Toll-Like Receptor 4 Inhibitor TAK-242 Attenuates Acute Kidney Injury in Endotoxemic Sheep

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ABSTRACT

Background: This study was conducted to investigate the role of toll-like receptor 4 (TLR4) in mediating acute kidney injury in endotoxemic sheep using the selective TLR4 inhibitor TAK-242.

Methods: A randomized, controlled, experimental study was performed with 20 adult Texel crossbred sheep. Before an Escherichia coli lipopolysaccharide infusion (3 μg·kg⁻¹·h⁻¹ for 24 h), sheep were randomized to receive a bolus dose (2 mg/kg), followed by a continuous infusion (4 mg·kg⁻¹·24 h⁻¹) of either TAK-242 (n = 7) or vehicle (n = 7). A third group of lipopolysaccharide-treated sheep (n = 6) received norepinephrine, titrated to maintain baseline arterial blood pressure.

Results: Endotoxin infusion established a state of hyperdynamic circulation, with an increased cardiac index, hypotension, and tachycardia. Urine output and creatinine clearance decreased throughout the experiment, together with increasing plasma creatinine, blood urea nitrogen, and arterial lactate concentrations. After 24 h, TLR4 inhibition had significantly (P ≤ 0.001) attenuated the mean ± SEM decrease in arterial pressure (97 ± 3 vs. 71 ± 4 mmHg), urine output (1.16 ± 0.15 vs. 0.13 ± 0.05 ml·kg⁻¹·h⁻¹), and creatinine clearance (126 ± 13 vs. 20 ± 7 ml/min) compared with vehicle-treated animals. Furthermore, arterial lactate, plasma creatinine, and blood urea nitrogen concentrations were significantly lower in the TAK-242 group versus the vehicle-treated animals. Compared with TLR4 inhibition, norepinephrine caused similar effects on arterial pressure, cardiac index, and heart rate; however, it did not attenuate the decrease in urine output or creatinine clearance.

Conclusions: These results indicate a critical role for TLR4 in impairing renal function during ovine endotoxemia that is independent of changes in central hemodynamics.

What We Already Know about This Topic:
- Acute kidney injury contributes to mortality in patients with sepsis.
- Lipopolysaccharide from Gram-negative bacterial cell walls may cause kidney injury by binding to toll-like receptor 4 in the kidney.

What This Article Tells Us That Is New:
- In this sheep model, a toll-like receptor antagonist decreased hypotension and improved renal function during lipopolysaccharide infusion.
However, recent data suggest that interaction between Gram-negative bacteria and both systemic and renal-bound TLR4 may contribute to septic AKI. The presence of TLR4 has been displayed in both human and murine renal tissue, and sepsis increases the expression of TLR4 in renal vascular and epithelial cells in both the Bowman capsule and the tubulus. Circulating endotoxin reaches these renal sites. Furthermore, activation of extrarenal TLR4 results in the stimulation of proinflammatory cytokines that may harm the kidney by inducing the release of reactive oxygen species and neutrophil granule proteins. These data provide a theoretic basis for TLR4-induced AKI, but studies investigating the impact of TLR4 activation on renal function during endotoxemia or sepsis have never been evaluated. Recently available selective pharmacologic antagonists for the TLR4 suitable for iv administration have made it possible to explore further the importance of TLR4 in sepsis and endotoxemia in a large animal model mimicking the clinical scenario and facilitating extensive cardiovascular monitoring with repeated blood and urine sampling. This study was conducted to investigate the role of TLR4 in mediating AKI in endotoxemic sheep. Conscious animals were pretreated with the selective TLR4 inhibitor TAK-242 or vehicle and subjected to 24 h of endotoxemia, during which renal function was monitored. To explore if any effect of TAK-242 was because of the prevention of hypotension, a third group of lipopolysaccharide-treated animals receiving norepinephrine titrated to maintain baseline blood pressure was added.

Materials and Methods
The study protocol was approved in advance by the Regional Ethics Committee for Experiments in Animals, Stockholm, Sweden. The experiments were conducted in accordance with the European Convention for Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe No. 123, Strasbourg, France, 1985).

