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Differential regulation of glomerular arginine transporters (CAT-1 and CAT-2) in lipopolysaccharide-treated rats. Am J Physiol Renal Physiol 284:F788–F795, 2003. First published December 10, 2002; 10.1152/ajprenal.00221.2002.—The decrease in glomerular filtration rate (GFR) that is characteristic of sepsis has been shown to result from inhibition of glomerular endothelial nitric oxide synthase (eNOS) by nitric oxide (NO) generated by NO biosynthesis. Although l-arginine is the sole precursor for NO biosynthesis, its intracellular availability in glomeruli from septic animals has never been investigated. Arginine uptake was measured in freshly harvested glomeruli from the following experimental groups: 1) untreated rats; 2) rats pretreated with LPS (4 mg/kg body wt, 4 h before experiments); 3) rats treated with LPS as above with either l-N6-(1-iminoethyl)lysine hydrochloride (l-NIL), a selective iNOS antagonist, or 7-nitroindazole, a selective neuronal NO antagonist; and 4) rats treated with l-NIL only. Both glomerular and mesangial arginine transport characteristics were found compatible with a y<sup>+</sup> system. Arginine uptake was augmented in glomeruli from LPS-treated rats. Treatment with l-NIL completely abolished this effect whereas l-NIL alone had no effect. Similar results were obtained when primary cultures of rat mesangial cells were preincubated with LPS (10 μg/ml for 24 h) with or without l-NIL. Using RT-PCR, we found that in vivo administration of LPS resulted in a significant increase in glomerular cationic amino acid transporter-2 (CAT-2) mRNA expression whereas CAT-1 mRNA was undetected. Northern blotting further confirmed a significant increase in glomerular CAT-2 by LPS. In mesangial cells, the expression of both CAT-1 and CAT-2 mRNA was augmented after incubation with LPS. In conclusion, in vivo administration of LPS augments glomerular arginine transport through upregulation of steady-state CAT-2 mRNA while downregulating CAT-1 mRNA. These results may correspond to the changes in glomerular iNOS and eNOS activity in sepsis.

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mRNA expression and the activity of eNOS and iNOS during sepsis.

METHODS

All animal experiments described in this study were conducted in accord with the protocol approved by the institutional committee on ethics in animal experiments. Studies were performed using male Wistar rats weighing 200–250 g.

Four hours after a single intraperitoneal injection of LPS (4 mg/kg body wt, serotype 0111:B4, Sigma) or saline, animals were allocated to different experimental groups. The above time course was chosen based on the observation demonstrating that one dose of LPS exerts its maximal effect on glomerular arginine uptake after 4–6 h (see Fig. 3).

Experimental groups. The experimental groups were as follows. In group 1, control rats were untreated (n = 6). Group 2 rats were treated with LPS only (n = 5). Group 3 rats were treated LPS + L-N^6-(1-iminoethyl)lysine hydrochloride (L-NIL), a selective inhibitor of iNOS (26) (5 injections at a dose of 3 mg/kg ip, 36, 24, and 12 h before LPS and simultaneously with and 2 h after the administration of LPS (n = 5); Sigma). Group 4 rats were treated with L-NIL only (n = 5) as in group 3 without LPS. Group 5 rats received LPS + 7-nitroindazole (7-NI), selective neuronal (brain) NOS (bNOS) inhibitor (5) (5 injections at 3 mg/kg, every 48 h, whereas adherent cells were retained. After cells reached confluence (in ~4 wk) they were passaged using trypsin/EDTA. The cells utilized in these experiments exhibited typical morphological characteristics of mesangial cells and stained uniformly positive for α-smooth muscle actin. Cells between passages 2 and 3 were used for subsequent experimental procedures.

