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Research article

Human adrenomedullin and its binding protein attenuate tissue injury and inflammation following hepatic ischemia reperfusion in rabbits

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ABSTRACT

Background: Liver injury caused by ischemia reperfusion (I/R) during surgical procedures, such as liver resection or liver transplantation, is a major cause of liver damage and graft failure. The current method of treatment is mostly preventative (i.e., ischemic preconditioning). While a number of pharmacological modalities have been studied to reduce hepatic I/R injury, none have been entirely successful. It has been demonstrated that the administration of adrenomedullin (AM) in combination with AM-binding protein (AM/AMBP-1) exerts significant protective effects in various pathological conditions. In an effort to develop AM/AMBP-1 as a novel therapeutic for hepatic I/R injury, the present study examined the effect of a low dose of human AM, which does not induce hypotension, in combination with human AMBP-1 in a rabbit model of hepatic I/R (i.e., non-rodent species).

Methods: Ischemia of 70% of the liver was induced by placing a microvascular clip across the hilum of the left and median lobes for 60 min. The clip was then removed to commence reperfusion. At 15 min following clip removal (i.e., reperfusion), human AM/AMBP-1 was administered intravenously via the ear marginal vein continuously for 30 min. At 20 h, blood and tissue samples were collected for various measurements.

Results: The serum levels of liver enzymes (alanine aminotransferase and aspartate aminotransferase) and lactate dehydrogenase, were elevated following hepatic I/R. The administration of AM/AMBP-1 significantly decreased these levels by 58, 44, 41%, respectively. Hepatic I/R increased the direct and total bilirubin levels, whereas treatment with human AM/AMBP-1 decreased these levels by 60% and 69%, respectively. Treatment with AM/AMBP-1 also inhibited interleukin-6 gene expression by 95%. There were no changes in tumor necrosis factor-α (TNF-α) gene expression and myeloperoxidase activity (MPO), lactate and Suzuki scores after treatment. The treatment, however, reduced apoptosis post-hepatic I/R in the ischemic portion of the liver.

Conclusion: Additional experiments with AM and AMBP-1 alone are needed to completely interpret the experimental results in this non-rodent species of hepatic I/R injury. The present study suggests that human AM/AMBP-1 may be developed as a novel therapeutic to attenuate hepatic I/R associated inflammation and liver injury.

1. Introduction

Hepatic ischemia-reperfusion (I/R) injury, occurring during surgical procedures including hepatic resection and liver transplantation, is the major cause of liver damage, graft dysfunction and post-transplantation failure [1]. Hepatic I/R injury is first caused by an initial reduction of blood flow to the liver resulting in hypoxia and cellular damage. Subsequently, as blood is reperfused to damaged tissue, the initial injury becomes exacerbated [2]. The pathophysiology of hepatic I/R injury is complex and involves two distinct phases [3]. During the early phase (i.e., 2 h after reperfusion), Kupffer cells (i.e., the liver macrophages), are activated and this leads to the production of extracellular reactive oxygen species (ROS) and pro-inflammatory cytokines. These ROS and cytokines not only exert direct cytotoxic effects on hepatocytes and endothelial

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cells but they also facilitate neutrophil infiltration to the tissues. These activated neutrophils then release more ROS and proteases, thereby further inducing oxidative stress during the late phase (i.e., 3–48 h post-reperfusion). To date, while a number of pharmacological modalities have been studied to reduce hepatic I/R injury, despite these efforts, there is still an unmet need for an effective hepatic I/R therapeutic strategy.

