Pleiotropic effects of angiopoietin-2 deficiency do not protect mice against endotoxin-induced acute kidney injury

Neng F. Kurniati1, Matijs van Meurs1,2, Franziska vom Hagen3, Rianne M. Jongman1,4, Jill Moser1,2, Peter J. Zwiers1, Michel M.R.F. Struys4, Johanna Westra5, Jan G. Zijlstra2, Hans-Peter Hammes3, Grietje Molema1 and Peter Heeringa1

1Department of Pathology and Medical Biology, Medical Biology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands, 2Department of Critical Care, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands, 35th Medical Department, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany, 4Department of Anesthesiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands and 5Department of Rheumatology and Clinical Immunology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Correspondence and offprint requests to: Peter Heeringa; E-mail: p.heeringa@umcg.nl

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ABSTRACT

Background. In sepsis and various other inflammatory conditions, elevated circulating levels of angiopoietin-2 (Ang2) are detected, but the precise functional role of Ang2 in these conditions is not well understood. Here, we investigated the contribution of Ang2 to the inflammatory response and renal function impairment in a mouse model of endotoxaemia.

Methods. Ang2-deficient mice and wild-type littermates were challenged with lipopolysaccharide [LPS; 1500 EU/g, intraperitoneal (i.p.)]. In additional experiments, wild-type C57Bl/6 mice were depleted of circulating neutrophils by antibody treatment (NIMPR14) prior to LPS challenge to study the role of neutrophils in regulating LPS-induced cytokine release. After 8 or 24 h of LPS challenge, the mice were sacrificed and organs were harvested. Quantitative reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay were performed for endothelial adhesion molecules (P-selectin, E-selectin, VCAM-1 and ICAM-1) and plasma cytokines (TNF-α, IL-6, KC, MIP-2), respectively. To assess renal function, blood urea nitrogen levels in plasma and albumin-to-creatinine ratio in urine were measured.

Results. Upon LPS challenge, expression levels of various endothelial adhesion molecules in Ang2-deficient mice were reduced in an organ-specific manner. In contrast, in these mice, plasma levels of TNF-α and IL-6 were significantly increased compared with their wild-type littermates, possibly due to decreased neutrophil glomerular influx. Importantly, the absence of Ang2 did not protect the mice from acute kidney injury (AKI) upon LPS challenge.

Conclusions. The absence of Ang2 release upon LPS challenge induces pleotropic effects with regard to endothelial activation and systemic inflammation, but does not protect mice from LPS-induced AKI.

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INTRODUCTION

The angiopoietin/Tie2 system has been identified as a vascular receptor tyrosine kinase system controlling vascular homeostasis [1]. The family of angiopoietins, ligands of the Tie2 receptor, consist of Ang1, Ang2 and Ang3/4. Ang1 and Ang2 have been extensively studied and identified as an agonist and antagonist of the Tie2 receptor, respectively [2, 3]. The binding of Ang1 to Tie2 induces phosphorylation of the kinase domain of Tie2, leading to vascular stabilization [4]. In contrast, the binding of Ang2 results in vascular destabilization and vascular leakage [5]. Recently, a number of studies have provided evidence that Ang2 can also function as a partial agonist/antagonist ligand [6, 7]. In vitro studies by Yuan et al. [7] showed that in human umbilical vein endothelial cells, low concentrations of Ang2 induce phosphorylation of the Tie2 receptor, whereas high Ang2 concentrations inhibit phosphorylation. Although Ang2 is a weaker agonist than Ang1, endogenous Ang2 has been shown to be important to maintain the level of Tie2 activation [7].

Ang2 can be rapidly released from Weibel–Palade bodies in endothelial cells upon stimulation with pro-inflammatory stimuli [8]. Moreover, increased levels of Ang2 have been reported in many inflammatory diseases, including acute lung injury/acute respiratory distress syndrome (ALI/ARDS) [9], acute kidney injury (AKI) [10], sepsis [11–14] and rheumatoid arthritis [15]. In ALI/ARDS and chronic kidney disease patients, a positive correlation between circulating Ang2 levels and mortality has been found [16, 17], suggesting that Ang2 is associated with disease severity and outcome. The exact functional role of Ang2 during acute critical illness development is not yet well understood.