Animals and Surgical Preparation
Twenty adult Texel crossbred ewes, weighing a mean ± SEM of 58 ± 3 kg, were used. They had free access to water and were housed individually in pens. Two times per day, they were fed hay and 75-g commercial pellets with the addition of 6-g NaCl. Before the experiments, one carotid artery was surgically exteriorized and placed in a cervical skin loop, as previously described.

Experimental Preparation
The experiments were performed with conscious animals placed in individual pens with access to water and hay. Intravascular catheterizations were performed under local anesthesia (lidocaine hydrochloride, 5 mg/ml) at least 4 h before starting the experiments. The exteriorized carotid artery was cannulated and used for measurement of arterial blood pressure and arterial blood sampling. Venous blood sampling was performed via a catheter placed in the left jugular vein. A balloon-tipped pulmonary artery catheter (7.5F Swan-Ganz; Edwards Lifesciences, Irvine, CA) was inserted into the pulmonary artery via an introducer in the right jugular vein. The position in the pulmonary artery was determined by pressure guidance on a monitor. The iv infusions were performed in a catheter placed in the right jugular vein. A retention catheter (14F) was inserted into the bladder via the urethra and used for urine collection.

Hemodynamic Recordings
Hemodynamic measurements were acquired online with a sampling rate of 50 Hz via a multichannel interface (MP150; Biopac Systems, Goleta, CA) with acquisition software (AcqKnowledge 3.8.1.; Biopac Systems) and stored on a computer. The different blood pressures were measured via pressure transducers (DPT-6003; PVB Medizin Technik, BMBH, Kirchseen, Germany). The pressure transducers were calibrated to atmospheric pressure at the level of the heart and to 100 or 25 mmHg using a saline column. The pulmonary artery catheter was connected to a monitoring system (Vigilance Monitor; Edwards Lifesciences), cardiac output was calculated every 30–60 s, and core temperature was measured continuously. When the body temperature exceeded the upper range for automatic measurements (i.e., 41°C), manual cardiac output measurements were performed by three consecutive injections (10 ml) of isotonic saline. Mean arterial pressure (MAP) and mean pulmonary arterial pressure were calculated and displayed online.

Blood, Plasma, and Urine Analyses
The venous blood was portioned into prechilled tubes with heparin or EDTA as anticoagulant and immediately centrifuged at 4°C (3000 rpm) for 10 min. Plasma aliquots were stored at −20°C until assayed for creatinine (Jaffe method) and blood urea nitrogen (Synchron LX; Beckman Instruments, Richmond, CA). Other portions of plasma were taken for determination of hematocrit and protein concentration by refractometry (Atago Co, Tokyo, Japan). The carotid blood samples were used for immediate arterial blood gas analyses (ABL 77; Radiometer, Copenhagen, Denmark). In addition, lactate (Accu-trend Lactate; Roche Diagnostics, Basel, Switzerland) was analyzed using arterial blood. Urinary concentrations of N-acetyl-B-D-glucosaminidase (U-NAG) were measured by a colorimetric assay (Cobas Mira; Hoffmann–La Roche AG, Basel, Switzerland) in groups receiving TAK-242 or vehicle.

Experimental Protocol
Sheep were randomized to receive a bolus dose (2 mg/kg), followed by a continuous infusion (4 mg·kg⁻¹·24 h⁻¹) of...
either the selective TLR4 inhibitor, TAK-242 (10 mg/ml) (n = 7), or vehicle (n = 7) (0.6 ml · kg⁻¹ · h⁻¹). Randomization was blinded and performed with random alphabetic letters assigned to each animal. The treatment remained blinded for the investigators until after the experiments were finished. Endotoxemia was started after the bolus dose was given by iv infusion of Escherichia coli lipopolysaccharide (serotype 0111:B4; 600,000 endotoxin units · μg; Sigma–Aldrich Sweden AB, Stockholm, Sweden). The infusion rate was slowly increased during the first 30 min to reach 3 μg · kg⁻¹ · h⁻¹. This rate was then maintained for the remainder of the experiment (24 h). In an additional group, lipopolysaccharide was administered as described; however, to prevent hypotension, norepinephrine (200 μg/ml) was titrated to maintain MAP at baseline levels. In all groups, baseline values were collected 60 min before the start of lipopolysaccharide infusion. Fluid volume support was administered as Ringer’s acetate solution (B. Braun Melsungen, AG, Melsungen, Germany) iv at 0.5 ml · kg⁻¹ · h⁻¹. In the nor-epinephrine group, additional volume support was given as bolus doses (200–250 ml) of hydroxyethyl starch (130/ 0.4) if MAP did not increase as expected in response to norepinephrine. Blood samples (approximately 20-ml venous blood and 1-ml arterial blood) were drawn at baseline and at 6, 12, 18, and 24 h after commencement of endotoxemia. Urinary output was measured, and urine samples were collected every second hour.