Adrenomedullin (AM), first isolated from a human pheochromocytoma, is a 52-amino acid peptide belonging to the calcitonin gene related peptide family [4, 5]. AM is widely expressed in virtually all tissues; however, the highest concentrations of the peptide are found in the adrenal medullae, heart, aorta and mesenteric arteries [5]. AM is produced by many cells including endothelial cells, monocytes, renal parenchymal cells and macrophages [5]. AM exerts its effect by binding to the calcitonin receptor-like receptor (CLR) complexed with a specific receptor activity-modifying protein (RAMP) [6]. The primary biological effect of AM is vasodilation, leading to hypotension and reduced peripheral resistance [4]. These vasodilatory effects of AM are mediated by binding to its receptor on the endothelial cells and vascular smooth muscle cells [7]. Circulating levels of AM have shown to be increased in patients with acute myocardial infarction [8], hemorrhagic and cardiogenic shock [9] and septic shock [10, 11], and following major surgery or hypoxia [12, 13]. While the primary biological feature of AM is its potent and long-lasting vasodilatory effect [4], AM also exerts several other biological actions. AM has been well described as an anti-inflammatory agent in various pathological conditions [14, 15, 16]. AM also inhibits neutrophil activation and migration [17]. Lastly, AM has antioxidant properties which allow it to protect organs from damage induced by ischemia [18, 19]. Therefore, AM targets several pathways associated with hepatic I/R injury, including microcirculatory disturbances, Kupffer cell activation, neutrophil infiltration and oxidative stress.

The beneficial effects of AM, however, are limited due to its potent hypotensive effect. Furthermore, the protective effect of a low dose of AM, which does not induce significant hypotension, is rather limited. In fact, a very little was known about the mechanism through which activity of AM was regulated until the discovery of the novel binding protein of AM, AMBP-1, which was later determined to be complement factor H [20, 21]. *In vitro*, AM has shown to suppress the secretion of tumor necrosis factor (TNF)-α and interleukin (IL)-6 from murine macrophage-like RAW264.7 cells stimulated by endotoxin; while AM alone has been shown to decrease endotoxin-induced TNF-α production in Kupffer cells by half, low concentrations of AM in combination with AMBP-1 (AM/AMBP-1) reduce TNF-α production by 90% [14]. The administration of AM/AMBP-1 produces significant benefits under various organ injury conditions [22, 23]. A previous study demonstrated that during hepatic I/R in rats, the plasma levels of AMBP-1 and its gene expression in the liver decreased significantly, while plasma AM levels increased [24]. The administration of AM/AMBP-1 immediately following the onset of reperfusion, decreased systemic inflammatory cytokines, hepatic neutrophil infiltration, liver damage and mortality following hepatic I/R in rats [24]. In an effort to develop AM/AMBP-1 as a novel therapeutic for hepatic I/R injury, the present study examined the effect of AM/AMBP-1 treatment in hepatic I/R in a non-rodent species (i.e., rabbits).

2. Materials and methods

2.1. Experimental animals

A total of 17 New Zealand white rabbits (4-month old males weighing approximately 3.0 kg) were purchased from Charles River, Burlington, MA, and housed in individual cages where standard rabbit chow (100–120 g/day) and water were provided. The rabbits were acclimatized to their environment for five days after their arrival and then fasted for 12 h prior to experimentation. The experiments were performed in accordance with the National Institutes of Health and USDA guidelines for the use of Experimental Animals. The present study was approved by the Institutional Animal Care and Use Committee of the Feinstein Institutes for Medical Research (Protocol #2011-043).

2.2. Rabbit model of hepatic I/R

Hepatic I/R in rabbits were performed as described with some modifications [25]. The rabbits were administered buprenorphine 0.02–0.05 mg/kg body weight (BW) subcutaneously (SQ) twice; once pre-operatively and a second dose, post-operatively. All surgical procedures were conducted under sterile conditions. The rabbits were pre-medicated with 3 mg/kg morphine SQ plus 0.2 mg/kg glycopyrrolate SQ. After 5–10 min, they were anesthetized with intramuscular injection of 50 mg/kg ketamine and 5 mg/kg xylazine [26]. While anesthetized, endotracheal intubation was performed and the rabbits were maintained on 100% oxygen initially and then 1–5% isoflurane to maintain anesthetic depth. Mechanical ventilation was employed to ensure that peripheral oxygen saturation (SpO_2) remained $\geq$95% throughout the procedure. The ear marginal vein was then cannulated with a 22-gauge catheter for the administration of fluids and drugs. A right subcostal incision was made parallel to the rib cage. The hepatic triad was isolated and the left hepatic artery and hepatic portal vein were separated from the bile duct and lymphatics. The complete ischemia of the medial and left lateral lobes of the liver was induced by clamping the left portal vein, hepatic artery and biliary radicles via an atraumatic microvascular clip. This method produced ischemia to the left and median lobes of the liver (~70% of the liver), while leaving the blood supply to the right and caudate lobes uninterrupted. Following 60 min of ischemia, the microvascular clip was removed to allow reperfusion. The abdominal incision was closed in two layers. The sham-operated (sham) rabbits were treated in a similar manner with the exception of clamping the blood vessels. Normal saline was infused at a rate of 10 ml/kg BW per hour to replace the intra-operative fluid loss. The rabbit groups in the study included were sham (n = 3), vehicle (n = 6) and AM/AMBP-1 treatment (n = 6). Additional rabbits (n = 2) were used in training by the laboratory personnel to establish the anesthesia procedure in rabbits.

