Simvastatin improves sepsis-induced mortality and acute kidney injury via renal vascular effects

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Acute kidney injury (AKI) occurs in about half of patients in septic shock and the mortality of AKI with sepsis is extremely high. An effective therapeutic intervention is urgently required. Statins are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors that also have pleiotropic actions. They have been reported to increase the survival of septic or infectious patients. But the effect of simvastatin, a widely used statin, on sepsis-induced AKI is unknown. The effects of simvastatin and tumor necrosis factor (TNF)-α neutralizing antibody were studied in a clinically relevant model of sepsis-induced AKI using cecal ligation and puncture (CLP) in elderly mice. Simvastatin significantly improved CLP-induced mortality and AKI. Simvastatin attenuated CLP-induced tubular damage and reversed CLP-induced reduction of intrarenal microvascular perfusion and renal tubular hypoxia at 24 h. Simvastatin also restored towards normal CLP-induced renal vascular protein leak and serum TNF-α. Neither delayed simvastatin therapy nor TNF-α neutralizing antibody improved CLP-induced AKI. Simvastatin improved sepsis-induced AKI by direct effects on the renal vasculature, reversal of tubular hypoxia, and had a systemic anti-inflammatory effect.

Acute kidney injury (AKI) is a common life-threatening disease, whose mortality has remained at about 45% over three decades, despite advances in supportive care. Sepsis is a contributing factor in about half of patients of severe AKI.[1] Septic shock is the most common contributing factor to AKI in intensive care unit.[2] Acute kidney injury occurs in half of septic shock patients whose blood cultures are positive.[3] The mortality is higher in AKI patients with sepsis (75%) than in those without sepsis (45%).[4] Acute kidney injury independently increases the morbidity and mortality, although other organ failures also contribute.[5] Thus, the strategy of treatment for sepsis-induced AKI is urgently required. Activated protein C decreases mortality from severe sepsis,[6] and intensive insulin therapy or early goal-directed therapy, including early resuscitation, is beneficial in patients with severe sepsis or septic shock.[7,8] However, there are no drugs to prevent or treat sepsis-induced AKI.[9,10]

We have recently developed a clinically relevant sepsis-induced AKI model based on a classic cecal ligation and puncture (CLP) model of polymicrobial sepsis that can be used to screen drugs and investigate the pathogenesis of sepsis. CLP differs from endotoxin injection models because there is bacterial infection that mimics human sepsis.[11–13] Serum creatinine starts to increase at 12 h but not 6 h after CLP, although tubular damage can be detected at 6 h by magnetic resonance imaging techniques[14] and renal cyr61 expression, a tubular damage marker.[15] The renal pathophysiology after CLP is currently unknown.

3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) such as simvastatin have pleiotropic effects independent of lipid lowering.[16–18] Statin therapy has clinically beneficial effects on cardiovascular, cerebrovascular and acute and chronic kidney diseases via diverse effects.[17,19–21] The protective effects of statins on both human and animal sepsis have been recently shown. A retrospective study in humans reported that statin therapy reduced both overall and attributable mortality in patients with bacteremia.[22] A controlled study revealed that prior statin therapy was associated with a reduction of severe sepsis and intensive care unit admission.[23] In animals, simvastatin improved survival in a murine CLP model.[24,25] Despite these observations, the possible role of statins on sepsis-induced AKI remains unknown.

In the present study, we investigated whether simvastatin has an effect on sepsis-induced AKI and studied its

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mechanism of action. Specifically, we investigated renal vascular permeability, microperfusion, tubular hypoxia and histologic damage. As simvastatin decreased circulating tumor necrosis factor (TNF)-α during sepsis, treatment with anti-TNF-α antibody was examined.

