“Subclinical” gentamicin nephrotoxicity: a potential risk factor for exaggerated endotoxin-driven TNF-α production

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Zager RA. “Subclinical” gentamicin nephrotoxicity: a potential risk factor for exaggerated endotoxin-driven TNF-α production. Am J Physiol Renal Physiol 293: F43–F49, 2007. First published April 18, 2007; doi:10.1152/ajprenal.00144.2007.—This study sought to determine whether gentamicin, a mainstay in treating Gram-negative sepsis, alters endotoxin (lipopolysaccharide; LPS)-driven TNF-α increases. CD-1 mice received 1 day of gentamicin treatment. Either 0, 24, or 72 h later, gentamicin-treated and control mice were injected with LPS. Renal cortical and plasma TNF-α, as well as MCP-1, protein levels were measured 2 or 24 h post-LPS injection. Renal cortical mRNAs for TNF-α, MCP-1, IL-10, and inducible nitric oxide synthase (iNOS) were also determined. Finally, gentamicin’s potential impact(s) on TNF-α/MCP-1 mRNA levels in nontraditional “target” organs (liver, spleen) was assessed. Gentamicin, when administered alone, slightly increased renal cortical TNF-α and MCP-1 mRNAs, but without changing plasma or renal TNF-α/MCP-1 protein levels. The gentamicin protocol induced no overt renal damage (assessed by blood urea nitrogen, creatinine, and histology). Nevertheless, gentamicin augmented LPS responsiveness, as manifested, in part, by a doubling of LPS-induced plasma TNF-α increases (vs. LPS injection alone). Plasma and renal cortical MCP-1 protein levels were also selectively enhanced. Gentamicin augmented LPS-driven renal mRNA increases (TNF-α, MCP-1, IL-10, iNOS). However, this was not an entirely renal-specific response, since gentamicin also enhanced basal and LPS-stimulated hepatic TNF-α mRNA levels. Subclinical gentamicin toxicity can potentiate LPS-driven TNF-α increases. Alterations in multiple proinflammatory (TNF-α; MCP-1; iNOS) and anti-inflammatory (IL-10) genes in the kidney, and possibly in extrarenal organs, may be involved. Thus gentamicin’s activity in Gram-negative sepsis may extend beyond its traditional antimicrobial effect.

A GROWING BODY OF CLINICAL information indicates that the presence of acute renal failure (ARF) is an independent risk factor for mortality in hospitalized patients (4, 5, 7, 8, 12, 13). Recent experimental work from this laboratory has suggested one potential mechanism for this pathophysiological link: renal injury-induced hyperresponsiveness within the kidney’s Toll receptor (TLR)/inflammatory response pathway (16–19). The evidence underlying this conclusion is as follows: when mice with established ischemic-, obstructive-, endotoxemic-, cisplatin-, or rhabdomyolysis-induced ARF were challenged with a TLR4 (endotoxin; LPS) or a TLR2 (lipoteichoic acid) ligand, exaggerated renal production of TLR-dependent inflammatory mediators (e.g., TNF-α, MCP-1, nitric oxide) resulted. Heightened renal TNF-α production culminated in increased circulating TNF-α levels, presumably due to renal venous efflux (16, 18, 19). Thus these observations imply that the acutely damaged kidney, when challenged with proinflammatory TLR ligands, is poised to participate in multiorgan “cross talk” via systemic cytokine release. Such a sequence could potentially worsen multiorgan failure and possibly decrease patient survival.