2.3. Administration of AM and AMBP-1

At 15 min following the initiation of reperfusion, 24 μg/kg BW human AM in combination with 80 μg/kg BW AMBP-1 or normal saline (Vehicle) were administered via the ear marginal vein catheter. Human AM was purchased from Phoenix Pharmaceuticals, Belmont, CA and, human AMBP-1 was purchased from CompTech Complement Technology, Inc. Blood pressure and heart rate were monitored using a blood pressure analyzer at the time of anesthesia and for the duration of 1 h thereafter. After 2 h, the rabbits were returned back to their cages. After 20 h, rabbits were sedated with 50 mg/kg ketamine. Afterwards, a full surgical plane of anesthesia was induced by 4–5% isoflurane (2L/min 100% oxygen flow) via a face mask using an anesthesia machine (IMPAC^6, VETEQUIP). The anesthesia was maintained with 2–3% isoflurane and was confirmed by the absence of response to both toe pinch and palpebral reflex. The rabbits were then euthanized by initially performing a bilateral thoracotomy followed by a laparotomy for exsanguination via the inferior vena cava (AVMA Guidelines for the euthanasia of Animals: 2020 Edition). Blood was collected and the ischemic liver biopsies and lung biopsies were harvested for use in further analyses.

2.4. Measurement of liver injury markers and bilirubin levels

Blood was centrifuged at 2,000 x g for 10 min at 4 °C and the serum was collected and stored at -80 °C until further analysis. Serum levels of aspartate aminotransferase (AST; cat. no. 7561), alanine aminotransferase (ALT; cat no. 7526), lactate dehydrogenase (LDH; cat no. 7572), lactate and bilirubin levels were measured using commercial assay kits according to manufacturer's instructions (Pointe Scientific, Inc.).

2.5. Measurement of liver IL-6 and TNF-α mRNA by reverse transcription-quantitative (RT-qPCR)

Total RNA was extracted from 100 mg ischemic liver tissues using Trizol™ Reagent (cat. no. 15596026, Invitrogen: Thermo Fisher Scientific Inc.) and was reverse transcribed into cDNA using murine leukemia virus reverse transcriptase (cat. no. 2805-013, Invitrogen: Thermo Fisher Scientific, Inc.). The PCR reaction was conducted with 2.5 μl cDNA, 0.2 μM each forward and reverse primers, 12.5 μl SYBR Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a final volume of 25 μl. qPCR was performed at 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The gene expression was expressed as the fold change from the β-actin level which was calculated as $2^{-\Delta\Delta Cq}$ method [27]. The following rabbit primers were used: Rabbit IL-6: 5′-CTT CAG GCC AAG TTC AGG AG-3' (forward), 5′-GAG GGT GGC TTC TTC ATT CA-3' (reverse); rabbit TNF-α: 5′-GTC ACC CTC AGA TCA GCT TC-3' (forward), 5′-GTG AGC TTC ATG, CCG TTG-3' (reverse); rabbit β-actin: 5′-ATC CTG ACG CTC AAG TAC CC-3' (forward) and 5′-CAT GAT CTG GGT CAT CTT CTC-3' (reverse).

2.6. Myeloperoxidase (MPO) activity

Ischemic liver biopsies were homogenized in lysis buffer containing 0.5% hexadecyltrimethylammonium bromide, centrifuged at 12,000 x g at 4 °C for 15 min and the supernatant assayed for MPO activity using reaction buffer containing o-dianiside hydrochloride and hydrogen peroxide as previously described [28].