RESULTS
Effect of simvastatin on sepsis-induced mortality and acute kidney injury
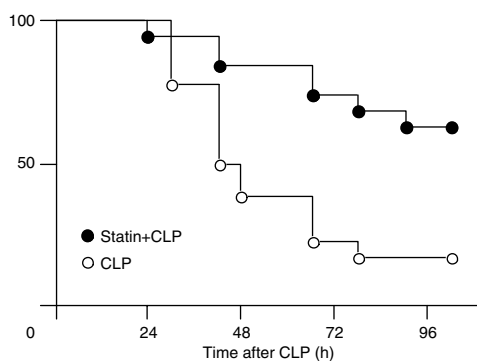
To determine whether simvastatin had an effect on CLP-induced mortality and renal dysfunction in aged mice treated with fluid and antibiotics, we measured survival and renal function. The survival for mice treated with saline was 100% at 24 h, 42% at 48 h and 26% at 72 h after CLP. The survival for aged mice treated with simvastatin was 95% at 24 h, 84% at 48 h and 73% at 72 h (Figure 1). Simvastatin significantly improved survival after CLP. This survival advantage is consistent with the previous reports[24,25] of the effect of simvastatin on sepsis in mice. However, previous studies did not evaluate renal function. Serum creatinine and blood urea nitrogen (BUN) were significantly increased at 6 h after CLP compared to sham and further worsened at 24 h. Prior statin treatment significantly prevented the renal dysfunction at 24 h but not 6 h after CLP, as detected by BUN and high-performance liquid chromatography serum creatinine (Figure 2).

Effect of simvastatin on sepsis-induced tubular damage

As reported previously, CLP caused very subtle changes in renal histology consisting of patchy tubular vacuolization but no thrombosis, tubular necrosis or cast formation.[15] The renal histology in both of the cortex and the outer stripe of the outer medulla (OSOM) worsened significantly after CLP (Figure 3). Simvastatin significantly prevented the deterioration of tubular damage induced by CLP in both the cortex and the OSOM (Figure 3).

Effect of simvastatin on sepsis-induced vascular permeability

Changes in vascular permeability are thought to be important in the pathogenesis of sepsis-induced organ injury.[26]

Lipopolysaccharide injection, which is another septic model, increases vascular permeability in various organs, including kidney, lung, liver and heart.[24,27–29] However, the renal vascular permeability after CLP is unknown. Therefore, vascular permeability was assessed by Evans blue dye (EBD) leakage. Since CLP did not significantly change vascular permeability at 2 h after CLP (data not shown), we evaluated
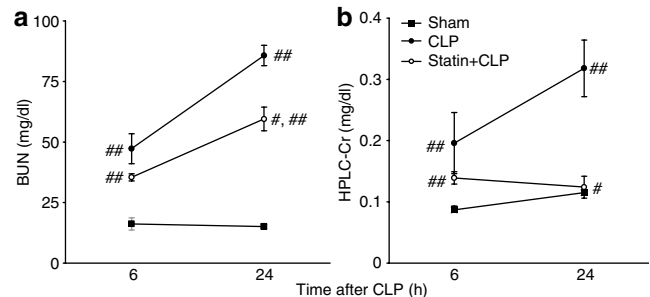
Figure 2 | Effect of simvastatin on renal function following CLP surgery. Mice were treated as in Figure 1. Mice were killed at indicated times for measurement of (**a**) serum BUN and creatinine by (**b**) HPLC serum creatinine. Closed circles indicate CLP group. Open circles indicate statin + CLP group. Closed squares indicate sham group. Values are mean ± s.e. ($N = 6$–16 per group). $^\#P < 0.05$ vs CLP. $^{\#\#}P < 0.05$ vs sham.

Figure 3 | Effect of simvastatin on renal histology following CLP surgery. Mice were treated as in Figure 1. Mice were killed at 24 h after surgery. Histology of cortex in (**a, d**) Sham group, (**b, e**) CLP group, (**c, f**) statin + CLP group; original magnification: (**a–c**) × 200, (**d–f**) × 400. (**g**) The tubular damage score (see Materials and Methods section) was measured in the cortex (left panel) and the OSOM (right panel). Values are mean ± s.e. ($N = 6$–16 per group). $^\#P < 0.05$ vs CLP. $^{\#\#}P < 0.05$ vs sham.
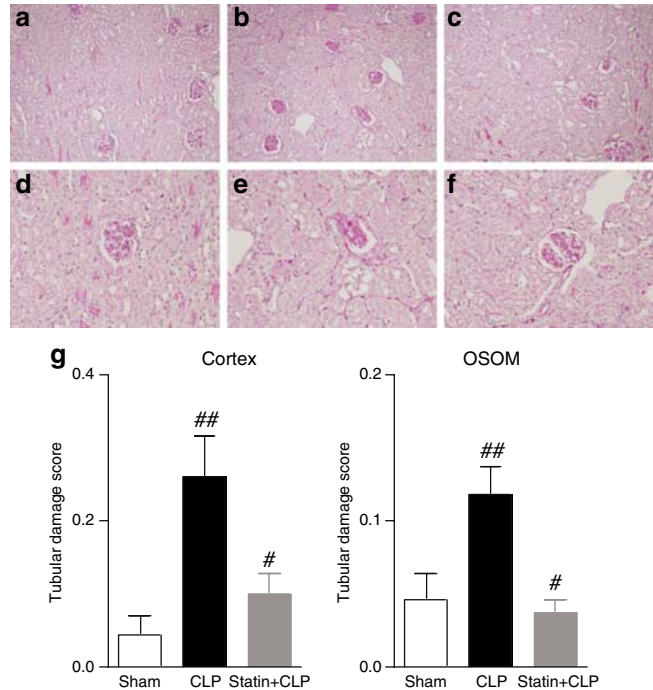

