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Abstract

Acute renal failure (ARF) is a frequent complication of Gram-negative sepsis, with a high risk of mortality. Lipopolysaccharide (LPS)-induced ARF is associated with hemodynamic changes that are strongly influenced by the overproduction of nitric oxide (NO) through the cytokine-mediated up-regulation of inducible NO synthase. LPS-induced reductions in systemic vascular resistance paradoxically culminate in renal vasoconstriction. Collagen XVIII is an important component of the extracellular matrix expressed in basement membranes. Its degradation by matrix metalloproteases, cathepsins and elastases results in the formation of endostatin, claimed to have antiangiogenic activity and to be a prominent vasorelaxing agent. We evaluated the expression of endostatin/collagen XVIII in an endotoxemic ARF model. ARF was induced in C57BL/6 mice by intraperitoneal injection of LPS (10 mg/kg) followed by sacrifice 4 and 12 h later. Kidney tissue was the source of RNA and protein and the subject of histological analysis. As early as 4 h after LPS administration, blood urea, creatinine and NO levels were significantly increased compared to control. Endostatin/collagen XVIII mRNA levels were 0.71 times lower than sham-inoculated mice 4 h after LPS inoculation, returning to normal levels 12 h after LPS inoculation. Immunohistological examination revealed that acute injury caused by LPS leads to an increase of endostatin basement membrane staining in association with the decrease of CD31 endothelial basement membrane staining. These results indicate that in the early phase of endotoxemic ARF the endostatin levels were not regulated by gene expression, but by the metabolism of collagen XVIII.

Key words: Acute renal failure; Lipopolysaccharide; Gene expression; Endostatin; CD31

Introduction

Acute renal failure (ARF) due to endotoxins is a common problem in clinical medicine. Endotoxins are released from the outer membrane of the Gram-negative bacterial envelope and are composed of lipopolysaccharides (LPS). ARF occurs in 20% of patients with severe sepsis and in 50% of patients with septic shock (1) and is clinically defined as a deterioration of glomerular filtration rate (GFR) and tubular function (2). Endotoxemia causes systemic release of cytokines (3,4) as well as the overproduction of nitric oxide (NO) through the cytokine-mediated up-regulation of inducible NO synthase (iNOS) (5-7).

Septic shock is characterized by generalized systemic vasodilation and hypotension accompanied by vascular hyporesponsiveness, leading to multiple organ failure and death (8,9). Although systemic hypotension is often present, LPS-induced ARF is characterized by marked intrarenal vasoconstriction (10,11). These hemodynamic changes alter renal blood flow, intrarenal hemodynamics and GFR. Intravital videomicroscopy has revealed that changes in peritubular capillaries can have a major impact on renal function (12). In fact, Wu et al. (12) demonstrated in a mouse model of endotoxemic ARF that renal peritubular capillary perfusion was significantly compromised as early as 2 h after LPS administration.

Collagen XVIII, a member of the heparan sulfate proteoglycan family, is an extracellular matrix (ECM) protein associated with basement membranes that is found in the basal laminae of the retina, epidermis, pia, heart and skeletal muscle, kidney, lung, and endothelial cells (13-15). Collagen XVIII molecules contain 10 triple-
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Material and Methods

Animals

Experiments were conducted on adult C57BL/6 mice (2 to 4 months old, 22-28 g body weight) maintained under specific pathogen-free conditions at the National Pharmacology Institute, Federal University of São Paulo, São Paulo, SP, Brazil. The experimental procedure was approved by the Federal University of São Paulo Committee for the Use of Live Animals in Teaching and Research.

ARF model

Mice were injected intraperitoneally with 10 mg/kg *Escherichia coli* LPS serotype 0111:B4 (Sigma-Aldrich, USA). At 4 and 12 h, respectively, after LPS inoculation the animals were anesthetized with ketamine (125 mg/g body weight, Ketalar; Parke-Davis, USA) and xylazine (12.5 mg/g body weight; Phoenix Scientific, Inc., USA), and blood was collected via the orbital sinus. This procedure was followed immediately by cervical dislocation and kidney harvest. One kidney was snap-frozen in liquid nitrogen and used for protein and total RNA extraction, and the other was fixed in 10% phosphate-buffered formalin for immunohistochemistry, and also stained with hematoxylin-eosin and periodic acid-Schiff.

Chemicals and renal function

Approximately 0.2 mL blood was collected from each animal into EDTA used as an anticoagulant.

Blood urea nitrogen and plasma creatinine levels were determined using commercial kits (Urea UV Liquiform, Cat. 104, and Creatinine K, Cat. 96, respectively; Labtest Diagnostics, Brazil).

