Ischemia-Induced Exocytosis of Weibel-Palade Bodies Mobilizes Stem Cells

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ABSTRACT
Recruitment of various stem and progenitor cells is crucial for the regeneration of an injured organ. Levels of uric acid, one of the prototypical "alarm signals," surge after ischemia-reperfusion injury. Exogenous uric acid rapidly mobilizes endothelial progenitor cells and hematopoietic stem cells and protects the kidney from ischemia. The relatively fast responses to uric acid suggest that preformed second messengers may be released from a storage pool. Here, it is reported that monosodium urate (MSU) results in exocytosis of Weibel-Palade bodies in vitro and in vivo, leading to the release of IL-8, von Willebrand factor, and angiopoietin 2 in the culture medium or circulation. Confocal and immunoelectron microscopy confirmed depletion of von Willebrand factor in MSU-treated aortic endothelial cells. Angiopoietin 2 alone induced exocytosis of Weibel-Palade bodies, mobilized hematopoietic stem cells and depleted splenic endothelial progenitor cells, partially reproducing the actions of MSU. In addition, pretreatment with angiopoietin 2 protected the kidneys from an ischemic insult, suggesting that the previously reported renoprotection conferred by MSU likely results from exocytosis of Weibel-Palade bodies. Furthermore, experiments with toll-like receptor 4 (TLR-4)– and TLR-2–deficient mice demonstrated that uric acid–induced exocytosis of Weibel-Palade bodies is mediated by TLR-4 and that uric acid–induced release of IL-8 requires both TLR-2 and TLR-4. In summary, these results suggest that exocytosis of Weibel-Palade bodies links postischemic repair with inflammation and mobilization of stem cells.


Tissue regeneration is a finely tuned and well-orchestrated process requiring recruitment of stem and progenitor cells to the sites of injury. Mobilization of stem cells and endothelial progenitor cells (EPC) has been extensively documented in myocardial infarction, limb ischemia, vascular trauma, and acute renal ischemia.1–4 The very fact that stem and progenitor cells are mobilized and consistently found at the sites of injury, regardless of previous institution of any pharmacologic stimulation of their mobilization, alludes to the existence of intrinsic factor(s) generated during organ damage that may be responsible for their mobilization. Identification of such factors could expand the arsenal of pathogenetically relevant pharmacologic maneuvers hastening regenerative processes.

One of the default mechanistic responses to...
ischemia-reperfusion injury is represented by the activation of xanthine oxidoreductase and metabolism of purines. Potential role of metabolic products of this pathway as extracellular signaling molecules has been envisaged by Szent-Gyorgyi and pursued by Burnstock’s laboratory. Having demonstrated renoprotective effects of ischemia-mobilized EPC in the setting of acute renal ischemia, we next performed a follow-up study that incriminated a product of xanthine oxidase metabolic pathway, uric acid (UA), as being a representative alarm signaling molecule discharged from the ischemic tissue and capable of downstream mobilization and recruitment of stem cells and EPC. Because the observed stem cells/EPC-mobilizing response to UA was relatively fast, we reasoned that putative second messenger(s) should preexist in a storage form, rather than depend on emergency on-demand synthesis. The organelle meeting this requirement is the Weibel-Palade body (WPB). These rod-shaped organelles contain an array of proteins, peptides, and cytokines that can be released urgently on demand. A list of compounds known to induce exocytosis of WPB is long and includes thrombin, histamine, peptido-leukotrienes, complement components, angiopoietin 2, IL-8, superoxide anion, vascular endothelial growth factor (VEGF), sphingosine-1-phosphate, ceramide, purine nucleotides, serotonin, vasopressin, and epinephrine. Here, we inquired whether WPB are exocytosed by the acutely and transiently elevated levels of UA, as it universally occurs after ischemia, and showed that this product of activated xanthine oxidase induces exocytosis of WPB at least in part via action on Toll-like receptor 4 (TLR-4), which was accompanied by the release of angiopoietin 2. The latter partially recapitulates the EPC-mobilizing effect of UA, raising the possibility of using UA and/or angiopoietin 2 for pharmacologic preconditioning.

