Mini-Abstract

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Structured Abstract

Objectives: To evaluate the potential changes in the plasma levels of resolvin D1 (RvD1) in patients with trauma and hemorrhage. Having found that trauma results in a profound reduction in plasma RvD1 in patients, we have then investigated the effects of RvD1 on the organ injury and dysfunction associated with hemorrhagic shock (HS) in the rat.

Summary Background Data: HS is a common cause of death in trauma due to excessive systemic inflammation and multiple organ failure. RvD1 is a member of the resolvin family of pro-resolution mediators.

Methods: Blood samples were drawn from critically injured patients (n=27, ACITII-prospective observational cohort study) within two hours of injury for targeted liquid chromatography tandem mass spectrometry. HS rats (removal of blood to reduce arterial pressure to 30±2 mmHg, 90 minutes, followed by resuscitation) were treated with RvD1 (0.3 or 1 μg/kg i.v.) or vehicle (n=7). Parameters of organ injury and dysfunction were determined.

Results: Plasma levels of RvD1 (mg/dl) were reduced in patients with trauma+HS (0.17±0.08) when compared to healthy volunteers (0.76±0.25) and trauma patients (0.62±0.20). In rats with HS, RvD1 attenuated the kidney dysfunction, liver injury, and tissue ischemia. RvD1 also reduced activation of the NF-κB pathway and reduced the expression of pro-inflammatory proteins such as iNOS, TNF-α, IL-1β and IL-6.

Conclusions: Plasma RvD1 is reduced in patients with trauma-HS. In rats with HS, administration of synthetic RvD1 on resuscitation attenuated the multiple

organ failure associated with HS by a mechanism that involves inhibition of the activation of NF-κB.

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Resolvin D1 attenuates the organ injury associated with experimental hemorrhagic shock

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Running head: Resolvin D1 in hemorrhagic shock

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Conclusions: Plasma RvD1 is reduced in patients with trauma-HS. In rats with HS, administration of synthetic RvD1 on resuscitation attenuated the multiple organ failure associated with HS by a mechanism that involves inhibition of the activation of NF-κB.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; iNOS, inducible nitric oxide synthase; HS, hemorrhagic shock; IL-6, interleukin 6; MAP, mean arterial pressure; MODS, multiple organ dysfunction syndrome; MPO, myeloperoxidase; NF-κB, nuclear factor kappa B; TNF-α, tumour necrosis factor-α.

INTRODUCTION

Despite improvements in trauma care, the morbidity and mortality associated with trauma remains very high. Approximately 40% of trauma deaths are due to excessive bleeding and occur in the first few hours after injury[1,2]. Severely injured patients that do survive the initial insult often develop persistent organ dysfunction associated with poor outcome[1,3]. Multiple organ dysfunction syndrome (MODS) is considered the leading cause of death and of poor-quality life after severe trauma[3]. The resuscitation after severe hemorrhage improves tissue perfusion, but also aggravates injury and triggers a systemic inflammatory response, both of which contribute to the development of MODS[4,5]. Although the precise mechanism responsible for MODS remains to be elucidated, inflammation plays a key role, and the increased release of inflammatory cytokines and nitric oxide (NO) importantly contribute to the pathophysiology of HS[6,7]. We have recently demonstrated that pharmacologic interventions which either reduce organ injury or increase the resistance of target organs against injury attenuate the MODS associated with HS[8–11].

Resolution of inflammation is a physiological active process, which is highly coordinated and regulated by several endogenous mediators of protein and lipid nature. As such, specialized pro-resolving mediators (SPMs), including lipoxins, protectins, maresins and resolvins, do not completely inhibit the inflammatory responses, but reprogram the immune response to accelerate resolution of inflammation and repair[12–14]. SPMs exhibit protective roles in several inflammatory conditions, including arthritis[15,16], sepsis[17,18], burn injury[19], uveitis[20] and edema[21].

Resolvin D1 (RvD1), a bioactive lipid mediator synthesised from docosahexaenoic acid, is member of the resolvin family that mediates resolution of inflammation[22,23].

RvD1 reduces the inflammation and lung injury caused by lipopolysaccharide[24], it reduces the inflammation in endometriosis[25], and improves survival in experimental sepsis[26]. However, therapeutic approaches that mimic or amplify these endogenous efforts to resolve inflammation and to initiate repair have not yet been investigated in trauma-hemorrhage. Thus, the aim of the present study is to evaluate the changes in the plasma levels of RvD1 in patients with severe trauma. Having found that trauma and hemorrhagic shock (HS) leads to a significant fall in the plasma levels of RvD1, we have then used a reverse-translational approach aimed at investigating the potential beneficial effects of synthetic RvD1 in a rat model of HS and MODS.

METHODS

Use of Human Subjects—Ethic Statement

Healthy Volunteers: Volunteers gave written consent in accordance with a Queen Mary Research Ethics Committee (QMREC 2014:61) and the Helsinki declaration. Venous peripheral blood was collected at indicated intervals from fasting volunteers (n=8) that declared not taking NSAIDS for at least 14 days, caffeine and alcohol for at least 24h and fatty fish for 48h.

Trauma Patients: Patients recruited into the Activation of Coagulation and Inflammation and Trauma (ACITII) study, a platform prospective observational study at an urban major trauma centre, were eligible for inclusion. The National Health Service (NHS) Research Ethics Committee (REC) provided ethical approval for this study (REC reference 07/Q0603/29). Informed consent for participation was obtained from the patient or their next of kin. All adult patients meeting criteria for trauma team activation were screened for inclusion. Exclusion criteria consisted of presentation

more than two hours after injury, administration of more than 2000 ml crystalloid prior to blood sampling, major burns and known bleeding diathesis or immunocompromise.

Patient Selection: We selected a subset of critically injured patients from the ACITII cohort for inclusion into this study based on their injury profile and clinical course. Patients with an injury severity score (ISS) of ≥25 resulting from blunt force trauma were included (n=27). We excluded patients with severe traumatic brain injury (abbreviated injury score >3) and those who died within 48 hours of admission, to eliminate the confounding effects of severe head injury on the immune response and to focus on patients at risk of MODS.

Data collection and Blood Sampling: Blood samples were collected immediately on arrival in the emergency department and within two hours of injury. Demographic data, admission physiology, mechanism of injury and blood product utilisation were collected prospectively on admission to hospital by a trained research fellow. HS was defined as a base deficit of >6mmol/L on arterial blood gas analysis at the time of sampling for RvD1 measurement, as previously described[27,28]. All patients were followed up daily over the first 28 days unless death or hospital discharge occurred sooner. Scores were calculated on each day of critical care unit stay. MODS was defined as a sequential organ failure assessment (SOFA) score of ≥5 occurring on two or more consecutive days, excluding the first 48h of admission, as previously described[29–31]. Additional outcome variables included ventilator and vasopressor utilisation, critical care and hospital length of stay, and 28-day mortality.