The occurrence of AKI was determined by reduced renal function, measured as decreased urine output and creatinine clearance.

Statistical Analysis
Cardiovascular parameters were averaged off-line. Cardiac output was indexed to body surface area (0.09 × body weight⁰·⁶). Creatinine clearance was calculated as follows: (Urine Flow) × (Urine Creatinine Concentration)/Plasma Creatinine Concentration.

All statistical calculations were performed using computer software (Statistica 8.0; Statsoft Inc., Tulsa, OK), and the graphs were created with a computer program (Pr Plot 11.0; SPSS Inc., Chicago, IL). Data are expressed as mean ± SEM. Urine production values were transformed to follow a normal distribution by taking the logarithm of the raw data. Changes in parameters over time were analyzed according to a two-way repeated-measures ANOVA, which included the time points of 0, 12, and 24 h after the start of lipopolysaccharide infusion as within effects and treatment (control, TAK-242, or norepinephrine) as a between effect. In case of a significant group or interaction effect, group differences were analyzed by a one-way ANOVA and a Tukey test at each time point included in the initial ANOVA. Furthermore, a one-way repeated-measures ANOVA was performed for each treatment to investigate if that group changed significantly over time. The result of this analysis is not displayed in the figures and is only referred to in the Results section. The significance level was set at P ≤ 0.05.

Results
Endotoxemia resulted in a hyperdynamic response, with a decrease in MAP and an increase in cardiac index and tachycardia (fig. 1, A–C; P < 0.05). TAK-242 attenuated the change in MAP and heart rate (fig. 1, A and C, respectively) but had no significant effect on cardiac index (fig. 1B). Body temperature increased and remained higher than baseline values throughout the experiment (fig. 1D, P < 0.001). TAK-242 had no significant effect on the fever response (P = 0.8). At the end of the experiment, animals in the TAK-242 group showed normal behavior, with adequate intake of hay and water. The sheep in the vehicle group had a significantly more affected general state of health and were often lying down, without eating or drinking.

After an early peak, urine output decreased significantly as a result of the lipopolysaccharide infusion (P < 0.001, fig. 2A). However, TAK-242 abolished the reduction in diuresis (fig. 2A). This was also reflected in the creatinine clearance; TAK-242 prevented the reduction seen in the vehicle-treated animals (fig. 2B). Furthermore, the reduced renal function caused by endotoxia caused a significant increase in plasma creatinine and blood urea nitrogen concentrations (P < 0.001 for both; fig. 2, C and D, respectively). These effects were abolished by pretreatment with TAK-242 (fig. 2, C and D, respectively). Moreover, TAK-242 reduced the hyperlactemia produced by the lipopolysaccharide infusion (fig. 2E). As an estimate of tubular cell injury, the urinary NAG concentration was analyzed. In the vehicle group, U-NAG remained close to baseline levels during the initial 12 hours of endotoxia; thereafter, it increased significantly (P < 0.001, fig. 2F). TAK-242 abolished the increase in U-NAG (fig. 2F).

Systemic vascular resistance decreased and mixed venous oxygen saturation increased as a result of the lipopolysaccharide infusion, without intergroup differences (P > 0.001, table 1). The mean pulmonary artery pressure increased progressively during the experiment in the vehicle-treated animals (P < 0.001, table 1), but this increase was significantly attenuated by TAK-242 (table 1). Furthermore, TAK-242 prevented the decrease in PO₂ seen in the vehicle-treated animals (table 1). The arterial partial pressure of carbon dioxide, base excess, pH, plasma protein, and hematocrit were not significantly changed by either endotoxia or treatment (table 1). However, for an unknown reason, the norepinephrine-treated sheep had a significantly lower pH at baseline.