2.7. Histological evaluation

Biopsies from the ischemic liver and the lungs were fixed in 10% formalin and embedded in paraffin. Sections of 4 μm thickness were cut and stained with hematoxylin and eosin (AML Laboratories). Both liver and lung sections were examined by light microscopy (Nikon Eclipse Ti, Nikon Corporation) and the liver sections were graded using Suzuki's score [29, 30]. No changes in sinusoidal congestion and hepatocyte vacuolization, and absent necrosis were marked as 0, whereas severe congestion/vacuolization and >60% necrotic areas were marked as 4.

2.8. Liver apoptosis

Apoptosis in the liver was assessed using the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay kit (cat. no. 11684795910, Roche Molecular Systems, Inc.). Briefly, ischemic liver biopsies were fixed in 10% formalin, paraffin embedded and sectioned. Sections of 6-μm thickness were de-waxed, rehydrated and incubated at room temperature for 20 min with proteinase K (20 μg/ml) and rinsed. The sections were then reacted with a mixture of the label and the enzyme solution supplied in the kit in 37 °C incubator for 1 h. After washing with Tris-buffered saline (TBS), the sections were mounted with VectaShield medium containing DAPI stain (Vector Laboratories, Inc.). The sections were examined using a Nikon Eclipse Ti microscope with a fluorescent attachment. TUNEL-positive cells exhibited green fluorescence and DAPI-stained nuclei showed blue fluorescence.

2.9. Statistical analysis

Data are expressed as means ± SEM. Shapiro-Wilk test was used to check if the data followed a normal distribution. Additional statistical analysis was performed using one-way analysis of variance (ANOVA) and the differences between groups were analyzed using the Student-Newman-Keuls (SNK) test with the exception of the Suzuki's scores. Suzuki's scores were analyzed using the Kruskal-Wallis test followed by the Dunn's test (a non-parametric post hoc test). The data for the Suzuki's scores are presented as the median and interquartile range. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Human AM/AMBP-1 treatment decreased tissue injury in a rabbit model of hepatic I/R

Following hepatic I/R, the serum levels of liver enzymes, AST and ALT increased by 19- and 12- fold, respectively. The administration of AM/AMBP-1 at 15 min following reperfusion significantly attenuated serum levels of AST and ALT by 58 and 44%, respectively (Figures 1A–B). The serum LDH and lactate also increased by 4.9- and 2.5- fold, respectively, following hepatic I/R. Treatment with AM/AMBP-1 significantly attenuated serum levels of LDH by 41% whereas lactate levels did not change (Figure 1C–D). In the vehicle-treated rabbits following hepatic I/R, the serum levels of direct and total bilirubin were increased by 12.4- and 7.6-fold, respectively (Figure 2). Following treatment with human AM/AMBP-1, these levels were significantly attenuated by 73 and 60%, respectively (Figure 2).

3.2. Human AM/AMBP-1 down-regulated IL-6 mRNA expressions in the ischemic liver tissue after rabbit hepatic I/R

To determine whether human AM/AMBP-1 treatment regulates cytokine-induced liver injury, IL-6 mRNA levels in the ischemic liver were measured following hepatic I/R and post-treatment. In the vehicle-treated rabbits following hepatic I/R, IL-6 mRNA levels were increased by 39.0-fold, while the administration of AM/AMBP-1 decreased the IL-6 mRNA levels by 95% (Figure 3A).

3.3. Human AM/AMBP-1 decreased TNF-α gene expression and neutrophil infiltration to the ischemic liver tissue after rabbit hepatic I/R

To examine whether human AM/AMBP-1 alters hepatic I/R-induced oxidative stress leading to ROS and cytokine production, TNF-α gene expression was assessed in ischemic liver tissue following hepatic I/R and post-treatment. The results revealed that TNF-α mRNA expression was increased by 3.6-fold following hepatic I/R and AM/AMBP-1 treatment did not significantly decrease the expression (Figure 3B). Neutrophil accumulation in the liver is a consequence of oxidative stress in hepatic I/R. MPO activity, which is a measure of neutrophil infiltration was assessed in the ischemic liver tissues following hepatic I/R and post-treatment. MPO activity was increased by 2.1-fold following hepatic I/R and AM/AMBP-1 treatment did not show any significant change in these levels (Figure 3C).