RvD1 measurement using targeted liquid chromatography tandem mass spectrometry

Peripheral blood from healthy volunteers and patients was drawn into vacutainers containing 3.2% sodium citrate (Becton, Dickinson and company, Plymouth, UK) and

centrifuged at 1500 x *g* for 10 minutes at room temperature. The plasma fraction was isolated and centrifuged again using the same settings before immediate storage at -80°C. All samples were extracted using solid-phase extraction columns as in[32,33]. Prior to sample extraction, deuterated labelled RvD2 (500 pg per sample) was added to facilitate quantification in 4 volumes of cold methanol. Samples were kept at -20°C for a minimum of 45 min to allow protein precipitation. Supernatants were subjected to solid phase extraction, methyl formate fraction collected, brought to dryness and suspended in phase (methanol/water, 1:1, vol/vol) for injection on a Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector, paired with a QTrap 6500 plus (Sciex). An Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 μm) was kept at 50°C and mediators eluted using a mobile phase consisting of methanol-water-acetic acid of 20:80:0.01 (vol/vol/vol) that was ramped to 50:50:0.01 (vol/vol/vol) over 0.5 min and then to 80:20:0.01 (vol/vol/vol) from 2 min to 11 min, maintained till 14.5 min and then rapidly ramped to 98:2:0.01 (vol/vol/vol) for the next 0.1 min. This was subsequently maintained at 98:2:0.01 (vol/vol/vol) for 5.4 min, and the flow rate was maintained at 0.5 ml/min. QTrap-6500+ was operated using a multiple reaction monitoring method using ion pairs 375 > 141 and 375 > 215 as in[32,33]. RvD1 was identified using established criteria including matching retention time to synthetic and authentic materials and at least 6 diagnostic ions[32,33]. Calibration curves were obtained using synthetic compound mixtures at 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 pg that gave linear calibration curves with an r^2 values of 0.98–0.99.

Use of Experimental Animals—Ethic Statement

All animal procedures were approved by the Animal Welfare Ethics Review Board (AWERB) of Queen Mary University of London (PCF29685) in accordance with Home Office guidance on Operation of Animals (Scientific Procedures Act 1986) and Guide

for Care and Use of Laboratory Animals of the National Research Council. Male Wistar rats (Charles River Ltd, UK) weighing 250 to 280 g receiving a standard diet and water *ad libitum* were used.

Hemorrhagic Shock and Quantification of Organ Injury and Dysfunction

HS was performed as previously described[8,34]. Briefly, rats were anesthetized with sodium thiopentone (120 mg/kg i.p. maintained using 10 mg/kg i.v.). Blood was withdrawn from the right carotid artery and collected with 2 IU/ml of heparin until the mean arterial pressure (MAP) reach 30±2 mmHg, which was maintained for 1.5 h. The shed blood was kept between 6-10°C. After 1.5h of initiation of hemorrhage, resuscitation was performed with the shed blood over a period of 5 min. An infusion of Ringer Lactate (1.5 mL/kg/hour; i.v.) was maintained throughout the experiment for a total of 4h. The last 3h urine was obtained for the estimation of creatinine clearance. Four hours after resuscitation, blood was collected from carotid artery for measurement of lactate (Accutrend Plus Meter, Roche Diagnostics, UK) and organ injury parameters (IDEXX Ltd, UK), and tissue samples were taken, placed on liquid nitrogen and stored at -80°C. Sham-rats were used as control and underwent identical surgical procedures, but without hemorrhage.

Experimental Design

Forty-two rats were randomly divided into the following groups (n=7 per group): sham+vehicle; sham+RvD1 (0.3 μg/kg); sham+RvD1 (1 μg/kg); HS+vehicle; HS+RvD1 (0.3 μg/kg) and HS+RvD1 (1 μg/kg). RvD1 was diluted in saline (vehicle) and rats were treated (i.v.) upon resuscitation.

Immunohistochemistry

Myeloperoxidase positive cells and ICAM-1 were detected through immunohistochemistry. Briefly, kidney and liver samples were obtained, fixed and processed to obtain 4μm sections. Sections were incubated with 0.03% H_2O_2 for inactivation of endogenous peroxidase (Dako EnVision+ System-HRP-DAB, K4010) and were blocked with 10% goat serum (15 min). The slides were then incubated with rabbit anti-ICAM-1 antibody (1:100; Cat# ab124760, Abcam, Cambridge, UK, 1 h, room temperature) or with rabbit anti-myeloperoxidase antibody (1:25; Cat# ab9535, Abcam, Cambridge, UK) for 1 h at 37°C. After washing with PBS, slides were incubated with labelled polymer-HRP antibody (Dako EnVision+ System-HRP-DAB, K4010), washed and incubated with DAB chromogen solution until a brown precipitate could be observed. A negative control was performed through the omission of primary antibody (data not shown). Reaction was stopped by immersing slides in water. Counter-staining was performed with Harris haematoxylin. Images were acquired using NanoZoomer Digital Pathology Scanner (Hamamatsu Photonics K.K., Japan) and analysed using the NDP Viewer software. The relative quantification of ICAM-1 immunostaining was achieved through densitometry analysis in 5 randomly selected fields (x400) per animal (5 per group) using NIH ImageJ 1.36 imaging software (NIH, Bethesda, MD, USA) and it is expressed as arbitrary units. The number of MPO positive cells was counted in 10 randomly selected fields (x400) in a double-blinded manner.

Determinations of Cytokines

Serum and tissue cytokines TNF-α, IL-1β, IL-6 and IL-10 were determined using commercial immunoassay kits (R&D Systems, Minneapolis, MN).

Western Blot Analysis

Briefly, kidney and liver samples were homogenized in buffer and centrifuged (4000 rpm, 5 min, 4ºC). To obtain the cytosolic fraction, supernatants were centrifuged (14000 rpm, 4ºC, 40 min). The pelleted nuclei were re-suspended in extraction buffer and centrifuged (14000 rpm, 20 min, 4ºC). Protein content was determined on both nuclear and cytosolic extracts using bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc, Rockford, IL). Semi-quantitative immunoblot analyses of the phosphorylation of IKKα/β, IκBα, nuclear translocation of p65 and expression of iNOS were carried out. Equal amounts of proteins were separated by SDS-PAGE and electrotransferred to PVDF membrane. After blocking (1h in 5% dry milk solution), membranes were incubated with primary antibodies (rabbit anti-NF-kB [1: 1000], rabbit anti-IKKα/β [1:1000], rabbit anti-Ser$^{176/180}$ IKKα/β [1:5000], mouse anti-IκBα [1:1000], mouse anti-Ser$^{32/36}$ IκBα [1:1000], rabbit anti-iNOS [1:1000]) followed by incubation with appropriated HRP-conjugated secondary antibodies. Proteins were detected with ECL detection system and quantified by densitometry using analytic software (Quantity-One, Bio-Rad, Hercules, CA, USA). Results were normalized with respect to densitometric value of tubulin for cytosolic proteins or histone H3 for nuclear proteins.