There is increasing evidence that Ang2 contributes to endothelial activation and inflammation [18]. We demonstrated that an increase of circulating Ang2 in LPS-challenged healthy volunteers associates with an increased level of soluble E-selectin [12]. In in vitro studies, Ang2 promotes the expression of adhesion molecules in activated endothelial cells by inhibiting the recruitment of the A20-binding inhibitor of NF-kappa B activation-2 (ABIN-2, an inhibitor of NF-kB) [19]. Moreover, Ang2 can act as a chemoattractant for leukocytes [20] through the activation of the PI3K pathway [21].

Other evidence suggests that Ang2 also promotes vascular leakage [5, 14]. In vivo studies have shown that systemic injection of recombinant Ang2 alone promotes vascular leakage in the lungs, and have demonstrated that Ang2/Tie2 binding regulates endothelial cell permeability in part by the modulation of the small GTPase RhoA-mediated pathways that activate myosin–actin filament (F-actin) interaction [14].

Previously, we observed the development of AKI in LPS-challenged mice [22]. Given the potential role of Ang2 in promoting inflammation and vascular leakage, we hypothesized that Ang2 is an important contributing factor in AKI. To test this hypothesis and explore the mechanism, we challenged Ang2-deficient mice and wild-type littermates with LPS and compared systemic and local inflammatory parameters, and kidney function.

MATERIALS AND METHODS

Animals

Specific pathogen-free Ang2LacZ (Ang2-deficient) mice [23] on a C57BL/6J background were obtained from Regeneron Pharmaceuticals (Tarrytown, NY, USA). Wild-type littermates were used as controls. For neutrophil depletion studies, C57BL/6 mice were obtained from Harlan (Horst, the Netherlands). All mice were maintained on chow and tap water ad libitum in a temperature-controlled chamber at 24°C with a 12-h light/dark cycle. All animal experiments were approved by the local Animal Care and Use committee of Regierungspräsidium Karlsruhe (protocol number 35-9185.81/G-148/09), Germany, and the University of Groningen (protocol number 4360A), the Netherlands, and were performed according to governmental and international guidelines on animal experimentation.

Endotoxaemia mouse model

Ang2LacZ (Ang2−/−, age 20–22 weeks, body weight 24.7 ± 2.8 g) mice and wild-type littermates (Ang2+/+, age 20–22 weeks, body weight 26.2 ± 3.9 g) were placed in metabolic cages for 8 h to collect urine (time point 0) 1 week before LPS (Escherichia coli, serotype 026:B6; Sigma, St. Louis, MO, USA) administration. Mice were divided into three groups, in casu, a control group [phosphate-buffered saline (PBS) injection, n = 7], and LPS-treated groups that were sacrificed at 8 (n = 7) and 24 h (n = 6) after LPS (1500 EU/g body weight, i.p.) injection. All mice received the same volume per gram body weight (5 μL/g). Mice were housed in metabolic cages for urine collection immediately after PBS or LPS administration.

An additional group of C57BL/6 mice (age 12 weeks) was i.p. injected with 0.5 mg of a rat anti-mouse neutrophil antibody (NIMP-R14, Hycult Biotechnology, Uden, The Netherlands) to selectively deplete neutrophils before LPS (Escherichia coli, serotype 026:B6; Sigma, St. Louis, MO, USA) administration. Mice were divided into three groups, in casu, a control group [phosphate-buffered saline (PBS) injection, n = 7], and LPS-treated groups that were sacrificed at 8 (n = 7) and 24 h (n = 6) after LPS (1500 EU/g body weight, i.p.) injection. All mice received the same volume per gram body weight (5 μL/g). Mice were housed in metabolic cages for urine collection immediately after PBS or LPS administration.

