Guanylyl Cyclase A in Both Renal Proximal Tubular and Vascular Endothelial Cells Protects the Kidney against Acute Injury in Rodent Experimental Endotoxemia Models

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ABSTRACT

Background: Natriuretic peptides are used, based on empirical observations, in intensive care units as antioliguric treatments. We hypothesized that natriuretic peptides prevent lipopolysaccharide-induced oliguria by activating guanylyl cyclase A, a receptor for natriuretic peptides, in proximal tubules and endothelial cells.

Methods: Normal Sprague-Dawley rats and mice lacking guanylyl cyclase A in either endothelial cells or proximal tubular cells were challenged with lipopolysaccharide and assessed for oliguria and intratubular flow rate by intravital imaging with multiphoton microscopy.

Results: Recombinant atrial natriuretic peptide efficiently improved urine volume without changing blood pressure after lipopolysaccharide challenge in rats (urine volume at 4 h, lipopolysaccharide: 0.6 ± 0.3 ml · kg^{-1} · h^{-1}; lipopolysaccharide + fluid resuscitation: 4.6 ± 2.0 ml · kg^{-1} · h^{-1}; lipopolysaccharide + fluid resuscitation + atrial natriuretic peptide: 9.0 ± 4.8 ml · kg^{-1} · h^{-1}; mean ± SD; n = 5 per group). Lipopolysaccharide decreased glomerular filtration rate and slowed intraproximal tubular flow rate, as measured by *in vivo* imaging. Fluid resuscitation restored glomerular filtration rate but not tubular flow rate. Adding atrial natriuretic peptide to fluid resuscitation improved both glomerular filtration rate and tubular flow rate. Mice lacking guanylyl cyclase A in either proximal tubules or endothelium demonstrated less improvement of tubular flow rate when treated with atrial natriuretic peptide, compared with control mice. Deletion of endothelial, but not proximal tubular, guanylyl cyclase A augmented the reduction of glomerular filtration rate by lipopolysaccharide.

Conclusions: Both endogenous and exogenous natriuretic peptides prevent lipopolysaccharide-induced oliguria by activating guanylyl cyclase A in proximal tubules and endothelial cells. **(ANESTHESIOLOGY 2018; 129:296-310)**

THE kidney loses its function in sepsis.[1,2] Notably, acute kidney injury increases the risk of death and need for renal replacement therapy in patients with sepsis.[1–3] Currently, there are no specific treatments for either kidney injury or oliguria in septic acute kidney injury.[1]

Recent advances in microscopy technology have enabled visualization of tubular flow in a real-time manner, as well as examination of renal structure at a subcellular level, in the kidney of living animals, using multiphoton laser microscopy. Using functional microscopic analysis, we previously reported that lipopolysaccharide decreased tubular flow rate in proximal tubules even during the early phase of acute kidney injury, before changes in glomerular filtration rate and blood pressure were evident.[4] This indicates that lipopolysaccharide decreases urine flow rate not only by regulating glomerular filtration rate but also by influencing tubular flow rate.

What We Already Know about This Topic

- The physiologic role of natriuretic peptides is increasingly understood in critical illness, but how these peptides might impact sepsis-induced oliguria is not known

What This Article Tells Us That Is New

- In an *in vivo* study of experimental sepsis, fluid resuscitation restored glomerular filtration, but recombinant atrial natriuretic peptide restored renal tubular flow and glomerular filtration

Corresponding article on page 235. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

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The natriuretic peptides, atrial natriuretic peptide and brain natriuretic peptide, were discovered as natriuretic factors secreted from the atrium or ventricle during heart failure.[5–7] Their receptor, guanylyl cyclase A, is expressed on many cell types including vascular endothelial cells,[8] renal glomerular podocytes,[9] collecting ducts,[10] and proximal tubules.[11] It has been reported that plasma concentrations of proatrial natriuretic peptide, the precursor of atrial natriuretic peptide, are increased during acute renal failure.[12] In addition, recombinant human atrial natriuretic peptide is used, based on empirical observations, in intensive care units to increase urine flow and prevent or treat acute kidney injury. Recently, a meta-analysis and several randomized controlled trials reported that low-dose human atrial natriuretic peptide decreased the risk of acute kidney injury.[13–16]

In this study, we hypothesized that natriuretic peptides regulate urine flow in the early phase of lipopolysaccharide-induced acute kidney injury through its receptor, guanylyl cyclase A, expressed on both proximal tubular and endothelial cells, which were targeted based on the above evidence addressing the causes of lipopolysaccharide-induced oliguria and the site of atrial natriuretic peptide action.[4,17]

Materials and Methods

All experiments were approved by the Institutional Animal Care and Use Committee of Kagawa University, Kagawa, Japan, and followed standard guidelines for the humane care and use of animals in scientific research. The animals were housed in an animal room under the following conditions: temperature, 24 ± 1°C; relative humidity, 55 ± 5%; and 12-h light/dark cycle. The animals received standard chow (MF; Oriental Yeast Co., Ltd., Japan) and drinking water *ad libitum*. The animals were assigned to the different experimental groups unsystematically but without a formal randomization protocol. All animals that were assigned to the study groups were included in the analysis, and there were no exclusions. Experimental analysis of anesthetized animals, such as blood pressure changes, were performed in a nonblinded fashion, while all other *post hoc* analyses were performed in a blinded fashion.

Chemicals

Lucifer yellow and isoflurane were from Invitrogen (USA) and Mylan (Japan), respectively. Lipopolysaccharide (O-55), Evans blue, and thiobutabarbital (inactin) were from Sigma-Aldrich (USA). Calperitide (human atrial natriuretic peptide) was a gift from Daiichi-Sankyo, Inc. (Japan).

Surgical Procedure and Experimental Protocol for Rat Studies

Sprague-Dawley rats were from CLEA (Japan). In this study, Sprague-Dawley rats weighing 250 to 350 g (8 to 10 weeks old) were used. Rats were selected as they can tolerate the observation of changes in blood pressure and renal function for several hours during anesthesia,[18,19] unlike mice, which were used in the later part of this study. The rats were divided into 4 groups (n = 5 per group): (1) control group, untreated; (2) lipopolysaccharide group, receiving only lipopolysaccharide; (3) lipopolysaccharide and fluid resuscitation group (lipopolysaccharide + fluid resuscitation); and (4) lipopolysaccharide + fluid resuscitation and human atrial natriuretic peptide group (lipopolysaccharide + fluid resuscitation + human atrial natriuretic peptide). Inactin (0.1 mg/kg) was injected intraperitoneally as anesthesia. All animals were warmed on a heating pad during all experimental procedures. After tracheal intubation, a PE50 polyether catheter (Natsume, Japan) was inserted into the femoral artery to monitor blood pressure with a PowerLab data acquisition system (AD Instruments, New Zealand) and to collect plasma samples, for measurement of creatinine and neutrophil gelatinase-associated lipocalin, at baseline and 4 h after lipopolysaccharide injection. Blood pressure was monitored throughout the experimental period. Two other PE50 catheters were inserted into the cervical and femoral veins. Maintenance fluid (saline, 0.9 ml/h during surgery and 0.6 ml/h during the recovery and experimental periods) was infused through a cervical catheter. A PE160 catheter (Natsume) was inserted into the bladder to collect urine every 30 min. After a 30-min recovery period, baseline urine collection (2 × 30 min) was started. Lipopolysaccharide (15 mg/kg) was injected intraperitoneally. At 2 h after lipopolysaccharide injection, fluid resuscitation (saline, 6 ml/h) through the femoral vein was initiated in the lipopolysaccharide + fluid resuscitation and lipopolysaccharide + fluid resuscitation + human atrial natriuretic peptide groups, and human atrial natriuretic peptide (1.8 $\mu g \cdot kg^{-1} \cdot h^{-1}$) was added to the saline for fluid resuscitation in the lipopolysaccharide + fluid resuscitation + human atrial natriuretic peptide group. *In vivo* imaging was performed at 4 h after the injection of lipopolysaccharide. The procedure for the *in vivo* imaging is described below (see "Experimental Procedure for *In Vivo* Imaging"). Additionally, five separately prepared rats were used for *in vivo* imaging analysis at 2 h after lipopolysaccharide injection to assess the tubular flow rate before beginning fluid resuscitation or human atrial natriuretic peptide treatment. It has been reported previously that lipopolysaccharide induces only modest histologic changes in the kidney,[20,21] which was confirmed in preliminary studies. Thus, histopathologic examination was not performed in this study.

To investigate the effects of human atrial natriuretic peptide initiated in the later phase of lipopolysaccharide-induced oliguria, another set of rats received fluid resuscitation with and without human atrial natriuretic peptide at 18 h after lipopolysaccharide (5 mg/kg). The dosage of lipopolysaccharide was reduced in this study as the original dosage (15 mg/kg) induced unacceptably high mortality at 18 h. Rats underwent the same surgical procedure and were maintained in an equivalent manner as per the other study.