In each of the above experiments, the employed ARF models induced injury throughout the kidney, as well as causing extrarenal tissue damage. For example, in the case of renal ischemia, urinary tract obstruction, or endotoxemic ARF, overt or covert injury occurs throughout the nephron and its vasculature. With endotoxemic-, rhabdomyolysis-, or cisplatin-induced ARF, extrarenal tissue damage also results. Finally, the abdominal surgery that is required to induce the ischemic or the obstructive ARF models introduces additional stress variables, with the potential to influence subsequent renal injury responses. These considerations raise two critical questions: First, can proximal tubule-specific injury independently incite heightened responsiveness within the kidney’s inflammatory pathways? Second, can this hyperresponsive state be expressed in the absence of massive tubular injury/concomitant ARF (as was induced in each of the above-described experiments)? If so, this would suggest that even subclinical renal damage can potentially set the stage for organ cross talk, thereby expanding the potential clinical relevance of the above-described phenomenon.

The present study was undertaken to address these two issues. Toward this end, mice were subjected to 1 day of gentamicin treatment, followed at variable time points by Escherichia coli LPS injection. Gentamicin was chosen as the renal injury model because its uptake, and hence the injury it evokes, is presumably limited to the proximal tubule (i.e., because its binding protein, megalin, is restricted to this nephron segment) (e.g., Refs. 6, 9, 11). Finally, the potential for gentamicin to alter LPS responses has obvious clinical relevance to Gram-negative sepsis.

METHODS

All experiments were conducted using male CD-1 mice (25–30 g) obtained from Charles River Laboratories (Wilmington, MA). They were housed under standard vivarium conditions with free food and water access. The animal protocols used in this study were reviewed and approved by the institution’s Institutional Animal Care and Use Committee.

Independent Effects of Gentamicin on TNF-α/MCP-1 Protein and mRNA Levels

Eighteen mice were injected with 80 mg/kg gentamicin (ip or im, 2 equally divided doses ~6 h apart, Sigma, St. Louis, MO). (As a point of...
reference, the gentamicin dosage range that is typically used in rodents to simulate clinical gentamicin toxicity is 40–120 mg/kg × 7–10 days). An equal number of control mice received an equal volume of vehicle (saline; 100 μl). Twenty-four or seventy-two hours post-gentamicin or vehicle injection, one-half of the mice in each group were anesthetized with pentobarbital sodium (50 mg/kg) and subjected to a midline abdominal incision. A plasma sample was obtained from the inferior vena cava, followed by a bilateral nephrectomy. The kidneys were immediately iced, and renal cortices were dissected. Left and right kidney samples from each mouse were used for either protein or total RNA extraction, and then the samples were assayed for TNF-α/MCP-1 protein (ELISA) (16) or TNF-α/MCP-1/GAPDH mRNA (RT-PCR) (16). Plasma samples were assayed for TNF-α, MCP-1 (ELISA), and blood urea nitrogen (BUN)/creatinine concentrations (17–19). In addition, sections of renal cortex from three mice 3 days post-gentamicin treatment and from three control mice were fixed in 10% formalin, and 4-μm sections were cut and stained with hematoxylin/eosin.

One-Day Gentamicin Pretreatment: Impact on LPS-Renal TNF-α/MCP-1 Responses

Control mice and mice 24 h post-gentamicin treatment (n = 8/group) were injected via the tail vein with 2 mg/kg of E. coli LPS (strain 0111:B4, in 80 μl of saline, cat. number L-2630, Sigma). Two hours later, the mice were anesthetized and subjected to plasma and renal cortical protein/RNA collections and analyses, as above.

Three-Day Gentamicin Pretreatment: Impact on LPS-Renal TNF-α/MCP-1 Responses

Three days post-gentamicin treatment, 12 mice were subjected to LPS injection (iv). Two hours later, plasma and renal cortical samples were obtained for TNF-α/MCP-1 protein/mRNA analyses. Results were contrasted to those obtained from 12 LPS-injected controls.

To further gauge potential effects of gentamicin on renal inflammatory mRNA levels, the above renal cortical samples were assayed for inducible nitric oxide synthase (iNOS) and IL-10 mRNA (2 h post-LPS injection) (16, 18, 19). The impact of 3-day gentamicin pretreatment in the presence and absence of LPS on renal iNOS/IL-10 mRNAs was also assessed (n = 4 mice each).