Gene expression analysis by quantitative reverse transcription polymerase chain reaction

Total RNA from organs was isolated with RNeasy Mini Plus Kit (Qiagen, Leusden, the Netherlands), according to the manufacturer’s instructions. Integrity of RNA was analyzed by gel electrophoresis, while RNA concentration and purity were measured using an ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). cDNA was synthesized using SuperScript III Rnase H-Reverse Transcriptase (Invitrogen, Breda, the Netherlands) in 20 μL final volume...
containing 250 ng random hexamers (Promega) and 40 units RNase Out inhibitor (Invitrogen). The Assay-on-Demand primers purchased from Applied Biosystems (Nieuwekerk a/d Issel, the Netherlands) were GAPDH (Mm99999915_g1), Ang2 (Mm00454822_m1), Ang1 (Mm00456503_m1), Tie2 (Mm00432424_m2), neutrophil gelatinase-associated lipocalin (NGAL/Lcn2, Mm01324470_m1) P-Selectin (Mm00441295_m1), E-selectin (Mm00441278_m1), VCAM-1 (Mm00449197_m1) and ICAM-1 (Mm00516023_m1). Taqman real-time polymerase chain reaction was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems, Foster City, CA, USA). Amplification was performed using the following cycling conditions: 15 min 95°C and 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. Duplicate real-time PCR analyses were executed for each sample, and the obtained threshold cycle values (C_T) were averaged. Gene expression was normalized to housekeeping gene GAPDH, yielding the ΔC_T value. The relative mRNA level was calculated by 2^-ΔC_T.

Quantitation of TNF-α, IL-6, KC and MIP-2 protein in mouse plasma by ELISA

TNF-α (Biolegend, San Diego, CA, USA), IL-6 (Biolegend), KC (R&D Systems, Minneapolis, MN, USA) and MIP-2 (R&D Systems) levels were measured in heparinized plasma using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions.

Immunohistochemistry

Frozen kidneys were cryostat cut at 5 μm, mounted onto glass slides and fixed with acetone for 10 min. After drying, sections were incubated with primary antibody for 1 h at room temperature followed by washing in PBS. Primary rat anti mouse antibodies, Ly-6G (BD Pharmingen, San Diego, CA, USA) were diluted to 1:50 in 5% fetal calf serum (FCS) in PBS. Endogenous peroxidase was blocked by incubation with peroxidase blocker from the DAKO envision kit (Cambridgeshire, UK) for 5 min. Detection was performed with rabbit anti-rat secondary antibodies (preabsorbed, Vector Laboratories, Burlingame, CA, USA) diluted to 1:300 in PBS/5% FCS supplemented with 2% normal mouse serum (Sanquin, Amsterdam, the Netherlands), which was followed by anti rabbit-HRP incubation (DAKO envision kit). Between antibody incubations, slides were extensively washed with PBS. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (DAKO envision kit), and sections were counterstained with Mayer’s haematoxylin (Merck, Darmstadt, Germany).

Renal function

To assess renal function, blood urea nitrogen (BUN, BioAssay Systems, Hayward, CA, USA) levels in plasma and albumin (Bethyl Laboratories, Montgomery, TX, USA) to creatinine (Exocell, Philadelphia, PA, USA) ratio in urine were measured using commercially available kits according to the manufacturer’s instructions.

RESULTS

Modulation of Ang1, Ang2 and Tie2 mRNA levels upon LPS challenge

At baseline, the expression of Ang1, Ang2 and Tie2 mRNA in the lungs was the highest compared with liver and kidneys (Figure 1). Upon LPS challenge, Ang2 mRNA levels increased significantly compared with control in all organs analysed (lungs, liver and kidneys) at 8 h, and returned to baseline levels at 24 h (Figure 1). In Ang2−/− mice, as expected, no Ang2 mRNA could be detected irrespective of treatment or time point, confirming the Ang2 deficiency in these mice. In lungs and livers, administration of LPS reduced mRNA expression levels of Ang1 at both time points in Ang2−/− and in Ang2+/+ mice although the difference did not always reach statistical significance when compared with baseline levels. In the kidney, Ang1 mRNA levels were significantly downregulated in Ang2−/− but not in Ang2+/+ mice. Finally, Tie2 mRNA expression levels were markedly downregulated in all organs at 8 h after LPS administration and tended to return to baseline levels at 24 h in both Ang2+/+ and Ang2−/− mice.