Serum nitrate/nitrite (NOx) levels were determined by the Griess test. Briefly, serum samples diluted in water were deproteinized by treatment with 1.84% sulfuric acid (1:1 dilution), 10% sodium tungstate (1:1), and deionized water (2:1). The samples were then centrifuged at 2000 g for 10 min and the supernatants were collected. Nitrite levels were estimated in the supernatants using the Griess reagent [equal volumes of a solution of 1% sulfanilamide-0.8% N-(1-naphthyl)-ethylenediamine in water and 0.5 N HCl]. After 20 min of incubation at room temperature (28), test and standard curve absorbance were read at 550 nm. The results were compared against an NaNO₂ standard curve, and the nitrite concentration (µM) was calculated.

Quantitative real-time polymerase chain reaction

The reverse transcription polymerase chain reaction (RT-PCR) was performed for endostatin in RNA extracted from the kidneys of normal and ARF mice at 4 and 12 h after injection of 10 mg/kg LPS. Total RNA was harvested from the renal tissue using the Trizol reagent (Life Technologies, USA). Total RNA samples of 2 µg were used to accurately detect changes in the number of endostatin...
gene copies. The target gene expression was normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) levels. The primer for mouse endostatin was Mm00487129_m1 (TaqMan® Gene Expression Assays, Applied Biosystems, USA) and the primer for mouse HPRT was Mm03024075_m1 (TaqMan® Gene Expression Assays, Applied Biosystems). Quantitative RT-PCR was carried out in a thermocycler (7300 Real-Time PCR System; Applied Biosystems) according to manufacturer recommendations (29). Relative quantification was performed to describe the change in endostatin expression in LPS-treated mice compared to untreated controls.

Western blot analysis
Kidneys harvested from normal and ARF mice were homogenized in warmed protein extraction buffer (0.1 M Tris, 0.01 M EDTA, 1% SDS, and 0.01 M DTT, pH 8.0). After 2 min of incubation at 95°C, the homogenate was centrifuged at 13,000 rpm for 15 min and the supernatant was stored at -80°C. The samples (50 µg protein per lane) were run under reducing conditions on sodium dodecyl sulfate-polyacrylamide gels (4% stacking gel, 12% separating gel). The gels were then electroblotted onto nitrocellulose membranes. After blocking, the blots were incubated overnight with a rabbit anti-mouse endostatin polyclonal antibody (work dilution 1:100, Chemicon International, USA) and a horseradish peroxidase-conjugated goat anti-rabbit antibody as the secondary antibody (work dilution 1:2000, Chemicon International, USA). Bands were detected using enhanced chemiluminescence system (Amersham Biosciences, USA) according to manufacturer guidelines.

Immunohistochemical staining
Immunohistochemical analyses were performed at the University of São Paulo School of Medicine. Paraffin-embedded sections of mouse kidneys (4-µm thick) were mounted onto SuperFrost Plus slides (Fisher Scientific, USA), baked, deparaffinized and rehydrated. Heat-induced epitope retrieval was performed by immersion of the slides in 1 mM EDTA, pH 8.0, and incubation in an electric pressure cooker (Decloaking Chamber; BioCare Medical, USA) for 3 min at 25 psi. The slides were then placed in an automated immunohistochemical stainer (Ventana Medical Systems, USA) for endostatin staining. Endostatin labeling was performed using the CI 1837.46 monoclonal antibody (Upstate Biotechnology, USA) at 1:100 dilution for 28 min at 42°C, an amplification kit and a basic diaminobenzidine detection system (Dako, USA). CD31 labeling was performed using a monoclonal antibody (Chemicon International, USA) at 1:50 dilution for 28 min at 42°C, following the same procedure as described above. The images were obtained with a digital camera (DXM1200F; Nikon Instruments Inc., USA) and analyzed using the EclipseNet software.

Statistical analysis
Statistical analysis of the experimental groups was performed using the Student t-test with the level of significance set at P < 0.05. Data are reported as mean ± SD.

Results
Renal function
LPS induced significant renal failure, as shown by the approximate doubling of plasma creatinine (normal: 1.14 ± 0.3; 4 h: 2.16 ± 0.2; 12 h: 3.44 ± 0.3) and blood urea nitrogen (normal: 35.1 ± 4; 4 h: 84 ± 11.1; 12 h: 153.5 ± 24.5) levels compared to normal mouse values (P < 0.01). Renal histology revealed tubular dilatation and loss of brush border (Figure 1A and B).

Serum NOx levels
LPS is known to induce iNOS and to increase systemic NO synthesis. Serum NOx, which are NO metabolites,
indicate NO generation. The NOx levels were significantly elevated 4 and 12 h after LPS administration. At 4 h, NOx levels showed a 10-fold increase from 45 ± 6 µM (N = 4) in the control group to 425 ± 83 µM (N = 4) in the LPS group (P < 0.001). At 12 h, NOx levels were decreased (325 ± 12 µM), but significantly higher than in the control group.

Quantitative RT-PCR

After 4 h of LPS treatment, endostatin mRNA level was down-regulated (0.18 ± 0.15). At the end of 12-h treatment, endostatin mRNA level returned to normal (0.91 ± 0.21).

