in Materials and Methods. **, $P < 0.01$ versus control; ○○ and ○, $P < 0.01$ and $P < 0.05$, respectively, versus each cytokine alone (ANOVA). Each value is the mean ± standard error of six determinations from two different cultures.

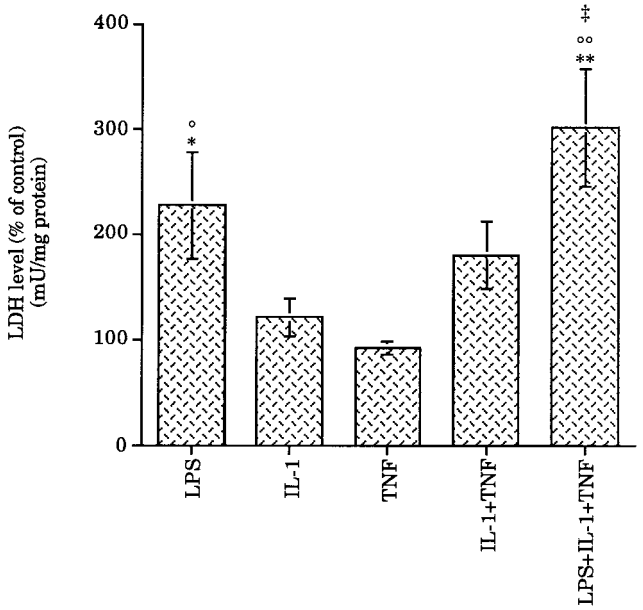

FIG. 3. LDH leakage following kidney PTC exposure to LPS, IL-1β, and TNF-α. Over a period of 7 days, cells were treated with LPS (100 μg/ml), IL-1β (10 ng/ml), and TNF-α (10 ng/ml) alone or combined together. The treatments were repeated every 48 h. Supernatants were harvested at the end of incubation time, and the LDH level was determined as described in Materials and Methods. ** and *, $P < 0.01$ and $P < 0.05$, respectively, versus control; ○○ and ○, $P < 0.01$ and $P < 0.05$, respectively, versus each cytokine alone; ‡, $P < 0.05$ versus IL-1β plus TNF-α (ANOVA). Each value is the mean ± standard error of six determinations from two different cultures.

MGP uptake by PTC (64% ± 11%, 74% ± 3%, and 77% ± 4% of the control level, respectively; $P < 0.01$) (Fig. 2). Furthermore, this decrease was more important with both IL-1β–TNF-α (47% ± 5% of the control level; $P < 0.01$) and LPS–IL-1β–TNF-α (54% ± 2% of the control level; $P < 0.01$).

LDH measurement. No significant accumulation of LDH in the supernatants was measured following incubation with IL-1β (10 ng/ml) or TNF-α (10 ng/ml) alone (121% ± 18% and 91% ± 6% of the control level, respectively) (Fig. 3). Treatment with both IL-1β and TNF-α did not lead to a significant rise of the LDH level in the supernatants (180% ± 32% of the control level). Interestingly, a significant increase of the LDH level was reached with LPS (100 μg/ml) alone (228% ± 51% of the control level; $P < 0.01$) and when LPS was combined with IL-1β and TNF-α (302% ± 56% of the control level; $P < 0.01$).

MTT assay. Cellular viability was assessed over 72 h of incubation time (Table 2). Four nanograms of IL-1β per milliliter and 20 ng of TNF-α per ml used in this experiment were determined as the lowest concentrations needed to decrease significantly PTC viability following 72 h of exposure time, with an addition of fresh material every 12 h. IL-1β and TNF-α used alone reduced significantly cell viability (87% ± 2% of the control level [$P < 0.01$] and 91% ± 4% of the control level [$P < 0.05$], respectively). Both IL-1β–TNF-α and LPS–IL-1β–TNF-α led to a significant decrease in cell viability (65% ± 5% and 75% ± 3% of the control level, respectively; $P < 0.01$).

DISCUSSION

The findings presented in this study demonstrate that LPS- and cytokine-induced NO release by PTC is associated with a cellular impairment characterized by an inhibition of the sodium-dependent uptake process and cytosolic enzyme leakage.