Ang2-deficient mice display a diminished expression of endothelial adhesion molecules upon LPS challenge

To investigate the contribution of Ang2 to the LPS-induced expression of endothelial adhesion molecules, we analysed the mRNA expression of P-selectin, E-selectin, VCAM-1 and ICAM-1 in the lungs, liver and kidneys of LPS-challenged Ang2−/− and Ang2+/+ mice (Figure 2). In general, mRNA expression levels of all adhesion molecules studied increased upon LPS challenge in both Ang2−/− and Ang2+/+ mice. However, the extent of induction varied between organs and genotype of the mice. Eight hours after LPS challenge, we found that in the lungs, the expression of P-selectin and VCAM-1 was significantly less in Ang2−/− mice compared with that in Ang2+/+ mice, whereas E-selectin and ICAM-1 mRNA levels were similar in both groups. In the kidneys, P-selectin, E-selectin and VCAM-1 mRNA levels were lower in LPS-challenged Ang2−/− mice. In contrast, in the liver mRNA levels for all adhesion molecules studied were similar between both groups (Figure 2). Twenty-four hours after LPS challenge, none of the organs studied showed significant differences between Ang2−/− and Ang2+/+ mice in the expression levels of the endothelial adhesion molecules. Overall, these data suggest that endogenous Ang2 transiently affects LPS-induced expression of endothelial adhesion molecules in an organ-specific manner.

Statistical analysis

Statistical significance of differences was studied by means of the Student’s t-test or analysis of variance with post hoc comparison using Bonferroni correction. All statistical analyses were performed using the GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA, USA). Differences were considered to be significant when P < 0.05.
Ang2-deficient mice display higher systemic and tissue cytokine levels upon LPS challenge

In humans injected with LPS and in sepsis patients, circulating levels of Ang2 positively correlate with systemic TNF-α levels [12] and IL-6 [25], respectively. To investigate a possible direct effect of Ang2 on LPS-induced pro-inflammatory cytokine release, we analysed systemic levels of pro-inflammatory cytokines (TNF-α, IL-6) and chemokines (KC, MIP-2). Eight hours after the LPS challenge, increased plasma levels of TNF-α, IL-6, KC and MIP-2 were detected in both Ang2−/− and Ang2+/+ mice compared with the control-treated mice. However, the extent of induction varied between Ang2−/− and Ang2+/+ mice. LPS-challenged Ang2−/− mice had significantly higher levels of TNF-α and IL-6 compared with Ang2+/+ mice (Figure 3A and B), whereas plasma levels of KC and MIP-2 were similar in both groups (Figure 3C and D). At the organ level, mRNA levels of TNF-α, IL-6, KC and MIP-2 were induced in both Ang2−/− and Ang2+/+ mice. However, we found that the expression of IL-6 mRNA in the kidneys of Ang2−/− mice was significantly higher compared with Ang2+/+ mice upon LPS challenge (Supplementary material Figure S1). Furthermore, significantly increased levels of IL-6 protein were detected in tissue homogenates of lungs, liver and kidneys of Ang2−/− mice 8 h after LPS injection compared with Ang2+/+ littermates (Supplementary material Figure S2). Taken together, these data demonstrate that ablation of Ang2 is related to an increase in the release of pro-inflammatory cytokines upon LPS challenge, suggesting that an increase of Ang2 during endotoxaemia might have a role in inhibiting an exaggerated release of cytokines.

Circulating neutrophils affect the release of cytokines upon LPS challenge

Previous studies have shown that in the absence of Ang2, leukocyte-endothelial interactions are impaired, leading to decreased neutrophil migration [18]. Corroborating previous studies, we observed that Ang2−/− mice had significantly less glomerular neutrophil influx compared with Ang2+/+ mice (Figure 4). Interactions between leukocytes and endothelial cells or other intrinsic tissue cells can regulate the release of cytokines [26–28]. Thus, we hypothesized that the increase of cytokine levels in Ang2-deficient mice might be due to decreased interactions between neutrophils and endothelial cells or other intrinsic tissue cells. To test this hypothesis, we depleted circulating neutrophils (which are the first invading inflammatory cells in the kidney upon LPS administration [22]) by injecting anti-neutrophil antibodies (NIMPR14) prior to LPS administration. Twenty-four hours after administration of the NIMP antibody, the mice were found to be severely neutropenic [22]. Eight hours after LPS administration, neutrophil-depleted mice had significantly elevated plasma levels of TNF-α compared with control IgG-treated mice. IL-6 levels showed a tendency toward increased levels in neutrophil-depleted mice, but this did not reach statistical significance (Figure 5).