A separate set of rats underwent an experiment to examine survival rate after lipopolysaccharide (15 mg/kg). The rats received fluid resuscitation, with and without human

atrial natriuretic peptide, *via* the tail vein from 2 to 4 h after lipopolysaccharide injection during isoflurane anesthesia. After the fluid infusion, rats were allowed to recover from anesthesia and were maintained under conscious conditions.

Surgical Procedure and Experimental Protocol for Mouse Studies

Mice with conditional knockout of the human atrial natriuretic peptide receptor, guanylyl cyclase A, in either proximal tubules (NDRG1^{CreERT2}:guanylyl cyclase A floxed mice: proximal tubular guanylyl cyclase A-knockout mice[22]) or endothelial cells (Tie2^{Cre}:guanylyl cyclase A floxed mice:endothelial guanylyl cyclase A-knockout mice[23]) were selected based on our previous data addressing the causes of lipopolysaccharide-induced complications.[4,17] All mice were from our breeding colony.[9,22,23] Cre recombinase in NDRG1^{CreERT2} mice was activated by injecting tamoxifen (10 mg · kg^{-1} · day^{-1}, intraperitoneally) for 5 consecutive days when the mice were 6 weeks of age. Animals were used for the experiments 6 weeks after tamoxifen injection. Lipopolysaccharide (5 mg/kg) was injected intraperitoneally in both conditional knockout mice and control guanylyl cyclase A floxed mice. At 5 h after injection, the mice were anesthetized with isoflurane and received tracheal intubation and a venous catheter in the internal cervical vein. The mice, both conditional knockout mice and control guanylyl cyclase A floxed mice, received either fluid resuscitation (saline at 0.6 ml/h) or human atrial natriuretic peptide (1.8 μg · kg^{-1} · h^{-1}) added into the saline for fluid resuscitation, respectively. *In vivo* imaging was performed at 6 h after the lipopolysaccharide injection.

A separate set of mice (n = 4 to 5) underwent an experiment to examine plasma and tissue markers of renal damage (neutrophil gelatinase–associated lipocalin and kidney injury molecule–1) or inflammation (tumor necrosis factor–α). The mice underwent the same surgery and treatments as above. The plasma and kidney tissue were harvested at 6 h after lipopolysaccharide injection for further assay and messenger RNA (mRNA) analysis.

Experimental Procedure for In Vivo Imaging

Both rats and mice were used for *in vivo* imaging experiments. The left kidney was exteriorized through a small flank incision and was affixed to a coverslip. The microscope stage and animals were warmed using a heating pad during all experimental procedures. Intravital multiphoton microscopy was performed using an FV1000MPE multiphoton confocal fluorescence imaging system (Olympus, Japan) powered by a Chameleon Ultra-II MP laser at 720 and 860 nm (Coherent Inc., USA). The microscope objective was a 25× water-immersion lens with a 1.05 numerical aperture. The imaging setting for the microscope (gain and offset for all three channels; blue, green, and red) was fixed throughout the experiment. After a 30-min recovery period, two-dimensional time-lapse images were taken at a depth of 25 μm from the surface of the kidney.

Tubular flow rate was estimated by timing the appearance of injected fluorescent dyes in distal nephron segments. The distal nephron in the kidney was identified based on its autofluorescence under multiphoton microscopy with excitation at 720 and 860 nm without exogenous fluorescent dye in all animals. Existence of tubules, especially tubules that show little autofluorescence, was confirmed with the 720 nm excitation laser and a 420- to 460-nm wavelength filter, which detects autofluorescence of nicotinamide adenine dinucleotide distributed ubiquitously in both proximal and distal tubules. After confirming the position of distal nephrons in each imaging window, Lucifer yellow (100 μg/kg, intravenous bolus), a dye freely filtered by glomeruli, was injected to visualize tubular fluid flow. Time-lapse images for this tubular flow measurement were taken at 512 × 512 pixels. The Lucifer yellow flow was continuously recorded from 0 s until 600 s of injection for rats and until 300 s for mice. We shortened the recording time in mice because of the weaker tissue resistance to potential phototoxicity in mice.

The inflow time of Lucifer yellow was defined as the time to peak fluorescence intensity, whereas outflow time was defined as the time from peak to half-peak fluorescence intensity, as previously described.[4] Inflow and outflow times were measured in five spatially separated tubular lumens that showed Lucifer yellow the earliest in each imaging window. Since these tubules were considered as early segments of proximal tubules, the inflow time reflects glomerular filtration rate. Outflow time is influenced by both inflow time and loss of water through tubular walls. Prolonged inflow time, indicating less tubular flow pressure, simultaneously prolongs outflow time that could be masked/exaggerated by decreased/increased water loss through tubular walls, respectively. More water loss through the tubular wall prolongs outflow time regardless of inflow time. Therefore, we did not give any interpretation of the outflow data from the mice showing reduction of inflow rate.

Vascular Permeability Test

Vascular permeability was measured to evaluate the contribution of endothelial guanylyl cyclase A to the lipopolysaccharide-induced increase in vascular permeability, using mice lacking guanylyl cyclase A under the control of Tie2-driven Cre recombinase. Evans blue (2%, 4 ml/kg; Sigma-Aldrich) was injected intravenously into mice that had received lipopolysaccharide 5 h before. After 1 h of Evans blue injection, ascites and right lung tissue were harvested. To collect ascites samples, we injected 60 ml/kg of sterilized saline intraperitoneally, kneaded the abdomen lightly for 10 s, and recovered the fluid.[24] The absorbance of formamide extracts of each sample were measured in a spectrophotometer, model SH-9000 (Corona, Japan).

Real-time Reverse Transcription

Quantitative real-time reverse transcription was performed to confirm Cre recombinase-mediated conditional gene deletion in

the two strains of conditional knockout mice, proximal tubular guanylyl cyclase A knockout and endothelial guanylyl cyclase A knockout mice, and to evaluate renal damage and inflammation. The right kidney from proximal tubular guanylyl cyclase A knockout and floxed control mice and descending thoracic aorta from both endothelial guanylyl cyclase A knockout and floxed control mice were harvested and the tissue samples immersed overnight in RNA-later (Sigma-Aldrich) and used for real-time reverse transcription analysis. Concentrations of 18S, guanylyl cyclase A, neutrophil gelatinase–associated lipocalin, kidney injury molecule–1, and tumor necrosis factor–α mRNAs were analyzed with a 7300 Fast Real-Time PCR System (Applied Biosystems, USA). The mouse primer sequences (forward and reverse) were as follows: 18S: 5′-GTAACCCGTTGAACCCCATT-3′, 5′-CCATCCAATCGGTAGTAGCG-3′; guanylyl cyclase A: 5′-CTTCATATACAGGAAGATGCAGCTGG-3′, 5′-GCTGTCTTGGCAAAGACTTGGAACTG-3′; neutrophil gelatinase–associated lipocalin: 5′-GGCCAGTTCACTCTGGGAAA-3′, 5′-TGGCGAACTGGTTGTAGTCC-3′; kidney injury molecule–1: 5′-TTGCCTTCCGTGTCTCTAAG-3′, 5′-AGATGTTGTCTTCAGCTCGG-3′; and tumor necrosis factor–α: 5′-TGGCACCACTAGTTGGTTGTCT-3′, 5′-AGCCTGTAGCCCACGTCGTA-3′. Relative mRNA concentrations were determined using the $2^{-\Delta\Delta}$Ct method, and the ΔΔCt value was calculated using data from saline-injected floxed control mice.

Plasma Creatinine and Neutrophil Gelatinase–associated Lipocalin Measurement

Plasma creatinine and neutrophil gelatinase–associated lipocalin were measured using commercially available assay kits (LabAssay Creatinine, Wako, Japan, and mouse NGAL ELISA kit, BioPorto Diagnostics A/S, Denmark, respectively) according to the manufacturers' instructions.

Statistical Analysis

An *a priori* power analysis was not performed for this study. Instead we used sample sizes that previous studies indicated as sufficient to identify biologically meaningful differences. Results are expressed as means ± SD. Statistical significance was assessed using one-way repeated-measures or two-way factorial or repeated-measures ANOVA followed by the Tukey multiple comparison test to evaluate the differences between groups in mouse experiments. Student's *t* tests were performed to compare the differences in guanylyl cyclase A mRNA expression in the mice with and without Cre recombinase. All experiments were performed by using inbred colony mice and a well-established lipopolysaccharide-induced oliguric model. Several preliminary experiments had been conducted before initiating the experiments to confirm the reproducibility of each phenomenon. All values were normally distributed in the normal control group, while skewing was observed for oliguric groups. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., USA), and two-tailed *P* values of $P < 0.05$ were considered statistically significant.