Materials

Unless otherwise stated, all compounds were from Sigma-Aldrich Company Ltd (Poole, Dorset, U.K.). Ringer's Lactate was from Baxter Healthcare Ltd.; sodium thiopentone (Thiovet©) from Link Pharmaceuticals (Horsham, UK). The BCA protein assay kit and SuperBlock blocking buffer were from ThermoFisher Scientific Inc. (Rockford, USA).

Statistics

All figures are expressed as box-and-whisker format showing medians, inter-quartile range and full range. The distribution of the data was verified by Shapiro-Wilk normality test, and the homogeneity of variances by Bartlett test. When necessary, values were transformed into logarithmic values to achieve normality and homogeneity of variances. Data were assessed by one-way ANOVA followed by Newman-Keuls post-hoc test. Data that were not normally distributed were analysed with Kruskal-Wallis followed by Dunn's test. Clinical samples were analysed by Mann Whitney test. A p-value of less than 0.05 was considered significant. Statistical analysis was carried out using GraphPad Prism 5.03 (GraphPad Software, USA).

RESULTS

Resolvin D1 is significantly Reduced in Plasma from Trauma Patients who Develop Organ Dysfunction.

Plasma was collected from healthy volunteers (n=8), severely injured trauma patients (n=12) and trauma + HS patients (n=15) and the concentrations of RvD1 were determined using LC-MS/MS. The median age and sex distribution of the three groups was similar. Trauma patients were critically injured [median ISS 38 (IQR 33-44)] but received minimal volumes of crystalloid [median 0mL (IQR 0-300)] prior to blood sampling. Detailed patient demographics and injury characteristics are reported in supplemental table 1 (see Table, Supplemental Digital Content 1). When compared to healthy volunteers (HV) and trauma patients (T), patients with concomitant trauma and HS (T+HS) had significantly lower plasma levels of RvD1 (HV: 0.76±0.25; T: 0.62±0.20 and T+HS 0.17±0.08, p=0.02; Fig. 1).

Resolvin D1 Reduces Multiple Organ Failure Induced by HS in Rats

Having demonstrated that patients with T+HS bear marked reduction in RvD1 plasma levels, we next explored whether pharmacological intervention with synthetic RvD1 attenuates the MODS associated with HS in rats.

Rats subjected to HS developed renal dysfunction as observed by an increase in serum creatinine (Fig. 2A) and a decrease in creatinine clearance (Fig. 2B). HS-rats also developed significant increases in serum AST (Fig. 2C) and ALT (Fig. 2D), indicating the development of liver injury; increases of creatine kinase (CK; Fig. 2E) and lactate (Fig. 2F) indicating the development of muscular injury and tissue/organ ischemia, respectively. Administration of RvD1 upon resuscitation (after a period of prolonged hemorrhage) resulted in a dose-dependent reduction in the organ injury/dysfunction. No differences in heart rate and urine output were observed among any of the groups studied (see Supplemental Table 2, Supplemental Digital Content 2). As the higher dose of 1 µg/kg of RvD1 showed the largest beneficial effect, we used kidney and liver biopsies of HS-animals treated with either vehicle or 1µg/kg of RvD1 to investigate the mechanism(s) of the observed beneficial effects of RvD1 in experimental HS. Administration of RvD1 to sham-animals had no significant effect on any of the parameters evaluated (data not shown).

Resolvin D1 Reduces MPO Positive Cells and ICAM-1 Expression in Liver and Kidney

When compared to sections of both kidneys and livers of sham rats, those of HS-rats treated with vehicle showed a significant increase in cells expressing MPO (a specific marker of local neutrophil accumulation) and a significant upregulation of the adhesion molecule ICAM-1, which is the endothelial ligand for the neutrophil receptor CD11b/CD18, in both kidney (Figs. 3A, B, E and Figs. 4A, B, E) and liver (Figs. 3F, G,

J and Figs. 4F, G, J). The treatment of HS-rats with RvD1 significantly reduced the markers of neutrophil infiltration (Figs. 3D, E, I, J and Figs. 4D, E, I, J).

Resolvin D1 Attenuates the Activation of the NF-κB Pathway

When compared to sham animals, rats subjected to HS developed a significant increase in the phosphorylation of IKKαβ (Figs. 5A and D), IκBα (Figs. 5B and E) resulting in a nuclear translocation of the p65 NF-κB subunit (Figs. 5C, F) in the kidney (Figs. 5A-C) and liver (Figs. 5D-F) suggesting significant activation of the NF-κB pathway in both organs. Treatment of the HS-rats with RvD1 on resuscitation significantly attenuated the phosphorylation of IKKαβ, IκBα and the translocation of the p65 NF-κB subunit to the nucleus in both organs (Fig. 5).

Resolvin D1 Reduces Pro-inflammatory proteins Induced by HS

As RvD1 decreased the nuclear translocation of p65 NF-κB, we investigated the effects of RvD1 on the expression of NF-κB-dependent pro-inflammatory proteins. Kidney biopsies from HS rats exhibited a significant increase in iNOS expression (Fig. 6A) and in the concentrations of the pro-inflammatory cytokines TNF-α (Fig. 7E), IL-1β (Fig. 7F) and IL-6 (Fig. 7G). In the liver, only iNOS expression (Fig. 6B) and IL-1 β (Fig. 1J) levels were significantly higher than in the livers obtained from sham-animals. When compared to sham animals, HS also resulted in a significant increase in the pro-inflammatory cytokines TNF-α (Fig. 7A), IL-1β (Fig. 7B) and IL-6 (Fig. 7C), and in the anti-inflammatory cytokine IL-10 (Fig. 7D) in the blood. Treatment of HS-rats with RvD1 significantly attenuated the observed increases in tissue/blood cytokines and iNOS expression caused by HS (Fig. 6 and Fig. 7). It should be noted that treatment of sham-rats with RvD1 had no significant effect on any of the parameters measured.

DISCUSSION

We report here for the first time that trauma patients who experienced significant hemorrhage had a significant reduction in the plasma levels of the pro-resolution mediator RvD1. As there is good evidence that RvD1 is a powerful, pro-resolving mediator in man[12–14,35], we postulated that a reduction in endogenous levels of RvD1 could contribute to excessive systemic inflammation and organ injury/dysfunction. To investigate this hypothesis, we have used a reverse-translational approach (from humans to rodents) in which we have investigated the potential beneficial effects of synthetic RvD1 in a well-established rat model of HS, previously reported in this journal[8,11]. Most notably, we report here that administration of RvD1 on resuscitation (following a 90 min period of severe hemorrhage) attenuated HS-induced systemic inflammation and organ injury/dysfunction (renal dysfunction, liver injury, skeletomuscular injury and tissue hypoxia).