Ang2-deficient mice develop AKI upon LPS challenge

To investigate the role of Ang2 in LPS-induced AKI, we measured BUN levels in plasma and albumin:creatinine ratios (ACRs) in urine of Ang2−/− and Ang2+/+ mice after LPS challenge. We observed a significant increase in BUN levels in Ang2−/− mice compared with Ang2+/+ mice (Figure 6A). Similarly, we found a significant increase in ACRs in Ang2−/− mice compared with Ang2+/+ mice (Figure 6B). These results suggest that ablation of Ang2 is related to the development of AKI upon LPS challenge.
FIGURE 2: Effects of Ang2 on mRNA expression of endothelial adhesion molecules in lungs, liver and kidneys upon LPS challenge. mRNA expression of P-selectin, E-selectin, VCAM-1 and ICAM-1 in lung, liver and kidney tissue was determined by quantitative RT-PCR. Black bars represent Ang2+/+ mice, white bars represent Ang2−/− mice. Values are mean ± SD of 5–7 mice. *P < 0.05 control group versus treated group; #P < 0.05 Ang2+/+ versus Ang2−/− mice.

FIGURE 3: Effects of Ang2 on cytokine production upon LPS challenge. Mouse plasma levels of TNF-α (A), IL-6 (B), KC (C) and MIP-2 (D) were determined by ELISA. Individual values and mean are shown for each group, n = 4–6 mice. *P < 0.05 control group versus treated group; #P < 0.05 Ang2+/+ versus Ang2−/− mice.
challenge. Eight hours after LPS challenge, increased BUN levels and ACRs were detected in both Ang2−/− and Ang2+/+ mice. However, LPS-challenged Ang2−/− mice had significantly higher levels of BUN and ACRs compared with Ang2+/+ mice (Figure 6A and B). No differences in BUN levels were detected at 24 h (Figure 6A). Similarly, increased renal NGAL mRNA levels were detected in both Ang2−/− and Ang2+/+ mice at 8 and 24 h after LPS challenge. At 8 h, NGAL mRNA levels were significantly higher in LPS-challenged Ang2−/− mice compared with their wild-type littermates (Figure 6C). These data suggest that the absence of Ang2 during LPS challenge leads to transient renal dysfunction.

DISCUSSION

In humans, high levels of circulating Ang2 are detected in various inflammatory conditions and have been associated with increased disease severity and mortality [10, 12, 17]. The exact functional role of increased Ang2 levels in disease conditions including sepsis is not yet well understood. The aim of the present study was to investigate the role of Ang2 in regulating vascular inflammation and vascular leakage, employing a well-characterized model of endotoxaemia in mice that lack Ang2 expression.
Upon LPS challenge, we found that expression levels of endothelial adhesion molecules in wild-type littermates were higher compared with the levels in Ang2-deficient mice. Interestingly, these differences were not observed in all organs analysed, suggesting that there is organ heterogeneity in response to LPS with regard to the expression of endothelial adhesion molecules in the absence of Ang2.

We observed that LPS-challenged Ang2-deficient mice had higher plasma cytokine levels compared with wild-type littermates, indicating a direct or indirect effect of circulating Ang2 on LPS-induced cytokine release. It has been reported that Ang2-deficient mice show decreased recruitment of neutrophils in the abdominal cavity after an intraperitoneal challenge with a pro-inflammatory stimulus [18]. Moreover, in the current study, we observed decreased glomerular infiltration of neutrophils in LPS-challenged Ang2-deficient mice. The impairment of neutrophil migration in Ang2-deficient mice could be due to the observed decrease in expression of endothelial adhesion molecules (Figure 2 and [18]). Moreover, previous research has shown that Ang2 can act as a chemottractant for neutrophils, a property that may have contributed to the observed effects as well [20]. Finally, our observation of decreased renal neutrophil infiltration is in agreement with a recent report demonstrating that endothelial overexpression of Ang2 promotes myeloid cell infiltration in a β2-integrin dependent manner [29]. Taken together, these data identify Ang2 as a factor that contributes to leukocyte recruitment in inflammatory conditions.