RESULTS

Dynamics of Uric Acid–Induced Mobilization of Hematopoietic Stem Cells and EPC
In the previous studies, we demonstrated that renal ischemia induces transient elevation in the level of UA. To mimic it, here we injected monosodium urate (MSU) into male FVB mice and killed mice at various times after injection. The amount of injected MSU (50 mg) was sufficient to almost double its plasma concentration of UA, which was comparable to that detectable in the immediate postischemic period. As shown in Figure 1, mobilization of hematopoietic stem cells (HSC) occurred at 1 h and peaked at 3 h; mobilization and splenic sequestration of EPC was detectable at 1 h. Concurrent with the mobilization of stem/progenitor cells, the dynamics of cytokines in the peripheral blood of mice treated with MSU was examined. Notably, the plasma level of keratinocyte chemoattractant (KC), a murine analog of IL-8, which is stored in WPB and released upon exocytosis, was elevated already 1 h after injection and peaked at 3 h (from 9.5 ± 3.0 to 248.9 ± 46.8 pg/ml; Figure 2). Dynamics of other cytokines are shown in Table 1.

Uric Acid Triggers Exocytosis of WPB In Vitro and In Vivo
To gain insights into the cellular mechanism of the observed EPC-mobilizing effect of MSU, we considered the urgency of this reaction as an indicator of a release of an already synthesized messenger compound(s), intracellularly stored and awaiting activation or exocytosis. The previous demonstration of the surge in KC level after administration of MSU prompted us to examine other constituents of WPB. Application of MSU to cultured human umbilical vein endothelial cells (HUVEC) resulted in the rapid depletion of von Willebrand factor (vWF) and angiopoietin 2, markers of WPB, from endothelial cells (Figure 3). Immunocytochemical detection of vWF and angiopoietin 2 in HUVEC revealed that this effect occurred already at 5 min of exposure (Figure 3B). This effect was detectable even with the lowest concentration of MSU used (10 μg/ml), which resulted in the depletion of angiopoietin 2 from the HUVEC (Figure 3, C and D). Exocytosis of WPB was independently confirmed by FACS analysis. As shown in Figure 4, side scatter of HUVEC was reduced after MSU treatment, indicative of the
Figure 2. Plasma levels of KC after monosodium urate injection. Here and below, * indicates \( P < 0.05 \), ** indicates \( P < 0.01 \), and *** indicates \( P < 0.001 \) unless otherwise indicated.

A reduction in cell volume and consistent with the degranulation reaction.

Western blot analysis of cell lysates (Figure 5A) and immunocytochemical staining (data not shown) revealed that the most prominent decrease of angiopoietin 2 abundance occurred at 15 min after application of MSU. Reciprocal changes in angiopoietin 2 were observed in the culture medium (Figure 5B). The concentration of angiopoietin 2 in the culture medium exhibited a mild elevation in response to 10 and 50 \( \mu \text{g/ml} \) UA and showed a five-fold increase to 100 \( \mu \text{g/ml} \) already after 15 min of incubation.

In the in vivo study of a highly vascularized organ, the kidney, the decrease of angiopoietin 2 was also observed 15 min after the injection of MSU (Figure 6A). In addition, the serum level of angiopoietin 2 in MSU-treated mice (50 \( \mu \text{g intraperitoneally} \)) was elevated already after 20 min with a significant two-fold increase documented after 40 min (Figure 6B).

Immunohistochemical staining of en face aortic preparations obtained from mice pretreated with MSU (Figure 7A) showed a rich pattern of immunodetectable vWF in control but a scarce expression of this main component of WPB after administration of MSU. Immunoelectron microscopy of vWF and angiopoietin 2 in aortic endothelium was next performed. Mice administered an injection of MSU or a vehicle and studied 15 min later. As shown in Figure 7B, 10 nm of gold nanoparticles labeling vWF were abundant in control endothelia but decreased significantly after in vivo administration of MSU. Immunodetectable angiopoietin 2 was expressed at a much lower level, not always co-clustering with vWF, and could not be quantified. Collectively, these data amply complement the results of in vitro study in establishing that MSU acutely induces exocytosis of WPB and release of vWF and angiopoietin 2 into the circulation.

