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## Angiopoietins in renal replacement therapy

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# CHAPTER

# 8

Modulation of the angiotensin/  
Tie2-system does not prevent brain  
death induced kidney injury

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## ABSTRACT

### *Background*

Organs derived from deceased brain dead (DBD) donors show worse function than those derived from living donors, possibly due to the brain dead-induced inflammatory response. Our study aimed to evaluate if modulating the Ang/Tie2-system in favor of angiotensin-1 (Ang1) and inhibiting angiotensin-2 (Ang2) would lead to a decrease in brain death-induced inflammation in brain dead (BD) rats.

### *Methods*

In an experimental model, rats (n=7 per group) were either exposed to 4hr of brain death or a sham-operation. BD was induced in anesthetized and ventilated rats by inflating a subdurally placed balloon catheter. rhAng1 or the synthetic Tie2 receptor agonist Vasculotide (VT) as well as the Ang2 inhibiting peptibody AMG386, or vehicle were administered 30 min prior to BD induction. After four hours of BD, serum, kidney and lung tissues samples were collected and stored. Routine biochemistry, RT-qPCR, and immunohistochemistry were performed.

### *Results*

Plasma creatinine levels were higher in BD rats compared to the sham-operated rats. Plasma injury markers were increased after BD induction compared to the sham-operated rats ( $p < 0.001$ ). A clear increase in influx of PMNs was observed in renal tissue in BD animals. rhAng1, VT and AMG386 treatment did not show a protective effect on functional plasma levels or renal mRNA expression of injury markers when compared to controls. Tie2 mRNA expression was however significantly decreased in BD rats ( $p = 0.01$ ), which was not affected by rhAng1, VT or AMG386 treatment.

### *Conclusion*

This rat study failed to demonstrate a beneficial effect of rhAng1, VT or AMG386 in experimental BD. A remarkable down regulation of renal Tie2 was observed in the BD rats which justifies further studying the Ang/Tie2-mechanisms and Tie2 preserving strategies in BD.

## INTRODUCTION

In patients with end-stage renal disease (ESRD) renal transplantation is the preferred replacement therapy as it is associated with better outcomes and results in superior quality of life and reduced mortality compared to dialysis<sup>1-3</sup>. In renal transplantation, grafts are retrieved from living, deceased brain death (DBD) and deceased cardiac death (DCD) donors. Worldwide most donor kidneys are derived from DBD donors. Although mechanistically not fully elucidated, it is commonly accepted that the pathophysiological processes initiated by brain death are responsible for the inferior quality and survival of these donor kidneys compared to those from living related donors<sup>4,5</sup>. Experimental and clinical studies have shown that brain death (BD) causes hemodynamic instability, hormonal dysregulation, a marked inflammatory response and various other processes<sup>6-8</sup>. BD induces an immediate procoagulatory and pro-inflammatory activation of vascular endothelium together with inflammatory responses in potential donor organs<sup>9-11</sup>. Activated endothelium disrupts the constitutive Angiopoietin/Tie2-signaling, an important system in maintaining vascular quiescence<sup>12</sup>.

Angiopoietins are natural ligands of the tyrosine kinase receptor Tie2 and are important mediators of angiogenesis and maintenance of vascular integrity<sup>13</sup>. Of the angiopoietin family, angiopoietin-1 (Ang1) to -4, Ang1 and Ang2 are best characterized<sup>14,15</sup>. Both angiopoietins bind Tie2 in a competitive manner with similar affinity<sup>16,17</sup>. The Tie2 receptor is primarily expressed on endothelial cells and early hematopoietic stem cells. Angiopoietin/Tie2-signaling cascades are next to angiogenesis involved in vascular stabilization and remodeling, as well as recruitment of pericytes and smooth muscle cells<sup>14,18</sup>. In healthy adults, Ang1 is expressed by pericytes and vascular smooth muscle cells at relatively constant levels and acts in a paracrine agonistic manner maintaining the Tie2 receptor in an activated state<sup>19</sup>. Ang1-mediated Tie2 phosphorylation provides an anti-apoptotic and anti-inflammatory signal to the endothelium, thereby leading to vessel stabilization and preventing vascular leakage<sup>14,20-22</sup>. Ang2 is highly produced and expressed by endothelial cells at sites of normal and pathological angiogenesis<sup>23</sup>. In contrast, Ang2 acts as an autocrine antagonist of Ang1-mediated Tie2 activation, competitively inhibiting Tie2 signal transduction<sup>24,25</sup>. Thereby, inflammatory responsiveness and vascular leakage increases and endothelial function impairs<sup>16</sup>. Ang2 can prime the endothelium to respond to cytokines and other inflammatory mediators<sup>25-27</sup>. Little is known about the exact mechanisms of Ang2 function on Tie2 and some studies presented Ang2 as a partial agonist, activating Tie2<sup>16,18,23</sup>. Endothelial storage granules, Weibel Palade bodies (WPB), store Ang2 and quickly release it into the systemic circulation upon pro-inflammatory stimulation<sup>27-29</sup>. It impairs endothelial function and increases inflammatory responsiveness and induces vascular leakage<sup>30</sup>. Thereby Ang2 primes the vascular endothelium to

respond to exogenous cytokines leading to vascular destabilization. Given the endothelial activation, inflammatory environment and hemodynamic instability in DBD donors, appropriate pretreatment of the DBD donor to counteract the endothelial destabilization by enhancing Ang1 mediated Tie2 phosphorylation or inhibiting the antagonistic properties of Ang2, may therefore be a tool to improve donor organ quality and subsequently transplant outcome.

In human sepsis, which has pathophysiological similarities to brain death, the Ang/Tie2- system has been shown to play a critical role<sup>31</sup>. Ang2 serum levels are increased up to 20fold during sepsis, which have also been associated with mortality<sup>32</sup>. Counteracting endothelial activation and the inflammatory response in the DBD donor by inhibiting Ang2 or enhancing Ang1-mediated Tie2 signaling may be of therapeutical value to improve donor organ quality and subsequently transplant outcome. In acute kidney and lung injury treatment with either acute administration of recombinant human Ang1 (rhAng1) or Ang1 gene transfer prevented capillary leakage and was shown protective<sup>33-35</sup>. Furthermore, Ang1 and an Ang1 variant prevented vascular permeability in experimental endotoxemia<sup>36,37</sup>, improved survival in endotoxic shock<sup>38</sup>. In human sepsis the Ang/Tie2-system has also been shown to play a critical role<sup>39</sup> and in murine sepsis, administration of rhAng1 improved survival and sepsis- associated organ dysfunctions<sup>33</sup>. Vasculotide (VT), a synthetic Tie2 receptor agonist, counteracted microvascular endothelial dysfunction in murine abdominal sepsis<sup>40</sup>. The frequent occurrence of endotoxemia in brain death donors makes Ang1 an interesting candidate to improve graft quality in DBD donors as well<sup>41</sup>. Several preclinical and clinical studies have investigated anti-Ang2 therapy in the treatment of chronic rejection in rat cardiac allografts, malignant tumors including monoclonal antibodies and siRNA<sup>42</sup>. None of them is evaluated in the setting of the DBD donor.