Simultaneous Gentamicin ± LPS Injection: Impact on 24-h Renal TNF-α/MCP-1 Responses

The following experiment evaluated the impact of LPS on renal cortical TNF-α/MCP-1 expression at a delayed time point (24 h) and whether simultaneous gentamicin administration alters these results. Twenty mice were injected with 10 mg/kg of LPS. One-half of the mice received simultaneous gentamicin or saline (ip, control) injections. Twenty-four hours later, plasma and renal cortical protein/RNA samples were collected and assayed, as noted above.

Hepatic and Splenic Responses to Gentamicin/LPS Injections

As described below (see RESULTS), gentamicin altered renal responses to LPS. To ascertain whether this was a kidney-specific response, six control mice and six mice that were 72 h post-gentamicin injection were subjected to the 2-h 2 mg/kg LPS challenge. This was followed by hepatic and splenic resection with subsequent TNF-α/MCP-1 mRNA analysis. Gentamicin’s potential independent impact on hepatic and splenic TNF-α/MCP-1 mRNAs (i.e., in the absence of LPS) was also assessed (6 gentamicin/6 control mice, 3 days post-gentamicin injection/sham injections).

Statistics

All values are presented as means ± SE and tested for significance (p < 0.05) by either a paired or an unpaired Student’s t-test.

RESULTS

Effects of Gentamicin in the Absence of LPS

As shown in Fig. 1 (left), gentamicin treatment, in the absence of LPS, increased renal TNF-α mRNA, as assessed at...
either the 24- or 72-h time point. Renal MCP-1 mRNA also rose post-gentamicin treatment, but the increase was observed only at the 72-h time point. Despite these mRNA increases, no significant changes in renal cortical TNF-α or MCP-1 protein levels were observed (Fig. 1, right).

Gentamicin did not alter plasma TNF-α concentrations, which remained at undetectable levels (<2 pg/ml) in control and postgentamicin plasma samples (not shown). Plasma MCP-1 levels were also unaltered by gentamicin treatment (controls, 69 ± 11 pg/ml; gentamicin, 72 ± 10 pg/ml; 24- and 72-h time points combined).

Gentamicin induced no evidence of renal histological damage, with kidney sections appearing identical to those obtained from normal controls. BUN and creatinine remained at control values at both 24 and 72 h (BUN: 28 ± 1, 28 ± 2, 29 ± 1 mg/dl at baseline, 24 h, and 72 h, respectively; creatinine: 0.3 ± 0.05, 0.3 ± 0.1, and 0.3 ± 0.1 mg/dl, respectively).

One-Day Gentamicin Pretreatment: Effects on LPS-Induced TNF-α/MCP-1 Changes

TNF-α. LPS induced marked renal TNF-α mRNA increases in all mice (vs. normal control values, as shown in Fig. 1). However, the degree of increase was comparable for the control and 24-h gentamicin-pretreated groups (Fig. 2A). LPS induced slightly (~20%), but significantly (P < 0.015), greater renal cortical TNF-α protein increases in gentamicin-pretreated mice, compared with controls (Fig. 2B).

Most dramatically, plasma TNF-α levels were twice as high in the gentamicin-pretreated/LPS-challenged mice vs. LPS-challenged controls (P < 0.03; Fig. 2C). As noted above, gentamicin, alone, failed to raise plasma TNF-α levels.

MCP-1. Renal MCP-1 mRNA and protein responses to LPS paralleled the above-noted changes in TNF-α: gentamicin pretreatment did not alter the LPS-induced MCP-1 mRNA increases (Fig. 2D); however, gentamicin pretreatment modestly (~25%), but significantly, increased MCP-1 protein levels in both renal cortex (Fig. 2E; P < 0.015) and in plasma (Fig. 2F; P < 0.001).