Cytokines and LPS have been shown to cause morphological and functional alterations in numerous cell types (6, 18, 23). The underlying mechanisms involved in these deleterious events are unclear; however, a potential mediator has recently been identified as NO, a highly reactive compound that spontaneously decomposes to nitrates and nitrites in culture medium (24, 30, 37, 38). NO is synthesized from L-arginine by nitric oxide synthase (NOS), a dioxygenase that exists in several isoforms which may principally be classified as inducible and constitutive (27, 32). NOS catalyzes the oxidation of one of the terminal guanidine nitrogen atoms of L-Arg to NO via an NADPH-dependent citrulline-forming enzyme (32, 46, 50). Experiments performed to induce NO production by PTC following exposure to cytokines and LPS demonstrated that sin-

TABLE 2. PTC viability following 72 h of exposure to LPS, IL-1β, and TNF-α alone or in combination

Treatment[a]	Cell viability (% of control)[b]
LPS, 100 μg/ml	98 ± 3
IL-1β, 4 ng/ml	87 ± 2**
TNF-α, 20 ng/ml	91 ± 4*
IL-1β + TNF-α	65 ± 5**#
LPS + IL-1β + TNF-α	75 ± 3**

[a] The treatments were repeated every 12 h. At 72 h, cell viability was assessed by the MTT reduction assay as described in Materials and Methods.

[b] ** and *, $P < 0.01$ and $P < 0.05$, respectively, versus control; #, $P < 0.01$ versus each agent used alone and $P < 0.05$ versus LPS plus IL-1β plus TNF-α (ANOVA). Each value is the mean ± standard error of at least five determinations.

gle cytokines, but not LPS, were capable of inducing a significant amount of total nitrite in the supernatants at the doses mentioned above. Both IL-1β and TNF-α acted synergistically and led to a further elevation of total nitrite. Contrary to its effects on macrophages, LPS alone is considered a weak inducer of inducible NOS in renal epithelium, and its combination with cytokines is required for full expression of inducible NOS (1, 2, 35, 49). Recently, the NF-κB proteins, a ubiquitous group of transcription factors that exist mainly as inactive dimers in the cytoplasm of cells, have been reported to be involved in the control of renal epithelial inducible NOS (1). These NF-κB proteins would be necessary for the transcriptional induction of renal epithelial inducible NOS by LPS and cytokines. However, it has been demonstrated that in renal epithelium NF-κB activation by LPS alone is not sufficient for induction of inducible NOS activity (1). It is important to note that these significant increases of total nitrite occurred only with a 7-day exposure, since 24- and 72-h-treated cells did not release a significant amount of nitrite (data not shown). In a previous study it was reported that at least 96 h of exposure to cytokines was required to induce a significant nitrite level in the supernatants of renal epithelial cells (29). In contrast, NO induction by macrophages and other cell types occurs more rapidly after exposure to cytokines (6–8, 13, 19, 36). Thus, it seems that NO production by PTC requires a long period of stimulation. Furthermore, it is important to mention that, in our experiments, NO determination was achieved by the indirect method, NO being measured in its final metabolites, nitrate and nitrite. Unfortunately, this method is not specific and is less sensitive than direct methods. Indirect measurement of NOS activity by incubating radiolabeled L-arginine with PTC and measuring the production rate of L-citrulline (45) did not appear to be a more specific method compared to the Griess reaction, since there is competition between NOS and arginase which catalyzes the formation of ornithine and urea from arginine (32). The direct measurement of gaseous NO by chemiluminescence, because of its greater specificity and ability to quantitate NO levels as low as 1 pmol/liter (11, 48), is another suitable technique for a better appreciation of the PTC ability to generate NO.

L-NMMA, an L-Arg analog, is a well-known NOS inhibitor (19, 23). However, there are several analogs of L-Arg capable of inhibiting NOS activity as L-NMMA does (21, 23). The most likely mechanism of action of these substances is competitive inhibition of the conversion of L-Arg to NO (16). It is interesting that both the inducible and the constitutive isoforms of NOS revealed characteristic differences in susceptibility to inhibition by various L-Arg analogs (21). Unfortunately, inhibitors with true isoform specificity have not yet been reported, although it has been demonstrated that N^{ω}-nitro-L-arginine, another L-Arg analog, was more potent than L-NMMA as a specific inhibitor in an LLC-PK_1 porcine kidney epithelial cell model (25). As mentioned in Table 1, L-NMMA prevented nitrite accumulation in the supernatant, whereas an excess of L-Arg reversed partially but not significantly this blockade. This property of L-NMMA to inhibit nitrite induction in PTC has been already demonstrated with rat renal tubular cells stimulated with various concentrations of cytokine combinations (31). Indeed, L-NMMA has been demonstrated to be an effective inhibitor of cytokine-induced NO in a rat PTC model, and this NO generation occurred via expression of an inducible NOS isoform (29).

