Endotoxemia-related acute kidney injury in transgenic mice with endothelial overexpression of GTP cyclohydrolase-1

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toxin-related acute kidney injury has been shown to profoundly induce nitric oxide (NO), which activates sympathetic and renin-
angiotensin system, resulting in renal vasoconstriction. While vascular
muscle cells are known to upregulate inducible NO synthase
(iNOS), less is known about the endothelium as a source of NO during
endotoxemia. Studies were, therefore, undertaken both in vitro in
mouse microvascular endothelial cells and in vivo in transgenic mice
with overexpression of endothelial GTP cyclohydrolase, the rate-
limiting enzyme for tetrahydrobiopterin, a cofactor for NO synthase.
LPS significantly induced endothelial cell iNOS expression and NO
concentration in the culture media, with no change in endothelial NO
synthase expression. GTP cyclohydrolase-1 transgenic (Tg) mice
demonstrated a significant increase in baseline urine NO-to-creatinine
ratio and a more significant increase in renal iNOS expression and
serum NO levels with LPS treatment compared with the wild-type
(WT) mice. Glomerular filtration rate and renal blood flow decreased
significantly in Tg mice with 1.0 mg/kg LPS, while no changes were
observed in WT with the same dose of LPS. Serum IL-6 levels were
significantly higher in Tg compared with WT mice during endotox-
emia. The antioxidant tempol improved the glomerular filtration rate
in the Tg mice. Thus endothelium can be an important source of iNOS
and NO in the endothelium, which would result in worsening
of AKI. Reactive oxygen species appear to be involved in
this acute renal injury in Tg mice during endotoxemia.

THE ANNUAL DEATHS IN THE UNITED States related to sepsis are
estimated to be 210,000, a number in excess of deaths due to
acute myocardial infarction (2). The mortality with sepsis is
strikingly increased when associated with acute kidney injury
(AKI). In contrast to the approximate 45% mortality of AKI in
nonseptic patients, the association of sepsis and AKI has a 70%
(mortality (8, 15).

Bacterial endotoxin accounts for much of the hemodynamic,
neuroinflammatory responses to gram-negative sepsis
(14); therefore, the effects of endotoxemia with lipopolysac-
charide (LPS) on renal function have been examined in various
species. In recent years, endotoxemia-related AKI in mice has
been investigated because of the molecular advantages of
transgenic and knockout mice.

Endotoxin-related AKI has been shown to profoundly in-
duce nitric oxide (NO) (10, 11). The resultant increase in
systemic and renal NO has potential deleterious effects, includ-

ing systemic vasodilation with activation of the sympathetic
and renin-angiotensin system and resultant renal vasoconstric-
tion (17), as well as combining with oxygen radicals to produce
the injurious peroxynitrite compound (18). While vascular
smooth muscle cells are known to upregulate inducible NO
synthase (NOS) (iNOS) in response to endotoxin and cytokines
(3, 5), less is known about the endothelium as a source of NO
during endotoxemia.

Studies were, therefore, undertaken to examine the potential
role of the endothelium as a source of NO in response to
endotoxin. The renal effect of endotoxin in mice with endo-
thelial overexpression of GTP cyclohydrolase-1 (GTPCH), the
rate-limiting enzyme for tetrahydrobiopterin (BH4) and thus
NO, was, therefore, examined. The in vitro effect of endotoxin
on cultured endothelial cells to produce NO was also exam-
ined. The tested hypothesis was that endotoxin increases iNOS
and NO in the endothelium, which would result in worsening
of AKI. Therefore, overexpression of BH4 in the GTPCH
transgenic mice will exhibit an enhanced endothelial response
to endotoxin, with resultant increased sensitivity to AKI.

METHODS

Animals. The experimental protocol was approved by the Animal
Ethics Review Committee at the University of Colorado Health
Sciences Center. C57BL/6 mice were purchased from Jackson Labo-
ratories (Bar Harbor, ME). GTPCH transgenic mice were kindly
provided by Dr. Keith Channon at the University of Oxford, UK (1).
There were no apparent morphological abnormalities in GTPCH
transgenic mice. To characterize the transgenic mice, organs with
different proportions of the endothelial cells (lung, liver, and aorta)
were examined regarding their relative mRNA and protein levels of
the human GTPCH (the transgene) and native murine GTPCH. Trans-
gene levels were the highest in the lung, where there is an increased
proportion of endothelial cells. The human transgene was undetect-
able in the wild-type mice. The background strain of the GTPCH
transgenic mice is C57BL/6. Male mice aged 8–10 wk were used
throughout the study. Mice were maintained on a standard rodent
chow and had free access to water.