L-Arginine uptake in freshly harvested glomeruli. Arginine uptake was determined essentially as described by Gazzola et al. (10). Glomerular suspensions from the various experimental groups were incubated and shaken for 10 min in HEPES buffer, pH 7.4, at 37°C. L-[H^3]arginine and L-arginine, in a final concentration of 1 mM, were added to a total volume of 1 ml for an additional 4 min. The duration of 4 min was chosen because it was within the linear portion of uptake curves (data not shown). Transport activity was terminated by rapidly washing the cells with ice-cold PBS buffer (4 times, 2 ml/tube). The glomeruli were then dried and solubilized in 1 ml of 0.5% SDS in 0.5 N NaOH. To monitor radioactivity by liquid scintillation spectrometry (Betamatic, Kontron), 700 μl of the extract were used. The remaining 300 μl were used for protein content determination by using the Lowry method. To correct for nonspecific uptake or cell membrane binding, glomeruli were incubated with 10 mM unlabeled arginine in HEPES buffer, and the associated radioactivity was subtracted from each data point. The results are expressed as means ± SE of at least 5 different experiments.

L-Arginine uptake in mesangial cells. Cells were seeded on 24-well culture plates (Corning) at a density of 10⁶ cells/well. When confluent, cells were washed with 2 ml HEPES buffer, pH 7.4. L-[H^3]arginine and L-arginine, in a final concentration of 1 mM, were added to a total volume of 1 ml for 1 min (within the linear portion of the uptake curve). Transport assays were performed as described above in the following conditions: 1) control (untreated cells); 2) LPS (mesangial cells preincubated with 10 μg/ml LPS for 24 h); 3) LPS + L-NIL (LPS as in the previous group +50 μM L-NIL); and 4) L-NIL only.

Analysis of mRNA levels for CAT-1 and CAT-2 by RT-PCR. Total cellular RNA was extracted from glomeruli or mesangial cells following the method described by Chomczynski and Sacchi (5). RT was carried out for 1.5 h at 42°C, and PCR in 1× Jeffreys’ buffer (16), for 35 cycles each of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and 7 min (final cycle). The first pair of primers was designed to bind to a portion of the rat CAT-1 gene: 5′-GCCATGCTACTCTCCTCGT-3′ (forward 21-mer), and 5′-CCCTCCCTACCCGATTTTCAC-3′ (reverse 21-mer) (17). A second pair of primers, which hybridizes to a sequence common to both CAT-2 and CAT-2A, comprised 5′-AACGTCGTCCTCTCTGTT-3′ (forward 24-mer) and 5′-GGTGACCTGAGTCGCTCTT-3′ (reverse 23-mer) (9). To differentiate between the two variants of CAT-2 (CAT-2 and CAT-2A), a third pair of primers, which hybridizes to a sequence specific to CAT-2A, was used: 5′-CCCTACCGCGCGGCTGG-3′ (forward 21-mer) and 5′-AAATGACCCCTGCAGTCATCG-3′ (reverse 21-mer) (12) to exclude the possibility of contamination by genomic DNA amplification and to assess the adequacy of cDNA, experiments in the absence of RT were carried out and amplification of GAPDH was performed, respectively. PCR products were electrophoresed on a 1.6% agarose gel and visualized by UV-induced fluorescence. All PCR reactions resulted in the amplification of a single product of the predicted size for CAT-1, CAT-1, CAT-2A, and GAPDH.

Northern blot analysis. CAT-2 mRNA level was determined by Northern hybridization. Fifteen micrograms of total RNA were denatured and fractionated by size on 1.3% formaldehyde-agarose gels. RNA was transferred overnight, followed by hybridization, to a nylon membrane (Hybond-N, Amersham) and cross-linked by short-wave UV illumination. Purified end products of CAT-2 and GAPDH cDNA (5 ng/ml) were used directly for radiolabeling after electrophoresis in 1.5% (wt/vol) low-melting-point agarose gels. The probes used were labeled to a specific activity of >10⁹ cpm/μg with...
[α-32P]dCTP by a random primer labeling method (GIBCO BRL), where cpm is counts/min. After hybridization with the 32P-labeled cDNA overnight at 50 °C, the membranes were sequentially washed twice in 1/1000 SSC, 0.1% SDS for 15 min at room temperature, once in 1/1000 SSC, 0.1% SDS for 15 min at 50 °C, followed by 0.5/1000 SSC, 0.1% SDS at 55 °C for 30 min, and then washed at high stringency in 0.1/1000 SSC, 0.1% SDS at 57 °C for 15 min. Autoradiography was carried out with Kodak XAR film for 24–48 h at −70 °C. Relative mRNA abundance was quantified by measuring the density of the exposed film with a densitometer (B.I.S 202D). CAT-2 mRNA level was normalized to GAPDH mRNA and is expressed in arbitrary units as the ratio of CAT-2 to GAPDH expression of three different experiments.

