Endotoxin and cisplatin synergistically induce renal dysfunction and cytokine production in mice

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Ramesh G, Zhang B, Uematsu S, Akira S, Reeves WB. Endotoxin and cisplatin synergistically induce renal dysfunction and cytokine production in mice. Am J Physiol Renal Physiol 293: F325–F332, 2007. First published May 9, 2007; doi:10.1152/ajprenal.00158.2007.—A major toxicity of the cancer chemotherapeutic agent cisplatin is acute renal failure. Sepsis is a common cause of acute renal failure in humans and patients who receive cisplatin are at increased risk for sepsis. Accordingly, this study examined the interactions between cisplatin and endotoxin in vivo with respect to renal function and cytokine production. Mice were treated with either a single dose of cisplatin or two doses of LPS administered 24 h apart, or both agents in combination. Administration of 10 mg/kg cisplatin had no effect on blood urea nitrogen or creatinine levels throughout the course of the study. LPS resulted in a modest rise in blood urea nitrogen at 24 and 48 h, which returned to normal by 72 h. In contrast, mice treated with both cisplatin and LPS developed severe renal failure and an increase in mortality. Urine, but not serum, TNF-α levels showed a synergistic increase by cisplatin and LPS. Urinary IL-6, MCP-1, KC, and GM-CSF also showed a synergistic increase with cisplatin+LPS treatment. The renal dysfunction induced by cisplatin+LPS was completely dependent on TLR4 signaling and partially dependent on TNF-α production. Increased cytokine production was associated with a moderate increase in infiltrating leukocytes which was not different between cisplatin+LPS and LPS alone. These results indicate that cisplatin and LPS act synergistically to produce nephrotoxicity which may involve proinflammatory cytokine production.

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Acute Renal Failure can develop in a variety of clinical situations such as ischemia, sepsis, and administration of nephrotoxic agents. Studies using experimental models of acute renal failure have identified a large number of putative pathophysiological mediators (33, 35). The majority of these studies have employed models of acute renal injury subsequent to a single renal insult (20). However, acute renal failure in humans often occurs in complex clinical settings in which patients may be exposed to a number of different renal insults (35). It has been suggested that the failure in humans of therapeutic interventions based on single-insult animal studies may be due, in part, to the multiplicity of renal insults in human acute renal failure (12, 20).

Sepsis is a common cause of acute renal failure, accounting for up to 60% of cases in some series (2, 32). TNF-α is an important mediator of the systemic and renal effects of sepsis (3, 15). The production of TNF-α in sepsis results from the activation of TLR receptors by bacterial products such as endotoxin (1, 4, 5). In previous animal studies, we and others (27, 29, 30, 36, 37) also demonstrated an important role for TNF-α in the pathogenesis of cisplatin-induced renal injury. Inhibition of TNF-α production or action markedly reduced the nephrotoxicity of cisplatin (29, 30). Against this background, Zager et al. (44) reported that treatment of mice with both cisplatin and endotoxin resulted in enhanced production of proinflammatory cytokines, including TNF-α, by the kidney and speculated that this inflammatory response might be responsible for some of the extrarenal complications seen in patients with acute renal failure. We subsequently determined that the synergy between cisplatin and endotoxin in stimulating TNF-α production by proximal tubule cells derives from both an endotoxin-induced increase in TNF-α gene transcription (26) and a cisplatin-induced increase in TNF-α mRNA translation (26). The latter was critically dependent on activation of p38 MAPK (26).

While these studies (26, 44) clearly showed that cisplatin priming before endotoxin treatment enhances cytokine production by proximal tubule epithelial cells in vitro and kidney in vivo, the impact of this increased cytokine production on renal function is unknown. The present studies examined the effects of treatment with both cisplatin and endotoxin on renal function, renal cytokine production, and cytokine excretion. In addition, the signaling pathways which mediate these effects were explored. The results demonstrate a marked synergy between cisplatin and endotoxin in producing acute renal failure. This synergy was only partially dependent on TNF-α production but completely dependent on TLR4 signaling. These interactions between cisplatin and endotoxin might predispose patients to cisplatin nephrotoxicity in the presence of infection.