Results

Effects of Lipopolysaccharide, Fluid Resuscitation, and Human Atrial Natriuretic Peptide on Blood Pressure, Urine Volume, Plasma Creatinine, and 24-h Survival Rate

Figure 1A shows a schematic representation of the experimental protocol. Mean arterial pressure (MAP) gradually decreased in rats after injection of lipopolysaccharide (15 mg/kg, intraperitoneally; fig. 1B), although the average MAP value remained above 75 mmHg at 4 h after lipopolysaccharide injection. Neither fluid resuscitation nor human atrial natriuretic peptide treatment affected the lipopolysaccharide-induced decreases in MAP. Urine volume was decreased within 1 h of lipopolysaccharide injection. Fluid resuscitation, either with or without human atrial natriuretic peptide, increased urine volume within 1 h of its initiation (fig. 1C). Fluid resuscitation increased urine volume in lipopolysaccharide-treated animals to concentrations similar to those in the saline-injected control group not receiving lipopolysaccharide. Fluid resuscitation with human atrial natriuretic peptide significantly increased the urine volume to concentrations even higher than those in the control group. Lipopolysaccharide increased the plasma creatinine concentration within 4 h, and fluid resuscitation both with and without human atrial natriuretic peptide suppressed the increase in plasma creatinine concentration (fig. 1D).

Lipopolysaccharide decreased the 24-h survival rate to 25% from 100% in the control group. Fluid resuscitation, administered between 2 to 4 h after lipopolysaccharide injection, increased the 24-h survival rate to 37.5% (3 in 8 rats), and adding human atrial natriuretic peptide to the fluid resuscitation further increased it to 62.5% (5 in 8 rats).

In Vivo *Imaging of Tubular Flow*

Tubular flow analysis was performed by an *in vivo* imaging method using multiphoton microscopy.[4] A schematic diagram of the *in vivo* imaging experiment is provided in figure 2A. Tubular flow rate was estimated by timing the appearance of injected fluorescent dye, Lucifer yellow, in distal nephron segments, which were identified based on their relative autofluorescence strength with a 720- and 860-nm wavelength excitation laser (fig. 2B). Lucifer yellow is freely filtered by the glomeruli, and the flow rate of Lucifer yellow was similar to that of fluorescein isothiocyanate–inulin in normal rats (31.5 ± 3.7 *vs.* 36.9 ± 9.4 s, respectively, to reach distal nephrons). Real-time analysis of Lucifer yellow flow enables the visualization of the tubular flow in each segment, especially tubular fluid retained in the proximal tubular lumen in lipopolysaccharide-induced oliguric mice (right panel, fig. 2C). There was some green fluorescence remaining in the tubular wall and brush border of proximal tubules after Lucifer yellow flow as a portion of Lucifer yellow is secreted from proximal tubules,[25] which does not affect the flow rate as confirmed by the similarity with fluorescein isothiocyanate-conjugated inulin flow rate as stated above. Notably, Lucifer yellow–derived fluorescence was detected in

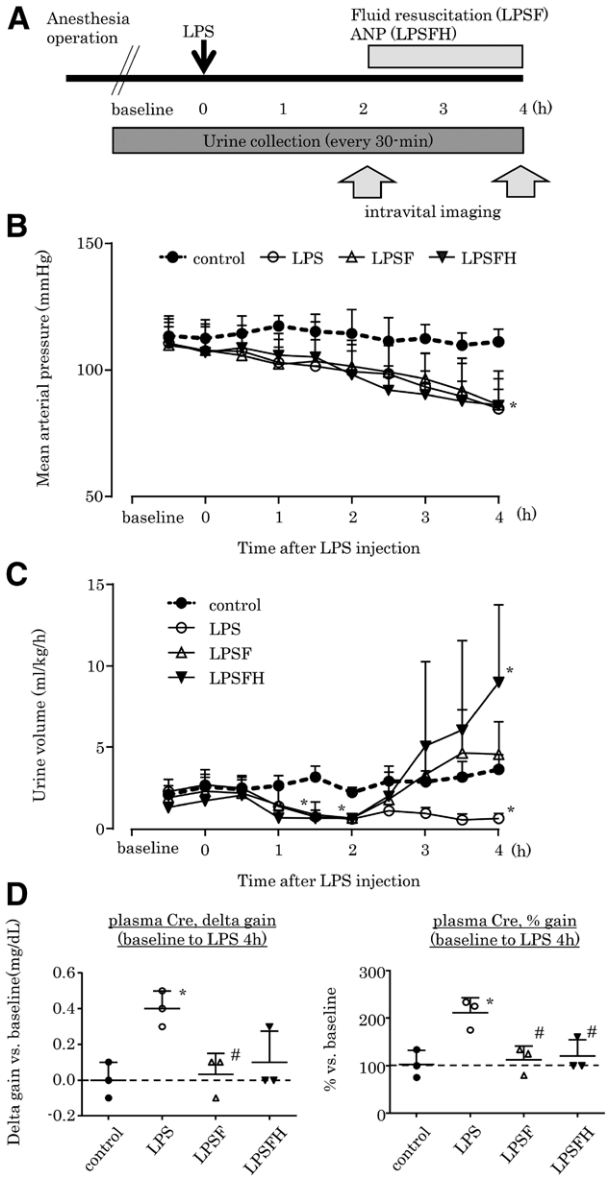

Fig. 1. Time course of changes in blood pressure, urine flow, and plasma creatinine concentration after lipopolysaccharide (LPS) injection in rats. (*A*) Schematic of the timeline of the experiment. (*B*) LPS gradually decreased mean arterial pressure. Fluid resuscitation, with and without human recombinant arterial natriuretic peptide (ANP) (LPSFH and LPSF, respectively), did not affect LPS-induced changes in blood pressure (n = 5). (*C*) LPS decreased the urine flow rate, an effect starting within 1 h after the injection. LPSF ameliorated the LPS-induced oliguria, and LPSFH further increased urine flow (n = 5). (*D*) LPS increased plasma creatinine (Cre) concentration in comparison with baseline values (both delta gain and % gain) within 4 h. Both LPSF and LPSFH attenuated this increase (n = 3). **P* < 0.05 *versus* control, #*P* < 0.05 *versus* LPS.

the "green" channel in low concentrations, and Lucifer yellow in distal nephrons is concentrated owing to loss of water from the tubular lumen, and therefore the fluorescence starts to be detected in the "red" channel, resulting in the change in color to yellow (green plus red).

Lucifer yellow was visible in the proximal tubules and peritubular capillaries within 10 s after its bolus injection through the jugular vein in the normal control rats (fig. 3A). After that, Lucifer yellow was detectable flowing into the distal nephrons within 60 s. Lucifer yellow fluorescence was dramatically less intense at 300 s after the injection, indicating washout from the distal tubules (fig. 3A), and became undetectable within 600 s after its injection (data not shown). At 4 h after lipopolysaccharide injection, the tubules maintained their normal luminal areas (0 s in fig. 3A). The Lucifer yellow flow rate was already slower in the untreated lipopolysaccharide-injected group at 2 h after the injection, before initiating the fluid resuscitation with and without human atrial natriuretic peptide, and it became even slower at 4 h after the injection (fig. 3B). Most of the distal nephrons (>80%) did not show Lucifer yellow fluorescence at 300 s after Lucifer yellow injection in rats that had received lipopolysaccharide 4 h before (fig. 3, A and B). As reported elsewhere,[4] there was a concentrated and saturated Lucifer yellow fluorescence observed in the proximal tubules in this group (fig. 3A), indicating slower tubular flow rates in the proximal tubules. The 2-h fluid resuscitation improved washout of Lucifer yellow from some of the proximal tubules, and Lucifer yellow fluorescence reached the distal nephrons earlier in this group than in the untreated lipopolysaccharide-injected group (0 to 150 s in fig. 3B). Nevertheless, the percentage of distal nephrons with no Lucifer yellow fluorescence within 600 s was not significantly affected by fluid resuscitation, indicating that the effect of fluid resuscitation was limited to only some nephrons. In contrast, when human atrial natriuretic peptide was combined with fluid resuscitation, there was a remarkable increase in tubular flow rate. The percentage of nephrons without Lucifer yellow fluorescence in the distal nephrons within 600 s was decreased from 43.1 ± 27.9% to 2.4 ± 7.5% in the presence of human atrial natriuretic peptide (fig. 3B, Supplemental Digital Content 1, http://links.lww.com/ALN/B692, and Supplemental Digital Content 2, http://links.lww.com/ALN/B693).