3.4. Human AM/AMBP-1 treatment improved histological integrity after rabbit hepatic I/R

Histological changes were graded from 0 to 4 based on the Suzuki's criteria [30]. No changes in sinusoidal congestion and hepatocyte vacuolization, and absent necrosis were marked as 0, while severe congestion/vacuolization and more than 60% necrotic areas were scored as 4. Following hepatic I/R, the vehicle-treated liver tissue exhibited structural abnormalities (Figure 4) and a high Suzuki's score as shown in Table 1. By contrast, these scores did not significantly change in AM/AMBP-1-treated rabbits (Figure 4 and Table 1). The vehicle-treated lung tissue exhibited notable abnormalities, hyperemia/congestion, exudates and neutrophil infiltrate. By contrast, these findings were notably attenuated in AM/AMBP-1 treated rabbits (Figure 4).

3.5. Human AM/AMBP-1 treatment decreased ischemic liver apoptosis after rabbit hepatic I/R

TUNEL assay was used to measure apoptosis. In the ischemic liver following hepatic I/R, a significant increase in the number of TUNEL-positive cells was observed in the vehicle-treated rabbits (Figure 5A).

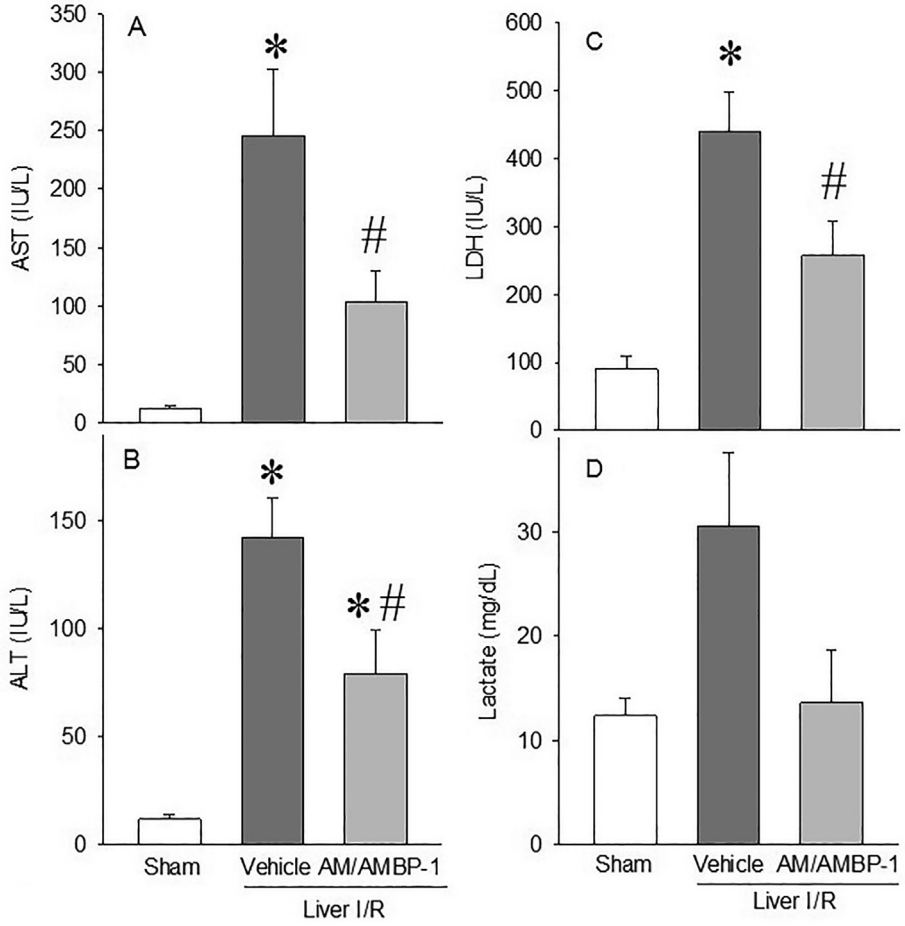

Figure 1. Hepatic I/R-induced tissue injury markers are attenuated following treatment with human AM/AMBP-1. Serum levels of (A) AST, (B) ALT, (C) LDH and (D) Lactate in sham-operated rabbits (Sham), hepatic I/R rabbits treated with normal saline (Vehicle) or hepatic I/R rabbits treated with human AM/AMBP-1 (AM/AMBP-1) at 20 h following reperfusion injury. Data are presented as the means ± SEM [Sham (n = 3), Vehicle (n = 6) and AM/AMBP-1 (n = 6)] and compared by one-way ANOVA and the SNK test. *P < 0.05 vs. Sham; #P < 0.05 vs. Vehicle. I/R, ischemia/reperfusion; AM, adrenomedullin; AMBP-1, AM-binding protein 1; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.