Statistical analysis. One-way ANOVA for comparison between groups (means ± SE) and Student’s t-test between two groups were computed and used to assess statistical significance. *P values < 0.05 were considered to be statistically significant.

RESULTS

While the membrane transport of arginine has been found to occur largely via a Na+—independent pathway, we wished to examine the possibility of a Na+—dependent transport component. Accordingly, initial experiments were designed to examine the possible contribution of Na+—dependent glomerular arginine transport. Figure 1, A and B, demonstrates that the presence of sodium had no effect on either glomerular or mesangial arginine transport. This indicates that arginine transport in glomeruli and mesangial cells occurs exclusively via Na+—independent pathways. In addition, an excess concentration of lysine strongly inhibited L-arginine uptake in both glomeruli and mesangial cells. In contrast, the neutral amino acid methionine was found to be a poor inhibitor (Fig. 1, A and B). These data establish that system y+ is the predominant arginine transport system in glomeruli and mesangial cells.

To characterize the kinetics of L-arginine transport in glomeruli and mesangial cells, the saturable uptake of L-arginine (0–1 mM) was measured. The plot of L-arginine uptake as a function of extracellular L-arginine concentration is shown in Fig. 2, A and B. A high-affinity transporter was present in both glomeruli and mesangial cells (glomeruli: $K_m$ 110 μM; $V_{max}$ 4.3 nmol arginine·μg protein−1·4 min−1; mesangial cells: $K_m$ 120 μM, $V_{max}$: 540 fmol arginine/μg protein). On the basis of previous reports, the kinetic properties of the arginine transport system in both glomeruli and mesangial cells resemble those of system y+, CAT-1, and CAT-2.
Effects of LPS on arginine transport in glomeruli and mesangial cells. In rats, a significant increase in glomerular arginine uptake was observed 2 h after LPS administration, peaking at 4–6 h and decreasing to control levels at 16 h (Fig. 3). These results correspond to the time course of glomerular iNOS activation after LPS (25). In vivo administration of LPS resulted in a significant increase in glomerular arginine uptake compared with untreated rats. The effect was completely abolished by the coadministration of L-NIL, a selective iNOS inhibitor. L-NIL alone had no effect on glomerular arginine transport. Because L-NIL could potentially block the effect of bNOS as well, 7-NI, a selective bNOS inhibitor, was also administered to LPS-treated rats. In contrast to L-NIL, 7-NI did not affect arginine uptake by glomeruli harvested from these animals (Fig. 4). We repeated the critical experiments using primary cultures of renal mesangial cells and found that mesangial cells exposed to LPS (10 μg/ml for 24 h) exhibited maximal nitrite production (data not shown). Therefore, we chose to use the above concentration and time course in all of the following experiments. Similar to our findings in glomeruli, incubating mesangial cells with LPS induced a significant increase in uptake of 1 mM extracellular arginine, an effect that was prevented by coincubating the cells with L-NIL. L-NIL alone had no effect on mesangial arginine uptake (Fig. 5). After the administration of LPS to both glomeruli and mesangial cells, arginine uptake remained sodium independent. Lysine, but not methionine, exerted an inhibitory effect on arginine uptake, implying that the γ system remains the predominant arginine transport system in tissues exposed to LPS (Fig. 6, A and B).

Differential regulation of CAT-1 and CAT-2 gene expression by LPS. To determine whether the observed LPS-induced changes in arginine uptake are associated with similar directional changes in levels for mRNA for the CAT family of transporters, total glomerular RNA was analyzed by RT-PCR to amplify portions of CAT-1 and CAT-2. RT-PCR identified cDNA encoding both CAT-1 and CAT-2 in glomeruli harvested from control rats. Surprisingly, in vivo administration of LPS abolished the expression of CAT-1 mRNA. In contrast, LPS significantly augmented the expression of mCAT-2 mRNA. The coadministration of L-NIL (a selective iNOS inhibitor) to LPS-treated rats reversed the effects of LPS on both transporters. Because a low-affinity component of the glomerular arginine transporter could not be excluded, specific primers to CAT-2A were used. The mRNA expression of CAT-2A was constitutively present but did not vary among the experimental groups (Fig. 7, A and B). Because CAT-2A was minimally expressed in glomeruli, positive controls from rat liver cells were performed (Fig. 7C). No RT-PCR products were obtained with RNA samples in the absence of RT or when cDNA was omitted from PCR.