Materials. Chemicals were purchased from Sigma (St. Louis, MO),
unless otherwise specified.

Endothelial cell culture. Mouse microvascular endothelial cells
(MS1 cells) were purchased from American Type Culture Collection.
This line expresses both factor VIII-related antigen and VEGF recep-
tors. The cells were grown to confluence in DMEM supplemented
with 5% fetal bovine serum. Experiments were performed in the
presence of 100 U/ml IFN-γ in six-well tissue culture dishes. LPS 10
µg/ml and/or 5 µM N-[3-(aminomethyl)benzyl]acetamide (1400W)
were incubated for 20 h before harvest.

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Animal protocol. Wild-type and GTPCH transgenic mice were injected intraperitoneally (ip) with 1.0 mg/kg dose of LPS (Escherichia coli 0111:B4) from LIST Biological Laboratories, Campbell, CA (19).

iNOS and endothelial NOS protein expression. Whole kidneys or endothelial cells were homogenized in 250 mM sucrose, 25 mM imidazole, 1 mM ethylenediaminetetraacetic acid, and 1/10 volume of a protease solution consisting of 25 μg/ml antipain, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.1 mg/ml soybean trypsin inhibitor, and 200 μM phenylmethylsulfonyl fluoride. SDS-PAGE/immunoblotting was performed on 50 μg (iNOS) of protein extract. Samples were electrophoresed through a 10% acrylamide gel for detection of iNOS and endothelial NOS (eNOS). iNOS and eNOS proteins were detected using a rabbit polyclonal antibody (Upstate, Charlottesville, VA) diluted 1:1,000 in Tris-buffered saline/0.1% Tween 20 containing 5% dry milk. The secondary antibodies were conjugated to horseradish peroxidase. Antigenic detection was made by enhanced chemiluminescence (Amersham, Arlington Heights, IL) with exposure to X-ray film.

Measurement of serum NO levels. Blood was taken through cardiac puncture 16 h after LPS ip injection. Cell supernatant and serum NO levels were measured using nitrate/nitrite colorimetric assay kit from Cayman (Ann Arbor, MI).

Measurement of glomerular filtration rate, renal blood flow, and mean arterial pressure. The animals were anesthetized with pentobarbital (60 mg/kg) and placed on a thermostatically controlled surgical table. A tracheotomy was performed in all mice. Catheters (custom pulled from PE-250) were placed in the jugular vein for maintenance infusion and in the carotid artery for blood pressure measurement. The kidney was exposed by a left subcostal incision and was dissected free from perirenal tissue, and the renal arteries were isolated for the determination of renal blood flow (RBF) using a blood flowmeter and probe (0.5v) (Transonic Systems, Ithaca, NY), as described by Traynor and Schnermann (16). Mean arterial pressure (MAP) was measured via a carotid artery catheter connected to a TranspacIV transducer and monitored continuously using Windaq Waveform recording software (Dataq Instruments). An intravenous maintenance infusion of 2.25% bovine serum albumin in normal saline at a rate of 0.25 μl/g body wt⁻¹/min⁻¹ was started 1 h before experimentation; 0.75% fluorescein isothiocyanate-inulin was added to the infusion solution for the determination of glomerular filtration rate (GFR), as described by Lorenz and Gruenstein (12). A bladder catheter (PE-10) was used to collect urine. Two 30-min collections of urine were obtained under oil and weighed for volume determination. Blood for plasma inulin determination was drawn between urine collections. Fluorescein isothiocyanate-inulin in plasma and urine

Fig. 1. Effect of lipopolysaccharide (LPS) on cultured endothelial cells to increase inducible nitric oxide (NO) synthase (iNOS) and NO. A: iNOS; B: NO₂; C: endothelial NO synthase (eNOS). Mouse microvascular endothelial cells (MS1 cells) were grown to confluence in DMEM supplemented with 5% fetal bovine serum. Experiments were performed in the presence of 100 U/ml IFN-γ in six-well tissue culture dishes. 10 μg/ml LPS and/or 5 μM N-[3-(aminomethyl)benzyl]acetamidine (1400W) was incubated for 20 h before harvest. Values are means ± SE. Con, control; NS, not significant.

Fig. 2. Baseline urine NO/creatinine levels in GTP cyclohydrolase-1 (GTPCH) mice. Urine NO levels were measured using nitrate/nitrite colorimetric assay kit. Values are means ± SE. WT, wild type; Tg, transgenic.
samples were measured using CytoFluor plate reader (PerSeptive Biosystems, Foster City, CA).