Since constitutive NOS enzymes are reported to be generally calcium-calmodulin dependent, whereas the inducible isoforms are calcium independent (32), we have conducted an experiment with EGTA, a potent calcium chelator currently used to determine the NOS isoform involved in NO synthesis (22). Although our results demonstrated that EGTA had no effect on the nitrite accumulation in the supernatants and a recent study with normoxic and hypoxic tubule models also showed that NO production was calcium independent (54), we think that further investigations are needed to confirm exactly which NOS isoform synthesized NO in the present study, because in kidney and other cell types a calcium-independent membrane-bound constitutive isoform of NOS has been found (22) and because both constitutive and inducible NOS mRNAs have been detected in the proximal tubule (29, 31, 51). Furthermore, an immunohistochemical study performed recently in our laboratory has demonstrated the expression of both inducible and constitutive NOS in rat epithelial tubular cells (unpublished data).

In the present study, the inhibition of sodium-dependent MGP uptake occurred in the early 24 h of exposure (data not shown). Each agent induced inhibition of MGP uptake when used alone and led to synergistic inhibition when combined together. It is known that the determination of sodium-dependent MGP uptake is a very sensitive and rapid method for assessment of the loss of cellular function at an early time point (9); furthermore, it has been demonstrated that sodium transport by cortical collecting tubular cells was inhibited by NO and that this inhibition was associated with an increase in cGMP (43). It is interesting that cGMP itself has been shown to act as an NO second messenger and that it can inhibit both sodium and water transport in the collecting duct (33, 34, 41, 52, 55). Moreover, Stoos et al. (44) demonstrated that NO release from cultured kidney cells or NO donors inhibits sodium reabsorption in the isolated kidney cells, as determined by decreases in intracellular sodium flux, and that this inhibition appears to occur via the blockade of sodium entry via apical membrane sodium channels. All the evidence mentioned above demonstrates that the inhibition of sodium transport induced by NO occurred via a mechanism involving cGMP and intracellular sodium regulation. Since in the present study sodium-dependent uptake of MGP markedly decreased when inducers were combined and this failure correlated with significant NO production, we can postulate that NO-induced inhibition of sodium-dependent uptake of MGP is involved in the alteration of this important physiological function of PTC.

LPS led to significant LDH release which was markedly enhanced when both IL-1β and TNF-α were associated with LPS, demonstrating a severe injury of PTC. This injury was parallel to an increased nitrite level in the supernatants. NO has been found to exert a cytotoxic activity directly, as demonstrated with macrophages and other cell types (6, 23, 36). It is known that the macrophage cytotoxic effector mechanism which is L-Arg dependent causes inhibition of mitochondrial respiration, aconitase activity, and DNA synthesis in tumor target cells (7, 14, 23, 42). Furthermore, this cytotoxic activity induced via the inhibition of mitochondrial respiration in target cells has been reported to be proportional to the concentration of L-Arg in the medium (19, 23). It has been reported also that NO can exert its cytotoxic activity through reaction with superoxide radical to yield peroxinitrite anion and its derivative products to initiate lipid peroxidation (54). Investigations about insulinoma β-cell damage revealed that cytokine-induced increases of nitrite levels were accompanied by increases in DNA fragmentation, followed by mitochondrial damage and cell destruction (47). These findings suggest that NO is involved in mediating the cytodestructive effects of cytokines in cells. Such mechanisms described above could probably explain the decrease of cell viability observed with PTC

exposed to cytokines over 72 h in the present study. Although weak, the increased nitrite level observed with an L-Arg excess confirmed that NO synthesis occurred via an L-Arg-dependent pathway. Thus, it is possible that the cellular impairment and damages associated with NO production are the consequence of its cytotoxic activity. However, cytokine-induced NO production did not appear to be the sole condition for cell destruction, since the cytodestructive action of cytokine combination could be prevented without inhibiting the NO-stimulating action (47). Moreover, in this present study, LPS alone, which was a weak inducer of NO, led to a significant increase of the LDH level as well as remarkably inhibited MGP uptake, while single or combined cytokines which induced important nitrite production failed to increase significantly the LDH level when not associated with LPS.