Therefore, in this translational study we investigated whether modulating the Ang/Tie2- system in favor of Ang1 would exert protective effects on the kidney during BD. Furthermore, we explored if inhibiting Ang2-mediated Tie2 signaling and would diminish the brain death-induced endothelial activation and inflammatory response. We hypothesize that rhAng1, VT or the Ang2 inhibiting peptibody AMG386 could preserve renal graft quality and function in an experimental brain dead rat model.

## METHODS

### *Animals*

Adult male Fisher F344 rats weighing 250 - 300 g (Harlan, Horst, the Netherlands) were used in all experiments. For a subanalysis C57Bl/6 wildtype (WT), and C5aR<sup>-/-</sup><sup>43</sup> and C5L2<sup>-/-</sup><sup>44</sup> mice were used both on C57Bl/6 background, kindly provided by B. Lu, Harvard Medical School, Boston, USA. Both rats and mice were housed in a light- and temperature-controlled environment and had free access to food and water. Mice

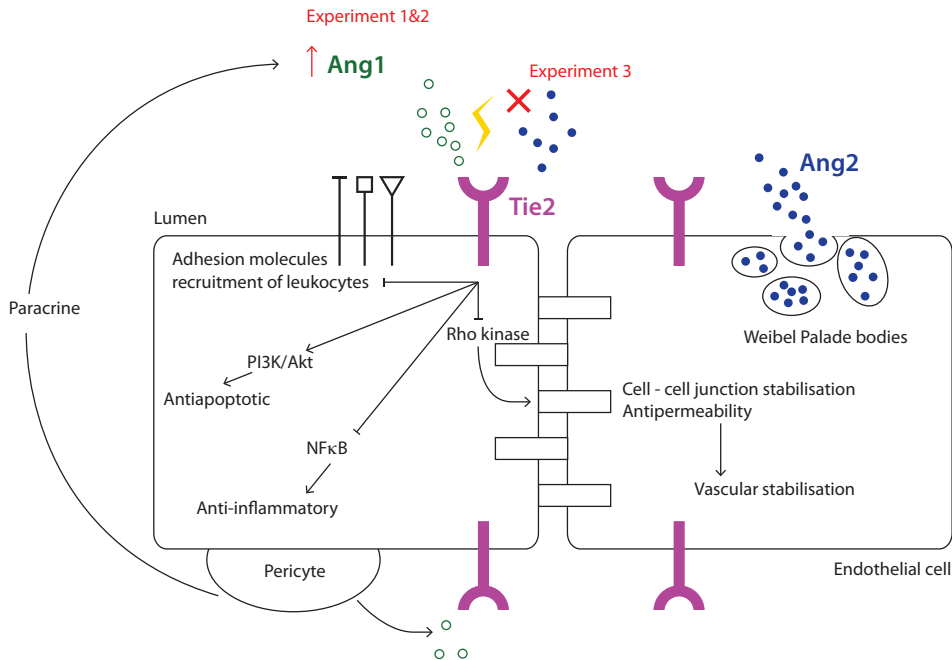
were bred in the local animal facility in the University Medical Center Groningen. The studies were carried out under a protocol approved by the Institutional Animal Care Committee of the University of Groningen (project numbers 6259ABC and 6279AF). All animals received care in compliance with the guidelines of the local animal ethics committee according to Experiments on Animals Act (1996) issued by the Ministry of Public Health, Welfare and Sports of the Netherlands.

### Study design

We used a two-step design to minimize the number of animals needed. Each experimental group consisted of 7 animals (table 1). In the first two experiments the aim was to increase the Ang1 availability and the third to inhibit the pro-inflammatory Ang2 properties (figure 1). In experiment 1, rats were randomly divided into four groups to study the effect of rhAng1 on BD and sham-operated animals. The number of animals was calculated using the method of Russ Lenth<sup>45</sup> with a meaningful difference of 50%, a variability ( $\sigma$ ) of 0.3 and a power of 0.9. Sham-operated rats served as controls and were ventilated for half an hour under anesthesia before termination. This was in accordance with the requirement of the local Animal Welfare Committee guidance for the use of sham controls in experiments. BD rats were sacrificed 4 h after BD induction. rhAng1 (R&D systems, Minneapolis, USA), or saline was administered IV 30 minutes before the start of BD induction (experiment 1). In a subsequent experiment, Vasculotide (VT)<sup>40</sup>, or PBS was administered IV 30 minutes before the start of BD induction (experiment 2). Dosages of rhAng1 (1  $\mu\text{g}/\text{kg}$ ) and VT (3  $\mu\text{g}/\text{kg}$ ) were based on previous experiments in rodents<sup>40,46</sup>. Vasculotide was provided by Sunnybrook Health Sciences Center, Toronto, Canada. In the third experiment, the anti-Ang2 antibody AMG386 (2.8 mg/kg subcutaneously, Amgen Inc., Thousand Oaks, USA) treatment was compared to saline treatment, both administered IV 30 minutes before the start of BD induction.

**Table 1.** Study design

Experiments	Group	Treatment	Number of animals
1	Sham-operated	Saline	7
	Sham-operated	1 $\mu\text{g}/\text{kg}$ rhAng1 in saline	7
	Brain dead	Saline	7
	Brain dead	1 $\mu\text{g}/\text{kg}$ rhAng1 in saline	7
2	Brain dead	PBS	7
	Brain dead	3 $\mu\text{g}/\text{kg}$ VT in PBS	7
3	Brain dead	Saline	7
	Brain dead	2.8 mg/kg AMG386 in saline	7



**Figure 1.** Overview of the Angiopoietin/Tie2-system at the endothelium. Adapted from van Meurs et al. Crit Care. 2009;13(2):207. Bench-to-bedside review: Angiopoietin signalling in critical illness - a future target? Experiment 1 & 2 were designed to increase Ang1 availability via rhAng1 and Vasculotide. In experiment 3 anti-Ang2 therapy of AMG386 was tested. Abbreviations: Ang1: angiopoietin-1, Ang2: angiopoietin-2, Tie2: tyrosine kinase receptor Tie2, NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells.