Measurement of serum cytokine levels. Serum was collected 16 h after the administration of LPS. Cytokines were measured using Bio-Plex cytokine assay kit (Bio-Rad, Hercules, CA).

Statistical analysis. Values are expressed as means ± SE. Multiple comparisons were assessed by ANOVA using the post hoc Newman-Keuls test. P < 0.05 was considered statistically significant.

RESULTS

Effect of LPS on cultured endothelial cells to increase iNOS and NO. As shown in Fig. 1, LPS increases iNOS protein expression and NO production in culture endothelial cells.

1400W was used as an iNOS inhibitor. It has been shown to inhibit human eNOS and neuronal NOS (nNOS) inefficiently, compared with its inhibition of iNOS (9). The selectivity ratio of 1400W for human NOS isoenzymes was reported to be 62 for iNOS vs. eNOS and 4 for iNOS vs. nNOS (4). In the present study, 1400W was shown to block this LPS-related increase in iNOS. There was no effect of the same dose of LPS on the eNOS protein in the endothelial cells.

Effects of LPS on renal iNOS and serum NO in wild-type and GTPCH transgenic mice. At baseline, the GTPCH transgenic mice demonstrated a significant increase in urine NO-to-urine creatinine ratio compared with wild-type mice (0.59 ± 0.1 vs. 0.26 ± 0.03, P < 0.05) (Fig. 2). In response to 1 mg/kg ip LPS, after 16 h, these transgenic mice demonstrated a significantly increase in renal iNOS expression (2.45 ± 0.08 vs. 1.52 ± 0.2; P < 0.05) (Fig. 3A). There was no effect of the same low dose of LPS in wild-type mice [1.12 ± 0.26 vs. 1.0 ± 0.11, P = not significant (NS)]. Similarly, serum NO increased dramatically in the transgenic mice (247.8 ± 16.2 vs. 38.2 ± 14.6 μM; P < 0.001), but only modestly in wild-type mice (51.4 ± 14.8 vs. 7.3 ± 1.4 μM; P < 0.05, Fig. 3B).

Fig. 3. Effect of LPS on renal iNOS (A) and serum NO levels (B) in GTPCH Tg mice compared with WT mice. LPS 1.0 mg/kg or vehicle (normal saline) was injected intraperitoneally (ip) in male GTPCH Tg mice. Whole kidneys and blood were collected 16 h after LPS injection. Renal iNOS expression was examined by Western blot. Serum NO levels were measured using nitrate/nitrite colorimetric assay kit. Values are means ± SE.

Fig. 4. Baseline and LPS response of glomerular filtration rate (GFR; A) and renal blood flow (RBF; B) in GTPCH Tg mice compared with WT mice. LPS 1.0 mg/kg or vehicle (normal saline) was injected ip, and all of the functions were measured 16 h after LPS injection. GFR was measured by FITC-inulin clearance, and RBF by renal flow probe. C57BL/6 mice were used as Con mice for GTPCH Tg. Values are means ± SE.
Effects of LPS on GFR, RBF, and MAP in GTPCH transgenic mice and wild-type mice. At baseline, GFR (157.0 ± 18.0 vs. 163.0 ± 11.8 μl/min, P = NS) (Fig. 4A) and MAP (87.1 ± 5.1 vs. 82.5 ± 1.5 μl/min, P = NS) were comparable in the wild-type and GTPCH transgenic mice. Baseline RBF was actually significantly higher in GTPCH mice than in wild-type mice (1.84 ± 0.29 vs. 0.98 ± 0.05 ml/min; P < 0.01) (Fig. 4B). With an ip dose of LPS (1 mg/kg) in wild-type mice, neither GFR (154.0 ± 22.0 vs. 157.0 ± 18.0 μl/min; P = NS) nor RBF (1.19 ± 0.05 vs. 0.98 ± 0.05 μl/min; P = NS) changed. However, with the same LPS dose in the GTPCH transgenic mice, GFR (26.6 ± 16.2 vs. 163.0 ± 11.8 μl/min; P < 0.01) and RBF (0.3 ± 0.08 vs. 1.84 ± 0.29 μl/min; P < 0.01) decreased significantly (Fig. 4, A and B, respectively) in the GTPCH transgenic mice. This renal effect in the transgenic mice occurred in the absence of a change in MAP (86.0 ± 4.4 vs. 87.1 ± 5.1 mmHg; P = NS).