To explore the possibility that the effects of TAK-242 on renal function were because of the prevention of hypotension, a third group of animals receiving lipopolysaccharide and norepinephrine was added. In this group, hypotension was abolished (fig. 1A); and cardiac index, heart rate, and temperature were not significantly different from the other groups (fig. 1, B–D, respectively). However, AKI still devel-
opened as indicated by a reduction in urine output and creatinine clearance \((P = 0.002\) and \(P < 0.001\), respectively; fig. 2, A and B, respectively) and an increase in plasma creatinine and blood urea nitrogen \((P < 0.001\) for both; fig. 2, C and D, respectively). Plasma lactate increased more in response to lipopolysaccharide compared with TAK-242 (fig. 2E) and was significantly increased over time \((P = 0.002\). The nor-epinephrine infusion was started after 7.3 \(\pm\) 0.4 h, and the amount infused was 229 \(\pm\) 83 \(\mu\)g/kg. The volume of infused hydroxyethyl starch was 20 \(\pm\) 5 ml/kg.

### Discussion

This study was conducted to investigate the role of TLR4 in mediating AKI in endotoxemic sheep. The main finding was that pretreatment with a TLR4 inhibitor reduced the renal dysfunction caused by lipopolysaccharide infusion. The effect was significantly greater compared with treatment with norepinephrine in a dose that prevented hypotension. Furthermore, systemic TLR4 inhibition abolished the lipopolysaccharide-induced elevation in urinary NAG, indicating a possible reduction of renal tubular damage.

Previous studies\(^8,9,18,21–23\) performed in rodents have not reported effects on blood pressure or cardiac output by TLR4 antibodies or TLR4 knockout during endotoxemia or sepsis. Herein, the continuous infusion of lipopolysaccharide resulted in hyperdynamic circulation, characterized by increasing cardiac output, hypotension, tachycardia, and reduced systemic vascular resistance. Anesthesia has the potential to severely affect cardiovascular performance\(^24\) and reduce renal function during endotoxemia\(^25\) but because these animals remained conscious throughout the experiment, there was no influence from any anesthetic agent on the results.

In the vehicle-treated animals, MAP reached concentrations that usually are considered to be less than the lower limit for renal blood flow autoregulation. In theory, this would cause a reduction in renal blood flow that could contribute to the decreased creatinine clearance and urine output. However, previous studies\(^{26,27}\) in conscious sheep have shown that renal blood flow actually increases during sepsis and endotoxemia. Although renal blood flow was not measured herein, the cardiovascular reaction to lipopolysaccharide, including increased cardiac output, tachycardia, and hypotension, is identical to that in previous studies.\(^{26–28}\) This indicates that the effect of TAK-242 in the current study is not because of prevention of prerenal azotemia caused by blood flow restriction. The attenuated decrease in MAP by TLR4 inhibition could still have had a positive effect on glomerular perfusion pressure, facilitating higher creatinine clearance and diuresis in the TAK-242 group. This may, in part, explain the findings in this study and

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**Fig. 1.** Changes in mean arterial blood pressure (A), cardiac index (B), heart rate (C), and body temperature (D) in response to lipopolysaccharide infusion and treatment with the selective toll-like receptor 4 antagonist, TAK-242 (6 mg/24 h, \(n = 7\)), vehicle (\(n = 7\)), or norepinephrine (NE, \(n = 6\), titrated to maintain mean arterial pressure (MAP) at baseline levels. Data are expressed as mean \(\pm\) SEM. Differences between treatments were evaluated by ANOVA using time points 0, 12, and 24 h after commencement of lipopolysaccharide infusion and were considered significant at \(P \leq 0.05\). *, a significant difference between TAK-242 and vehicle; and †, a significant difference between vehicle and NE.
previous investigations performed in rodents (in which effects on blood pressure were not reported), showing a role for TLR4 in mediating septic AKI. To explore further this question, we added a group of sheep subjected to endotoxemia treated with norepinephrine. The results show that these sheep still develop AKI, although MAP was the same as in the TAK-242 group, indicating that hypotension is of limited importance for renal dysfunction in this model of endotoxemia. Changes in renal blood flow by norepinephrine may have resulted in kidney ischemia, although blood pressure remained at baseline levels. However, norepinephrine causes no significant reduction in an already increased renal blood flow or change in renal microcirculation in septic hypotensive sheep. This and the results presented herein indicate that norepinephrine had only a minor effect on central and renal hemodynamics, in addition to maintaining MAP. Thus, the renal-protective effect of TLR4 inhibition does not depend on prevention of hypotension. Further studies are needed to clarify the exact mechanisms of action, but results from studies on renal ischemia/reperfusion injury suggest that TAK-242 may act directly on renal parenchyma.