Inflow and Outflow Rates of Tubular Flow in Proximal Tubules

The inflow rate of tubular flow was evaluated by calculating Lucifer yellow inflow and outflow times in the early segments of proximal tubules using multiphoton imaging as noted in Supplemental Digital Content 3, figure S1 (http://links.lww.com/ALN/B691). The duration required to fill the proximal tubular lumen with Lucifer yellow (inflow) was increased by lipopolysaccharide treatment in rats (fig. 4), indicating decreased glomerular filtration rate. Fluid resuscitation, either with or without human atrial natriuretic peptide, significantly shortened the time required for Lucifer yellow to fill the proximal tubular lumens, suggesting improved glomerular filtration rate. The outflow rate to assess washout of filtrate from the proximal tubules was not affected by fluid resuscitation alone, but was markedly improved by the

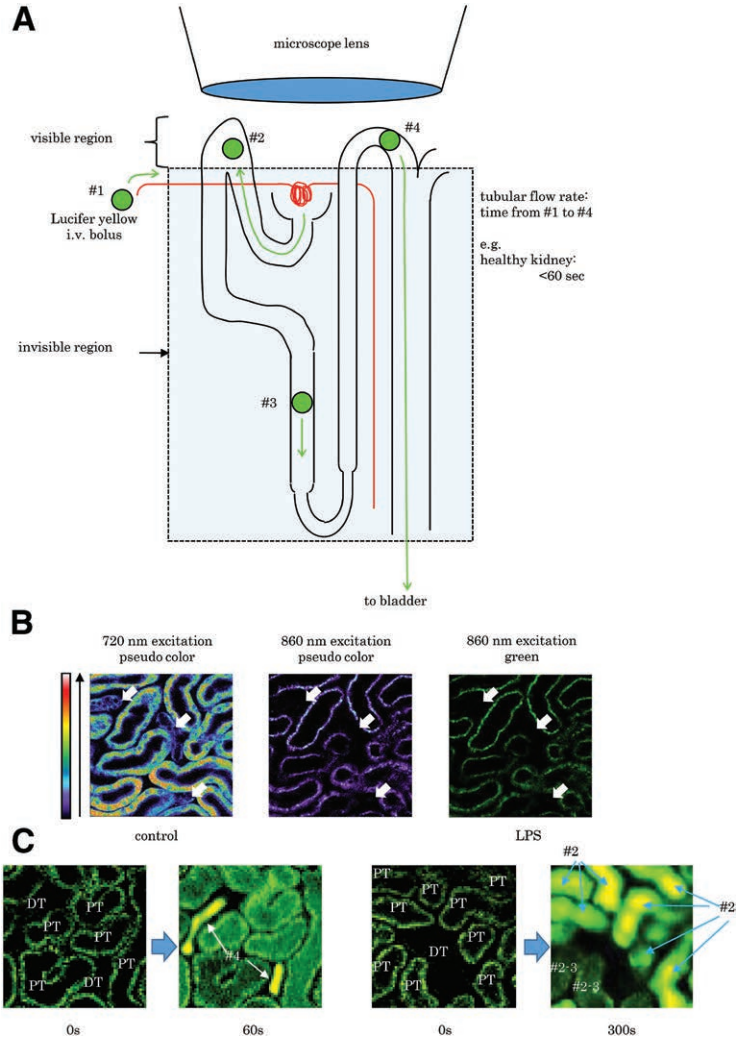

Fig. 2. How to measure tubular flow rate. Tubular flow rate was estimated by timing the appearance of injected fluorescent dye in distal nephron segments. (*A*) Schematic diagram of the nephron demonstrating the Lucifer yellow (*green circle*) flow as *#1* to *#4* and the visible depth of multiphoton microscopy. Intravenously (i.v.) injected Lucifer yellow (*#1*) is freely filtered by the glomerulus and reaches the convoluted proximal tubules (*#2*). Lucifer yellow flows to the downstream nephron (*#3*), which is too deep to visualize by microscopy (deepest visualization is approximately 80 μm in our system, and glomeruli exist greater than 150 μm) so that Lucifer yellow–derived fluorescence transiently disappears. Lucifer yellow flows into the distal nephron, again being visible by microscopy (*#4*). We hypothesized that the distance from jugular vein, where Lucifer yellow was injected, to the distal nephron of superficial nephron was comparable between the groups. Thus, time to visualize distal nephron lumens by Lucifer yellow reflects the tubular flow rate in the case where systemic blood pressure is similar between groups. Decline in blood pressure or glomerular filtration rate would be reflected by altered inflow rate to proximal tubules. In healthy animals (both rats and mice), the time to visualize distal nephrons by Lucifer yellow is less than 60 s. (*B*) Representative images of renal tubules with the excitation laser wavelength at 720 nm (*left*) and 860 nm (*center and right*) for identifying distal nephrons. Distal nephrons are indicated by *white arrows*. The autofluorescence detected through the 495- to 540-nm wavelength filter, which is used as the "green" filter in the following figures, is shown by pseudo color at *left and center panels*. Proximal tubules show much stronger autofluorescence than distal nephrons with the 720-nm excitation laser. Dot-like fluorescence in the *center* (*pseudo color*) and *right* (*green*) *panels* is autofluorescence of tubules with the 860-nm excitation laser. Distal nephrons (*white arrows*) show little autofluorescence in the tubular wall compared with proximal tubules with the 860 nm excitation laser. (*C*) The representative images for Lucifer yellow flow from control and lipopolysaccharide (LPS) groups in rats. The images are from regions of interest (*blue dashed square*) in figure 3A. The Lucifer yellow–derived fluorescence is depicted *green* at low concentrations and *yellow* at high concentrations. Lucifer yellow became visible in the distal nephron lumen (DT) within 60 s in the control rat. Note that there is some green fluorescence left in the tubular wall and brush border of proximal tubules after Lucifer yellow flow (60 s in control), which does not affect the flow rate as confirmed by the similarity with fluorescein isothiocyanate-inulin flow rate (values in the main text). In lipopolysaccharide-treated rats, Lucifer yellow was retained in the proximal tubular lumen (PT) even at 300 s after the injection (*position #2*). There are two proximal tubular lumens that are not filled with Lucifer yellow fluorescence, indicating that either the Lucifer yellow had flowed into the downstream nephron that is not visible with the current technology or Lucifer yellow was retained in the upstream proximal tubules, which can be judged by analyzing the movie if Lucifer yellow had shown up in the lumen between 0 s to 300 s. Since superficial proximal tubules are convoluted, there are multiple proximal tubular lumen detected in one xy imaging section, and it is possible that each of them connected as upstream or downstream segments.