Figure 1 | Effect of simvastatin on survival after CLP. Aged mice were subjected to CLP. Simvastatin (40 mg/kg) or vehicle was administered for 3 days before CLP. Open circles indicate CLP group ($N = 19$). Closed circles indicate statin + CLP group ($N = 19$) ($P < 0.05$).

it at 6 and 24 h after surgery. Renal vascular permeability was similar at 6 and 24 h in sham mice. CLP significantly increased renal vascular permeability at 6 h and remained high at 24 h (Figure 4). Simvastatin significantly decreased the CLP-induced increase in renal vascular permeability at 24 h but not at 6 h after CLP.

Effect of simvastatin on renal tubular hypoxia

Tissue hypoxia is thought to be a dominant factor in organ dysfunction of sepsis.[30] Therefore, we assessed renal tubular hypoxia by pimonidazole incorporation, which is considered to react with cellular proteins at oxygen tensions below 10 mm Hg.[31] Pimonidazole incorporation was detected in the tubules of the cortex and the OSOM, but not in the inner stripe of the OSOM or inner medulla after CLP (Figure 5). Pimonidazole incorporation was significantly increased in both the cortex and the OSOM at 24 h after CLP (Figure 5).
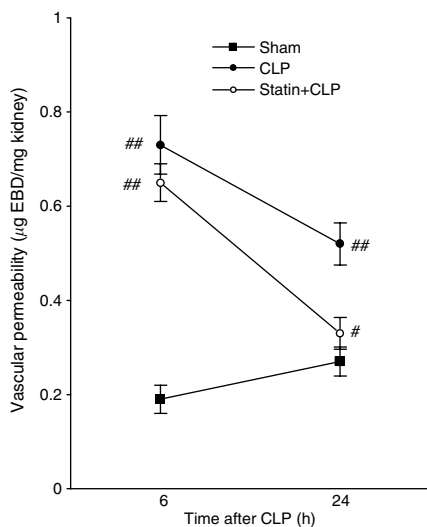
Figure 4 | Effect of simvastatin on renal vascular permeability. Mice were treated as in Figure 1. Mice were killed at 24 h after surgery. Evans blue dye (EBD) leakage in kidney tissue (see Materials and Methods) was measured at 6 and 24 h following surgery. Values are mean ± s.e. ($N = 4$–6 per group). $^{\#}P < 0.05$ vs CLP. $^{\#\#}P < 0.05$ vs sham.
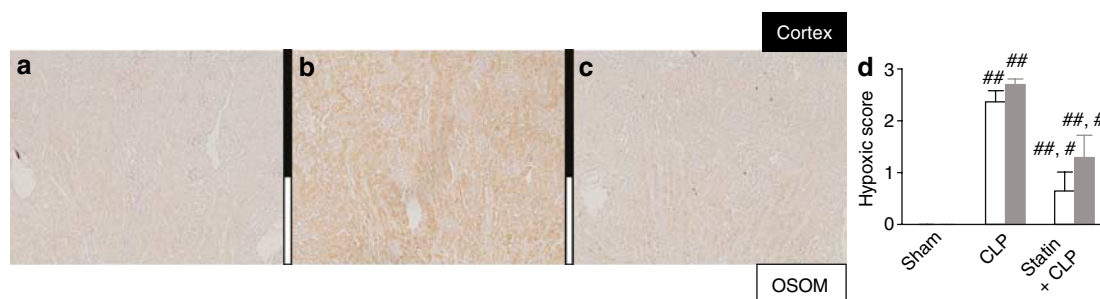
Simvastatin decreased pimonidazole incorporation in both the cortex and the OSOM at 24 h. These findings suggest that CLP caused renal hypoxia at 24 h and that simvastatin improved renal hypoxia.