In conclusion, this study demonstrates that NO generation by PTC in response to LPS and cytokines is closely related to cellular damage. Although NO is known to mediate cytodestructive effects of cytokines and LPS, its production did not account for the sole cytotoxic effects of cytokine and LPS, and other actions of these compounds might be involved in cell dysfunction, since cell impairment can occur even in the presence of weak induction of NO.

ACKNOWLEDGMENTS

We thank Yves Bergeron, Caroline Francoeur, and Denis Beauchamp for assistance.

REFERENCES

1. **Amoah-Apraku, B., L. J. Chandler, J. K. Harrison, S. S. Tang, J. L. Ingelfinger, and N. J. Guzman.** 1995. NF-κB and transcriptional control of renal epithelial-inducible nitric oxide synthase. Kidney Int. **48:**674–682.
2. **Amoah-Apraku, B., L. J. Chandler, K. M. Madsen, S. S. Tang, J. R. Ingelfinger, and N. J. Guzman.** 1993. Regulation of inducible nitric oxide synthase expression in murine proximal tubular epithelial cells. J. Am. Soc. Nephrol. **4:**544. (Abstract.)
3. **Baylis, C., P. Harton, and K. Engels.** 1990. Endothelial derived-relaxing factor controls renal hemodynamics in the normal rat kidney. J. Am. Soc. Nephrol. **1:**875–881.
4. **Beierwaltes, W. H., D. H. Sigmon, and O. A. Carretero.** 1992. Endothelium modulates renal blood flow but not autoregulation. Am. J. Physiol. **262:** F943–F949.
5. **Bello-Reuss, E., and M. Weber.** 1986. Electrophysiological studies on primary cultures of proximal tubule cells. Am. J. Physiol. **251:**F490–F498.
6. **Bergmann, L., K. D. Kröncke, C. Suschek, H. Kolb, and V. Kolb-Bachofern.** 1992. Cytotoxic action of IL-1β against pancreatic islets is mediated via nitric oxide formation and is inhibited by N^G-monomethyl-L-arginine. FEBS Lett. **299:**103–106.
7. **Billiar, T. R., R. D. Curran, D. J. Stuehr, J. Stadler, R. L. Simmons, and S. A. Murray.** 1990. Inducible cytosolic enzyme activity for the production of nitrogen oxides from L-arginine in hepatocytes. Biochem. Biophys. Res. Commun. **168:**1034–1040.
8. **Billiar, T. R., R. D. Curran, D. J. Stuehr, M. A. West, B. G. Bentz, and R. L. Simmons.** 1989. An L-arginine-dependent mechanism mediates Kupffer cell inhibition of hepatocyte protein synthesis in vitro. J. Exp. Med. **169:**1467–1472.
9. **Boogaard, P. J., G. J. Mulder, and J. F. Nagelkerke.** 1989. α-Methylglucose uptake as a sensitive parameter for mechanistic studies of acute toxicity by xenobiotics. Toxicol. Appl. Pharmacol. **101:**144–157.
10. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
11. **Chung, S. J., and H. L. Fung.** 1990. Identification of the subcellular site for nitroglycerin metabolism to nitric oxide in bovine coronary smooth muscle cells. J. Pharmacol. Exp. Ther. **253:**614–619.
12. **Cook, H. T., and R. Sullivan.** 1991. Glomerular nitrite synthesis in in situ immune complex glomerulonephritis in the rat. Am. J. Pathol. **139:**1047–1052.
13. **Curran, R. D., T. R. Billiar, D. J. Stuehr, K. Hofmann, and R. L. Simmons.** 1989. Hepatocytes produce nitrogen oxides from L-arginine in response to inflammatory products of Kupffer cells. J. Exp. Med. **170:**1769–1774.
14. **Curran, R. D., F. K. Ferrari, P. H. Kispert, J. Stadler, D. J. Stuehr, R. L. Simmons, and T. R. Billiar.** 1991. Nitric oxide and nitric oxide generating compounds inhibit hepatocyte protein synthesis. FASEB J. **5:**2085–2092.
15. **Drapier, J. C., and J. B. Hibbs.** 1988. Differentiation of murine macrophages to express non specific cytotoxicity for tumor cells results in L-arginine dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. J. Immunol. **140:**2829–2838.
16. **Furchgott, R. F., and P. M. Vanhoutte.** 1989. Endothelium-derived relaxing and contracting factors. FASEB J. **3:**2007–2018.
17. **Furchgott, R. F., and J. V. Zawadzky.** 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature **288:**373–376.
18. **Goldblum, S. E., B. Hennig, and M. Jay.** 1989. Tumor necrosis factor alpha-induced pulmonary vascular endothelial injury. Infect. Immun. **57:**1218–1226.
19. **Granger, D. L., J. B. J. Hibbs, J. R. Perfect, and D. T. Durack.** 1990. Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. J. Clin. Invest. **85:**264–273.