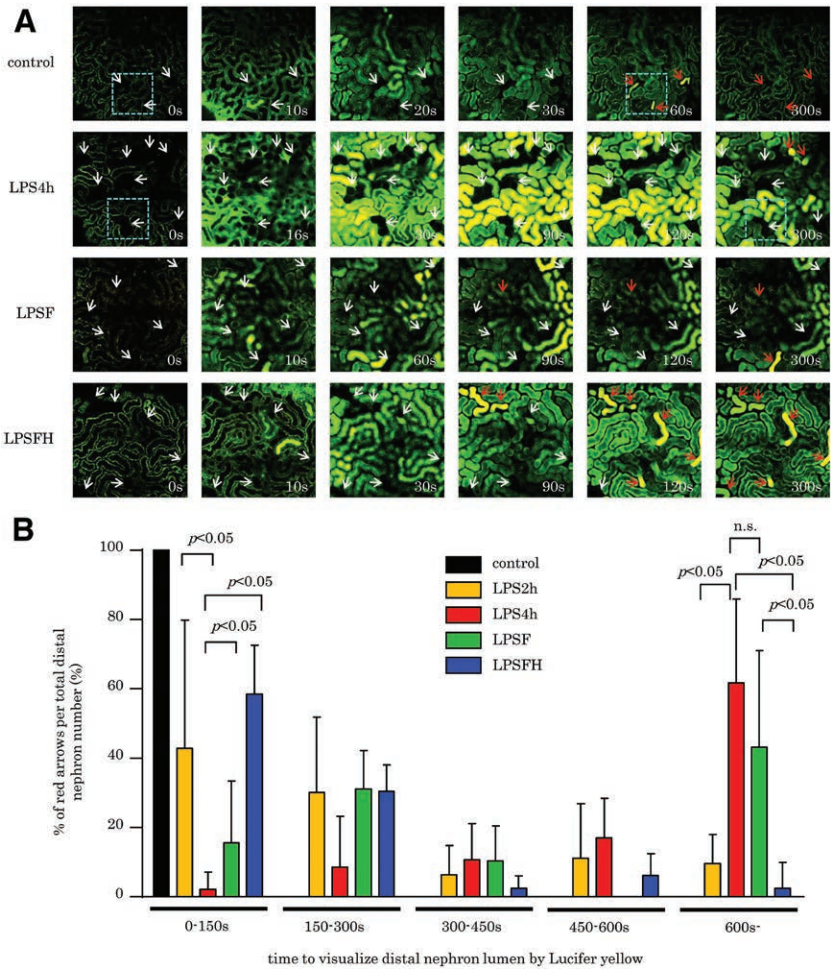

Fig. 3. Estimation of renal function by *in vivo* imaging. Tubular flow rate was measured by timing the appearance of injected fluorescent dye, Lucifer yellow, in distal nephron segments. The Lucifer yellow-derived fluorescence is depicted by green at low concentrations and yellow at high concentrations. The lipopolysaccharide (LPS)–treated group was separately analyzed at 2 and 4 h after LPS injection. The rest of the groups were analyzed only at 4 h after LPS injection. (*A*) Representative pictures from time-lapse imaging after Lucifer yellow injection. The images were taken using the 860-nm excitation laser. The distal nephrons that have not had Lucifer yellow in the lumen are indicated by *white arrows*, and the distal nephrons that have already had Lucifer yellow in the lumen are indicated by *red arrows*. In the control group, Lucifer yellow (*green*) appeared in the proximal tubules (tubules not indicated by arrows) at 10 to 20 s, and flowed into all distal nephrons in the focal plane within 60 s. In the LPS groups at 4 h, there were no distal nephrons with Lucifer yellow at 90 and 120 s after injection, and nearly half of the proximal tubules had Lucifer yellow (*yellow*) in the lumen at 300 s after injection. The Lucifer yellow fluorescence was saturated (*yellow*) in proximal tubules of the LPS-treated groups, indicating the reduction of tubular flow rate in the proximal tubules. In the LPS + fluid resuscitation (LPSF) group, many distal nephrons did not have Lucifer yellow in the lumens at 120 s after injection, and there were some proximal tubules with Lucifer yellow still in the lumens at 300 s after injection. In the LPSF + human recombinant arterial natriuretic peptide (LPSFH) group, most of the distal nephrons had Lucifer yellow in the lumens, and there was no Lucifer yellow in the proximal tubular lumens in the focal plane at 300 s. The image at 600 s looked similar to that at 300 s and so is not shown in the figure. (*B*) Time for Lucifer yellow to achieve the distal nephron, as tubular flow rate. The time to visualize distal nephron lumens by Lucifer yellow was categorized into five groups as shown on the *x-axis*, and percentage of *red arrows* per total distal nephron number in each category was the *y-axis*. LPS at 2 h already started slowing tubular flow, which was further enhanced at 4 h. Fluid resuscitation partially improved tubular flow rate, whereas the percentage of distal nephrons that did not show Lucifer yellow in the lumen within 600 s was unaffected. Adding human atrial natriuretic peptide increased the percentage of distal nephron lumens that showed Lucifer yellow in the lumen within 150 s, and decreased the percentage of distal nephrons that did not show Lucifer yellow in the lumen within 600 s. n.s. = Not significant. n = 5 per group.

addition of human atrial natriuretic peptide, supporting the tubular flow data shown in figure 3.

Late Initiation of Human Atrial Natriuretic Peptide Infusion

Our study demonstrated that human atrial natriuretic peptide, initiated at 4 h after lipopolysaccharide injection, efficiently improved both tubular and urine flow rates in rats, but timing of the administration may be crucial.[26] Therefore, we next examined the effects of human atrial natriuretic peptide infusion started at 18 h after lipopolysaccharide injection (fig. 5A). Lipopolysaccharide at 15 mg/kg markedly increased mortality. Thus, for this experiment, the

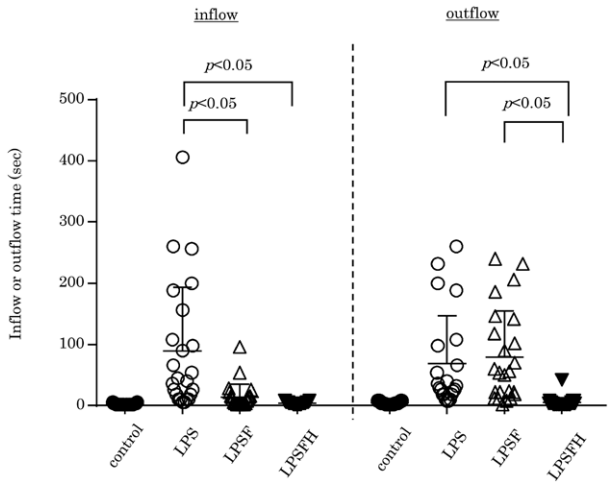

Fig. 4. Inflow and outflow times of Lucifer yellow in the proximal tubules after lipopolysaccharide (LPS) injection. The inflow time, which reflects glomerular filtration rate, was analyzed by how quickly Lucifer yellow appeared and reached the peak concentration in the proximal tubules after the injection. Five spatially dissociated proximal tubules in imaging windows that showed Lucifer yellow right after the injection were employed to count the inflow time. The inflow time of Lucifer yellow was prolonged in the LPS-treated groups, whereas fluid resuscitation both with (LPSFH) and without (LPSF) human recombinant arterial natriuretic peptide ameliorated this effect (n = 25 tubules in 5 rats in each group). The outflow time reflects the washout of tubular fluid from proximal tubules to downstream tubules. The outflow time was analyzed in the nephrons that were used for the inflow time analysis, and was defined as the time from peak to half peak of fluorescence intensity. The outflow time of Lucifer yellow was prolonged in the LPS group. The LPSF group also had a delayed outflow time, whereas it was normalized in the LPSFH group.

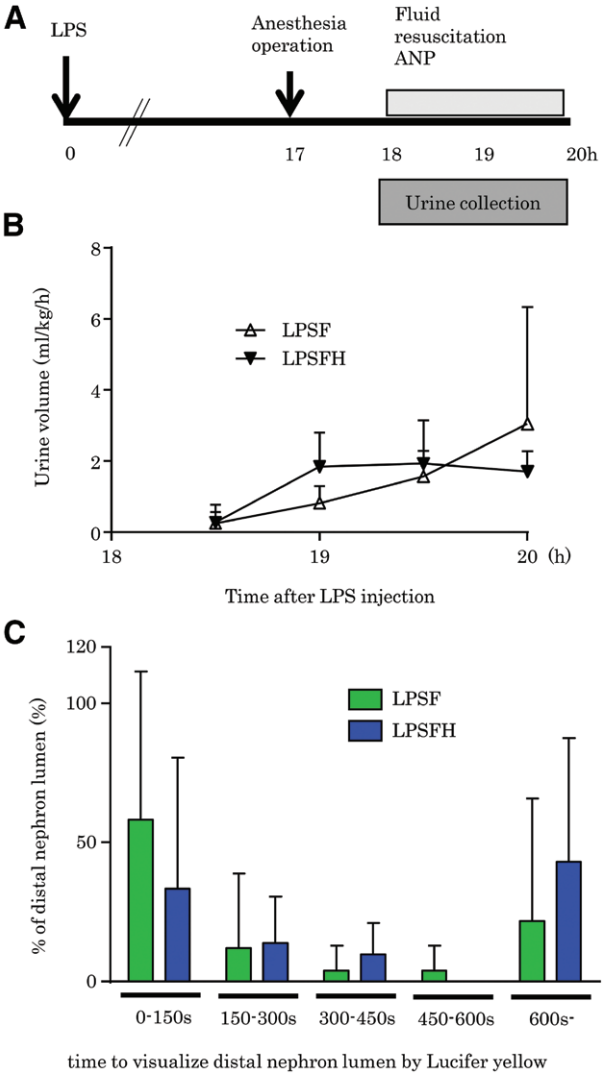

Fig. 5. Effects of recombinant human arterial natriuretic peptide (ANP) initiated at 18 h after lipopolysaccharide (LPS) injection. (*A*) Schematic of the timeline of the experiment. (*B*) Changes in urine flow rate. Administration of human arterial natriuretic peptide with fluid resuscitation (LPSFH) failed to increase urine flow rate compared to LPS with fluid resuscitation only (LPSF; n = 5). (*C*) Time for Lucifer yellow to achieve the distal nephron, as tubular flow rate. The time to visualize distal nephron lumens by Lucifer yellow was categorized into five groups as shown on the *x-axis*, and percentage of *red arrows* per total distal nephron number in each category was set as the *y-axis*. Administration of human arterial natriuretic peptide with fluid resuscitation failed to increase tubular flow rate (n = 5).

lipopolysaccharide dose was decreased to 5 mg/kg. The late initiation of 2 h human atrial natriuretic peptide infusion, in addition to fluid resuscitation, ameliorated neither impaired urine flow (fig. 5B) nor decreased tubular flow rate (fig. 5C).

Proximal Tubular Guanylyl Cyclase A Knockout Mice

Guanylyl cyclase A mRNA concentrations in the whole kidney of proximal guanylyl cyclase A knockout mice were decreased to approximately 60% of those in floxed control mice after activation of Cre recombinase by tamoxifen (Supplemental Digital Content 3, figure S2, http://links.lww.com/ALN/B691).

In the floxed control mice, Lucifer yellow in the proximal tubules was retained for more than 120 s at 6 h after lipopolysaccharide with 1 h fluid resuscitation (fig. 6A). Human atrial natriuretic peptide treatment for 1 h (started at 5 h after lipopolysaccharide injection) dramatically accelerated the tubular flow rate in the floxed control mice, similar to the observations in rats (fig. 6, A and B). Deletion of proximal tubular guanylyl cyclase A decreased the percentage of distal nephrons with Lucifer yellow in the lumens within 60 s of the Lucifer yellow injection to 0, compared with 23.3 ± 19.4% in the floxed control mice (fig. 6B). However, the percentage of distal nephrons without Lucifer yellow in the lumens within 300 s was similar in the floxed control and proximal tubular guanylyl cyclase A knockout mice (fig. 6B). Thus, human atrial natriuretic peptide failed to improve tubular flow rates in proximal tubular guanylyl cyclase A knockout mice, indicating the importance of proximal tubular guanylyl cyclase A on modulation of tubular flow rate by human atrial natriuretic peptide.