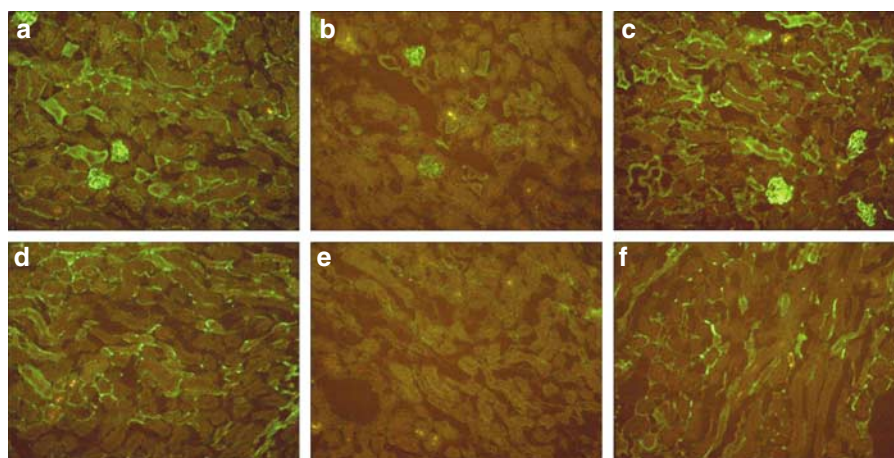## Effect of simvastatin on intrarenal microcirculation

Since simvastatin improved CLP-induced renal hypoxia at 24 h, we hypothesized that simvastatin might have a vascular effect during the late phase of CLP. Therefore, we evaluated perfusion of the renal microcirculation by physiologic infusion of fluorescein isothiocyanate-labeled lectin, which binds to the capillary endothelium. In sham-surgery animals, peritubular capillaries were clearly detected in the cortex and in the OSOM. Perfused capillaries were decreased in the cortex and the OSOM at 24 h after CLP (Figure 6). Simvastatin partially reversed the decrease in peritubular capillary flow in the cortex and in the OSOM at 24 h after CLP (Figure 6). As a control for lectin binding, we evaluated fluorescein isothiocyanate-labeled lectin binding to unperfused kidney sections to confirm the ability of endothelium to bind lectin after CLP. There was no difference in direct, topically applied lectin staining between CLP and sham sections (data not shown). Therefore, the changes shown here can be attributed to altered perfusion, and not to changes in lectin binding.

Effect of short-term administration of simvastatin on serum lipids

CLP did not significantly alter serum cholesterol or triglyceride at 24 h when compared to sham (serum cholesterol, 62 ± 6.2 vs 72 ± 4.9 mg/ml; serum triglyceride, 44 ± 6.0 vs 59.2 ± 8.3 mg/ml). There was no significant difference in serum cholesterol and triglyceride at 24 h between statin + CLP and CLP groups (serum cholesterol, 62 ± 6.2 vs 55.4 ± 3.9 mg/dl; serum triglyceride, 44 ± 6.0 vs 40 ± 2.0 mg/dl). CLP significantly increased serum CK at 24 h as reported previously [15] (data not shown); however, simvastatin did not alter serum CK at 24 h after CLP surgery (data not shown), which suggests that rhabdomyolysis, a potentially serious side effect of statin therapy,[32] did not occur.

Figure 5 | Effect of simvastatin on pimonidazole incorporation following CLP surgery. Mice were treated as in Figure 1. Mice were killed at 24 h after surgery. Histology of the cortex and OSOM in (**a**) sham group, (**b**) CLP group and (**c**) statin + CLP group. Original magnification, ×200. (**d**) The hypoxic score (see Materials and Methods) was measured in the cortex and the OSOM. Values are mean ± s.e. ($N = 3$–7 per group). $^{\#}P < 0.05$ vs CLP. $^{\#\#}P < 0.05$ vs sham.