By contrast, the number of TUNEL-positive cells was markedly decreased by 93% in the rabbits treated with human AM/AMBP-1 (Figure 5B).

4. Discussion

Hepatic I/R injury during liver resection or liver transplantation is associated with high morbidity and mortality [1]. During liver resection, it is essential to apply vascular occlusion to reduce blood loss. Several surgical techniques have been implemented in clinical practice to reduce blood loss. These include the Pringle Maneuver (PM), total hepatic vascular exclusion and the hemi-hepatic or segmental occlusion of the portal vein or hepatic artery [31]. However, these techniques have been associated with hepatic I/R injury, which is an unresolved problem in clinical practice. Hepatic I/R injury is a two-stage process in which initial hypoxia to the tissue results in cellular damage that later becomes further exacerbated when oxygenated blood is reperfused to the affected tissue. The current approaches towards this issue are mostly preventative (i.e., ischemic preconditioning) and cannot be routinely used in the clinical settings. However, despite many efforts, an unmet need still exists for an effective treatment to prevent hepatic I/R injury.

The primary objective of the current study is to develop human AM in combination with human AMBP-1 as a novel therapeutic strategy for hepatic I/R injury. Towards this end, the effect of a low dose AM (24 μg/kg), which has been shown to not induce hypotension, was examined in combination with AMBP-1 (80 μg/kg) in a rabbit model of hepatic I/R. Treatment with AM/AMBP-1 improved histological integrity by decreasing tissue injury, as evidenced by the reduction in liver enzymes (AST and ALT), and LDH following hepatic I/R. Treatment with AM/AMBP-1 also decreased IL-6 levels in the ischemic liver. However, there were no changes in TNF-α, MPO, lactate and Suzuki scores after AM/AMBP-1 treatment, possibly due to small sample size. Nevertheless, these results further highlight the role of low-dose AM in reducing inflammation and tissue injury. Treatment with AM/AMBP-1 also decreased liver apoptosis in hepatic I/R. In addition to the liver, remote organs such as the lungs can also be injured during hepatic I/R. The present study also demonstrated that in vehicle-treated rabbits, the lung histological architecture was notably altered and that the AM/AMBP-1 treatment attenuated such damage suggesting that the treatment not only benefited the local organ, but also reduced injury to remote organs such as the lungs.

Adrenomedullin is a 52-amino acid peptide first discovered from a pheochromocytoma originating from the adrenal medulla [4]. The primary function of AM is the vasodilation of vascular resistance and capacitance vessels. AM lowers blood pressure, but increases flow, thereby influencing vascular tone and preserving endothelial integrity [4]. Although AM was first discovered as a vasodilatory peptide, it has multiple biological functions. Among these, the anti-inflammatory property of AM has been well-studied in various pathophysiological conditions [14, 15, 24]. The authors have previously demonstrated that AM plasma levels are increased in various rodent models of organ injury, including hepatic I/R injury [24]. The elevation of AM levels during these conditions has been considered as a compensative mechanism to counteract the cardiovascular effects [18]. AM is a bioactive peptide with pleiotropic effects with a potential in therapeutics for a wide range of diseases [32].

Although AM is elevated in various conditions including hepatic IR, the beneficial effect of AM is highly dependent on its biological activity. The authors have previously demonstrated that AM plays an important