What, then, are the mechanisms by which RvD1 reduces both systemic inflammation and organ injury/dysfunction in experimental HS? There is now very good evidence that RvD1 has potent anti-inflammatory effects: specifically, RvD1 reduces polymorphonuclear leukocyte infiltration[36], inhibits activation of the NF-κB pathway[24,37] and reduces the formation of pro-inflammatory cytokines[38]. However, so far, none of these beneficial effects of RvD1 have been demonstrated in settings of HS. Here we demonstrated, that the MODS-related activation of NF-κB cascade (measured as IKKα/β phosphorylation, IκBα phosphorylation and nuclear translocation of p65) in kidney and liver, and the following enhanced formation of many NF-κB-dependent proteins including TNF-α, IL-1β, IL-6, IL-10 (kidney, liver, serum) and iNOS (in kidney, liver), are strongly inhibited by RvD1 administration. Taken together, these findings not only further confirm the key role of NF-κB activation in the pathophysiology of the

MODS in HS, but, most notably, form the basis for our current working hypothesis that the observed reduction in MODS afforded by RvD1 is secondary to inhibition of NF-κB. We have not investigated the detailed mechanism(s) by which RvD1 inhibits the activation of NF-κB, thus we cannot rule out potential indirect effects due to RvD1 interference with other inflammatory cascades. Nevertheless, the pro-resolving effects of RvD1 are known to be secondary to interactions with the receptors ALX/FPR2 and/or GPR32, whose activation has been demonstrated to directly downregulate NF-κB p65 nuclear translocation[39–41]. Indeed, there is evidence in both human endothelial and epithelial cells[42,43] which supports the view that activation of either ALX/FPR2 or GPR32 by RvD1 results in inhibition of nuclear translocation of p65, whereas the pre-treatment of these cells with specific ALX/FPR2 antagonists or a GPR32-neutralizing Ab abolished the inhibition of NF-κB afforded by RvD1 in these cells *in vitro.* Thus, we speculate that the inhibition of NF-κB afforded in our study by RvD1 administration is also secondary to activation of ALX/ FPR2 and/or GPR32.

There is good evidence that excessive formation of the proinflammatory cytokines TNF-α, IL-1β and IL-6 contribute to both development of MODS and mortality in HS[34,44–46]. We report here that administration of RvD1 on resuscitation attenuated the HS-induced formation of these proinflammatory cytokines. RvD1 treatment has also attenuated the increase of the anti-inflammatory cytokine IL-10 caused by HS in rats, and reduction in IL-10 improves outcome in experimental HS[9]. In patients with trauma-HS, increases in IL-10 are associated with immunosupression and delayed clinical recovery[47,48], Thus, the reduction of IL-10 may have contributed to the beneficial effects of RvD1 in HS. Our result corroborate previous studies reporting that RvD1 reduces IL-10 formation in septic shock[49].

The formation of proinflammatory cytokines including TNF-α and IL-1β drives the expression of iNOS in many disease states. There is good evidence that the excessive formation of nitric oxide by iNOS contributes to the development of MODS in rodents with HS and that inhibition of iNOS activity reduces MODS[6–9]. We report here that RvD1 abolishes the expression of iNOS caused by HS in the kidney and attenuates the expression caused by HS in the liver. Thus, we propose that prevention of the expression of iNOS importantly contributes to the beneficial effects of RvD1.

Neutrophils play a key role in the MODS associated with HS. Neutrophils bind to ICAM-1 expressed on endothelial cells and drives the recruitment of neutrophils into tissues; these neutrophils cause direct local cytotoxic cellular effects through the release of mediators such as reactive oxygen species, nitric oxide, cytokines and MPO[45]. We report here that the reduction in MODS afforded by RvD1 is associated with a reduced recruitment of neutrophils to target organs (kidney and liver) and this was due to reduced expression of ICAM-1 in these tissues. These protective actions of RvD1 are also in line with findings made during ischemia-reperfusion injury as well as with primary human leukocytes where RvD1 was found to regulate neutrophil recruitment and protect against neutrophil mediated organ injury[40,50].

Limitations of the study: Although the improvement of organ dysfunction by RvD1 is an interesting finding, our main efficacy endpoint (organ dysfunction) was only measured at a single time point after a relatively short resuscitation period (4 h after surgery). We have also not investigated the effects of RvD1 on other important organs involved in the MODS associated with HS including the lungs and the heart or (more importantly) on mortality rate. Thus, our preclinical results must be cautiously interpreted and carefully extrapolated to the clinical situation. Further preclinical studies evaluating survival after prolonged resuscitation periods are warranted. Moreover, clinical studies

in larger cohorts of trauma patients are also required to assess the relationships between RvD1 and clinical outcomes in humans, including measurement of RvD1 kinetics over time.

In summary, this paper shows that the development of MODS in patients with severe trauma/HS is associated with low plasma levels of RvD1. This finding formed the basis for our hypothesis that a restoration of higher plasma levels of RvD1 may reduce the MODS associated with trauma/HS. Indeed, administration of RvD1 on resuscitation (after a 90-min period of severe hemorrhage) reduced the organ dysfunction and injury as well as the local and systemic inflammation associated with HS. We propose that these beneficial effects of RvD1 are secondary to inhibition of the activation of NF-κB resulting in reduced formation of pro-inflammatory cytokines, NO from iNOS and neutrophil recruitment into target tissues (secondary to expression of ICAM-1). Thus, we believe that the reduction of MODS by RvD1 in the rodent model used here may be useful in the development of a preclinical dossier with the ultimate aim to evaluate RvD1 in patients with trauma-hemorrhage.

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FIGURE LEGENDS

Fig. 1: RvD1 is significantly reduced in plasma from trauma / hemorrhagic shock patients. Plasma was collected from healthy volunteers (n = 8), trauma patients (n=12) and trauma+HS patients (n=15) and the concentrations of RvD1 were determined using LC-MS/MS based lipid mediator profiling. (A) Multiple reaction monitoring chromatogram (B) MS-MS spectrum used for the identification of RvD1. (C) Plasma RvD1 concentrations. Data are presented as box and whiskers, showing medians, inter-quartile range and full range. * $p<0.05$ using Kruskal-Wallis followed by Dunn's test.

Fig. 2. RvD1 attenuates the organ injury and dysfunction induced by HS. (A) serum creatinine, (B) creatinine clearance, (C) serum aspartate aminotransferase (AST), (D) serum alanine aminotransferase (ALT), (E) serum creatine kinase (CK) and (F) lactate of HS-rats treated with vehicle or RvD1 (0.3 or 1.0 µg/kg) on resuscitation are shown. Sham rats were treated with vehicle or RvD1 (0.3 or 1.0 µg/kg). Data are presented as box and whiskers, showing medians, inter-quartile range and full range (n = 7 animals per group). Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls post hoc test. *p < 0.05 versus sham + vehicle and #p < 0.05 versus HS + vehicle.

Fig. 3. RvD1 reduces the increased number of MPO positive cells in kidney and liver tissue induced by HS. MPO positive cells recruitment in kidney (A-E) and liver (F-J) of sham (A, C, F, H) and HS-rats (B, D, G, I) treated with vehicle (A, B, F, G) or RvD1 (1.0 µg/kg; C, D, H, I) on resuscitation were determined by immunohistochemistry. Data are presented as box and whiskers, showing medians, inter-quartile range and full range (n = 5 animals per group; E, J). Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls post hoc test. *p < 0.05 versus sham + vehicle and #p < 0.05 versus HS + vehicle.