Figure 6 | Effect of simvastatin on renal microvascular perfusion following CLP surgery. Mice were treated as in Figure 1. Mice were killed at 24 h after surgery. (**a–c**) Histology of cortex and (**d–f**) OSOM in (**a, d**) sham group, (**b, e**) CLP group and (**c, f**) statin + CLP group. Original magnification, × 400.

Effect of tumor necrosis factor-α neutralizing antibody on sepsis-induced acute kidney injury

Tumor necrosis factor-α is a prototypical proinflammatory cytokine that is elevated in some sepsis models, and is considered to be one of the key mediators in developing AKI in several other renal injury models.[33] We found that simvastatin inhibited serum TNF-α elevation at 24 h after CLP (Figure 7). We evaluated if simvastatin might improve renal injury via reduction of circulating TNF-α. However, neutralization of TNF-α did not significantly reduce serum BUN and creatinine at 24 h after CLP (Figure 8).

### Effect of delayed simvastatin treatment on sepsis-induced acute kidney injury

We also examined delayed administration of simvastatin using two different protocols. (1) Simvastatin administered subcutaneously 40 mg/kg at 6 h after CLP did not improve serum BUN or creatinine (serum BUN, vehicle: 93.0 ± 6.5 vs simvastatin: 102.3 ± 2.7 mg/dl; serum creatinine, 0.49 ± 0.07 vs 0.69 ± 0.17 mg/dl, $N = 6$ mice per group). (2) Two doses of simvastatin administered subcutaneously (40 mg/kg) at 6 and 18 h after CLP did not improve serum BUN or creatinine (serum BUN, 87.2 ± 16.3 vs 89.7 ± 4.7 mg/dl, serum creatinine, 0.39 ± 0.08 vs 0.45 ± 0.16 mg/dl, $N = 6$ mice per group).

DISCUSSION

We demonstrated that simvastatin, a widely used lipid-lowering drug, improved sepsis-induced mortality and AKI. Simvastatin reversed the late renal microvascular injury and microvascular perfusion defect with corresponding improvement in tissue oxygenation, and reduced a serum proinflammatory cytokine (serum TNF-α). These results suggest that simvastatin has both vascular and anti-inflammatory effects in this model.

Effects of simvastatin in sepsis-induced acute kidney injury

We found that simvastatin significantly improved survival after CLP in mice, which is consistent with Merx's previous
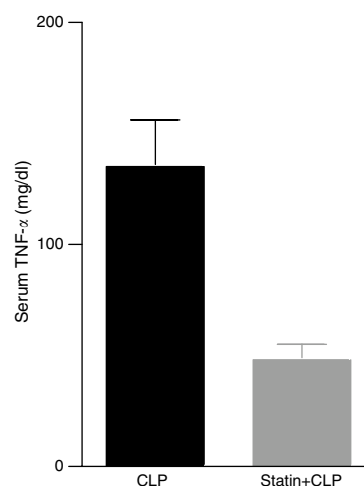
Figure 7 | Effect of simvastatin on serum TNF-α following CLP surgery. Mice were treated as in Figure 1. Mice were killed at 24 h after surgery. Values are mean $\pm$ s.e. ($N = 9$ per group). [#]$P < 0.05$ vs CLP.
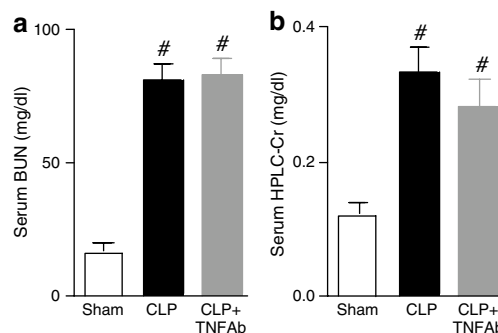
Figure 8 | Effect of TNF-α neutralizing antibody on renal function following CLP surgery. TNF-α neutralizing antibody or normal mouse Immunoglobulin G was administered at a dose of 800 μg/kg body weight immediately after CLP. Mice were killed at 24 h after CLP for measurement of (**a**) serum BUN and (**b**) creatinine by HPLC method. Values are mean $\pm$ s.e. ($N = 5$–16 per group). [#]$P < 0.05$ vs sham.