In neutrophil-depleted endotoxaemic mice, we found a marked increase in circulating TNF-α levels (Figure 5), an observation that is in line with previous reports [26–28, 30]. This exaggerated cytokine response suggests that neutrophils play an important role in regulating cytokine release in inflammatory conditions, although the underlying mechanisms are unclear. Taken together, we propose that the elevated plasma cytokine levels in LPS-challenged Ang2-deficient mice are due to the modulating effect of Ang2 on neutrophil recruitment. However, we cannot exclude that other pathways are affected by Ang2 as well. Therefore, additional experiments, including neutrophil depletion or systemic administration of (recombinant) Ang2 to LPS challenged Ang2-deficient mice, are necessary to establish a direct link between Ang2, neutrophil influx and cytokine production.

In our model, Ang2-deficient mice were not protected from acute renal injury. These results are in line with data reported by Hegeman et al. [31] who demonstrated that changing the Ang/Tie2 balance toward Ang1 in ventilator-induced lung injury does not prevent vascular leakage despite inhibition of inflammation. Although the exact mechanisms underlying the dissociation between inflammation and acute renal injury are unknown, several possibilities can be considered. First, a previous study has indicated a direct, inflammation-independent effect of LPS on the kidney barrier function, demonstrating that induction of urokinase receptor signaling in podocytes causes foot process effacement and proteinuria via a mechanism that includes activation of alphavbeta3 integrins [32]. To our knowledge, such a mechanism is not modulated by the Ang/Tie2 system. Secondly, other vascular receptor tyrosine kinase systems controlling vessel integrity and permeability are likely to be involved as well, in particular the VEGF system. It is well known that either deletion or overexpression of VEGF causes kidney

**Figure 6:** Effects of Ang2 on renal function after LPS challenge. Renal function was assessed by measuring plasma BUN (A) and ACR in urine (B). Additionally, renal NGAL mRNA levels were measured by quantitative PCR (C). BUN, ACR and renal NGAL mRNA levels were elevated in Ang2−/− mice compared with Ang2+/+ 6 h after LPS challenge. Individual values and mean are shown for each group. *P < 0.05 control group versus treated group; #P < 0.05 Ang2+/+ versus Ang2−/− mice.
barrier function impairment [33], indicating that tight regulation of VEGF expression is necessary to maintain proper renal function. As a first step to investigate the involvement of the VEGF system in our model, we analysed the expression of VEGF-A mRNA (Supplementary material Figure S3). There was no difference in VEGF-A mRNA expression between wild-type littermates and Ang2-deficient mice after LPS challenge.

Our studies have some limitations that need to be taken into account when interpreting the data. The first limitation concerns the fact that Ang2-deficient mice on a C57BL/6J background already display some abnormalities including lymphatic abnormalities [23]. Although Ang2-deficient mice on a C57BL/6J background survive to adulthood [23], the abnormalities of the lymphatic system may have contributed to the observed AKI [34] in these mice upon LPS challenge. Secondly, since we used conventional (i.e. non-conditional) Ang2-deficient mice, Ang2 has been lacking throughout development. We cannot exclude that mechanisms have evolved in these mice to compensate for the absence of Ang2. Finally, although LPS injection is a model with limited resemblance to human sepsis, we chose to use this model based on the fact that this model is highly standardized and frequently used. This standardization makes the results reproducible and allows comparison with published research.

In conclusion, the absence of Ang2 release upon LPS challenge can reduce endothelial activation with regard to the expression of endothelial adhesion molecules but leads to an increase in pro-inflammatory cytokines. Our initial hypothesis was that deletion of Ang2 would rescue renal function in an endotoxaemia model. However, as shown in our study and by others [35], the effect of whole body, non-conditional ablation of Ang2 has many effects including modulation of inflammation (neutrophil influx, cytokine production), endothelial cell activation and abnormalities in lymphatic vessel density. The result of all these effects is a transient decrease in renal function in our rodent LPS model in the absence of Ang2.

These studies suggest that influencing the Angiopoietin/Tie2 system in sepsis patients should not only focus on completely depleting or blocking Ang2 activities, but also on increasing Ang1 levels by recombinant protein or with designer peptides mimicking Ang1 [36]. Studies that focus on restoring the increased Ang2 levels to baseline or a combination of drugs intervening in the Ang/Tie2 system may be important to maximize the drug effects in the treatment of kidney dysfunction during sepsis.

CONFLICT OF INTEREST STATEMENT

None declared.


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SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.


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