### Brain death induction

Brain death (BD) was induced as follows: animals were anesthetized using 5% isoflurane with 100% O<sub>2</sub>. Cannulas were inserted in the femoral artery and vein for continuous mean arterial pressure (MAP) monitoring and fluid administration. Animals were intubated via a tracheostomy and ventilated throughout the experiment. A no. 4 Fogarty catheter (Edwards Lifesciences Co, Irvine, CA) was placed in the epidural space through a frontolateral burr hole, and slowly inflated (0.16 ml/min) with saline using a syringe pump (Terufusion, Termo Co, Tokyo, Japan). Inflation of the balloon was terminated once the MAP increased rapidly again after a characteristic period of hypotension, reflecting the autonomic storm. BD was confirmed by the absence of corneal and pupillary reflexes and a positive apnea test. Following confirmation of BD, anesthesia was terminated but ventilation continued. MAPs were maintained above 80mmHg using Hydroxyethyl starch (HAES) 10% (Fresenius Kabi AG, Bad Homburg, Germany) with a maximum rate of 1 ml/hr. If HAES was insufficient to maintain the MAP, noradrenaline 0.01 mg/ml was administered. To

maintain body temperature of the rats, a homeothermic blanket control system was used throughout the BD maintenance period. To achieve full muscle relaxation for abdominal surgery, rocuroniumbromide (0.6 mg/kg) was administered 15 minutes before the end of the brain death period of four hours. Five minutes before ending the brain death period, rats were heparinized with 500 IU heparin. A laparotomy was subsequently performed and blood was collected from the aorta. Organs were flushed with 0.9% saline and snap frozen in liquid nitrogen, another part was fixated in 4% paraformaldehyde. Collected plasma was stored at  $-80^{\circ}\text{C}$ .

#### *Plasma measurements*

Plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine and urea were measured in a routine fashion by the biochemistry lab of the University Medical Center Groningen (Mega, Merck, USA). Plasma levels of Ang2, Tie2 and IL-6 were determined by rat enzyme linked immunosorbent assays (ELISA) according to manufacturers' instructions (R&D Systems, Minneapolis, USA). All samples were analyzed in duplicate and read at 450 nm using a microplate spectrophotometer (Victor3, 1420 multi-label counter, Perkin Elmer, USA).

#### *RNA isolation and cDNA synthesis*

Total RNA was isolated from whole kidneys sections by using TRIzol (Life Technologies, Gaithersburg, MD). RNA samples were verified for absence of genomic DNA contamination by performing RT-PCR reactions in which the addition of reverse transcriptase was omitted, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. For cDNA synthesis, 1  $\mu\text{l}$  T11VN Oligo-dT (0.5  $\mu\text{g}/\mu\text{l}$ ) and 1  $\mu\text{g}$  mRNA were incubated for 10 min at  $70^{\circ}\text{C}$  and cooled directly after that. cDNA was synthesized by adding a mixture containing 0.5  $\mu\text{l}$  RnaseOUT Ribonuclease inhibitor (Invitrogen, Carlsbad, USA), 0.5  $\mu\text{l}$  RNase water (Promega), 4  $\mu\text{l}$  5 x first strand buffer (Invitrogen), 2  $\mu\text{l}$  DTT(Invitrogen), 1  $\mu\text{l}$  dNTPO's and 1  $\mu\text{l}$  MLV reverse transcriptase (Invitrogen, 200U). The mixture was held at  $37^{\circ}\text{C}$  for 50 min. Next, reverse-transcriptase was inactivated by incubating the mixture for 15 min at  $70^{\circ}\text{C}$ . Samples were stored at  $-20^{\circ}\text{C}$ .

#### *Real-Time PCR on renal tissue*

Fragments of several genes were amplified with the primer sets outlined in table 2. Pooled cDNA obtained from brain-dead rats were used as internal references. Gene expression was normalized with the mean of  $\beta$ -actin mRNA content. For each gene the expression was normalized relative to the mean CT value of the  $\beta$ -actin gene, using the  $\beta$ -actin signal from the same cDNA. Real-Time PCR was carried out in reaction volumes of 15  $\mu\text{l}$  containing 10  $\mu\text{l}$  of SYBR Green mastermix (Applied Biosystems, Foster City, USA), 0.4  $\mu\text{l}$  of each primer (50  $\mu\text{M}$ ), 4.2  $\mu\text{l}$  of nuclease free



**Table 2.** Primer sequences used

Gene	Primer sequences	Accession number	Amplicon size (bp)
$\beta$ -actin	5'-GGAAATCGTGCGTGACATTA-3' 5'-GCGGCAGTGGCCATCTC-3'	NM_031144.2	74
Angiotensin-1	5'-TTGGGAATCCCTCTGGTGAAT-3' 5'-CTGCCTCTGACTGGTTATTGCA-3'	NM_053546.1	67
Angiotensin-2	5'-TATGAGAAGGCCATGGTTAGAATC-3' 5'-CGTAACATTCTTGCTTGACAGA-3'	XM_344544.3	70
Tie2	5'-AAGAGCAGCAGGGAAGAGGG-3' 5'-TGGAATGATTTGGATGCTGTAGAA-3'	NM_001105737.1	71
IL-6	5'-CCAACCTCCAATGCTCTCCTAATG-3' 5'-TTCAAGTGCTTTCAAGAGTTGGAT-3'	NM_012589	89
TNF- $\alpha$	5'-AGGCTGTCGCTACATCACTGAA-3' 5'-TGACCCGTAGGCGCATTACA-3'	NM_012675.2	67
KIM-1	5'-AGAGAGAGCAGGACACAGGCTTT-3' 5'-ACCCGTGGTAGTCCCAAACA-3'	NM_173149.1	75
ICAM-1	5'-CCAGACCCTGGAGATGGAGAA-3' 5'-AAGCGTCGTTTGTGATCCTCC-3'	NM_012967.1	251
HO-1	5'-CTCGCATGAACACTCTGGAGAT-3' 5'-GCAGGAAGCGGTCTTAGC-3'	NM_012580.2	74

water and 10 ng of cDNA. All samples were analyzed in triplicate. Thermal cycling was performed on the Taqman Applied Biosystems 7900HT Real Time PCR System with a hot start for 2 min at 50°C followed by 10 min 95°C. Second stage was started with 15 s at 95°C (denaturation step) and 60s at 60°C (annealing step and DNA synthesis). The latter stage was repeated 40 times. Stage 3 was included to detect formation of primer dimers (melting curve) and begins with 15s at 95°C followed by 60 s at 60°C and 15 s at 95°C. Primers were designed with Primer Express software (Applied Biosystems, Foster City, USA) and primer efficiencies were tested by a standard curve for the primer pair resulting from the amplification of serially diluted cDNA samples (10 ng, 5 ng, 2.5 ng, 1.25 ng and 0.625 ng) obtained from brain-dead rats. Specificity of qPCR products was routinely assessed by performing a dissociation curve at the end of the amplification program and by gel electrophoresis on a 1.5% agarose gel. PCR efficiency was found to be  $1.8 < \epsilon < 2.0$ . Gene expression was normalized with the mean of  $\beta$ -actin mRNA content and calculated relative to controls using the relative standard curve method. Results were finally expressed as  $2^{-\Delta\Delta CT}$  (CT: Threshold Cycle), which is an index of the relative amount of mRNA expressed in each tissue.