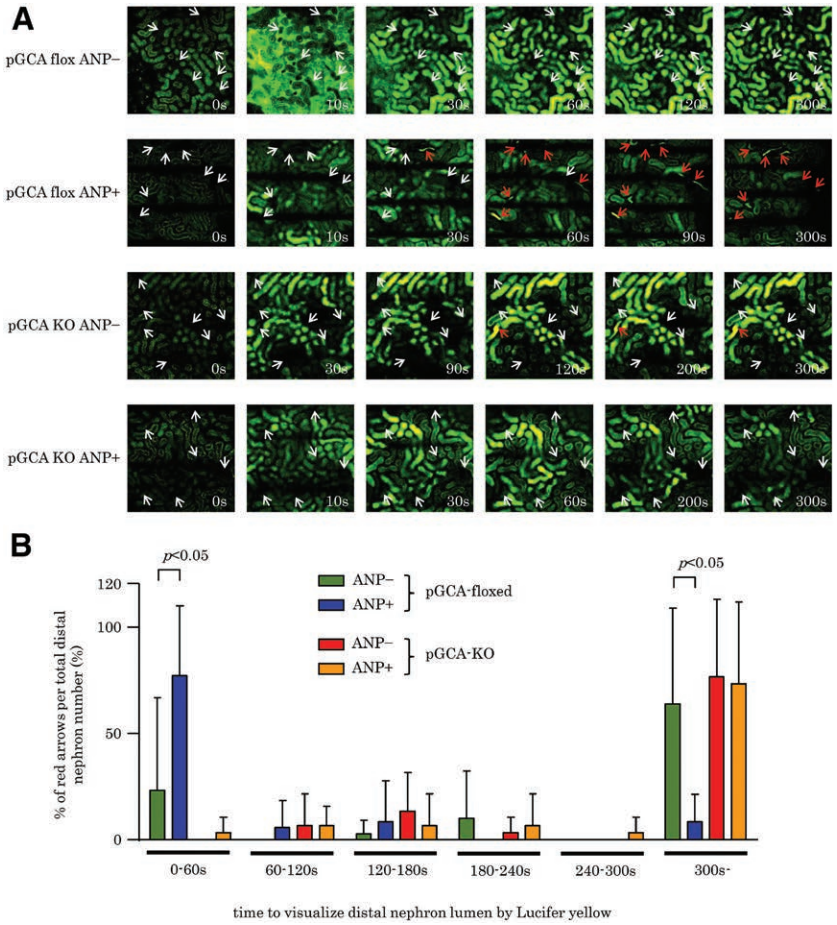

Fig. 6. Estimation of renal function by *in vivo* imaging in proximal tubular guanylyl cyclase A knockout (pGC-A-KO) mice. The analysis was performed as outlined in the legend for figure 2, except for the duration of observation; 600 s for rats and 300 s for mice because of the weaker tissue resistance to potential phototoxicity in mice. The distal nephrons that have not had Lucifer yellow in the lumen are indicated by *white arrow*s, and the distal nephrons that have already had Lucifer yellow in the lumen are indicated by *red arrows* in *A*. (*A*) Representative pictures from time-lapse imaging after Lucifer yellow injection. In guanylyl cyclase A floxed control mice, human recombinant arterial natriuretic peptide (ANP) improved Lucifer yellow flow, and all distal nephrons had Lucifer yellow in the lumens that were in the focal plane within 300 s. In pGC-A-KO mice, human recombinant ANP failed to improve tubular flow (n = 5). (*B*) Time for Lucifer yellow to achieve distal nephrons, as tubular flow rate. The time to visualize distal nephron lumens by Lucifer yellow was categorized into five groups as shown on the *x-axis*, and percentage of *red arrows* per total distal nephron number in each category was the *y-axis*.

There were no differences in Lucifer yellow inflow rates in proximal tubules between the groups (fig. 7), suggesting an absence of glomerular filtration rate changes in this murine model, as reported previously.[4] Deletion of proximal tubular guanylyl cyclase A itself did not affect outflow rate. Administration of human atrial natriuretic peptide significantly improved outflow rate from the proximal tubules in the floxed control mice, but had no significant effect in proximal tubular guanylyl cyclase A knockout mice.

There were no statistically significant differences in plasma creatinine and neutrophil gelatinase–associated lipocalin concentrations and renal mRNA concentrations of kidney injury molecule–1, neutrophil gelatinase–associated lipocalin, and tumor necrosis factor–α between the groups at 6 h after lipopolysaccharide injection (Supplemental Digital Content 3, figure S3, http://links.lww.com/ALN/B691).

Vascular Endothelial Cell–Specific Guanylyl Cyclase A Knockout Mice

Guanylyl cyclase A mRNA concentrations in the aortas of $Tie2^{Cre}$:guanylyl cyclase A floxed mice (endothelial guanylyl cyclase A knockout mice) were decreased to approximately 40% of those in the floxed control mice (Supplemental Digital Content 3, figure S2, http://links.lww.com/ALN/B691).

Lipopolysaccharide decreased tubular flow in the floxed control mice (fig. 8). Human atrial natriuretic peptide, administered for 1 h (started at 5 h after lipopolysaccharide injection), dramatically accelerated tubular flow rate in the floxed control mice. There were few distal nephrons with Lucifer yellow within 60 s in the endothelial guanylyl cyclase A knockout mice after lipopolysaccharide injection. There were no differences in tubular flow rates in endothelial guanylyl cyclase A knockout mice treated with and without human atrial natriuretic peptide

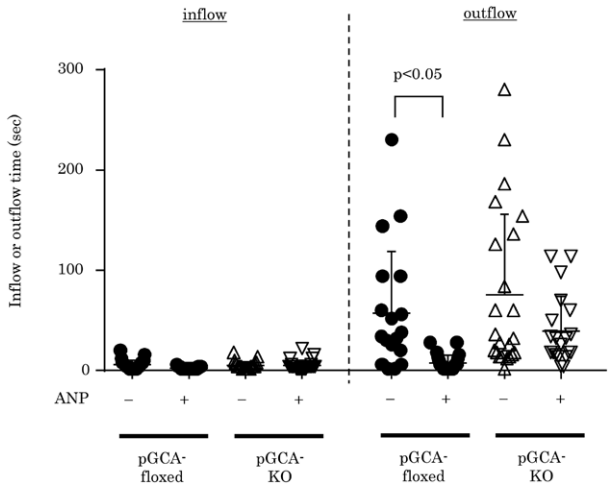

Fig. 7. Inflow and outflow times of Lucifer yellow in proximal tubules after lipopolysaccharide injection. The inflow time, which reflects glomerular filtration rate, was determined by how quickly Lucifer yellow appeared and reached the peak concentration in the proximal tubules after the injection. Five spatially dissociated proximal tubules that showed Lucifer yellow right after the injection were employed to count the inflow time. The inflow time of Lucifer yellow was similar in floxed control and proximal tubular guanylyl cyclase A knockout (pGC-A-KO) mice, with and without human recombinant arterial natriuretic peptide (ANP; n = 22 to 25 tubules in 5 mice). The outflow time reflects the washout of tubular fluid from proximal tubules to downstream nephron. The outflow time was analyzed in the nephrons that were used for the inflow time analysis, and was defined as the time from peak to half peak of fluorescence intensity. The outflow time of Lucifer yellow was prolonged in the floxed control mice, and this was shortened by human recombinant ANP treatment. The effect of human recombinant ANP was not significant in pGC-A-KO mice.

(fig. 8). This indicated that endothelial guanylyl cyclase A was also important for the effects of exogenous human atrial natriuretic peptide on tubular flow rate.

Infusion of human atrial natriuretic peptide in the floxed control mice did not induce significant changes in the inflow rates (fig. 9). However, guanylyl cyclase A deletion in the endothelial cells led to slower inflow rates, suggesting that endogenous natriuretic peptides, acting on endothelial guanylyl cyclase A, maintain glomerular filtration rate after lipopolysaccharide treatment. Infusion of human atrial natriuretic peptide did not improve inflow rate in the endothelial guanylyl cyclase A knockout mice. The outflow rate in the floxed control mice, however, was improved by human atrial natriuretic peptide. There were numerous proximal tubules not exhibiting a 50% decrease in Lucifer yellow fluorescence intensity within 300 s in the endothelial guanylyl cyclase A knockout mice (16/25 and 6/25 tubules, without and with human atrial natriuretic peptide, respectively), preventing calculation of the outflow rate in this strain.

There were no statistically significant differences in plasma creatinine and neutrophil gelatinase–associated lipocalin concentrations and renal mRNA concentrations of kidney injury molecule–1, neutrophil gelatinase–associated lipocalin, and tumor necrosis factor–α between the groups at 6 h after lipopolysaccharide injection (Supplemental Digital Content 3, figure S3, http://links.lww.com/ALN/B691).

Vascular Permeability in Vascular Endothelial Cell–Specific Guanylyl: Cyclase A Knockout Mice

The inflow rate in endothelial guanylyl cyclase A knockout mice was decreased by lipopolysaccharide injection at a dose that did not affect the inflow rate in floxed control mice. This suggested that glomerular filtration rate was more susceptible to lipopolysaccharide in mice lacking endothelial guanylyl cyclase A. To further clarify the effect of lipopolysaccharide on the vasculature in endothelial guanylyl cyclase A knockout mice, changes in vascular permeability after lipopolysaccharide injection were assessed. Lipopolysaccharide increased Evans blue–albumin leakage in the ascites and lung of floxed control mice (fig. 10). These changes were further augmented in the endothelial guanylyl cyclase A knockout mice.