### Angiopoietin 2–Induced Mobilization of HSC and EPC

Administration of angiopoietin 2 (1 \( \mu \text{g per mouse} \), intravenously) to intact mice resulted in the surge of the number of HSC in the blood and spleen detected after 3 h (Figure 8A). EPC levels decreased in the splenic niche (Figure 8B). A similar phenomenon was previously observed under conditions of ischemic preconditioning, when the mobilized EPC evaded splenic sequestration and were shunted directly to the ischemic site.

### Table 1. Serum cytokine/chemokine profile after injection of MSU

<table>
<thead>
<tr>
<th>Cytokine/ Chemokine</th>
<th>Control Mean SE</th>
<th>15 min Mean SE</th>
<th>1 h Mean SE</th>
<th>3 h Mean SE</th>
<th>24 h Mean SE</th>
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<td>G-CSF</td>
<td>2.300</td>
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<td>&lt;4.600</td>
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<td>&lt;0.700</td>
<td>&lt;0.700</td>
<td>&lt;0.700</td>
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<td>6.650</td>
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<td>IL-1( \alpha )</td>
<td>4.400</td>
<td>&lt;4.400</td>
<td>56.070</td>
<td>34.510</td>
<td>99.610</td>
<td>26.440</td>
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<tr>
<td>IL-1R</td>
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<td>1.683</td>
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<td>1.740</td>
<td>0.140</td>
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<td>&lt;10.300</td>
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<td>&lt;0.800</td>
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<td>&lt;6.000</td>
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<td>&lt;6.300</td>
<td>103.600</td>
<td>76.570</td>
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<td>&lt;0.900</td>
<td>1.146</td>
<td>0.2463</td>
<td>&lt;0.900</td>
<td>1.390</td>
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</table>

*aControl mice received intraperitoneal injection of the vehicle, which was treated in the same way as MSU. MCP-1, monocyte chemoattractant protein 1; MIP-1\( \alpha \), macrophage inflammatory protein 1-\( \alpha \).

\( bP < 0.05 \).
Because our previous studies showed that MSU pretreatment afforded renoprotection against ischemia-reperfusion injury, we next inquired whether this phenomenon could occur as a result of exocytosis of WPB; therefore, we tested the ability of angiopeitin 2–induced HSC/EPC mobilization to affect the severity of ischemic renal injury. Angiopeitin 2 (1 μg/25 g, intravenously) was administered 3 h before the induction of 30 min of bilateral renal ischemia in mice. Angiopeitin 2 pretreatment afforded renoprotection against ischemic insult, as judged from the stable plasma creatinine level in the treated group compared with the elevation in plasma creatinine in untreated ischemic mice (Figure 9). Thus, administration of a component of WPB, angiopeitin 2, released by a surge in UA level mimicked the effect of MSU administration. Similar results were obtained with intravenous injection of vWF (D.P. et al., manuscript in preparation).

TLR-4 Mediates the Action of Uric Acid on WPB

The question of cellular target(s) of UA that is (are) involved in the observed action of MSU was addressed next. There are data implicating both TLR-2 and -4 in mediating some cellular actions of UA. Moreover, a recent report suggested that TLR-2 activation triggers exocytosis of WPB. Consequently, we attempted to dissect relations between UA, TLR, and exocytosis of WPB in vivo using TLR-2– and TLR-4 knockout mice. As shown in Figure 10, administration of UA to TLR-2–deficient mice resulted within 30 min in an increase in plasma levels of vWF and angiopeitin 2. In contrast, this effect was curtailed in TLR-4–deficient mice, indicating that TLR-4 was necessary for the execution of MSU effect on the release of vWF and angiopeitin 2. This was not the case in the wild-type mice: C57Bl/6J (background for TLR-2 knockout mice) and Balb/CJ (background for TLR-4 knockout mice) both showed significant increases in vWF and angiopeitin 2 levels 30 and 180 min after MSU administration.