Methods

Drug administration. Experiments were performed using 8- to 10-wk-old male C57BL/6, TNF knockout (KO), and TLR4 KO mice weighing 25–30 g. The TNF-α-deficient mice on a C57BL6 background were obtained from B and K Universal (East Yorkshire, UK). The TLR 4 knockout mice, also on a C57BL6 background, have been described previously (11). Cisplatin (Sigma, St. Louis, MO) was dissolved in saline at a concentration of 1 mg/ml and filtered through a 0.2-μm syringe filter. Escherichia coli LPS (Sigma) was dissolved in saline at a concentration of 0.25 mg/ml. The protocol for drug administration is provided in Fig. 1. Mice were given a single intraperitoneal injection of either vehicle (saline) or cisplatin (10 mg/kg body wt) at t = 0. Mice were then administered either saline or
LPS (2.5 mg/kg body wt) by intraperitoneal injection at 6 and 24 h after the cisplatin injection. Blood and urine samples were collected at the indicated times for measurement of urea nitrogen, creatinine, and cytokines. Urine was harvested by bladder massage. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Penn State University College of Medicine.

Renal function. Renal function was assessed by measurements of blood urea nitrogen (BUN; VITROS DT60II Chemistry slides, Orthoclinical Diagnostics) and serum creatinine (DZ072B, Diazyme Labs).

Histology and histochemistry. Kidney tissue was fixed in buffered formalin for 24 h and then embedded in paraffin wax. Five-micrometer sections were stained with periodic acid-Schiff (PAS) or naphthol AS-D chloroacetate esterase (kit no. 91A; Sigma). The esterase stain identifies infiltrating neutrophils and monocytes. Twenty × 40 fields of esterase-stained sections were examined for quantitation of leukocytes. Tubular injury was assessed in PAS-stained sections using a semiquantitative scale (13) in which the percentage of cortical tubules showing epithelial necrosis was assigned a score: 0 = normal, 1 = <10%, 2 = 10–25%, 3 = 26–75%, 4 = >75%. The individuals scoring the slides were blinded to the treatment and strain of the animal.

Quantitation of mRNA by real-time RT-PCR. Real-time RT-PCR was performed in an Applied Biosystems 7700 Sequence Detection System (Foster City, CA). Total RNA (1.5 μg) was reverse transcribed in a reaction volume of 20 μl using Omniscript RT kit and random primers. The product was diluted to a volume of 150 μl and either 2-μl (actin) or 10-μl (all others) aliquots were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen) and gene-specific primers. The primer sets used were mouseACT-β (forward: TGAGTATCCGC-GACGTCGGA; reverse: AATACGGTTACACCATGG), TLR4 (forward: CTTCGTCACAGAGACCTT; reverse: TGTGGAAGCCTTCTTGAG), and HMG1 (forward: TGGCGCTTCTTCTTGTGT; reverse: CTTCCAGTGCCAGCTTCTT). The amount of DNA was normalized to the β-actin signal amplified in a separate reaction (forward primer: AGAGGAAATCTGTGCGTAC; reverse: CAATGATGACCCTGCG). Cytokine/chemokine quantitation. The levels of TNF-α in blood and urine were quantitated using an ELISA assay (Quantikine Mouse TNF-α kit; R&D Systems, Minneapolis, MN). Fifty microliters of serum were used for the TNF-α assay. Urine cytokines and chemokines [IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p70), IL-17, IFN-γ, IP-10, G-CSF, MIP-1α, MCP-1, RANTES] were measured using a bead-based multiplexed cytokine analysis kit (Linfco Research, St. Charles, MO) using a Luminex-100 system (Luminex, Austin, TX). Assays were run in duplicate according to the manufacturer’s protocol and data were collected and analyzed using MasterPlex QT 2.5 software (MiraBio, Alameda, CA). Amounts of cytokines were normalized to the creatinine concentration in the urine.