#### *HIS-48 staining on renal tissue cryosections*

The polymorphonuclear cell (PMN) count was used as a marker of inflammation. To detect PMNs in the kidney, immunohistochemistry was performed on 5  $\mu$ m renal tissue

cryosections of experiment 1. Sections were fixed for 10 minutes using acetone. Next, sections were stained with HIS-48 mAb (supernatant, two times diluted) using an indirect immunoperoxidase technique. Endogenous peroxidase was blocked using  $H_2O_2$  0.01% in PBS for 30 minutes. After thorough washing, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG as a secondary antibody for 30 minutes followed by goat anti-rabbit IgG as a tertiary antibody for 30 minutes (both from Dako, Glostrup, Denmark). The reaction was developed using 9-amino-ethylcarbazole as chromogen and  $H_2O_2$  as substrate. Sections were counterstained using Mayers' hematoxylin solution (Merck, Darmstadt, Germany). Negative antibody controls were performed. Localization of immunohistochemical staining was assessed by light microscopy. For each tissue section, positive cells were counted in 10 microscopic cortical fields of the glomerulus at 20x magnification. Results were presented as number of positive cells per cortical area ( $\mu m^2$ ).

#### *Quantification of Tie2 protein by Western Blot analysis*

To gain further insight in the Tie2 availability on protein level, we tried several techniques such as an ELISA and Western Blot (WB) analysis total Tie2. Unfortunately we experienced technical difficulties using the rat kidneys of our above-mentioned experiments leading to irreproducible, invalid results. In order to give an approximation of the Tie2 protein level in brain death compared to controls, we used BD and sham-operated mouse kidneys of another experiment. In brief, C57Bl/6 male mice (wildtype,  $C5aR^{-/-}$ , and  $C5L2^{-/-}$ ) aged 8 to 12 weeks, with a weight of 25-28 grams were used. Per mouse strain, 8 animals were subjected to 3h of brain death as described above. During brain death, a MAP above 60 mmHg was considered to be normotensive. To maintain stable blood pressure, 50  $\mu l$  saline with lepirudin was administered every 15 minutes via the jugular vein cannula. When blood pressure dropped below 60 mmHg, an additional 50  $\mu l$  of saline was administered. A maximum of 1200  $\mu l$  of saline was administered during the 3 hours brain death period. 30 minutes after declaration of brain death, ventilation was switched to a mixture of oxygen and medical air (50%-50%). Body temperature was continuously monitored and maintained at 37°C as described above. At time of sacrifice, blood and kidneys were collected for analysis. For total Tie2 WB analysis, kidneys of two experimental groups (sham+saline, n=3 and BD+saline, n=3) were homogenized in RIPA buffer (150mM NaCl, 50mM Tris pH 8.0, 0,5% Na-deoxycholate, 0,1% SDS, 1% IGEPAL) supplemented with protease and phosphatase inhibitors (Roche, Almere, The Netherlands) and 1 mM  $Na_3VO_4$ . Protein concentrations were determined by DC Protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

#### *Statistical analysis*

For statistical analyses of more than two groups, the Kruskal-Wallis test was performed, followed by the Mann-Whitney posttest. For comparison of two

groups, a Mann-Whitney test was performed. Results are presented as mean  $\pm$  SEM (standard error of the mean). All the statistical tests were 2-tailed with  $p < 0.05$  regarded as significant. Analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, USA).

## RESULTS

### *Brain death experiments*

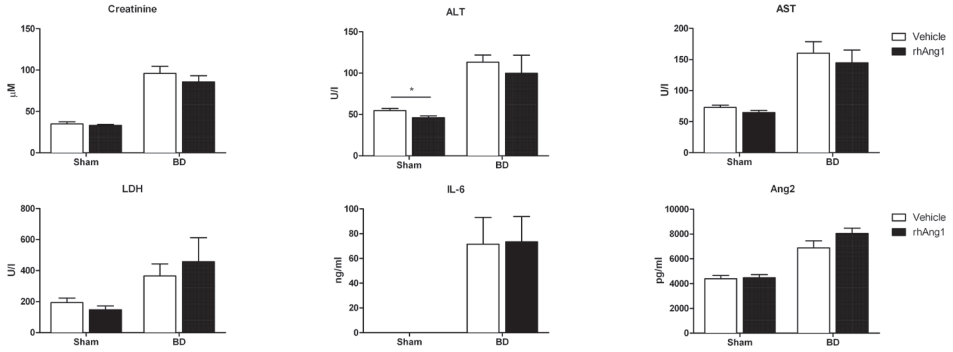
Induction of BD showed a consistent and uniform pattern in MAP alterations as described before<sup>47</sup> and took approximately 30 minutes after commencing balloon inflation. During the experiments, all animals were successfully kept at a MAP of at least 80 mmHg after the confirmation of BD. In experiment 1, saline treated animals required  $4.1 \text{ ml} \pm 1.1$  infusion of HAES to maintain MAP whereas in the rhAng1 treated group  $3.7 \text{ ml} \pm 0.3$  was needed (NS). Additional hemodynamic support by administration of noradrenaline also not differed between the groups ( $1.2 \text{ ml} \pm 0.6$  in the saline treated group vs.  $1.2 \text{ ml} \pm 0.3$  in the rhAng1 treated group). In experiment 2, in both the PBS and VT treated group  $1.4 \text{ ml} \pm 0.5$  infusion of HAES was needed. In the PBS treated group,  $1.8 \text{ ml} \pm 1.3$  of noradrenaline vs.  $2.3 \text{ ml} \pm 2$  in the VT treated group was needed. In the saline treated group of experiment 3 infusion of  $1.1 \text{ ml} \pm 0.7$  HAES infusion was needed to maintain MAP whereas in the AMG386 treated group  $1.2 \text{ ml} \pm 0.5$  was required. Administration of noradrenaline was  $0.9 \text{ ml} \pm 0.9$  in the saline treated group vs.  $1.7 \text{ ml} \pm 1.6$  in the AMG386 treated group.