Fig. 2. Changes in urine output (A), creatinine clearance (B), plasma (P) creatinine (C), blood urea nitrogen (BUN) (D), P-lactate (E), and urinary N-acetyl-β-D-glucosaminidase (U-NAG) (F) in response to lipopolysaccharide infusion and treatment with the selective toll-like receptor 4 antagonist, TAK-242 (6 mg/24 h, n = 7), vehicle (n = 7), or norepinephrine (NE, n = 6), titrated to maintain mean arterial pressure at baseline levels. Data are expressed as mean ± SEM. Differences between treatments were evaluated by ANOVA using time points 0, 12, and 24 h after commencement of lipopolysaccharide infusion and were considered significant at \( P \leq 0.05 \). * indicates a significant difference between TAK-242 and vehicle; and #, a significant difference between TAK-242 and NE.
Table 1. Systemic Variables

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<th>Variable</th>
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<th>12</th>
<th>18</th>
<th>24</th>
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<td>20.1±2.1</td>
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<td>10.0±1.4</td>
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<td>Vehicle</td>
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<td>23.8±4.4</td>
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<td>21.3±2.6</td>
<td>17±1.1</td>
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<td>NE</td>
<td>14±1.0</td>
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<td>19±1.2*</td>
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<td>19±2.2†</td>
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<td>MPAP, mmHg</td>
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<td>NE</td>
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<td>72±5.0</td>
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</table>

Data for TAK-242 (n = 7), vehicle (n = 7), and NE (n = 6) are given as mean ± SEM. Differences between treatments were evaluated by ANOVA using time points 0 (baseline) and 12 and 24 h after commencement of lipopolysaccharide infusion and were considered significant at P ≤ 0.05.

* A significant difference between TAK-242 and vehicle. † A significant difference between TAK-242 and NE. ‡ A significant difference between vehicle and NE.

MPAP = mean pulmonary artery pressure; NE = norepinephrine; PO2 = partial pressure of oxygen; PCO2 = partial pressure of carbon dioxide; SvO2 = mixed venous oxygen saturation, SVR = systemic vascular resistance.

glycosidase found in proximal tubular epithelial cell lysosomes, and an increase in urinary levels is commonly used as a marker of tubular damage.\(^{31}\) Interestingly, Good et al.\(^{32}\) have suggested a TLR4-dependent mechanism in which lipopolysaccharide inhibits H⁺ secretion and HCO₃⁻ absorption; this mechanism inhibits the renal correction of lipopolysaccharide-induced systemic acidosis, contributing further to tubular dysfunction. The drastic increase of U-NAG in the vehicle-treated animals may suggest a tubular injury. However, because creatinine clearance and urine production decreased before the increase in U-NAG, we believe that tubular damage is probably not the main cause of AKI in this model.

Lipopolysaccharide acts as the primary ligand for TLR4 and activates an intracellular signaling cascade that involves both myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent pathways. This results in up-regulation of nuclear factor κB and subsequent induced expression of genes encoding for inflammatory mediators, such as tumor necrosis factor α and interleukin 6.\(^{33}\) The pharmacologic substance used for inhibition of TLR4 in the current study, TAK-242, binds directly to the amino acid Cys747 in the intracellular domain of the receptor,\(^{34}\) thus inhibiting the activation of the downstream signaling pathway by lipopolysaccharide. Experiments to confirm the specificity of TAK-242 for TLR4 have mainly been