Effect of iNOS inhibition with 1400W on renal function in GTPCH transgenic mice during endotoxemia. 1400W (20 mg/kg) administered ip 30 min before LPS (1.0 mg/kg) significantly decreased serum NO levels (47.10 ± 7.0 vs. 135.8 ± 19 μM; P < 0.001), but did not improve either GFR (62.6 ± 9.0 vs. 71.5.7 ± 5.7 μl/min; P = NS) or RBF (1.25 ± 0.12 vs. 1.50 ± 0.05 ml/min; P = NS). MAP was also unchanged (92.7 ± 7.2 vs. 81.4 ± 3.1 mmHg; P = NS).

Effect of tempol on renal function in GTPCH transgenic mice during endotoxemia. Tempol (250 mg/kg) or vehicle (normal saline) was administrated ip 30 min before and 4 h after LPS (1.0 mg/kg ip). GFR was improved in mice treated with tempol compared with the vehicle group (110 ± 17 vs. 70 ± 8 μl/min; P < 0.05, Fig. 6).

Fig. 5. Serum cytokine profile in the WT and GTPCH Tg mice at baseline and during endotoxemia. A: TNF-α; B: IL-10; C: IL-1β; D: IL-6. Serum was collected 16 h after the administration of LPS 1.0 mg/kg or vehicle (normal saline). Cytokines levels were measured using Bio-Plex Cytokine assay kit. Values are means ± SE. BH4, tetrahydrobiopterin.
At baseline, urine NO-to-creatinine ratio was found to be significantly higher in the GTPCH transgenic mice than wild-type mice. Most importantly, renal iNOS protein expression and serum NO were dramatically higher in the transgenic than wild-type mice with endotoxin dose (1 mg/kg), which did not alter either GFR or RBF in the wild-type mice. This dose of endotoxin, however, had a highly significant effect of decreasing GFR and RBF in the GTPCH transgenic mice (Fig. 3). These results thus support the endothelium as a potentially important source of renal iNOS and circulatory NO during endotoxemia. Based on the in vitro effect of 1400W, a specific inhibitor of iNOS, studies were performed in vivo. Significant in vivo protection was, however, not detectable with this inhibitor. This result suggested that other factors, in addition to NO, could be involved in endotoxin-related AKI with GTPCH transgenic mice. This is supported by previous studies in which iNOS knockout mice still developed AKI in response to endotoxin (11). Studies of several cytokines, including TNF-α, IL-1β, IL-10, and IL-6, were shown to be increased in both the wild-type and GTPCH transgenic mice during endotoxemia. However, TNF-α, IL-1β, and IL-10 were significantly lower in the transgenic than the wild-type mice. Thus only IL-6 was not lower and indeed was significantly higher in the GTPCH transgenic than wild-type mice during endotoxemia. This observation is of interest, since elevated plasma concentration of IL-6 predicts AKI in patients with severe sepsis (6) and the protective effect of TNF antibodies in septic patients was only observed in those patients in the highest quartile of serum IL-6 concentrations (13). Soluble TNF receptor (11), decreased TNF production secondary to pentoxifylline (20), and TNF receptor 1 knockout mice (7) have also been found to be protective during experimental endotoxemia in mice.

Studies were then performed to examine whether reactive oxygen species (ROS) could be involved in this increased renal sensitivity to endotoxin in the GTPCH transgenic mice. Earlier studies had shown that larger amounts of endotoxin (2.5–5.0 mg/kg ip LPS) were associated with AKI in wild-type mice, and oxygen radical scavengers, e.g., tempol, attenuated this renal injury (18). Studies were, therefore, performed in which tempol treatment was shown to improve renal function significantly, as assessed by GFR, in the GTPCH transgenic mice receiving the low-dose LPS (1 mg/kg ip). While higher levels of NO in the transgenic mice would be expected to enhance the direct tubule damage (22), NO also also know to scavenge ROS, which occur in endotoxemia (18). Thus inhibition of iNOS with 1400W may abolish this beneficial ROS scavenger property. The role of ROS in the AKI of the transgenic mice was supported by the renal protective effect of ROS scavenging of tempol.

In summary, earlier results indicate that the endothelium affords renal protection against endotoxin insult involving the integrity of prostacyclin and eNOS. However, the present in vitro and in vivo studies demonstrate that the endothelium also can be an important source of iNOS and serum NO concentration during endotoxemia, thereby increasing the sensitivity to AKI. The present results further indicate that ROS are involved in this renal injury in GTPCH transgenic mice.
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