Statistical methods. All assays were performed in duplicate. The data are reported as means ± SE. Statistical significance was assessed by unpaired, two-tailed Student’s t-tests for single comparisons or ANOVA for multiple comparisons.

RESULTS

Mice were treated with either 10 mg/kg cisplatin, 2.5 mg/kg LPS, or both. The doses of cisplatin (preliminary data) and LPS (15) were selected to produce limited nephrotoxicity alone. As shown in Fig. 2, mice receiving 10 mg/kg cisplatin alone had only a slight increase in BUN at 72 h and no change in the serum creatinine concentration. Mice receiving two consecutive doses of LPS displayed modest increases in BUN and serum creatinine at 24 and 48 h which returned to baseline by 72 h. In contrast, mice which received both the cisplatin and LPS had an early rise in both BUN and creatinine which continued to increase throughout the course of observation. The mortality rate at 72 h for the cisplatin- or LPS-treated mice was 0% but was 44% for mice treated with both cisplatin and LPS.

The deleterious effects of the combination of cisplatin and LPS were also reflected by histologic changes. As shown in
Fig. 3, mice treated with either cisplatin or LPS alone, and killed at 72 h, displayed almost normal renal histology. In contrast, mice which received both the cisplatin and LPS had evidence of tubular injury such as dilation of tubules, vacuole formation, and necrosis. Semiquantitative scoring of tubular injury yielded scores of 0.3 ± 0.1 for saline-treated animals, 0.4 ± 0.07 for LPS-treated animals, 1.5 ± 0.5 for cisplatin-treated mice, and 2.6 ± 0.26 for mice receiving both cisplatin and LPS (n = 4–6). The tubular injury score of the cisplatin + LPS-treated mice was significantly higher than saline- or LPS-treated mice (P < 0.01).

Since TNF-α is known to contribute to the pathogenesis of both endotoxin and cisplatin-induced nephrotoxicity (3, 29, 30), we measured TNF-α levels in blood and urine (Fig. 4). The low dose of cisplatin had no effect on blood TNF-α levels. As expected, LPS produced a robust rise in serum TNF-α which returned to baseline by 48 h. Mice treated with both cisplatin and LPS had a similar magnitude increase in serum TNF-α. Although the duration of the TNF-α peak appeared to persist somewhat longer than in mice which received only LPS, this was not a consistent finding (see Fig. 9). Urinary excretion of TNF-α was not increased by cisplatin alone. LPS alone resulted in a transient increase in urine TNF-α. However, the combination of cisplatin and LPS resulted in significantly higher urinary TNF-α than did LPS alone.

TNF-α drives the expression of a variety of other cytokines during cisplatin toxicity (29). We measured the expression of various cytokine genes and protein levels within the kidney after treatment with cisplatin, LPS, or both. As noted in Fig. 5, several inflammatory genes, e.g., IL-1β, IL-6, MCP-1, and ICAM, were increased by LPS and the combination of cisplatin and LPS. However, there were no significant differences between the levels of expression between LPS and cisplatin plus LPS mice. Low-dose cisplatin had no significant effect on the expression of any of these genes. Levels of MCP-1 and MIP-2 protein were significantly elevated in kidney cortex from LPS-treated mice and cisplatin plus LPS mice. TNF-α levels in the medulla were increased by LPS and by LPS plus cisplatin.
There was no synergy noted between cisplatin and LPS in the kidney content of TNF-α, MCP-1, or MIP-2 proteins. Consistent with the upregulation of chemokines and adhesion molecules, leukocyte infiltration into the kidney was increased after LPS administration but there was no further increase seen in kidneys from cisplatin plus LPS-treated mice (Fig. 6).

Urinary cytokine excretion is elevated in humans with acute renal failure and may have prognostic significance (16, 22). We measured urinary cytokines using a multiplex immunoassay (Fig. 7). Urine was collected 3 h after each injection of LPS, i.e., at either 9 or 27 h after the initial cisplatin injection. At both times, a number of cytokines were markedly elevated in urine from LPS- and cisplatin plus LPS-treated mice. In particular, KC and G-CSF showed synergy between cisplatin and LPS at the 27-h time point.