animal studies.[24,25] Two clinical natural history studies showed that statin usage was associated with reduced mortality from sepsis and/or bacterial infection.[22,23] We also demonstrated for the first time that simvastatin had a beneficial effect on sepsis-induced AKI. Pretreatment with simvastatin improved renal function, as measured by serum BUN and creatinine (Figure 2). Miyaji *et al.*[15] showed that CLP caused subtle injury to the renal tubules of the cortex and the OSOM. Simvastatin also inhibited the patchy tubular vacuolar degeneration found after CLP in both the cortex and the OSOM (Figure 3). Statins have been shown to have beneficial effects in other kidney diseases, including ischemia–reperfusion injury, transplantation and chronic kidney disease.[16–18,34,35] Interestingly, these effects, including an effect on cardiovascular morbidity and mortality, were independent of lowering serum cholesterol. Although the site of action of simvastatin is unclear, simvastatin could have direct tubular effects. The mevalonate pathway or cholesterol synthesis pathways are activated in cortical tubules after sepsis induced by lipopolysaccharide or *Escherichia coli* injection; however, cholesterol accumulation was not reversed by statin therapy.[36–38]

Vascular effects

During to the rather subtle tubular changes in this model, and the known effects of statins on the vasculature, we focused our remaining studies on the renal vasculature. Renal vascular permeability measured by Evans blue dye (EBD) extravasation was increased after CLP, and the late increase at 24 h was reversed by simvastatin (Figure 4). Changes in vascular permeability are thought to be important in the pathogenesis of sepsis-induced organ injury.[26] Increased vascular permeability can cause compression of peritubular capillaries,[39] hemoconcentration[40] and reduced microvascular flow. Indeed, alteration of vascular permeability is thought to modulate renal function[41] and play an important functional role in the pathophysiology of ischemic AKI.[42,43] Vascular permeability may be altered by several multiple factors, including the interaction between activated leukocytes and the endothelium, endothelial NO synthase, vascular endothelial growth factor, angiopoietins, shear stress and matrix metalloproteinases,[42,44,45] which might in turn be therapeutic targets in sepsis. Statins have been shown to protect the endothelium with preservation of endothelial NO synthase function and to regulate several mediators associated with vascular permeability, such as vascular endothelial growth factor and matrix metalloproteinases.[17,34] Simvastatin enhanced endothelial NO synthase expression and improved vascular permeability in lungs subjected to ischemia–reperfusion injury.[46]

Interestingly, in our studies, simvastatin did not improve vascular permeability at 6 h, although vascular permeability was improved at 24 h (Figure 4). The late reversal was quite unexpected, and suggests that prolonged increases in vascular permeability may contribute to renal dysfunction as recently proposed in ischemic injury.[47]

We next focused on renal microperfusion and hypoxia, two consequences of altered vascular permeability. We detected decreased renal microvascular perfusion measured by fluorescein isothiocyanate-lectin infusion (Figure 6) and renal tubular hypoxia measured by pimonidazole staining (Figure 5) at 24 h after CLP. Interestingly, both microvascular hypoperfusion and tubular hypoxia were found in both the cortex and the OSOM at 24 h. A similar loss of global tubular function was detected by dendrimer contrast magnetic resonance imaging after CLP.[14] Taken together, our data suggest that increased microvascular permeability and global worsening of renal microperfusion and tubular hypoxia might be responsible for developing AKI after CLP, since tissue hypoperfusion and hypoxia are considered to be dominant factors in organ dysfunction of sepsis.[30,48] Also, the global pattern of tubular and vascular injury differs from ischemia–reperfusion injury, which is limited to the OSOM.[43,49,50] On the other hand, this reduced renal microvascular perfusion might contribute to reduced tubular damage in our model because reduced cellular metabolism might increase cell survival.[51]

Another late effect of simvastatin was that it reversed the microperfusion defect and tubular hypoxia at 24 h after CLP (Figures 5 and 6). This reversal might be caused by beneficial hemodynamic effects and/or local effects on renal tubular or microvasculature. For example, simvastatin preserved cardiac function and improved hemodynamic stability[24,25] in a CLP model, although renal end points were not measured. Simvastatin could decrease tubular oxygen consumption since it has been shown to reduce cardiac oxygen consumption.[52,53] However, that would not easily account for the reversal effect. Statins may also have direct vascular protective effects via preservation of endothelial function, in part, mediated by endothelial NO synthase.[54] It is likely that simvastatin has a direct effect on the vasculature since renal vascular permeability was restored towards normal after CLP by simvastatin (Figure 4).