Fig. 4. RvD1 attenuates the expression of ICAM-1 in kidney and liver tissue induced by HS. ICAM-1 expression in kidney (A-E) and liver (F-J) of sham (A, C, F, H) and HS-rats (B, D, G, I) treated with vehicle (A, B, F, G) or RvD1 (1.0 µg/kg; C, D, H, I) on resuscitation are shown. Data are presented as box and whiskers, showing medians, inter-quartile range and full range (n = 5 animals per group; E, J). Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. *p < 0.05 versus sham + vehicle and #p < 0.05 versus HS + vehicle.

Fig. 5. RvD1 attenuates the activation of NF-κB pathway induced by HS. The phosphorylation of IKK on $Ser^{176/180}$ (A and D), IκBα on $Ser^{32/36}$ (B and E) and the nuclear translocation of the p65 NF-κB subunit (C and F) in the kidney (A-C) and liver (D-F) of sham and HS rats treated with RvD1 were determined by western blotting. Protein expression was measured as relative optical density (O.D.), corrected for the corresponding β-actin or Histone contents and normalized using the related sham band. Data are presented as box and whiskers, showing medians, inter-quartile range and full range (n = 7 animals per group). Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. *p < 0.05 versus sham + vehicle and #p < 0.05 versus HS + vehicle.

Fig. 6. RvD1 attenuates iNOS expression in liver and kidney tissue induced by HS. The iNOS expression in the kidney (A) and liver (B) of sham and HS rats treated with vehicle or RvD1 (1.0 μg/kg) on resuscitation were determined by western blotting. Protein expression was measured as relative optical density (O.D.), corrected for the corresponding tubulin content and normalized using the related sham band. Data are presented as box and whiskers, showing medians, inter-quartile range and full range (n = 7 animals per group). Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. *p < 0.05 versus sham + vehicle and #p < 0.05 versus HS + vehicle.

Fig. 7. RvD1 attenuates cytokines production induced by HS. The serum (A-D), kidney (E-H) and liver (I-L) amounts of TNF-α (A, E, I), IL-1β (B, F, J), IL-6 (C, G, K) and IL-10 (D, H, L) were determined by ELISA in sham and HS-rats treated with vehicle or RvD1 (1.0 μg/kg) on resuscitation. Data are presented as box and whiskers, showing medians, inter-quartile range and full range (n = 7 animals per group). Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls

post hoc test. *p < 0.05 versus sham + vehicle and #p < 0.05 versus HS + vehicle.

List of Supplemental Digital Content

Supplemental digital content 1. Table that illustrates the patients' characteristics. Pdf

Supplemental digital content 2. Table that illustrates rat's urine output and heart rate. pdf

Figure 1

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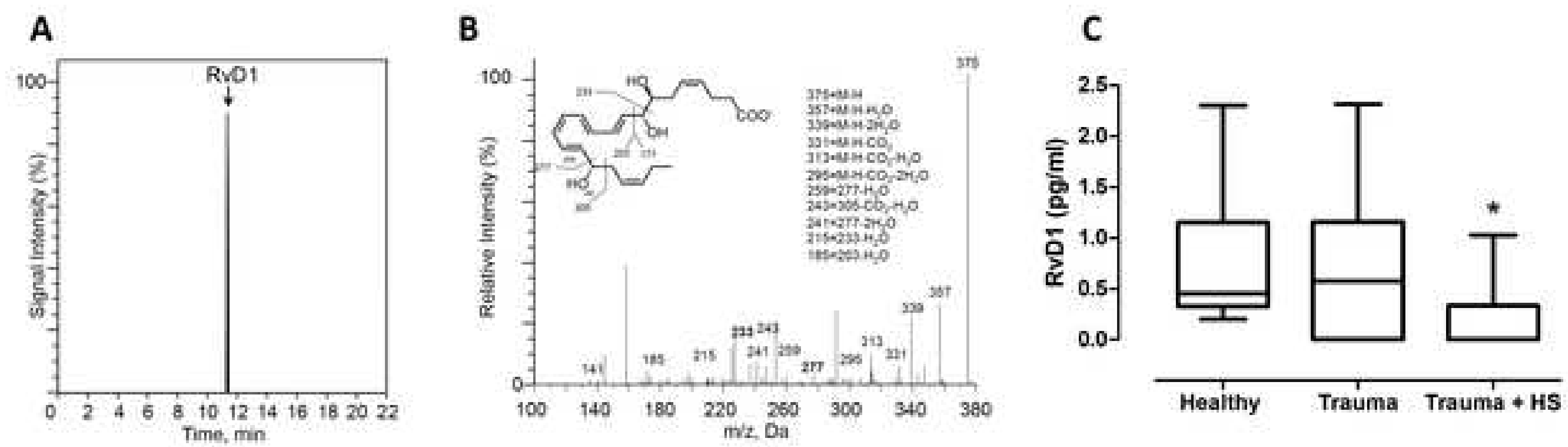

Figure 2

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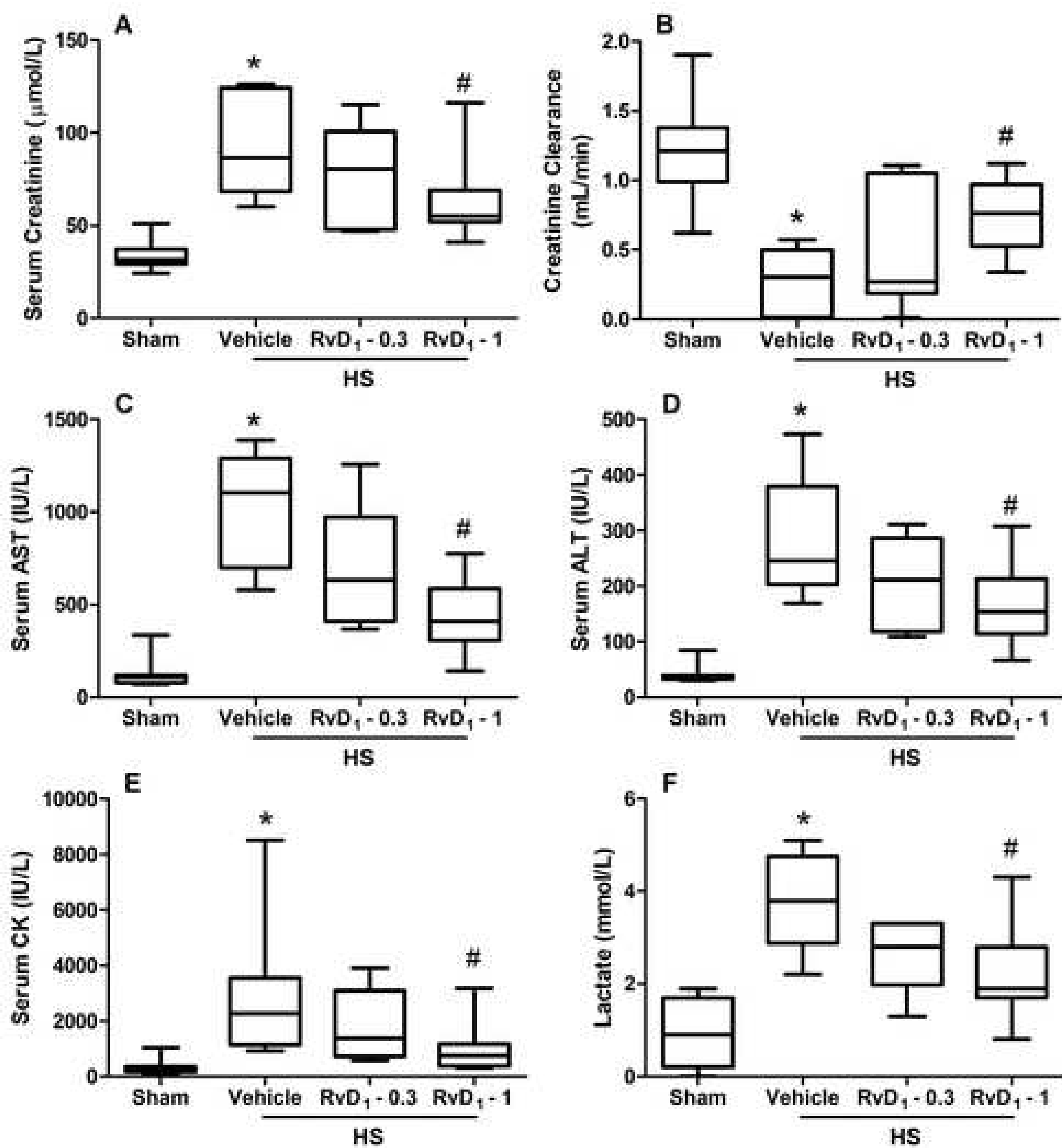