### *Plasma biochemistry after brain death*

Plasma levels of kidney function and injury markers are demonstrated in figure 2, (experiment 1), 3 (experiment 2) and 4 (experiment 3). Plasma creatinine levels were significantly higher in BD animals treated with saline ( $96 \pm 8.6 \text{ } \mu\text{mol/L}$ ) compared to  $35 \pm 2.7 \text{ } \mu\text{mol/L}$  in saline treated sham-operated animals ( $p = 0.0006$ ). Creatinine levels in BD animals treated with rhAng1 or VT did not significantly differ from BD animals treated with a vehicle.

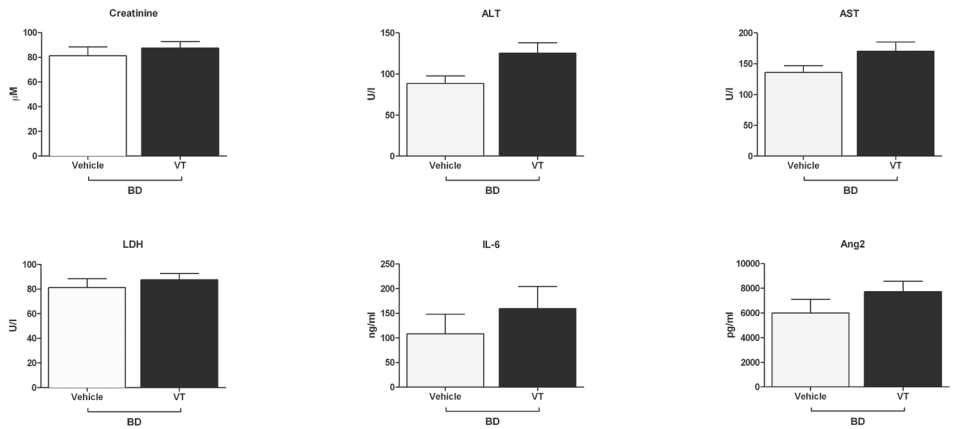
Plasma ALT, AST and LDH were significantly increased after BD induction and saline treatment compared to saline treated sham-operated animals (ALT:  $113 \pm 8.7$  vs.  $55 \pm 2.5 \text{ U/l}$ ,  $p = 0.0006$ ; AST:  $160 \pm 18.7$  vs.  $73 \pm 3.7 \text{ U/l}$ ,  $p = 0.006$ ; LDH:  $366 \pm 77.8$  vs.  $194 \pm 29.2 \text{ U/l}$ ,  $p = 0.04$ ). Plasma Ang2 and IL-6 were significantly increased in the saline treated BD group compared to the sham-operated animals treated with saline (Ang2:  $6878 \pm 556$  vs.  $4387 \pm 273 \text{ pg/ml}$ ,  $p = 0.006$ ; IL-6:  $71 \pm 21.8 \text{ ng/ml}$  vs. undetectable). Treatment with rhAng1 or VT did not significantly affect these plasma levels compared to their controls. In the third experiment, AMG386 treatment did not affect plasma creatinine levels (saline:  $75.5 \pm 5.1 \text{ } \mu\text{M}$  vs AMG386:  $72.5 \pm 2.2 \text{ } \mu\text{M}$ ). Also, ALT, AST and LDH levels did not differ between treatment

and controls. Plasma IL-6 and Ang2 were increased in the AMG386-treated group (saline:  $197.2 \pm 53.8$  vs AMG386  $392.1 \pm 60.1$  pg/ml,  $p=0.03$  and saline:  $5621 \pm 599.7$  vs AMG386:  $20234 \pm 375.1$  pg/ml,  $p < 0.001$  respectively).

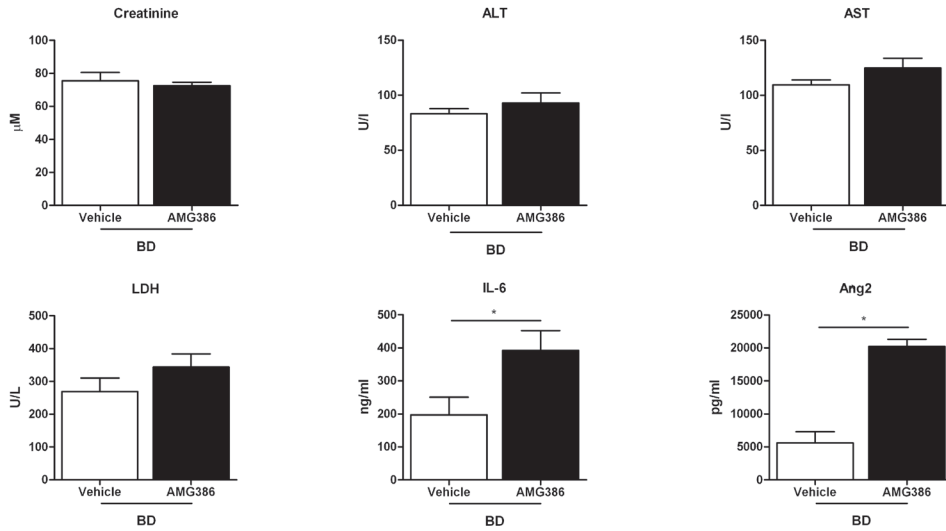


**Figure 2.** Plasma levels of renal function and cellular injury markers of experiment 1, administering rhAng1 (1 µg/kg) to sham-operated and BD rats. Data of 7 animals are presented as mean±SEM after 4h of brain death. Sham-operated animals and/or treatment with a vehicle, saline, represent the controls. Plasma levels were not affected by rhAng1 treatment ( $p > 0.05$ ).