Inflammatory effects

Statins also have anti-inflammatory effects.[17,20,21,34] We found that simvastatin inhibited serum TNF-α elevation at 24 h after CLP (Figure 7). Inhibition of the TNF-α pathway reduced kidney injury in lipopolysaccharide model.[55,56] However, anti-TNF-α treatment does not decrease mortality in CLP mice,[57,58] and did not improve sepsis-induced AKI in our studies (Figure 8). Although anti-TNF-α did improve sepsis-induced AKI in a small human study,[59] the role of TNF-α in sepsis is still controversial.

Effect of delayed administration of simvastatin on sepsis-induced acute kidney injury

A recent study found that delayed administration of simvastatin starting at 6 h improved mortality in a slightly different CLP model.[25] However, we found that delayed administration of simvastatin starting at 6 h after CLP did not improve renal function. How to reconcile these

observations is not clear. Conceivably, delayed administration of simvastatin could have a beneficial effect on mortality but not kidney injury. Alternatively, subtle differences between animal models might be responsible.

CONCLUSION

We showed that simvastasin improved sepsis-induced mortality and AKI. The mechanism of its renal protection may include effects on systemic circulation, direct effects on the renal vasculature and subsequent reversal of tubular hypoxia and a systemic anti-inflammatory action. The advantage of simvastatin in clinical settings is that it is clinically well established and already widely used, has an adequate safety profile in septic patients[22,23] and is relatively inexpensive. There are clinical situations where preventive therapy with simvastatin might be useful. For example, one could prophylactically administer simvastatin to septic patients who have been identified to be at high risk for sepsis-induced AKI by either clinical characteristics or biomarker findings. Thus, simvastatin may be a potential preventive intervention for sepsis-induced AKI.

MATERIALS AND METHODS
Animals

Animal care followed the National Institutes of Health criteria for the care and use of laboratory animals in research (K100-MDB-02). Male (38–44 week old) C57BL/6 mice (National Institutes of Health, Frederick, MD, USA) had free access to water and chow before and after surgery and were housed individually.

Polymicrobial CLP sepsis-induced acute kidney injury

CLP was performed as described previously with small modifications.[15] Briefly, a 4–0 silk ligature was placed 15 mm from the cecal tip after laparotomy under isoflurane anesthesia. The cecum was punctured twice with a 21-gauge needle and gently squeezed to express a small amount of fecal material, and then returned to the central abdominal cavity. In sham-operated animals, the cecum was located, but neither ligated nor punctured. The abdominal incision was closed in two layers with 6–0 nylon sutures. After surgery, 1 ml per 25 g body weight of prewarmed normal saline was given subcutaneously.[13] All animals received antibiotic and fluid therapy subcutaneously (imipenem/cilastatin; 14 mg/kg in 1.5 ml of 2/3 saline at 6 h and 7 mg/kg in 1.5 ml of 2/3 saline at 18 h after surgery). At 24 h after surgery, blood was collected from the abdominal aorta for measurement of serum creatinine, BUN and TNF-α. Serum creatinine was measured by high-performance liquid chromatography.[60] Serum BUN and TNF-α were measured as described previously.[15] Kidneys were fixed in 10% formalin or snap-frozen in liquid nitrogen before storage at -80°C until further study.

Treatment of simvastatin and TNF-α antibody

Simvastatin (EMD Biosciences, Inc., CA, USA) was prepared by dissolving 10 mg of simvastatin in 500 μl of ethanol and 0.407 μl of 1 N NaOH, incubating at 50°C for 2 h and then storing at -80°C. The stock solution was diluted with saline at a ratio of 1:20 and adjusted to pH 7.2 before use. The vehicle solution was prepared in the same way without simvastatin. For pretreatment studies, mice were given 40 mg/kg simvastatin or vehicle solution by oral gavage for 3 consecutive days before the surgery; simvastatin was not given after surgery. For post-treatment studies, mice were given 40 mg/kg or vehicle solution subcutaneously once at 6 h, or twice at 6 and 18 h after surgery.

TNF-α neutralizing antibody (R&D Systems, Inc., MN, USA) was administered as a single intraperitoneal injection at a dose of 800 μg/kg body weight[61] immediately after CLP. Normal mouse immunoglobulin G was used as a control.