To further confirm this observation, Northern blotting was performed. CAT-1 and CAT-2 mRNA were not detectable in glomeruli taken from untreated animals. Therefore, we were unable to confirm our PCR results on CAT-1. Administration of LPS resulted in a signif-
significant increase in steady-state glomerular CAT-2 mRNA, an increase that was abolished by coadministration of L-NIL (Fig. 8, A and B).

RT-PCR for the two arginine transporters was also performed in mesangial cells treated with LPS. In contrast to our findings in glomeruli from LPS-treated rats, incubating mesangial cells with LPS for 24 h significantly augmented the expression of both CAT-1 and CAT-2 mRNA, when normalized to GAPDH (Fig. 9, A and B).

DISCUSSION

Despite the enormous interest being focused on the various renal effects of arginine metabolites, most importantly NO, the present study is the first to investigate the regulation of arginine uptake by glomeruli. We demonstrate that both glomerular and mesangial arginine transport systems are sodium independent, subject to cis inhibition by lysine, but not methionine, and constitutively express the two major arginine transporters, namely, CAT-1 and CAT-2. Our results imply that arginine uptake in those tissues occurs largely via a $y^+$ system and the above transporters play a crucial role in glomerular arginine traffic. Therefore, changes in their levels or activity could potentially alter NO production under certain pathophysiological conditions, including sepsis.

We have also demonstrated that LPS-treated rats exhibit an augmented glomerular arginine uptake through modulation of CAT-2 mRNA. The observations above suggest that increased glomerular arginine transport in sepsis is related to the induction of iNOS by LPS and results from upregulation of CAT-2 mRNA. The fact that coadministration of L-NIL (a selective iNOS antagonist) but not 7-NI (a selective bNOS inhibitor) completely abrogated the increase in glomerular arginine uptake and CAT-2 mRNA expression after LPS administration further supports the above hypothesis. The association between activation of iNOS and CAT-2 has been previously described in nonrenal tissues. The first indication that CAT-2 may provide
iNOS with its substrate came from the observation that CAT-2 and iNOS transcripts were coinduced in concert with increased system \( \gamma \) activity after appropriate cytokine stimulation. Simmons et al. (29) have shown in cardiac myocytes that IL-1 and interferon simultaneously increase mRNA expression of both CAT-1 and CAT-2, thereby enhancing arginine transport into the cells. Nicholson et al. (23) have reported that iNOS activity was reduced in macrophages from CAT-2 knockout mice. We have recently demonstrated that tetrahydrobiopterin (BH4) acts as a cofactor that simultaneously upregulates both iNOS and CAT-2 mRNA (27). Together, these data support the view that on induction of iNOS, the excess arginine required is delivered by CAT-2. Whether the association between activation of iNOS and CAT-2 stems from dependency on the same regulatory cofactors, such as BH4, or relies on an adjacent intracellular localization remains elusive.