Interestingly, MSU-induced elevation in circulating KC levels was absent in either TLR-2 or TLR-4 knockout mice, suggesting that the presence of both receptors is necessary for release of KC and that separate mechanism(s) of regulation of release of different constituents of WPB may exist. Three hours after MSU administration, levels of vWF and angiopeitin 2 returned to baseline and were indistinguishable between TLR-4– and TLR-2–deficient mice.
DISCUSSION

Data presented herein provide the first demonstration of the effect of UA, a universal companion of ischemia-reperfusion tissue injury, on TLR-mediated exocytosis of WPB, resulting in increased circulating levels of angiopoietin 2, which seemed to be sufficient to induce mobilization of HSC and EPC. This latter action has been linked to induction of pharmacologic preconditioning in various cardiovascular and renal diseases. As such, the findings presented herein may serve as a blueprint toward design of novel strategies for pharmacologic preconditioning.

Distinct mechanistic pathways may be implicated in accomplishing local and systemic reactions to ischemia or other stressors. It has been shown that hypoxia per se activates exocytosis of WPB (although UA levels were not determined), the event that may take place within the ischemic organ. Distant regulation of exocytosis of WPB can be accomplished, according to the data presented here, via release of the product of xanthine oxidase activation in the ischemic organ—UA. The same mediator was previously implicated in the cell injury-induced activation of dendritic cells. This type of regulation may represent a novel paradigm of alarm signaling in response to injury. Data demonstrated (Figure 10) that this action of urate can be mediated via both TLR-4 and TLR-2: TLR-4 is necessary for the release of vWF and angiopoietin 2, whereas both receptors are required for release of KC. Intriguingly, vWF and angiopoietin 2 responses to urate in TLR-2 knockout mice seem to be somewhat exaggerated compared with the wild-type mice. It is not clear whether it reflects some antagonistic relations between TLR-2 and TLR-4 or other mechanisms contribute to this enhanced response. Recent demonstration of bacterial lipoteichoic acid recognition by TLR-2 resulting in exocytosis of WPB, although TLR-4 actions were not investigated, is in concert with our findings (Figure 11).

In addition to UA, other mediators may use a similar mechanism of exocytosis of WPB to induce mobilization of EPC. It was previously found that VEGF is a potent EPC-mobilizing agonist;
however, VEGF has been shown to induce exocytosis of WPB.17
On the basis of the present demonstration that angiopoietin 2 is
capable of mobilizing HSC and EPC, such a mechanism may ex-
plain at least in part the described effect of VEGF.

Angiopoietin 2 has been localized to WPB from which it
can be rapidly released.18 Competing with angiopoietin 1
for Tie-2 receptor, angiopoietin 2 has been implicated in
pleiotropic actions depending on the biologic context, from
proinflammatory with increased vascular permeability to
cytoprotective in stressed endothelial cells19–24; therefore, a
delicate balance could be envisaged between the proinflam-
atory actions of angiopoietin 2 (among other released
constituents of WPB) and its HSC- and EPC-mobilizing
action serving as a pharmacologic preconditioner. It is quite
possible that a more severe tissue injury and/or higher levels
of UA result in an intense angiopoietin 2 signaling with
proinflammatory actions prevailing over its HSC- and EPC-
mobilizing effect. In fact, angiopoietin 2 release has been
shown to be responsible for the sepsis-induced leakage of
pulmonary blood vessels.25 It was also suggested that angio-
poietin 2 participates in the recruitment of bone
marrow–derived endothelial precursors, as well as
stimulates EPC migration.26,27 It is quite possible
that the observed recruitment of stem cells in our
studies represents another facet of the same phe-
omenon—the stress-induced response.

On a broader scale, our findings provide further
experimental support to the “danger model”—a
conceptual link between immune- and non–im-
une-mediated tissue injury.28 The essence of this
theory is that many signaling elements initiating in-
flammation in response to invading organisms and
alarm signals released from injured/necrotic tissues
are shared and elicit similar responses at the tissue
level. A host of alarm signals, “alarmins,” has been
described in immunologic literature, UA being one
of them.15,29 Thus, our studies enrich the spectrum
of physiologic actions of the acute and reversible
surge in UA levels by demonstrating the ability of UA
to effect exocytosis of WPB and mobilize stem cells
in response to ischemic signaling.