The TLR4 receptor and TNF-α are believed to mediate many of the actions of endotoxin (1), including acute renal injury (3, 4). We examined the interactions between cisplatin and LPS in mice deficient of either TLR4 or TNF-α. TLR4-deficient mice (Fig. 8) developed only modest renal dysfunction in response to cisplatin plus LPS at day 3 and did not respond to LPS alone. The degree of renal dysfunction in the TLR4-deficient mice treated with cisplatin and LPS (BUN = 63 ± 13 mg/dl) was similar to that seen in wild-type mice treated with cisplatin alone (50 ± 18 mg/dl; Fig. 2), suggesting that LPS has no added effect with cisplatin in the absence of TLR4. Likewise, serum and urine TNF-α responses to LPS were absent in the TLR4-deficient mice (Fig. 9), also indicating that the TLR4 receptor is essential in mediating LPS actions on renal function and TNF-α production. TNF-α-deficient mice treated with LPS plus cisplatin sustained less renal dysfunction than wild-type mice (Fig. 10), but more than TNF-α-deficient mice treated with either cisplatin or LPS alone. Thus synergy between cisplatin and LPS in producing renal dysfunction is entirely dependent on TLR4 signaling, and partially dependent on TNF-α production.

**DISCUSSION**

The present study examined the interactions between two diverse renal insults, cisplatin and endotoxin, on renal function. Our results indicate that cisplatin and endotoxin exhibit remarkable synergy in producing renal dysfunction. Sepsis is a common cause of acute renal injury in humans (32). The pathophysiology of sepsis-induced acute renal failure is complex and involves hemodynamic factors (40, 42), inflammation (9, 19, 38), and cytotoxic injury (39). It is possible, based on the current results and the results of others (33–48), that a portion of the high incidence of acute renal failure in sepsis may be referable to sensitization of the kidney to other insults, such as nephrotoxins or ischemia, rather than to a direct effect of endotoxin. Another interpretation of these results is that cisplatin sensitizes the kidney to injury by endotoxin. Since both agents were required to produce severe renal injury, we cannot draw any conclusions regarding the primacy of the individual insults. Nonetheless, the observations support the view that interactions between disparate, mild renal insults may lead to profound renal failure. These types of interactions may account for what clinicians have long recognized as “multifactorial” acute renal failure.

The current study was predicated on previous in vitro and in vivo evidence that cisplatin and endotoxin exhibit synergy with respect to the production of TNF-α, an important mediator of renal injury (25, 26, 44). It was interesting, then, to find that the combination of cisplatin and endotoxin resulted in only a slight increase in serum TNF-α levels compared with those produced in response to endotoxin alone. Urinary TNF-α, however, was elevated to a much
The TLR4 receptor in conjunction with CD14 and MD-2 is the primary receptor for gram negative endotoxins (11, 41). Although endotoxin by itself can produce acute renal failure in animal models (3, 4, 15), the role of endotoxin in the pathogenesis of clinical septic acute renal failure is not clear. In both an animal model of sepsis, the cecal ligation and puncture model (5), and in human postsurgical sepsis (8), no clear dependence on TLR4 could be demonstrated. However, through the use of TLR4 knockout mice, we were able to show an absolute dependence on TLR4 signaling for the synergistic production of TNF-α and also functional renal injury resulting from the combination of cisplatin and endotoxin.

Enhanced TLR-mediated inflammatory responses in the setting of tissue injury have been reported previously. For example, acute thermal injury, lung injury, and hemorrhagic shock result in increased production of various proinflammatory cytokines by monocytes in response to TLR2 and TLR4 ligands (7, 24). In the kidney, Zager and colleagues (44, 45, 47) demonstrated enhanced TLR2- and TLR4-induced cytokine production in the setting of cisplatin, ischemia, obstruction, or glycerol-induced renal injury. The present study confirms the hyperresponsiveness to TLR4 agonists and demonstrates that the hyperresponsiveness contributes to the development of renal failure. The mechanism of the hyperresponsiveness is not understood. In the thermal injury model, no changes in monocyte TLR2 or TLR4 expression were demonstrated (24). Although El-Achkar et al. (6) reported an increase in renal TLR4 expression in sepsis, Zager et al. (46, 47) were unable to demonstrate any increase in