20. **Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum.** 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal. Biochem. **126:**131–138.
21. **Gross, S. S., D. J. Stuehr, K. Aisaka, E. A. Jaffe, R. Levi, and O. W. Griffith.** 1990. Macrophage and endothelial cell nitric oxide synthesis. Cell type selective inhibition by N^G-aminoarginine, N^G-nitroarginine, and N^G-methylarginine. Biochem. Biophys. Res. Commun. **170:**96–103.
22. **Hecker, M., D. T. Walsh, and J. R. Vane.** 1991. On the substrate specificity of nitric oxide synthase. FEBS Lett. **294:**221–224.
23. **Hibbs, J. B. J., V. Zdenek, and R. R. Taintor.** 1987. L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J. Immunol. **138:**550–565.
24. **Ignarro, L. J.** 1990. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu. Rev. Pharmacol. Toxicol. **30:**535–560.
25. **Ishii, K., J. F. J. Kerwin, and F. Murad.** 1990. N^{ω}-nitro-L-arginine: a potent inhibitor of the L-arginine-dependent soluble guanylate cyclase activation pathway in LLC-PK_1 cells. Can. J. Physiol. Pharmacol. **68:**749–751.
26. **Joly, V., Y. Bergeron, M. G. Bergeron, and C. Carbon.** 1991. Endotoxin-tobramycin additive toxicity on renal tubular cells in culture. Antimicrob. Agents Chemother. **35:**351–357.
27. **Lowenstein, C. J., and S. H. Snyder.** 1992. Nitric oxide, a novel biological messenger. Cell **70:**705–707.
28. **Lüscher, T. F., H. A. Bock, Z. Yang, and D. Diederich.** 1991. Endothelium-derived relaxing and contracting factors: perspectives in nephrology. Kidney Int. **39:**575–590.
29. **Markewitz, B. A., J. R. Michael, and E. D. Kohan.** 1993. Cytokine-induced expression of a nitric oxide synthase in rat tubule cells. J. Clin. Invest. **91:**2138–2143.
30. **Marletta, M. A., P. S. Yoon, R. Iyengar, C. D. Leaf, and J. S. Wishnok.** 1988. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry **27:**8706–8711.
31. **Mohaupt, M. G., J. L. Elzie, K. Y. Ahn, W. L. Clapp, C. S. Wilcox, and B. C. Kone.** 1994. Differential expression and induction of mRNAs encoding two inducible nitric oxide synthases in rat kidney. Kidney Int. **46:**653–665.
32. **Moncada, S., R. M. J. Palmer, and E. A. Higgs.** 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. **43:**109–142.
33. **Nonoguchi, H., J. M. Sands, and M. A. Knepper.** 1988. Atrial natriuretic factor inhibits vasopressin-stimulated osmotic water permeability in rat inner medullary collecting duct. J. Clin. Invest. **82:**1383–1390.
34. **Nonoguchi, H., J. M. Sands, and M. A. Knepper.** 1989. ANF inhibits NaCl and fluid absorbtion in cortical collecting duct of rat kidney. Am. J. Physiol. **256:**F179–F186.
35. **Nussler, A. K., and T. R. Billiar.** 1993. Inflammation, immunoregulation and inducible nitric oxide synthase. J. Leukocyte Biol. **54:**171–178.
36. **Nussler, A. K., M. Di Silvio, T. R. Billiar, R. A. Hoffman, D. A. Geller, R. Selby, J. Madariaga, and R. L. Simmons.** 1992. Stimulation of the nitric oxide synthase pathway in human hepatocytes by cytokines and endotoxin. J. Exp. Med. **176:**261–264.
37. **Palmer, R. M., and S. Moncada.** 1989. A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. Biochem. Biophys. Res. Commun. **158:**348–352.
38. **Palmer, R. M., D. D. Rees, D. S. Ashton, and S. Moncada.** 1988. L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochem. Biophys. Res. Commun. **153:**1251–1256.
39. **Romero, J. C., V. Lahera, M. G. Salom, and M. L. Biondi.** 1992. Role of endothelium-dependent relaxing factor nitric oxide on renal function. J. Am. Soc. Nephrol. **2:**1371–1387.
40. **Salom, M. G., V. Lahera, and J. C. Romero.** 1991. Role of prostaglandins and endothelium-derived relaxing factor in the renal response to acetylcholine. Am. J. Pharmacol. **260:**F145–F149.
41. **Shultz, P. J., A. E. Schorer, and L. Raij.** 1990. Effects of endothelium-derived relaxing factor and nitric oxide on rat mesangial cells. Am. J. Physiol. **258:** F162–F167.
42. **Stadler, J., T. R. Billiar, R. D. Curran, D. J. Stuehr, J. B. Ochoa, and R. L. Simmons.** 1991. Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. Am. J. Physiol. **260:**910–916.
43. **Stoos, B. A., O. A. Carretero, R. D. Fahry, G. Scichi, and J. L. Garvin.** 1992.