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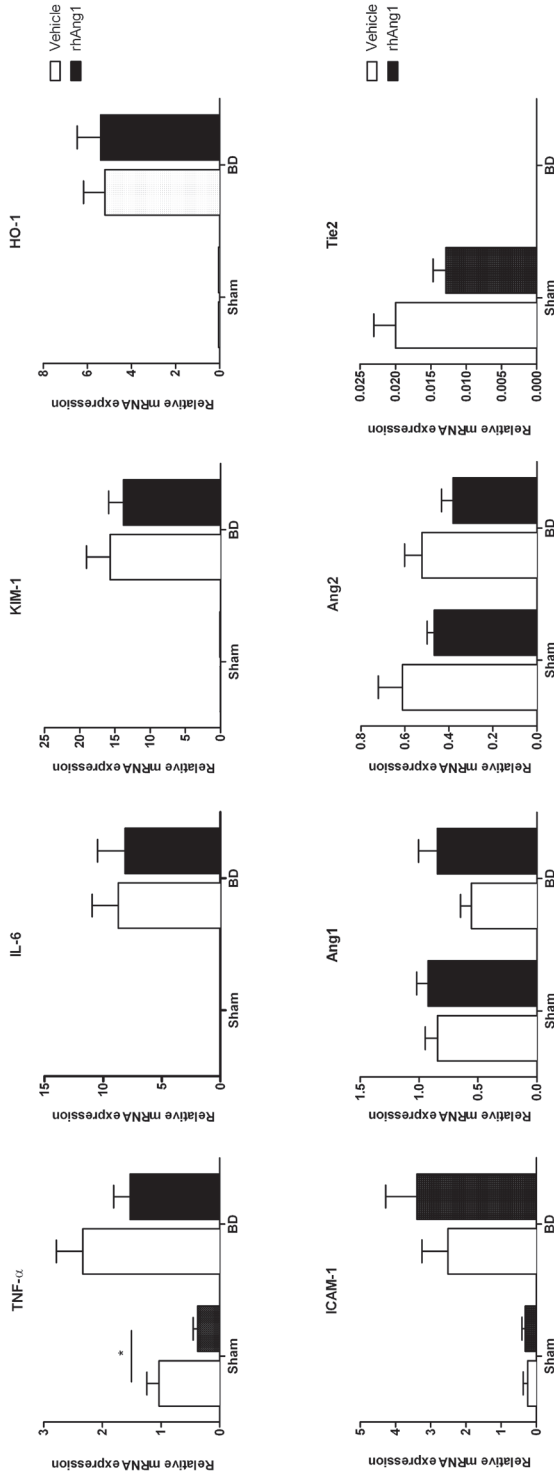
**Figure 3.** Plasma levels of renal function and cellular injury markers of experiment 2, administering VT (3 µg/kg) to BD rats. Data of 7 animals are presented as mean±SEM after 4h of brain death. BD animals treated with a vehicle, PBS, represent the controls. Plasma levels were not affected by VT treatment ( $p > 0.05$ ).



**Figure 4.** Plasma levels of renal function and cellular injury markers of experiment 3, administering anti-Ang2 antibody AMG386 (2.8 mg/kg) to BD rats. Data of 7 animals are presented as mean±SEM after 4h of brain death. BD animals treated with a vehicle, saline, represent the controls. Plasma levels of IL-6 and Ang2 were increased in the AMG386-treated group ( $p < 0.001$ ).

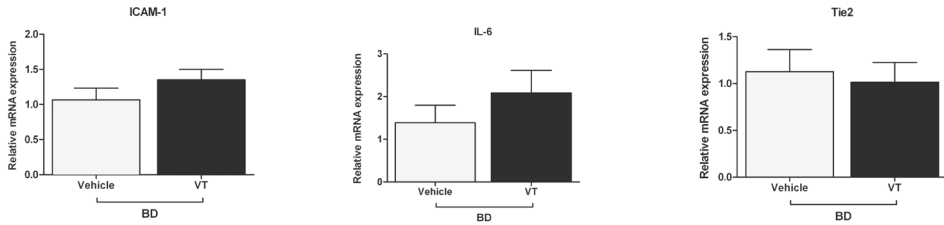
### Renal gene expression

As demonstrated in figure 5, quantitative RT-PCR of experiment 1 showed significantly increased gene expression of TNF- $\alpha$ , IL-6, KIM-1, HO-1 and ICAM-1 in kidneys as a consequence from BD treated with saline compared to sham-operated controls treated with saline (TNF- $\alpha$ :  $2.3 \pm 0.5$  vs.  $1 \pm 0.2$ , IL-6:  $8.7 \pm 2.2$  vs.  $0.02 \pm 0.007$ , KIM-1:  $16 \pm 3.4$  vs.  $0.04 \pm 0.009$ , HO-1:  $5.2 \pm 1.0$  vs.  $0.05 \pm 0.006$ , ICAM-1:  $0.70 \pm 0.2$  vs.  $0.07 \pm 0.04$ ,  $p < 0.05$ ). In the sham-operated animals, renal gene expression of TNF- $\alpha$  was down-regulated by rhAng1 treatment compared to saline ( $0.38 \pm 0.08$  vs.  $1 \pm 0.2$ ,  $p = 0.03$ ). Relative renal gene expression of Ang1 and Ang2 did neither significantly differ between BD and sham-operated animals nor between saline and rhAng1 treated animals. Tie2 gene expression was significantly decreased in BD induced animals compared to sham-operated animals. rhAng1 administration did not influence Tie2 gene expression. In the second experiment administering VT or PBS to BD induced rats, renal ICAM-1, IL-6 and Tie gene expression were measured (figure 6). Their gene expression was not affected by VT treatment. Quantitative RT-PCR of experiment 3 did not show a significant different gene expression of Ang1, Ang2, Tie2, IL-6 or ICAM-1 in kidneys as a consequence from BD rats treated with AMG386 compared to controls (figure 7).