Figure 3

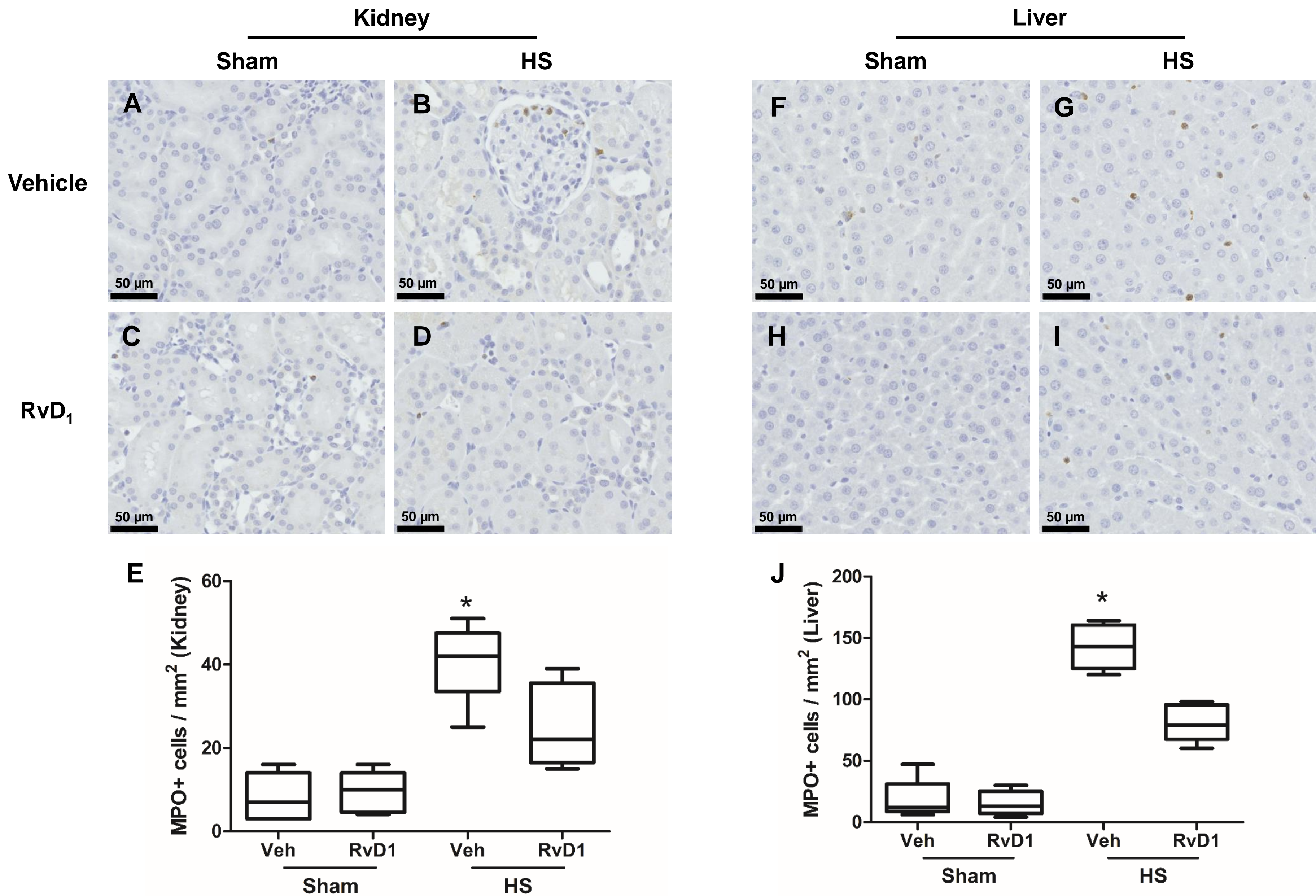


Figure 4

Kidney

Sham HS

Liver

Sham HS

Vehicle

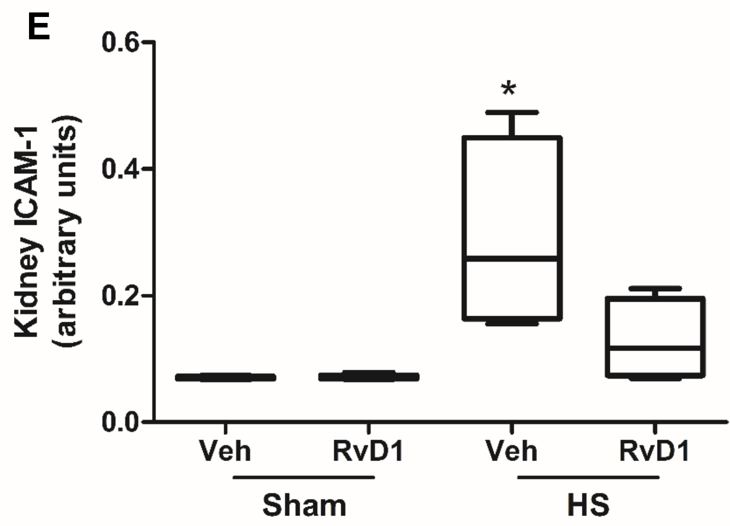
A B F G

50 µm 50 µm 50 µm 50 µm

RvD_1

C D H I

50 µm 50 µm 50 µm 50 µm



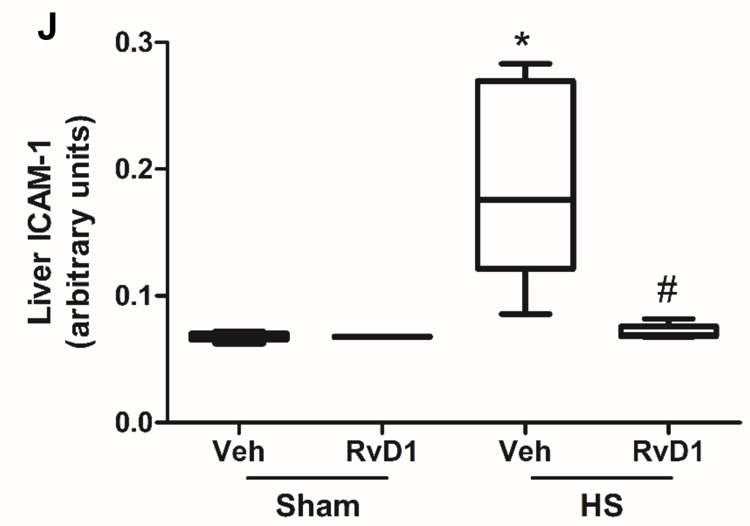
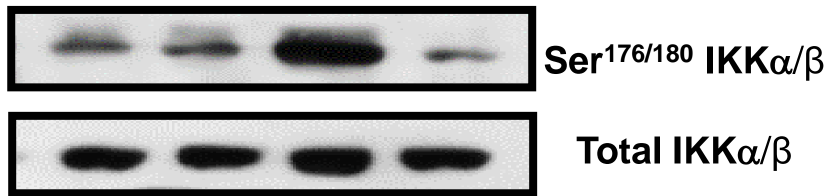