There was a significant decrease of endostatin mRNA levels at 4 h after LPS treatment (0.71 times lower than the levels of sham-inoculated mice; P < 0.01). Of interest, the signals of endostatin mRNA were not significantly changed in the normal mice kidneys.

Western blot analysis

Endostatin levels, examined by Western blot (Figure 2), were stable at 4 h (1.95 ± 0.12) after LPS treatment and at 12 h they were significantly reduced (1.7 ± 0.13) compared to the control group (1.8 ± 0.3; P < 0.05).

Immunohistochemistry analysis

Figure 3 shows the results of immunostaining for endostatin in normal and LPS-treated kidneys. In the normal kidney cells, we observed a diffuse staining for endostatin in the proximal and distal tubules; endostatin staining was positive in the basement membrane of Bowman’s capsule and in the glomerular capillaries (Figure 3A). Four hours after LPS treatment, the kidney showed marked staining...
in the tubular basement membranes (Figure 3B) and 12 h after LPS treatment a reduction in staining was evident (Figure 3C), with moderate to weak staining of tubules and glomeruli.

Immunohistochemical staining for CD31 revealed a progressive decrease of kidney endothelial CD31 expression (Figure 3D-F). As early as 4 h after LPS administration, the peritubular endothelial cells showed a decrease in the expression of CD31, while CD31 expression was unchanged in the glomerular endothelium (Figure 3E). Twelve hours after LPS inoculation, CD31 expression virtually disappeared (Figure 3F).

Semi-quantitative analysis of endostatin and CD31 expression in normal and endotoxemic kidneys demonstrated an opposite modulation by LPS. There was an increase in focal expression of endostatin and marked decrease in CD31 expression in both the tubular and glomerular endothelium (Table 1).

### Discussion

LPS-induced ARF is associated with hemodynamic changes that are strongly influenced by NO overproduction through the cytokine-mediated up-regulation of iNOS (5-7). These hemodynamic changes alter renal blood flow, intrarenal hemodynamics, and GFR and produce marked intrarenal vasoconstriction (10,11).

In a previous study, using a murine model of ischemia/reperfusion-induced and obstructive nephropathy, we reported that collagen XVIII/endostatin takes part in the physiopathology of ischemic ARF, as well as in a fibrotic process (18,27).

In the present study, we used an experimental endotoxemic animal model in order to analyze the expression of collagen XVIII/endostatin in the early phase of endotoxemic ARF.

Our results demonstrate that LPS injury caused a significant but transient decrease in endostatin mRNA levels 4 h after LPS treatment. This transient decrease had no marked reflection on protein levels. These data demonstrate that the endostatin detected by Western blot analysis and immunohistochemistry is a product of collagen XVIII cleavage, which in turn indicates that, in the early phase of endotoxemic ARF, the increase in endostatin is a consequence of collagen metabolism. This is consistent with studies showing the anti-angiogenic activity of noncollagenous domain proteolytic fragments of collagen types XVIII (endostatin) and XV (restin) and type IV collagen chains a1 (arrestin), a2 (canstatin), and a3 (tumstatin) (22,30,31).

Metalloproteases and other proteases are involved in the cleavage of collagens and, consequently, in ECM remodeling, wound healing, development, cancer invasion, and angiogenesis (19,20).

LPS-induced endotoxemia causes up-regulation of several MMP genes in the liver, spleen and kidney. The increased MMP activity present in the kidney might have a significant impact on the basement membrane components, including collagen XVIII degradation (25).

Immunostaining experiments have revealed an increase of endostatin on the basolateral tubular surface in association with a decreased number of positive endothelial cells, especially in the peritubular capillaries. Tiwari et al. (7), using intravital videomicroscopy, showed that peritubular capillary perfusion is dramatically reduced in the early phase of endotoxemic ARF. In an in vitro experiment, Yu et al. (31) demonstrated that LPS-activated endothelial cells were more endostatin-sensitive. HDMEC cells, upon activation by LPS or IL-1β, displayed a dose-dependent sensitivity to endostatin.

The increased levels of endostatin may result from gene expression regulation and/or collagen XVIII cleavage. We reported previously that collagen XVIII/endostatin gene expression as well as endostatin levels were up-regulated in an ischemic ARF and obstructive nephropathy model (18,27). These data demonstrated the presence of both genetic and metabolic control of collagen XVIII/endostatin in these nephropathy models.

The results of the present study indicate that in the early phase of endotoxemic ARF, the increased levels of renal endostatin were not associated with gene expression, but rather with the metabolism of collagen XVIII. These results could be explained by the LPS induction of major changes in the extracellular proteolytic balance of the murine kidney (32). Imbalanced extracellular proteolysis participates in the alterations of kidney function in septic shock, and we believe that endostatin release may play a role in the reduction of renal capillary density and, consequently, in the alterations of intrarenal hemodynamics.

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References