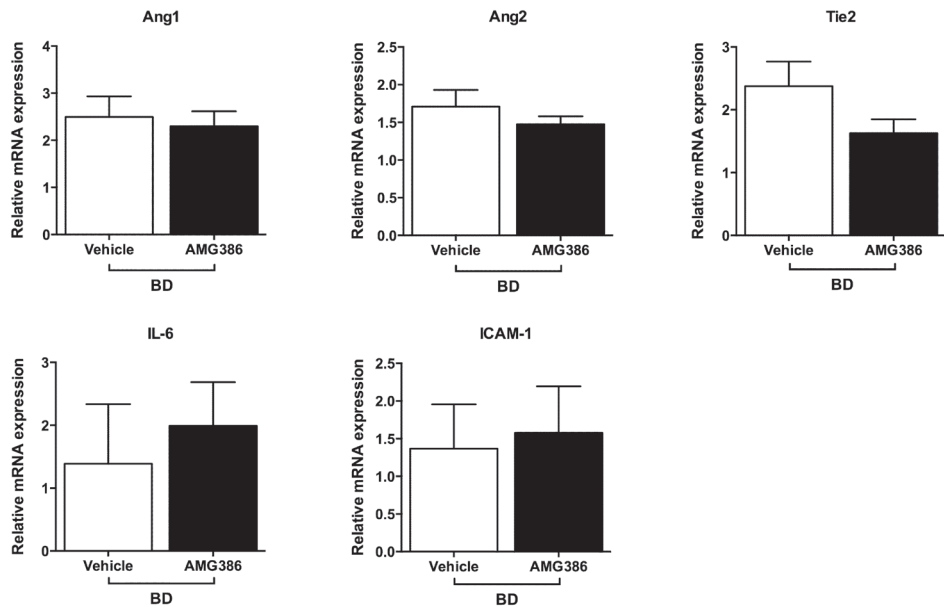


**Figure 5.** Relative renal gene expression of experiment 1 administering rhAng1 (1  $\mu$ g/kg) to sham-operated and BD rats. Renal mRNA levels of 7 animals per group presented as mean $\pm$ SEM after 4 h of brain death. TNF- $\alpha$  decreased in the rhAng1 treated sham-operated animals compared to the saline treated sham-operated animals ( $p=0.03$ ). Relative expression of all genes was increased after BD induction ( $p<0.05$ ) except for Tie2, which was markedly reduced ( $p<0.05$ ). mRNA levels were normalized to the  $\beta$ -actin signal of the same cDNA.





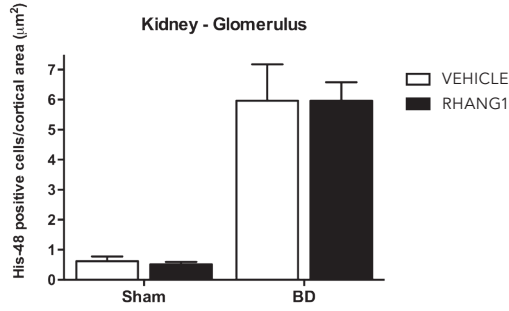
**Figure 6.** Relative renal gene expression of experiment 2 administering VT (3 µg/kg) to BD rats. Renal mRNA levels of 7 animals per group presented as mean±SEM after 4h of brain death. Renal ICAM-1, IL-6 and Tie2 mRNA expression was not affected by VT treatment in BD rats ( $p>0.05$ ). mRNA levels were normalized to the  $\beta$ -actin signal of the same cDNA.



**Figure 7.** Relative renal gene expression of experiment 3, administering anti-Ang2 antibody AMG386 (2.8 mg/kg) to BD rats. Renal mRNA levels of 7 animals per group presented as mean±SEM after 4h of brain death. Renal Ang1, Ang2, Tie2, IL-6 and ICAM-1 mRNA expression was not affected by AMG386 treatment in BD rats ( $p>0.05$ ). mRNA levels were normalized to the  $\beta$ -actin signal of the same cDNA.

### Renal PMN infiltration

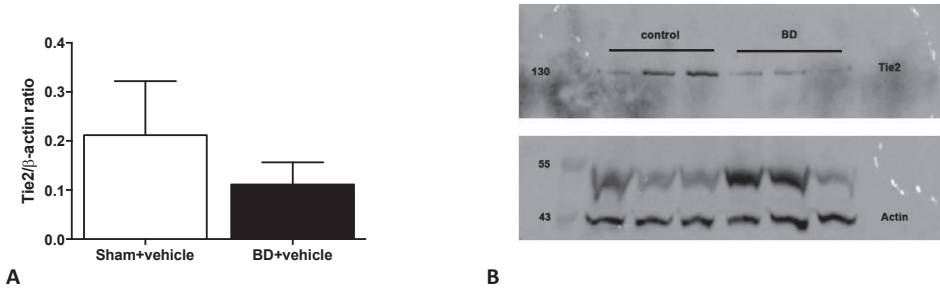
Renal cryosections of experiment 1 were stained with HIS-48 mAb to study the influx of PMNs (figure 8). The number of positive cells per cortical area in the glomerulus was  $0.6\pm 0.2$  in sham-operated controls and increased significantly to  $6\pm 1.2$  in the BD+saline group ( $p=0.004$ ).



**Figure 8.** Renal PMN infiltration, quantification and HIS-48 staining of experiment 1 administering 1 µg/kg rhAng1 to sham-operated (n=7) and BD rats (n=7). Quantification of PMN infiltration in cortical glomeruli of renal cryosections. PMN infiltration was increased after BD induction, but not affected by rhAng1 treatment (p>0.05).

*Quantification of Tie2 protein*

Renal Tie2 protein quantification of a mouse BD experiment is demonstrated in figure 9. The renal Tie2/β-actin ratio was not significantly different between sham- and BD operated rats (0.21±0.06 vs. 0.11±0.03, p=0.2).



**Figure 9.** Relative Tie2 protein quantification of sham-operated and BD mice. A) Renal total Tie2 protein expression of 3 mice per group after 4h of brain death presented as mean±SD, normalized to the β-actin signal. BD did not significantly alter the protein expression of total Tie2 in the mouse kidney. B) 100 µg protein input of mouse kidney was used for WB analysis. β-actin was used as a loading control.

**DISCUSSION**

To our knowledge, this is the first study investigating Ang/Tie2-axis intervention in experimental brain death. We aimed to modify the Ang1 levels in our BD rat model by administrating rhAng1 and the synthetic drug-like Tie2 agonist, VT. In the same model we studied administration of AMG386, an Ang2 inhibiting peptibody. Besides the finding that exogenous treatment with rhAng1, VT or



AMG386 did not attenuate brain death-induced inflammation nor improve renal function a major finding of this study is the remarkable decrease of Tie2 gene expression in the kidney after BD.

In the literature, no other (pre)clinical studies regarding donor pretreatment via Ang1 have been reported. Both rhAng1 and VT have been studied in various preclinical models including murine sepsis, which has pathophysiological similarities to brain death. Intravenous rhAng1 treatment showed significant improvement of sepsis-associated organ dysfunctions and survival time, possibly via preserving the endothelium<sup>33</sup>. In an abdominal sepsis model in mice, systemic VT administration protected against sepsis-induced endothelial barrier dysfunction and reduced mortality<sup>40</sup>. Inhibiting Ang2 gained more and more attention in preclinical studies, also outside anti angiogenesis therapy in tumors. Various studies have shown that it is possible to inhibit Ang2-induced Tie2 phosphorylation by antibodies in pre-clinical studies<sup>42,48-51</sup>. One study showed that Ang2 inhibition decreased the expression of adhesion molecules in an ischemic mouse hind limb model<sup>52</sup>. A peptibody, inhibiting the interaction between the Tie2 receptor and Ang1 and Ang2 was the first to enter a phase III clinical trial demonstrating promising results<sup>53</sup>. Although the strategy to inhibit Ang2 is rather novel in the field of transplantation, another anti-Ang2 antibody demonstrated promising results in rat cardiac allografts after brain death. *Ex vivo* treatment of the allografts with anti-Ang2 antibody reduced endothelial cell adhesion molecule expression, leukocyte infiltration and the activation of the innate immune response<sup>49</sup>.