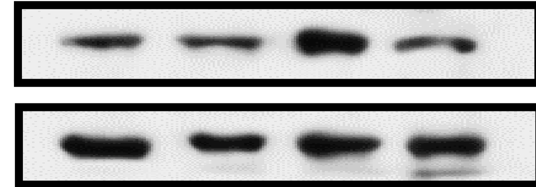
Figure 5

Kidney

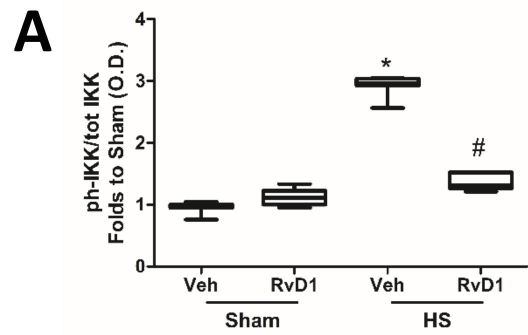
Liver

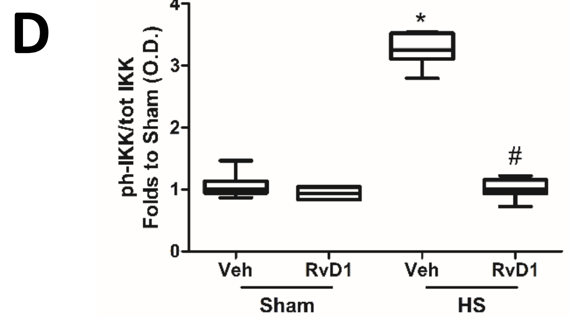


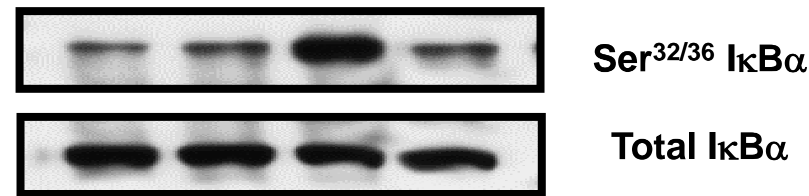
Ser$^{176/180}$ IKKα/β



Total IKKα/β

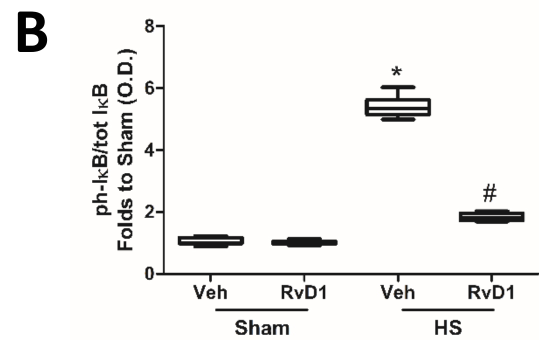


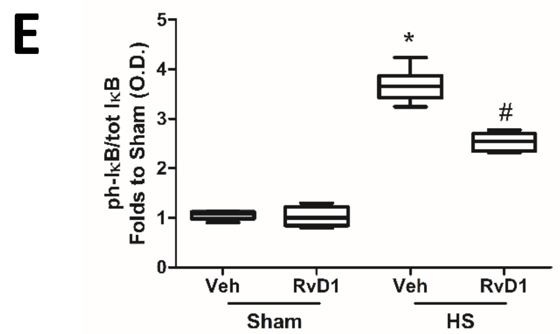


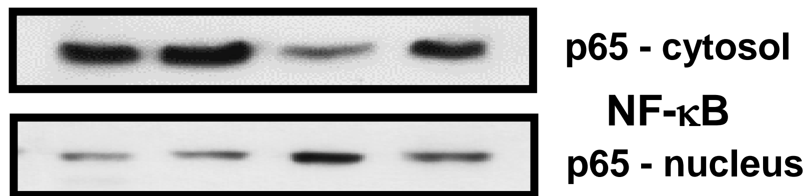


Ser$^{32/36}$ IκBα

Total IκBα



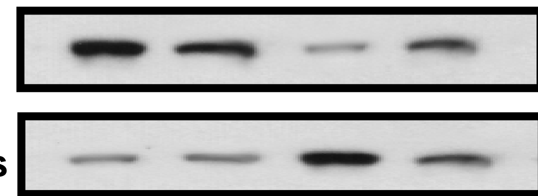


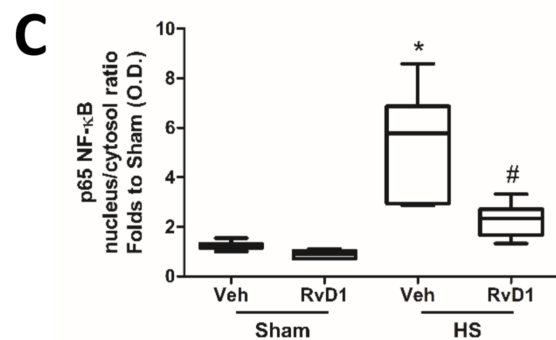


p65 - cytosol

NF-κB