**CONCISE METHODS**

**Endothelial Cell Culture and Treatments**

HUVEC were purchased from Clonetics (Walkersville,
MD) and used between passages 5 and 7. Cells were
cultured in EBM-2 medium (Cambrex Bio Science, Walkers-
ville, MD) with EGM-2 SingleQuots supplements (Cam-
brex Bio Science) and maintained at 37°C incubator with
5% CO2. For cell culture experiments, UA was prepared as
previously reported.30 Briefly, UA sodium salt (MSU), 10
to 100 μg/ml (Sigma-Aldrich, St. Louis, MO) was added to
prewarmed EBM-2 medium (37°C). The mixture was ag-
itated at 37°C for 30 min and then passed through a sterile 0.22-
m filter. Control media were treated similarly but with the omission of
UA. For animal experiments, MSU was dissolved in sterile water,
heated in a microwave, and filtered through a 0.22-μm filter.

**Immunocytochemical Staining**

HUVEC (approximately 10^4 cells) were seeded on chamber slides
(Lab-TekII Chamber Slide System; Nalge Nunc Int., Rochester, NY)
and grown in EGM-2 medium until at least 80% confluent. The cells
were treated with various concentrations of UA (0, 10, 50, and 100
μg/ml) for various periods of time (5, 15, and 60 min). After treatment,
cells were fixed with 4% paraformaldehyde, permeabilized with
0.25% Triton-X 100 in PBS (pH 7.4), and stained with antibodies
against vWF (1:200; F3520, rabbit anti-human vWF; Sigma) and
angiopoietin 2 (1:100; Sc-7017; Santa-Cruz Biotechnology, Santa Cruz,
CA). Nonspecific protein binding was blocked by 1% BSA (Sigma)
in PBS for vWF staining and 5% rabbit serum in PBS for angiopoietin 2
staining. Incubations with primary and secondary antibodies (FITC-conjugated rabbit anti-goat and
Texas Red dye–conjugated donkey anti-rabbit; Jackson Immunore-

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Figure 7. Laser-scanning confocal microscopy of en face aorta and trans-
mission immunoelectron microscopy of aortic endothelium. (A) Representa-
tive views of laser-scanning confocal microscopy of the endothelial layer
of en face aorta from control and MSU-treated mice. (B) Immunoelectron
microscopy of vWF in aortic endothelial cells. Note the labeling of vWF
within WPB (insert) and reduction of immunodetectable vWF after MSU.
vWF (10 nm gold) and angiopoietin 2 (6 nm gold particles) labeling of
aortic endothelium at baseline (left) and 15 min after administration of MSU
(right). Central panel provides a zoomed view (arrows) of WPB with vWF
labeling. Angiopoietin 2 labeling is too sparse to be quantified. Quantifi-
cation of vWF labeling showed a significant decline after UA. Magnifi-
cation, ×29,000.
search, West Grove, PA) were performed for 1 h at room temperature. For visualization of the nuclei, cells were co-stained with Hoechst 33342 (Invitrogen, Carlsbad, CA). Slides were mounted using an antifade reagent (Slowfade; Invitrogen). Each experiment was performed at least three times with fluorescence intensity of approximately 100 cells digitally quantified using MetaMorph image analysis routines.

**Western Blot Analysis**

Passages 5 through 7 HUVEC were cultured in 100-mm tissue culture dishes (BD Falcon, San Jose, CA) until confluent. After washing twice with PBS, cells were cultured in serum-free EBM-2 medium with various concentrations of MSU (0, 10, 50, and 100 μg/ml) for various times (5, 15, and 60 min). The harvested cells were lysed in RIPA...
buffer (1% Triton-X 100, 0.1% SDS, and 0.5% sodium deoxycholate in PBS) with proteinase inhibitors (Complete tablet; Roche, Basel, Switzerland). Cell lysates were dissolved in Laemmlı buffer, boiled at 95°C for 5 min, and separated on 4 to 12% SDS-PAGE (Invitrogen). The proteins were electrotransferred to a polyvinylidene difluoride membrane (Millipore, Medford, MA). After blocking with 5% wt/vol nonfat dry milk, membranes were incubated at 4°C overnight on a microplate (Maxisorp; Nunc [Fisher Scientific, Rochester, NY]) and incubating at room temperature for 1 h with goat anti–angiopoietin 2 (Sc-7017; 1:50; Santa-Cruz Biotechnology), followed by incubation with horseradish peroxidase–conjugated anti-goat IgG (Sc-2020; 1:10,000; Santa-Cruz Biotechnology) for angiopoietin 2. Detection was performed using enhanced chemiluminescence (Pierce, Rockford, IL) and exposure to x-ray film. The same membrane was reprobed with β-tubulin–specific antibody (T 5293; Sigma) to ensure equal protein loading. Relative protein levels were calculated as densitometric ratios to β-tubulin.