The current study was designed as a proof of principle study to evaluate the effect of modulating the Ang/Tie2-system in favor of Ang1, by enhancing Ang1 availability or inhibiting Ang2, on the pro-inflammatory response caused by BD. We expected a rapid brain death-induced endothelial activation quickly triggering the WPB Ang2 release since previous studies using our BD rat model showed increased expression of endothelial adhesion molecules, endotoxins and cytokine levels such as IL-6 at 4 hours of BD compared to sham-operated rats<sup>47,54-57</sup>. Furthermore, including profound ICAM-1, HO-1, TNF- $\alpha$ , KIM-1 mRNA expression and renal influx of PMNs were shown to indicate BD-induced inflammation<sup>54,55,57,58</sup>. In another BD rat model it was demonstrated that circulatory inflammatory molecules influence the state of peripheral organs in which expression of lymphocyte- and macrophage associated products were increased<sup>6</sup>. In line with previous studies using an identical experimental brain death model, we found increased functional and inflammatory markers in the BD animals compared to sham-operated controls<sup>6,59-61</sup>. Unfortunately, no beneficial effects of rhAng1, VT or AMG386 donor pretreatment on these injury parameters were found.

Our study did not show increased Ang1 mRNA expression in the rhAng1 or VT treated groups indicating no feed-back on the native Ang1 release. This could

be explained by the used low dosages (1 µg/kg and 3 µg/kg respectively), which were based on previous sepsis experiments in mice. We can therefore not exclude a potential anti-inflammatory effect when higher dosages are used. However an alternative and more eligible explanation may be the marked decrease in Tie2 mRNA expression, also suggested by the trend in reduced protein levels via Western Blot analysis, which we observed in all BD groups. Under normal conditions, Tie2-phosphorylation mediated by Ang1 prevents apoptosis<sup>20</sup> and leads to anti-inflammatory survival signaling, stabilizing the endothelium<sup>14,22</sup>. However if Tie2 expression is diminished due to BD induction, Ang1-mediated Tie2 phosphorylation may be futile regardless of the availability of endogenous or exogenous Ang1. As demonstrated by *in vitro* experiments, Tie2 expression can be regulated by its ligands Ang1 and Ang2<sup>18,62</sup>. One could speculate that in response to rhAng1 or VT, Tie2 is rapidly internalized and targeted for degradation and thereby diminishing the ability of the cell to respond to further stimulation<sup>18</sup>. Receptor internalization and degradation is considered to be a mechanism attenuating downstream signal transduction resulting in an overall loss of receptors from the cell surface, thereby diminishing the ability of the cell to respond to further stimulation<sup>63,64</sup>. In human umbilical vein endothelial cells (HUVECs), Tie2 was rapidly internalized and targeted for degradation in response to Ang1<sup>18</sup>. Therefore, exogenous Ang1 treatment may not be the preferential route to manipulate the Ang/Tie2-system in BD. To date, the mechanism regulating Tie2 internalization is unknown. Another explanation for the absence of an effect of rhAng1 or VT may be the capability of Ang1 to bind to other surface receptors such as Tie1 and integrins<sup>65,66</sup>. In that way, the observed downregulation of Tie2 mRNA in BD may be caused by BD related cascades independent of exogenous Ang1. However, as Ang1 and Ang2 are released into the medium after binding to endothelial cells<sup>18</sup>, they are capable of rebinding Tie2 on fresh endothelial cells.

The increased plasma Ang2 we found in the AMG386-treated animals compared to the saline-treated controls in the third experiment may be caused by the used dosage. Ang2 has been characterized as a context-dependent antagonist since in some studies Tie2 binding by high Ang2 levels resulted in receptor activation with similar outcomes to Ang1<sup>67,68</sup>. Since we measured increased Ang2 levels in the AMG386 treated group, one could speculate that Ang2 could thereby act as a Tie2 agonist, inducing anti-inflammatory and anti-apoptotic survival signals. Except looking at the increased plasma IL-6 mRNA expression, that could hardly have been the effect. This upregulation is more likely caused by BD-induced inflammation itself<sup>69</sup> with IL-6 playing an important role in activating the inflammatory response what presumably caused enhanced WPB exocytosis of Ang2. Regardless of the pathophysiological effects caused by BD, there seems to be a role for the Ang/Tie2-system in renal failure and repair. Ang2 over-expression in mice causes proteinuria and apoptosis of glomerular endothelial cells<sup>70</sup>. In a rat

model of glomerulonephritis, Ang1 and Ang2 are overexpressed by podocytes and Tie2 is overexpressed by endothelial cells, all in a time-dependent manner during the repair phase<sup>71</sup>.

Despite efforts made, several questions remain unsolved. In this study due to the absence of an appropriate rat Ang1 ELISA, no definite conclusions on available systemic Ang1 can be made. Next, we were unable to determine the phosphorylated and systemic Tie2 availability due to the absence of a working method in our lab. Several anti-bodies and methods were tested, unfortunately without satisfactory results. Also we do not know how quickly Tie2 expression is affected in the BD setting since we have not evaluated the effect of BD on Tie2 expression at earlier time points after BD induction. Despite these limitations, our data reveal a remarkable effect of BD on Tie2 mRNA expression and protein level which underlines the suggestion of the Ang/Tie2-axis playing a role in BD. This finding is to some extent with in line with the reported decreased Tie2 expression in post-mortem renal biopsies of patients with sepsis<sup>72</sup>. As the pathophysiology of sepsis and BD are alike, perhaps Tie2 preserving strategies, may be another therapeutical target in BD.

In conclusion, this rat study was not able to show any beneficial effect of rhAng1, VT or AMG386 in terms of improved renal function, lower inflammation, enhanced Ang1 or decreased Ang2 expression in experimental brain death. However based on the remarkable down regulation of Tie2 we feel that further studying the mechanism of the Ang/Tie2-system in BD is justified. Better mechanistic understanding of the Ang2/Tie system is warranted before translation of this endothelial signaling system as a therapeutic target in human BD studies is feasible.

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