Three-Day Gentamicin Pretreatment: Effects on LPS-Induced TNF-α/MCP-1 Changes

TNF-α. As shown in Fig. 3, A and B, mice that had been primed with gentamicin 3 days earlier mounted significantly greater renal cortical TNF-α mRNA and protein increases in response to LPS than did sham-treated controls.

Plasma TNF-α levels were again twice as high in the LPS-gentamicin group vs. LPS-challenged controls (Fig. 3C). Of note, assay of these plasma samples for gentamicin demonstrated undetectable levels (<0.05 μg/ml; University of Washington Clinical Laboratory, Seattle, WA). Thus circulating gentamicin was not a prerequisite for these results.

MCP-1. Gentamicin-pretreated mice had slightly, but significantly, higher MCP-1 mRNA increases in response to LPS than did the LPS-injected controls (P < 0.05; Fig. 3D). However, this did not translate into significantly higher MCP-1 protein levels [in either renal cortex (Fig. 3E) or plasma (Fig. 3F)].

Fig. 2. Twenty-four-hour gentamicin pretreatment: effect on TNF-α/MCP-1 responses to LPS. Gentamicin did not alter TNF-α or MCP-1 mRNA responses to LPS (A and D, respectively). However, it slightly augmented renal cortical TNF-α and MCP-1 increases, assessed 2 h post-LPS injection (B and E). Most dramatically, gentamicin pretreatment doubled LPS-mediated TNF-α increases in plasma (C). A very slight, but highly significant, increase in plasma MCP-1 levels was also observed (F).
IL-10 and iNOS mRNAs. Three-day prior gentamicin treatment sensitized to LPS mediated IL-10 and iNOS mRNA increases in the kidney (Fig. 4) without exerting an independent effect (i.e., in the absence of LPS).

TNF-α/MCP-1 Assessments at 24 h Post-LPS

Both TNF-α and MCP-1 mRNAs were about two times higher at 24 h post-LPS injection in mice that had received simultaneous gentamicin treatment (vs. 24-h post-LPS injected controls; Fig. 5, A and D). Renal cortical TNF-α protein levels did not differ between the gentamicin+LPS-injected mice vs. LPS control groups (Fig. 5B). However, renal cortical MCP-1 levels were about two times higher in mice that had received combined gentamicin/LPS treatment (vs. LPS alone; Fig. 5E).

Plasma TNF-α levels returned to normal (<2 pg/ml) by 24 h post-LPS injection in both the gentamicin+LPS and control LPS groups (Fig. 5C). However, plasma MCP-1 levels were about three times higher at 24 h post-LPS/gentamicin injection vs. LPS injection alone (Fig. 5F).

By 24 h post-LPS injection, significant BUN and creatinine elevations had developed. The degree of plasma creatinine elevation was significantly greater with gentamicin+LPS vs. LPS alone (0.73 ± 0.1 vs. 0.47 ± 0.07 mg/dl; P < 0.025), indicating a worsening of LPS-mediated ARF. BUN levels...
were also higher (but not statistically so) in the former group (87 ± 10 vs. 68 ± 16 mg/dl).

**Hepatic and Splenic Responses to Gentamicin/LPS Injections**

Gentamicin sensitized the liver to LPS-mediated TNF-α mRNA (and to a lesser extent MCP-1) mRNA increases (Fig. 6). Conversely, in the spleen, gentamicin pretreatment suppressed TNF-α mRNA responses to LPS without impacting MCP-1 mRNA. In the absence of LPS, gentamicin slightly increased hepatic (but not splenic) TNF-α mRNA (gentamicin, 0.42 ± 0.02 vs. controls, 0.10 ± 0.12; \( P < 0.035 \)). Conversely, gentamicin had no independent effect on splenic TNF-α/MCP-1 mRNA levels.
DISCUSSION