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performed in rodents and humans. However, sheep TLR4 corresponds well to human TLR4,35 and TAK-242 abolished the cardiovascular effects of TLR4 activation in sheep. This makes us confident that the effects of TAK-242 on renal function were because of TLR4 inhibition. Although we did not measure cytokines, we believe that reduced production of these inflammatory mediators may be a large contributing mechanism for the results obtained in the current study. This idea is supported by findings from Cunningham et al.18 in a 6-h model of endotoxemia in C3H/HeJ mice with loss of function in TLR4. Their results indicate that TLR4-mediated renal injury depends on induction of tumor necrosis factor α production and renal neutrophil infiltration within the kidney.18 On the other hand, C3H/HeJ mice, lacking functional TLR4, subjected to polymicrobial infections still develop sepsis,22 suggesting that TLR4 activation by lipopolysaccharide may not be the sole mechanism in septic AKI. TLR4 inhibition significantly attenuated the increase in mean pulmonary artery pressure, prevented the decrease in P<sub>p</sub>, and reduced lactate production by endotoxemia. These results are probably a reflection of attenuated cytokine production because reduced cytokine levels in the lung have been reported in TLR4-deficient mice36 and after TAK-242 treatment in endotoxemic mice.37

In contrast to findings from previous studies reporting that activation of TLR4 by lipopolysaccharide causes changes in body temperature, TAK-242 did not reduce fever in the current study. In mice, TAK-242 reduced lipopolysaccharide-induced hypothermia21; and C3H/HeJ mice showed an absent temperature reaction after lipopolysaccharide challenge.38 TLR4-dependent production of prostaglandin E2 in macrophages has been proposed as a mechanism for this lipopolysaccharide-induced fever,39 but this theory has not been confirmed in sheep. Therefore, the current results could be because of incomplete inhibition of TLR4 or because other fever-inducing mechanisms (vs. those that are TLR4 dependent) are important in conscious endotoxic sheep. Another likely explanation is that the lipopolysaccharide infused was not perfectly pure and contained pyrogens activating receptors other than TLR4.

There are some limitations to this study that we would like to acknowledge. TLR4 inhibition was administered before endotoxemia and the subsequent development of AKI. Therefore, this study did not elucidate whether TLR4 inhibition may improve renal function when renal injury is already present. However, these data may still be of potential interest for clinical practice because prophylactic treatment with a TLR4 inhibitor may prove useful before certain invasive and surgical procedures. For instance, during major abdominal surgery, there is a risk for infection with Gram-negative bacteria and subsequent sepsis,40 for which antibiotics are usually administered perioperatively or preoperatively. Furthermore, the renal-protective benefit of pretreatment with TLR4 inhibitors may include disorders other than sepsis because TLR4 can be activated by lipopolysaccharide and as a response to tissue damage.41,42 Another limitation is that these experiments were performed with an observation period of 24 h and may, therefore, be too short to represent the common clinical manifestations of sepsis. Moreover, to our knowledge, this is the first study investigating the effect on renal function during sepsis after TLR4 inhibition; at present, these findings are limited to this specific experimental setting and need to be confirmed by others before any interpretation to clinical practice can be performed. A recent clinical trial43 investigating the effects of TAK-242 on human sepsis was terminated because TAK-242 failed to decrease serum interleukin 6 levels. The treatment was well tolerated but did not significantly improve survival. Because the enrollment of patients was discontinued prematurely, definite conclusions about the survival efficacy of TAK-242 in humans are still lacking. Direct comparisons with the current study are difficult because only 40% of the patients had Gram-negative infections and there was no special emphasis on renal function. However, the result of the current study suggests that septic patients with AKI would be interesting to investigate in future clinical trials with TAK-242.

Based on the findings from the current study, we conclude that activation of TLR4 may mediate the development of endotoxemic AKI in conscious sheep. This effect is not only because of a reduction in blood pressure because renal function was not maintained merely by preventing hypotension in animals treated with norepinephrine. Further studies are needed to investigate the full mechanism of activated TLR4-induced AKI, if TLR4 inhibition can reverse an already present AKI and if blockade of TLR4 is efficient during renal dysfunction induced by bacterial infection.

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References


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