Discussion

Summary

This study demonstrates that natriuretic peptides, both endogenous and exogenous, are important in maintaining tubular and urine flow during the early phase of endotoxemic acute kidney injury in rats and mice also receiving fluid resuscitation. The findings indicated that stimulation of proximal tubular guanylyl cyclase A with a subdepressor dose of human atrial natriuretic peptide prevented lipopolysaccharide-induced proximal tubular flow retention. Furthermore, our results in endothelial guanylyl cyclase A knockout mice suggested that endogenous natriuretic peptides, acting on endothelial guanylyl cyclase A, are involved in maintaining vascular permeability, and can prevent vascular dehydration and decreased glomerular filtration rate during endotoxemia. Early initiation of human atrial natriuretic peptide, at 2 and 5 h after lipopolysaccharide injection in rats and mice, respectively, ameliorated acute kidney injury. However, late initiation of human atrial natriuretic peptide showed no benefits on tubular or urine flow rates, indicating that there is a time window for effectiveness of human atrial natriuretic peptide administration.

Role of Guanylyl Cyclase A in the Proximal Tubules and Fluid Sensitivity

The floxed controls for both proximal tubular guanylyl cyclase A and endothelial guanylyl cyclase A knockout mice showed either nephrons whose tubular flow rates were almost normal (0 to 60 s) or nephrons whose tubular flow rates were decreased (>300 s) after lipopolysaccharide with fluid resuscitation (figs. 6B and 8B). Similar heterogeneity was observed in rats. Fluid resuscitation increased the urine flow rate at the bladder (fig. 1), although there were nephrons with either normal or very slow tubular flow rates observed by *in vivo* imaging (fig. 3). Human atrial natriuretic peptide markedly decreased the numbers of nephrons resistant to

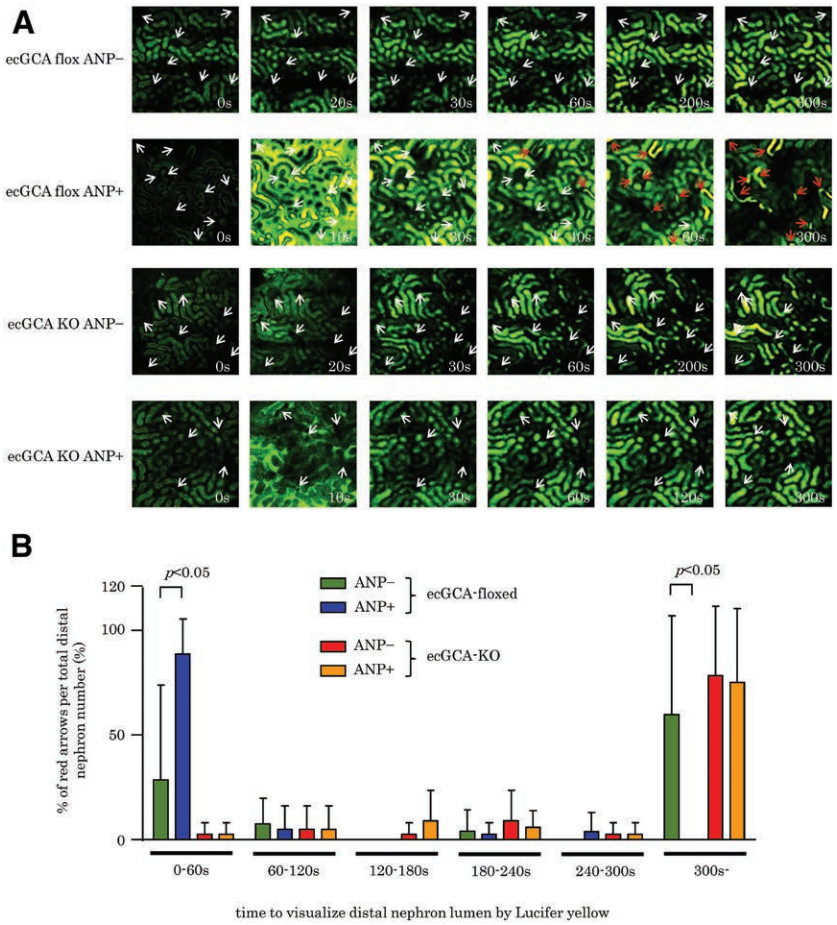

Fig. 8. Estimation of renal function by *in vivo* imaging in endothelial cell guanylyl cyclase A knockout (ecGCA KO) mice. The analysis was performed as outlined in the legend for figure 2, except the duration of observation: 600 s for rats and 300 s for mice because of the weaker tissue resistance to potential phototoxicity in mice. The distal nephrons that have not had Lucifer yellow in the lumen are indicated by *white arrows*, and the distal nephrons that have already had Lucifer yellow in the lumen are indicated by *red arrows* in *A*. (*A*) Representative pictures from time-lapse imaging after Lucifer yellow injection. In ecGCA floxed control mice, human recombinant arterial natriuretic peptide (ANP) improved Lucifer yellow flow, and most of the distal nephrons had Lucifer yellow in the lumens that were in the focal plane within 300 s. In ecGCA-KO mice, human recombinant ANP failed to improve tubular flow (n = 5). (*B*) Time for Lucifer yellow to achieve distal nephrons, as tubular flow rate. The time to visualize distal nephron lumens by Lucifer yellow was categorized into five groups as shown on the *x-axis*, and percentage of *red arrows* per total distal nephron number in each category was the *y-axis*.

fluid resuscitation. This effect was suppressed in the proximal tubular guanylyl cyclase A knockout mice. Moreover, deletion of proximal tubular guanylyl cyclase A itself decreased the numbers of nephrons sensitive to fluid resuscitation (0 to 60 s). Taken together, guanylyl cyclase A, activated by either endogenous or exogenous agonists, in proximal tubules may play an important role in the development of fluid resistance in the proximal tubules during endotoxemia.

There was no difference in glomerular filtration rate among the groups in proximal tubular guanylyl cyclase A mice, and human atrial natriuretic peptide accelerated the outflow rate from proximal tubules and the tubular flow rate in the control floxed group. Therefore, proximal tubules could be one of the sites of human atrial natriuretic peptide–induced action, ameliorating the reduction of tubular flow rate and suppressing the development of fluid resistance. This should be independent of the classical natriuretic actions of human atrial natriuretic peptide in the distal nephron[27,28] (#4 in fig. 2A to the medullary collecting duct) since the majority of nephron segments that we analyzed were upstream of this action (#2 to #4). In our study, the mechanisms by which human atrial natriuretic peptide ameliorated the slowing of tubular flow rate in proximal tubules were unclear. It was reported previously that activation of tubular guanylyl cyclase A inhibited sodium–hydrogen antiporter 3,[29] which increases both water and sodium delivery from the proximal tubules to the downstream nephrons. However, lipopolysaccharide was also reported to decrease expression of the sodium–hydrogen antiporter 3.[30] Thus, the contribution of human atrial natriuretic peptide–induced effects on the sodium–hydrogen antiporter 3 to our findings remain unclear.

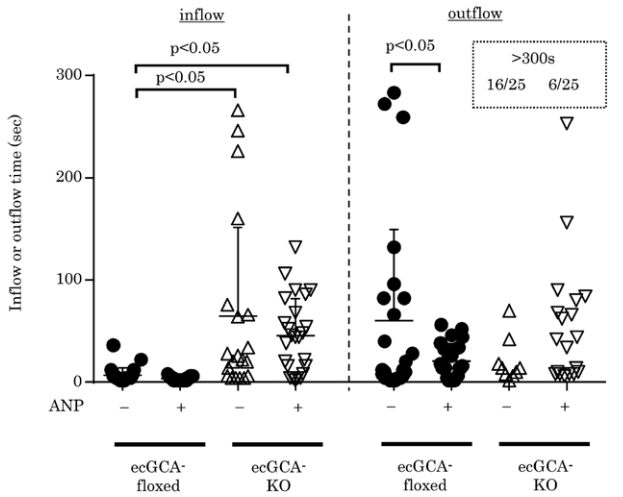

Fig. 9. Inflow and outflow times of Lucifer yellow in proximal tubules after lipopolysaccharide injection. The inflow time, which reflects glomerular filtration rate, was analyzed by how quickly Lucifer yellow appeared and reached the peak concentration in the proximal tubules after the injection. Five spatially dissociated proximal tubules that showed Lucifer yellow right after the injection were employed to count the inflow time. The inflow time of Lucifer yellow was prolonged in endothelial cell guanylyl cyclase A knockout (ecGCA-KO) mice, and human recombinant arterial natriuretic peptide (ANP) treatment did not affect this delay (n = 9–25 tubules in 5 mice). The outflow time reflects the washout of tubular fluid from proximal tubules to downstream nephron. The outflow time was analyzed in the nephrons that were used for the inflow time analysis, and was defined as the time from peak to half peak of fluorescence intensity. The outflow time of Lucifer yellow was prolonged in the floxed control mice, and this was shortened by human recombinant ANP. There were numerous proximal tubules not showing a clear washout of Lucifer yellow within 300 s; the exact number of tubules is noted in the *dashed box* as greater than 300 s.