![Fig. 7. Urinary cytokine excretion in mice treated with cisplatin and/or LPS. Urine samples were collected at 9 (A) and 27 (B) h after the initial injection, i.e., 3 h following each LPS injection. All values are indexed to the creatinine concentration in the urine sample, n = 2–4. *P < 0.05 vs. saline or LPS. +P < 0.05 vs. saline.](http://ajprenal.physiology.org/)

![Fig. 8. Role of TLR4 in the response to cisplatin and/or LPS treatment. Wild-type (WT) or TLR4-deficient mice were treated with saline, cisplatin, and/or LPS according to the protocol in Fig. 1. Blood urea nitrogen (BUN; A) and serum creatinine (B) were measured as indexes of renal function, n = 3–5. *P < 0.01 vs. all other groups. +P < 0.01 vs. LPS/TLR4 knockout.](http://ajprenal.physiology.org/)
kidney TLR4 protein levels after either cisplatin or endotoxin treatment. Likewise, we did not detect any impact of cisplatin, endotoxin, or combined treatment on kidney TLR4 mRNA expression (Fig. 5). Rather, changes in the downstream signaling pathways mediating TLR4 responses, such as p38 MAPK, may be responsible.

Evidence for an enhanced inflammatory response within the kidney also emerged from an examination of urinary cytokine excretion. In recent work, we reported that several urinary cytokines/chemokines, including IL-2, IL-6, and RANTES, were increased at 72 h after injection of high-dose cisplatin (49), i.e., during established renal injury. The current results indicate that, in addition to TNF-α, several urinary cytokines were markedly elevated by endotoxin treatment at early time points, 9 and 27 h after cisplatin injection. Of these, KC, IL-6, G-CSF, and GM-CSF were increased to a greater extent by the combination of endotoxin and cisplatin vs. either agent alone. Increased renal expression of KC, a neutrophil chemotactic factor, was first reported following renal ischemia by Safirstein et al. (31). Likewise, increased urine and serum KC has been found after renal ischemia (10, 21). Based on the current results, urinary KC may be a more general marker of renal injury. An increase in G-CSF expression in the kidney has been demonstrated after ischemia (50), but elevations in urine G-CSF levels in acute renal failure have not been previously reported. IL-6 has been incriminated in the pathogenesis of ischemic acute renal failure (14, 23). Increases in urine and serum IL-6 in human acute renal failure correlate with duration of renal failure and mortality (16, 34). We also observed large increases in urine IL-6 following endotoxin injection, but not after the low dose of cisplatin alone. Taken together, these observations support the notion that renal inflammation is present during endotoxin- and cisplatin-induced acute renal injury and that measurements of urinary cytokines may be useful as early markers of injury.

We speculate that activation of p38 MAPK may account for the synergistic toxicity noted between endotoxin and cisplatin. We previously demonstrated that cisplatin increases renal p38 MAPK activity and that this is critical to cisplatin-induced production of TNF-α by renal epithelial cells and the resulting renal injury (27). We also found that the activation of p38 MAPK is crucial, via an increase in mRNA translation, to the synergy between cisplatin and endotoxin in stimulating TNF-α production by cultured renal proximal tubule cells (25). Since TNF-α is an important mediator of the renal injury observed here (Fig. 10), the activation of p38 MAPK and subsequent TNF-α production may account for the synergistic renal injury from cisplatin and endotoxin in vivo.

Finally, based on the present results, one can speculate that coexisting infections might influence an individual’s susceptibility to developing cisplatin nephrotoxicity. Likewise, acquired or genetic differences in TLR4 signaling or downstream effector pathways, such as NF-κB activation or TNF-α production and action, might also influence the risk of cisplatin nephrotoxicity. If so, knowledge of these...
determinants could be used to guide the clinical use of cisplatin.

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