ELISA for Angiopoietin 2 and vWF

Confluent HUVEC were washed with PBS, and medium was exchanged to serum-free EBM-2 with additions of UA and collected at various times for measurements of secreted angiopoietin 2 by ELISA Quantikine (R&D Systems, Minneapolis, MN) kit. For determination of angiopoietin 2 in the serum of mice, indirect ELISA was performed by coating standard antigen (Recombinant Ang2; R&D Systems) or serum at 4°C overnight on a microplate (Maxisorp; Nunc [Fisher Scientific, Rochester, NY]) and incubating at room temperature for 1 h with goat anti–angiopoietin 2 (Sc-7017; 1:40; Santa-Cruz Biotechnology), followed by incubation with horseradish peroxidase–conjugated anti-goat IgG (Sc-2020; 1:500; Santa-Cruz Biotechnology). Detection was performed by adding peroxidase substrate (SureBlue TMB; KPL, Gaithersburg, MD) and TMB stop solution (KPL). For detection of vWF, capture antibodies were used at 1:200 dilution and reaction with 100 μl of the plasma sample (1:32 dilution), followed by secondary antibodies at 1:750 dilution with extensive washings between these procedures. Plates were read at a wavelength of 450 nm.

FACS Analysis

For quantification of peripheral circulating and splenic EPC and HSC by FACS, mononuclear cells were isolated either from 500 μl of peripheral blood or from tissue homogenates by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) solution. For preparing tissue homogenates, the whole organ was placed in 2 ml of RPMI 1640 (Invitrogen) at 4°C. The tissue was minced and immediately homogenized, according to a previously published technique with minor modifications. Cells were incubated for 30 min on ice with FITC-conjugated anti-mouse CD34 (RAM34; eBioscience, San Diego, CA) and PE-conjugated anti-mouse Flk-1 (Avas12a1; BD Biosciences, Rockville, MD) or with FITC-conjugated anti-mouse CD117 (c-Kit; BD Biosciences) and PE-conjugated anti-mouse CD150 (eBioscience). After incubation, cells were washed with PBS and fixed in 4% parafomaldehyde. Data were acquired using a FACSscan cytometer equipped with a 488-nm argon laser and a 635-nm red diode laser and analyzed using CellQuest software (Becton Dickinson, San Jose, CA). The setup of FACSscan was performed using unstained cells. For quantification of EPC, the number of CD34/Flk-1 double-positive cells within the monocytic cell population was counted. Side-scatter parameter was used to assess exocytosis of WPB in HUVEC after UA treatment (100 μg/ml) and compared with nontreated cells.

Surgical Procedures

Male FVB/NJ mice were obtained from Jackson Laboratory and used at the age of 11 to 12 wk. UA (50 μg/100 μl in distilled water) was injected intraperitoneally. Mice (n = 4 in each group) were killed at 5 and 15 min and 1, 3, and 24 h after injection.

Male TLR-2 knockout (B6.129-Tlr2tm1Kir/J) and TLR-4 knock out (C3-Tlr4tm1Klr/J) mice were purchased from Jackson Laboratory. UA (50 μg/100 μl in distilled water) was injected intraperitoneally. Mice (n = 5 in each group) were killed at 30 min and 3 h after injection. Background strains Balb/CJ (for TLR-4 knockout) and C57Bl/6J (for TLR-2 knockout) were treated and examined similarly.