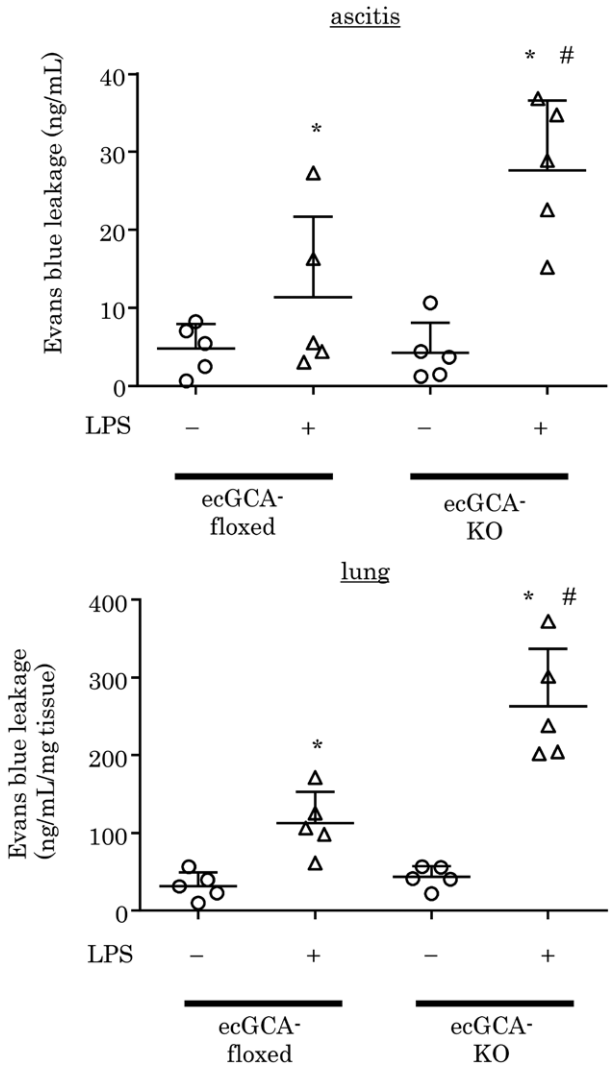

Fig. 10. Vascular permeability after lipopolysaccharide (LPS). LPS increased Evans blue leakage into ascites and lung. Notably, guanylyl cyclase A deletion in endothelial cells (ecGCA-KO) increased this response to LPS (n = 5 per group). **P* < 0.05 *versus* saline-injected non-LPS mice, *#P* < 0.05 *versus* floxed mice.

NDRG1 Cre recombinase was reported to be active in most S1 and S2 segments of proximal tubules, as well as in a part of the S3 segment and, occasionally, in collecting ducts.[22] Infused human atrial natriuretic peptide might have stimulated guanylyl cyclase A in the collecting duct, as previously reported.[31–34] Therefore, the absence of a human atrial natriuretic peptide–induced benefit on tubular flow rate in the NDRG1^{CreERT2}:guanylyl cyclase A floxed mice, which we denoted as proximal tubular guanylyl cyclase A knockout mice in this study, could be partially explained by guanylyl cyclase A deletion in the collecting duct. Also, the potential diuretic and natriuretic actions by human atrial natriuretic peptide in collecting ducts could have accelerated urine flow rates in lipopolysaccharide-injected animals in our study. However, importantly, a collecting duct–dependent mechanism could not explain all the effects of human atrial natriuretic peptide on tubular flow rate because our *in vivo* imaging analysis focused on tubular flow in the upstream nephron segments of the collecting ducts.

One-hour infusion of fluid resuscitation with human atrial natriuretic peptide did not significantly ameliorate the increase in plasma creatinine and neutrophil gelatinase–associated lipocalin concentrations and renal expression of tissue damage and inflammatory markers. Fluid resuscitation alone could improve plasma creatinine concentration, as it did in the rat study, and might affect the other parameters as well. Another possibility is that 1-h treatment with human atrial natriuretic peptide might not be sufficient to influence these markers in the kidney that had been exposed to lipopolysaccharide for 5 h.

Role of Guanylyl Cyclase A in the Vasculature

Our study revealed that both endogenous and exogenous natriuretic peptides induced protective effects through their receptor on vascular endothelial cells. The effects of exogenous human atrial natriuretic peptide on glomerular

filtration rate were previously reported. Intravenous infusion of rat recombinant atrial natriuretic peptide increased both whole kidney and single nephron glomerular filtration rate.[35] Lanese *et al.*[36] reported that atriopeptin III, a recombinant human atrial natriuretic peptide, dilated afferent arterioles but constricted efferent arterioles. Our study demonstrated that endogenous natriuretic peptides/endothelial guanylyl cyclase A were important for maintaining vascular permeability under endotoxemia *in vivo,* as had been suggested previously.[37–39] Notably, lipopolysaccharide can disrupt the endothelial glycocalyx and, thus, increase its permeability.[40,41] The increased permeability can then induce loss of intravascular fluid, so called intravascular dehydration, which induces sympathoexcitation and decreases organ perfusion. In addition, natriuretic peptides were reported to act as antipermeability factors, preserving cell–cell junctions.[42] Therefore, endogenous natriuretic peptides could counteract lipopolysaccharide-induced increased vascular permeability to maintain organ perfusion. Taken together, we speculate that orchestration of endogenous and exogenous natriuretic peptides resulted in a remarkable improvement of tubular flow rate in our study.

There are potential interactions between proximal tubules and peritubular capillary endothelium in the microenvironment of endotoxemia-induced oliguria. Contributions of the peritubular capillary endothelial human atrial natriuretic peptide/guanylyl cyclase A pathway to such interactions were not clearly identified in our study because endothelial guanylyl cyclase A knockout mice showed a decreased glomerular filtration rate. This prevented reliable comparisons between the effects of human atrial natriuretic peptide on proximal tubular guanylyl cyclase A and on tubular flow rate.

Limitations of Fluid Resuscitation

Fluid resuscitation is a standard procedure used to treat acute kidney injury and was reported to decrease renal microvascular resistance and cytokine concentrations in rats.[43] Urine flow in lipopolysaccharide-treated rats receiving fluid resuscitation was increased to concentrations similar to those in the saline control group not receiving lipopolysaccharide. In addition, fluid resuscitation improved glomerular filtration rate (plasma creatinine and inflow rate) after lipopolysaccharide injection. Therefore, fluid resuscitation did attenuate acute kidney injury. However, microscopy analysis demonstrated that fluid resuscitation did not significantly ameliorate the slowing of tubular flow rates and, in particular, the heterogeneity of tubular flow rate among nephrons, in our study, as well as in previous reports.[4] The fluid resuscitation–resistant nephrons, which absorbed lipopolysaccharide,[4] might have sustained risk, thus influencing the prognosis of acute kidney injury. Stronger fluid resuscitation may overcome this resistance; however, it is not recommended to continuously increase fluid infusion rates, to prevent positive balance.[44] Efficient therapies should target the fluid-resistant nephrons by eliminating factors creating fluid resistance. These might include therapies incorporating early initiation of human atrial natriuretic peptide, as investigated in our study.

Conclusions

Our study demonstrated that human atrial natriuretic peptide improved lipopolysaccharide-induced oliguria in the early phase. Human atrial natriuretic peptide induced its effects through its receptor, guanylyl cyclase A, expressed in both vascular endothelium and proximal tubules. Endothelial guanylyl cyclase A was important for maintaining glomerular filtration rate and vascular permeability while proximal tubule guanylyl cyclase A was important for improving tubular flow rates.

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Competing Interests

Carperitide, a synthetic human atrial natriuretic peptide, was provided by Dai-ichi Sankyo Inc. (Tokyo, Japan).

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Address correspondence to Dr. Nakano: Department of Pharmacology, Kagawa University, 1750–1 Ikenobe, Miki, Kita, Kagawa 761–0793, Japan. dnakano@med.kagawa-u.ac.jp. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. Anesthesiology's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

Liebig's Trade Card for Numbing but Toxic Aconite

As advertised by a beef extract company named after chloroform codiscoverer Justus von Liebig, this portion of a trading card's obverse is from the Italian-language version of the 1904 "Poisonous Plants" series. A deadly member of *Ranunculaceae*, the Buttercup Family, the depicted aconite (*Aconitum napellus, right*) is better known as monkshood or wolf's bane. Also illustrated are tribesmen (*left*) carefully applying aconite to the tips of their arrows, darts, and spears. Brushing against this toxic plant can produce dizziness, vomiting, and even death. Historically, dilute quantities of aconite have been used as a neurotoxic local anesthetic to treat toothaches, but such practices are dangerous. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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