In contrast to the effect of LPS on glomerular CAT-2, the expression of CAT-1 was completely abolished. Although a direct comparison of mRNA levels does not necessarily reflect the amount or activity of the transport proteins, the fact that CAT-1 mRNA was undetectable makes the assumption of decreased activity conceivable. The mechanism responsible for decreased CAT-1 mRNA level is not clear. Could regulation of one transporter be dictated by changes in expression of another CAT protein? Similar findings were reported by us in a study of the effect of BH4 on CAT-1 and CAT-2 mRNA expression in rat cardiac myocytes (27). Nicholson et al. (24) demonstrated that CAT-3 can compensate for the loss of functional CAT-1 in cells from CAT-1 knockout mice. It was hypothesized that upregulation of an individual CAT decreases the steady-state mRNA of the other transporters via a direct effect or, alternatively, an increase in an individual CAT could reflect a compensatory response to a decrease in the expression of a different CAT isoform. It seems logical that NO or one of its metabolites, most importantly peroxynitrite, plays a role in the attenuation of CAT-1 expression. This led us to conduct a series of in vitro experiments in which we exposed mesangial cells to LPS in an attempt to explore a possible explanation for the decrease in CAT-1 mRNA. Surprisingly, in contrast to our findings in glomeruli from LPS-treated rats, when primary cultures of mesangial cells were treated with LPS, mRNA expression of both transporters was increased, contradicting the above assumption. On the basis of the discrepancy...
between the results in freshly harvested glomeruli and mesangial cell cultures, one could speculate that certain events occurring in the kidney after systemic administration of LPS are responsible for the downregulation of CAT-1 mRNA. Such a critical event after LPS administration is renal vasoconstriction and ischemia. Preliminary data from our laboratory suggest that peroxynitrite, a toxic oxidant formed from the reaction of NO and superoxide during ischemia-reperfusion, increased rather than decreased the expression of both CAT-1 and CAT-2 when administered to mesangial cells (Schwartz and Iaina, unpublished observations). Therefore, its production could not serve as a possible explanation of the above-mentioned phenomenon. At this juncture, the mechanism for downregulation of glomerular CAT-1 mRNA by LPS remains enigmatic.

A major question posed by this observation is whether selective inhibition of CAT-1, while total glomerular uptake of arginine is significantly increased, has any pathophysiological meaning. In endothelial cells, extracellular arginine seems to drive NO production even when excess intracellular levels are available. This phenomenon has been termed “the arginine paradox.” One paradigm that could explain this observation is that intracellular arginine is sequestered in one or more pools that are poorly accessible to eNOS, whereas extracellular arginine transported into the cell is preferentially delivered to eNOS. It has recently been shown that CAT-1 and eNOS are colocalized in a caveolar complex (21). Such a complex has been suggested to serve as a mechanism for channeling newly acquired extracellular arginine to eNOS for NO synthesis. Thus selective delivery of transported arginine to membrane-bound eNOS could explain the arginine paradox discussed above. We have previously demonstrated that induction of iNOS by LPS induces selective eNOS inhibition (26). One can speculate that inhibition of CAT-1 after in vivo administration of LPS, as shown in the present experiments, may provide a possible mechanism for selective eNOS inhibition in the presence of increased arginine uptake and iNOS activity. In the aggregate, the effects of LPS on the expression of the two transporters described here parallel the changes in their corresponding NOS activity, as shown previously (26). Namely, increased activity/expression of CAT-2 and iNOS, while activity/expression of CAT-1 and eNOS is diminished, emphasizes the dependency of these two pairs of proteins.

Using glomeruli as a source of renal tissue in our experiments reveals a significant limitation because the specific cells in which the above events occur cannot be defined. One can argue that the induction of iNOS by LPS, which involves mainly macrophages and mesangial cells, would not influence NO metabolism in endothelial cells. Nevertheless, Shultz and Raij (28) demonstrated in LPS-treated rats that inhibition of NO generation induces glomerular capillary thrombosis, a purely endothelial event. We have found that induction of iNOS inhibits eNOS within the glomerulus (26). These publications suggest that the glomerulus exhibits a microenvironment, in which events in one cell can affect NO metabolism in neighboring cells. Moreover, the importance of using freshly harvested glomeruli rather than cell cultures is greatly supported by the above findings, which demonstrated an opposite effect of LPS on CAT-1 in glomeruli vs. mesangial cells.

Arginine transport velocities in our experiments were found to be significantly higher than the average values published by others. These differences can be partly explained by higher extracellular arginine concentrations and longer incubation periods used in the present studies, as well as improved accessibility of glomeruli in suspension to extracellular arginine compared with that of adherent cells. With regard to the high transport rates reported in our studies, equilibrium should have been reached earlier. Glomeruli tend to stick to tube walls, and therefore the participation of cells in a transport assay is probably unsynchronized. This phenomenon could have caused a deviation of the equilibrium point in our studies.

In summary, our findings suggest that administration of LPS to rats, as an experimental model of sepsis, augments glomerular arginine uptake through upregulation of CAT-2 mRNA while decreasing CAT-1 mRNA levels. The complex regulation of genes encoding proteins required for L-arginine transport by LPS could potentially play a role in altered NOS isoform activity in sepsis.

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REFERENCES