The present study demonstrates that a single day of gentamicin treatment can augment subsequent LPS-mediated TNF-α generation in mice. This phenomenon was most dramatically illustrated by the results of plasma TNF-α assays. By 2 h post-LPS injection, gentamicin pretreated mice manifested twofold higher plasma TNF-α concentrations vs. LPS-injected controls. The same result was obtained irrespective of whether the gentamicin was administered 1 or 3 days before the LPS challenge. Thus this LPS hyperresponsive state did not simply reflect an early, or transitory, gentamicin effect. MCP-1 appeared to participate in this phenomenon. For example, when mice were treated simultaneously with gentamicin + LPS, about three times higher plasma MCP-1 elevations were observed 24 h later vs. values in LPS-injected controls. Furthermore, in selected experiments, gentamicin treatment approximately doubled LPS-mediated renal cortical MCP-1 increases. That a chemokine (MCP-1) and a cytokine (TNF-α) might functionally interact is consistent with the notion that the chemokine system is involved in these cytokines are involved in these events.

In each of our prior studies of renal injury-induced sensitization to LPS, overt tubular injury/filtration failure existed (16–19). Furthermore, extrarenal tissue damage was present (e.g., glycerol-induced rhabdomyolysis; cisplatin-induced gut injury; abdominal surgery, as required to induce renal ischemia or ureteral obstruction). Given these potential confounding variables, it was not clear as to whether “subclinical” renal injury or a proximal tubular-specific injury could also induce the LPS hyperresponsive state. The present study resolves both issues, given that gentamicin recapitulated LPS sensitization under conditions of normal renal function (BUN, creatinine) and histology. The observation that covert tubular injury can enhance renal LPS responsiveness seemingly extends the potential clinical relevance of this phenomenon.

Gentamicin is known to inhibit ribosomal protein synthesis within renal tubular cells (2). This suggests a seeming paradox: tubular gentamicin loading appeared to sensitize to LPS-driven TNF-α and MCP-1 protein generation. This led us to question whether gentamicin treatment might exaggerate inflammatory gene transcription, an action that could potentially overcome a partial protein synthesis blockade. The data suggest that this may be the case, at least in some of the experiments, based on the following pieces of information: first, gentamicin was able to increase renal TNF-α and MCP-1 mRNA in the absence of LPS; second, 3 days after gentamicin loading, exaggerated LPS-driven renal TNF-α/MCP-1 mRNA accumulation resulted; and third, gentamicin also sensitized the kidney to LPS mediated IL-10 and iNOS mRNA increases. The latter underscores the potential broad based nature of the presently described gentamicin results.

Initially, given that sepsis is a multisystem disease, we questioned whether gentamicin could potentially alter extrarenal inflammatory responses. At first consideration, this seems unlikely, since gentamicin uptake is widely assumed to be restricted to proximal tubular (and inner ear) cells (3, 6, 9). However, some experimental evidence indicates that small amounts of gentamicin can accumulate in the liver (1, 3, 10) (via active secretion into bile) and that this may culminate in intrahepatic multilamellar “myeloid body” formation (1) (the classic morphological hallmark of aminoglycoside nephrotoxicity). Given these observations, it is notable that gentamicin loading in the present study increased basal hepatic TNF-α mRNA expression and augmented LPS-initiated TNF-α (and to a lesser extent, MCP-1) mRNA increases. However, this was not a generalized response, given that gentamicin tended to suppress LPS-stimulated splenic TNF-α mRNA levels. Taken together, the present observations suggest a potentially new and important pathophysiological concept: renal, as well as extrarenal, inflammatory responses may be impacted by gentamicin therapy. Gentamicin has been previously documented to worsen renal injury in the setting of endotoxemia (20), systemic hypotension (14), and ischemia (15, 21). The present results suggest that an augmentation of tissue inflammation might have contributed to these prior experimental results. Alternatively, the anti-inflammatory effects of heightened IL-10 activity could potentially dampen these processes. Thus further explorations of these possibilities, their implications, and their underlying molecular mechanisms are required.

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