p65 - nucleus





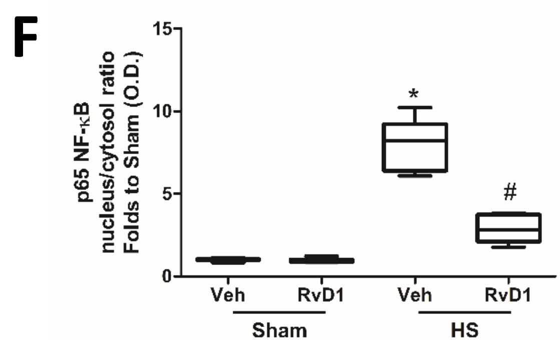
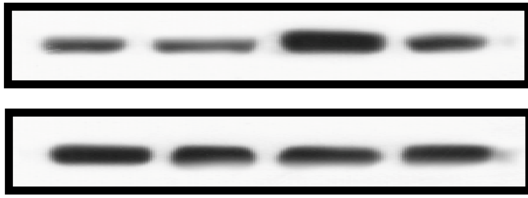
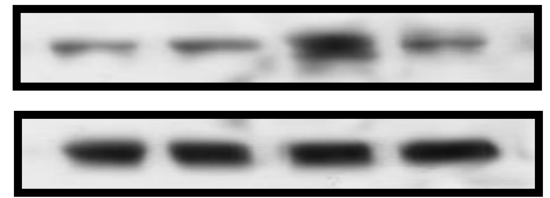
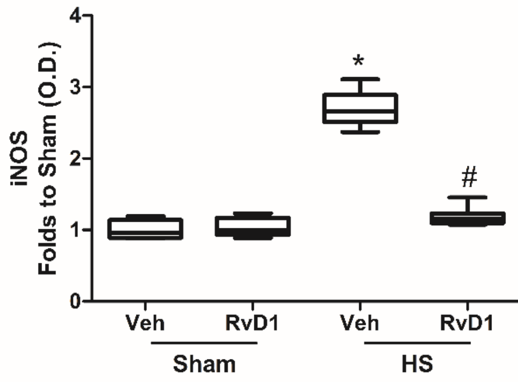


Figure 6







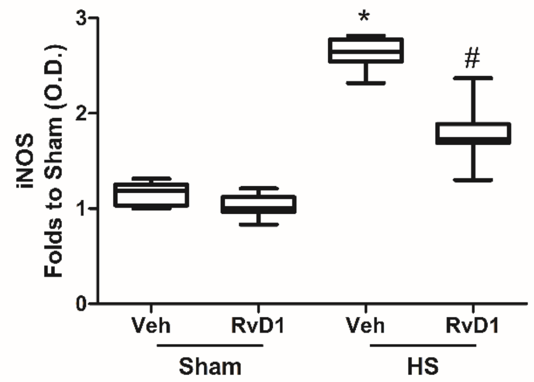


Figure 7

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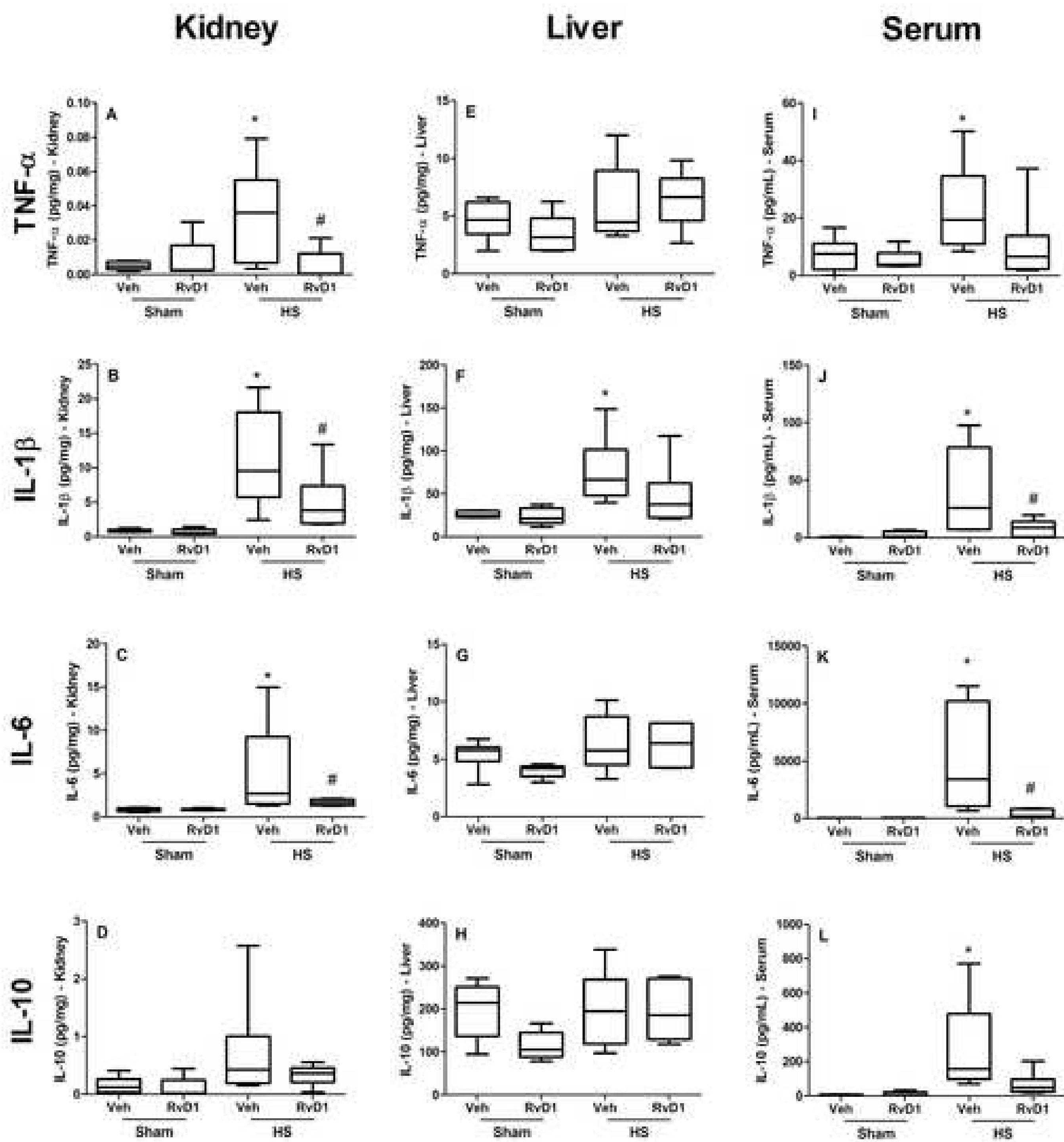

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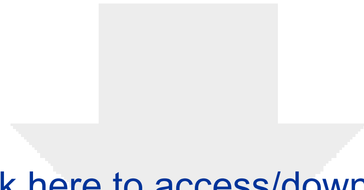
Supplemental Table



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