For renal ischemia induction, mice were anesthetized with intraperitoneal injection of ketamine hydrochloride (6 mg/100 g) and xylazine hydrochloride (0.77 mg/100 g) and placed on a heated surgical pad. Rectal temperature was maintained at 37°C. After a 1.5-cm mid-laparotomy, the kidneys were exposed and clamping of the renal pedicles performed with microserrefines (Fine Science Tools, Foster City, CA). After 25 min, the clamps were removed. Mice were killed 48 h after surgery, and blood, kidneys, and spleen were collected for further analysis. For sham operation, mid-laparotomy without vascular clamping was performed.

Chemokine and Cytokine Measurements

Multiplex assay kit (Linco Research, MCYTO-70K-PMX, Millipore Corp.) was used for the simultaneous quantification of the following mouse cytokines and chemokines: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, and 2321–2330, 2008
IFN-γ, IP-10, G-CSF, GM-CSF, TNF-α, KC, monocyte chemotractant protein 1, macrophage inflammatory protein 1-α, and RANTES. All analytes were tested individually and in combination to ensure that there were no cross-reactions. Briefly, the cytokine standards were resuspended in assay buffer and serially diluted. Twenty-five microliters of standard, quality controls, or sample was added to each well of a 96-well plate with 25 µl of the bead solution. The plate was sealed, covered with aluminum foil, and incubated overnight (16 to 18 h) with agitation on a plate shaker at 4°C. Then the plate was washed twice with 200 µl/well wash buffer, with removal of buffer by vacuum filtration between each wash. This was followed by addition of 25 µl of a detection antibody cocktail into each well and incubation at room temperature for 1.5 h. Streptavidin-phycocerythrin solution (25 µl) was added to each well and incubated at room temperature for 30 min. The plate was then analyzed on the Luminex IS100 analyzer (Luminex, Austin, TX). The data were saved and evaluated as median fluorescence intensity using appropriate curve-fitting software (Luminex 100IS 2.3). A five-parameter logistic method with weighting was used. All measurements were performed in duplicate.

**Immuno-electron Microscopy of vWF.**

Anesthetized mice received intraperitoneally injection of MSU (50 µg/mouse) or the equal amount of vehicle, killed after 15 min, and perfused with PBS followed by 4% PFA. Thoracic aortas were gently removed, sectioned, and postfixed. Tissue wedges were dissected and stored in PBS overnight at 4°C. Tissue wedges were further processed as follows: Dehydration in 50, 70, 85, 95, 100, 100, and 100% cold (4°C) ethanol, 15 min at each step; infiltration with LRWhite resin (resinethanol 1:1) for 90 min then pure resin for 24 h, both steps at 4°C; and embedding with fresh LRWhite resin and polymerization at 55°C overnight. Ultrathin sections were collected on Nickel grids. For immunolabeling, sections were incubated in the blocking buffer (BB) for donkey secondary antibodies (Aurion; Electron Microscopy Sciences, Hatfield, PA) for 15 min; anti-vWF 1:50 in BB or angio petrolin 2 1:50 in BB for 1 h; washed three times for 5 min each in BB, followed by the secondary antibodies donkey anti-rabbit IgG/10 nm gold or donkey anti-goat IgG/6 nm gold both at 1:100 in BB or angiopoietin 2 1:50 in BB for 1 h; washed three times for 5 min in PBS then two times for 1 min in water and counterstained with uranyl acetate (aqueous) for 15 min, followed by lead citrate for 5 min. Preparations were examined at 80 Kv in a JEOL (Peabody, MA) 100-cx II. Images were captured on Kodak (Rochester, NY) 4489 film, negatives were scanned into digital format on an Epson Expression 1600 professional flatbed scanner with transillumination, and the number of nano-gold particles per cluster was counted.

**Measurement of Serum Creatinine and UA.**

Serum creatinine concentration was measured using a commercially available kit (Raichem, San Diego, CA). Serum concentration of UA was measured using the Amplex Red UA/uricase assay kit (Invitrogen). Statistical Analysis

All of the values were expressed as means ± SE. Significant of difference between two groups were tested by two-tailed t test. One-way ANOVA and post test with Tukey multiple comparison test was used for three or more groups. P < 0.05 was considered as a significant difference.

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**DISCLOSURES**

None.

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