Survival study

Survival was assessed every 6–12 h starting 6 h after surgery. Antibiotic injection and fluid resuscitation were started 6 h after surgery by subcutaneous injection, and then repeated every 12 h for 4 days.

Renal histology analysis

Tissue was fixed in 10% formalin and embedded in paraffin. Sections of 4 μm were stained with periodic acid-Schiff reagent. Histologic changes in the cortex and the OSOM were assessed by quantitative measurements of tissue damage. As tubular damage was mainly vacuolization, the damage was defined as tubular vacuolar degeneration. The degree of kidney damage was estimated at $\times 400$ magnification using more than 100 randomly selected tubules for each animal by following the criteria: 0, normal; 1, area of damage $< 25\%$ of tubules; 2, damage involving 25–50% of tubules; 3, damage involving 50–75% of tubules; 4, 75–100% of tubules being affected.

Assessment of renal microvascular protein leak using Evans blue dye

The microvascular leakage of EBD was assessed as described previously[62–64] with slight modifications. At 30 min before killing, mice were injected intravenously with EBD (Sigma-Aldrich, MO, USA) 2 ml/kg at 1% in 0.9% sodium chloride via a tail vein. At killing, mice were perfused with phosphate-buffered saline (PBS) through the left ventricle until blood was totally eliminated. The kidneys were weighed, snap-frozen in liquid nitrogen, and stored at -80°C. The kidneys were homogenized in 1mL formamide and incubated at 55°C for 18 h. The supernatant was collected after centrifugation at $10\,000 \times g$ for 30 min. The amount of EBD in the supernatant was analyzed by measuring absorbance at 620 nm. Results were calculated from a standard curve of EBD and expressed as micrograms of EBD per gram of kidney (wet weight).

Assessment of renal hypoxia

Renal hypoxia was assessed by pimonidazole immunohistochemistry. Pimonidazole (Chemicon International, Inc. CA, USA) was administered administrated intraperitoneally at the dose of 60 mg/kg 2 h before killing. Kidneys were bisected and immersed, gently agitated in 10% formalin at room temperature for 24 h, and processed for paraffin embedding. sections of 4 μm were processed by the manufacturer's procedure. All procedures were conducted at room temperature. Briefly, the sections were incubated with 0.01% pronase (Biomeda, CA, USA) for 5 min after deparaffinization and peroxidase quenching. After washing with PBS, the sections were incubated with 10% normal donkey serum in 1% bovine serum albumin) in PBS for 20 min. The sections were sequentially incubated with Hypoxyprobe-1 Mab1 (1:100 dilution with PBS containing 1% bovine serum albumin) for 2 h, biotin-conjugated donkey anti-mouse immunoglobulin G (1:1000 dilution, Jackson Immunolaboratory, PA, USA) for 30 min, peroxidase-conjugated

streptavidin for 30 min, 3,3′-diaminobenzaidine, then counter-stained with hematoxylin. No staining was detected without Mab1 (not shown). Renal hypoxia was evaluated by semiquantitative measurements of pimonidazole staining on tubules of the cortex and the outer stripe of the OSOM. The degree of pimonidazole staining was estimated at ×400 magnification using more than five randomly selected fields for each animal by the following criteria according to the intensity and the extent of positive cells: 0, no staining; 1, slight, 2; moderate, 3; severe staining.

Analysis of renal microvasculature by fluorescein isothio-cyanate-labeled lectin perfusion

To identify vessels with intact blood flow, the renal vasculature was identified with a lectin that binds uniformly to the luminal surface of endothelial cells.[65–67] Fluorescein isothiocyanate-*Lycopersicon Esculentum* lectin (30 µg, Vector Laboratories, CA, USA) was injected via a tail vein 5 min before killing. The vasculature was perfused with PBS via the left ventricle, and the kidneys were harvested after intracardiac perfusion of PBS and fixed with 2% paraformaldehyde for 1 h, followed by freezing in optimum cutting temperature compound.

Cryosections of 5 µm were evaluated with a Leica DMRXE fluorescence microscope.

Statistical analysis

Differences between groups were examined for statistical significance by analysis of variance with a multiple comparison correction. Comparisons between survival curves were made using a log-rank test (Prism 4.0, Graphpad Software, Inc., CA, USA). A P-value <0.05 was accepted as statistically significant.

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