Endothelium-derived relaxing factor inhibits transport and increases cGMP content in cultured mouse cortical collecting duct cells. J. Clin. Invest. **89:** 761–765.
44. **Stoos, B. A., N. H. Garcia, and J. L. Garvin.** 1995. Nitric oxide inhibits sodium reabsorption in the isolated perfused cortical collecting duct. J. Am. Soc. Nephrol. **6:**89–94.
45. **Stuehr, D. J., and O. W. Griffith.** 1992. Mammalian nitric oxide synthases. Adv. Enzymol. **65:**287–346.
46. **Stuehr, D. J., and C. F. Nathan.** 1989. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. **169:**1543–1555.
47. **Suarez-Pinzon, W. L., K. Strynadka, R. Schulz, and A. Rabinovitch.** 1994. Mechanisms of cytokine-induced destruction of rat insulinoma cells: the role of nitric oxide. Endocrinology **134:**1006–1010.
48. **Sung, Y. J., J. H. Hotchkiss, R. E. Austic, and R. R. Dietert.** 1992. Direct measurement of nitric oxide in headspace gas produced by a chicken macrophage cell line in a closed culture system. Biochem. Biophys. Res. Commun. **184:**36–42.
49. **Szabo, C., J. A. Mitchell, S. S. Gross, C. Thiemermann, and J. R. Vane.** 1993. Nifedipine inhibits the induction of nitric oxide synthase by bacterial lipopolysaccharide. J. Pharmacol. Exp. Ther. **265:**674–680.
50. **Tayeh, M. A., and M. A. Marletta.** 1989. Macrophage oxidation of L-arginine to nitrite, and nitrate. Tetrahydrobiopterin is required as cofactor. J. Biol. Chem. **264:**19654–19658.
51. **Terada, Y., K. Tomita, H. Nonoguchi, and F. Mammo.** 1992. Polymerase chain reaction localization of constitutive nitric oxide synthase and soluble guanylyl cyclase messenger RNAs in microdissected rat nephron segments. J. Clin. Invest. **90:**659–665.
52. **Vanhoutte, P. M.** 1989. Endothelium and control of vascular function. Hypertension **13:**658–667.
53. **Vinay, P., A. Gougoux, and G. Lemieux.** 1981. Isolation of a pure suspension of rat proximal tubules. Am. J. Physiol. **241:**F403–F411.
54. **Yu, L., P. E. Gengaro, M. Niederberger, T. J. Burke, and R. W. Schrier.** 1994. Nitric oxide: a mediator in rat tubular hypoxia/reoxygenation injury. Proc. Natl. Acad. Sci. USA **91:**1691–1695.
55. **Zeidel, M. L., K. Deepak, P. Silva, M. Burrowes, and B. M. Brenner.** 1988. Atrial natriuretic peptides inhibit conductive sodium uptake by rabbit inner medullary collecting duct cells. J. Clin. Invest. **82:**1067–1074.