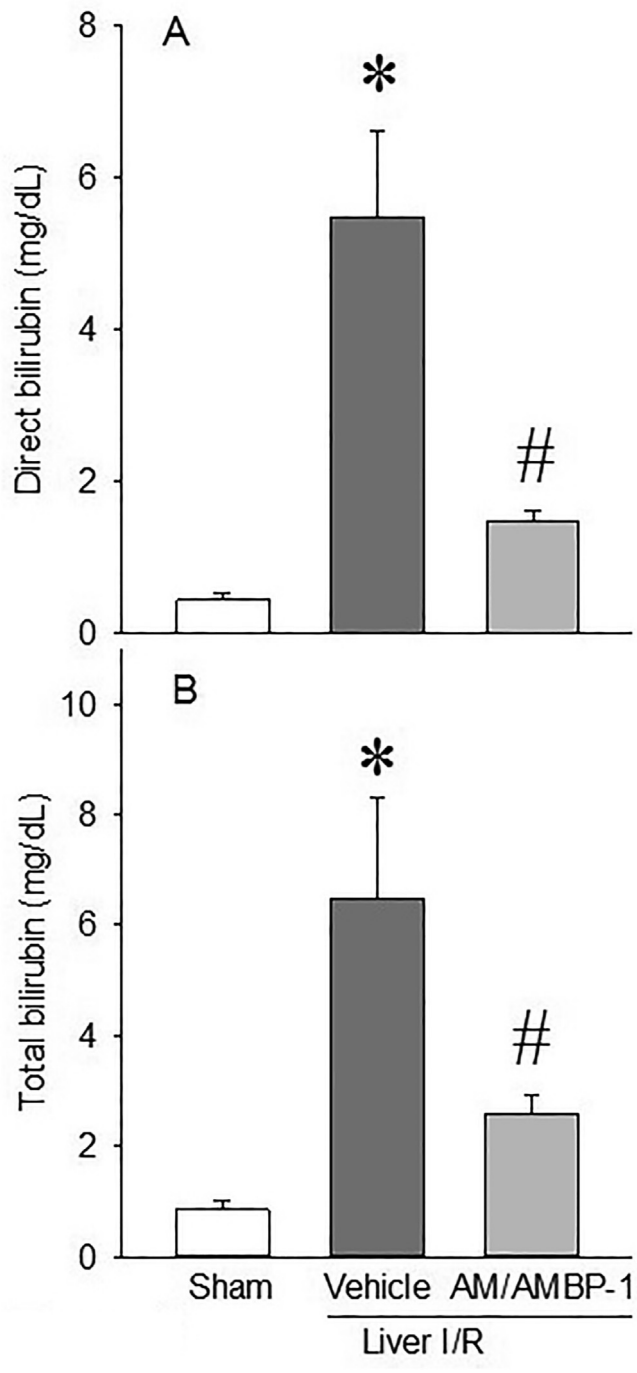

Figure 2. Hepatic I/R-induced bilirubin levels are reduced following treatment with human AM/AMBP-1. Serum contents of (A) direct and (B) total bilirubin following reperfusion in rabbits treated with the vehicle or human AM/AMBP-1. Data are presented as the means ± SEM [Sham (n = 3), Vehicle (n = 6) and AM/AMBP-1 (n = 6)] and compared by one-way ANOVA and the SNK test. *P < 0.05 vs. Sham; #P < 0.05 vs. Vehicle. I/R, ischemia/reperfusion; AM, adrenomedullin; AMBP-1, AM-bindng protein 1.

role in the initiation of the early hyperdynamic phase in sepsis and that a reduced vascular response to AM appears to be responsible for the transition from the early phase to the late phase in sepsis [33]. Subsequently, it was demonstrated that a reduction in the levels of AMBP-1 is responsible for the vascular hyporesponsiveness to AM in late sepsis [34]. Therefore, although AM is elevated in various conditions, its hyporesponsiveness to the vasculature could prevent the beneficial effect of AM in various diseases. Alternatively, others have shown that the plasma-half-life of AM injected intravenously is relatively short and when AM is infused continuously, the dose needs to be monitored carefully to avoid reduction in blood pressure [35, 36]. To overcome these acute effects, AM has been molecularly modified with polyethylene glycol which has been shown to increase the plasma half-life *in vivo* 7- to 8-fold without interfering with its pharmacological effect. Yet, others have developed a humanized non-neutralizing anti-AM antibody, adrecizumab, an antibody directed against the N-terminal part of AM without affecting its biological activity, to prevent AM from diffusing freely from circulation to the interstitium and thereby preventing its potent vasodilatory effects [37]. Therefore, although AM is elevated during various conditions including hepatic IR, its bioavailability and/or its hyporesponsiveness to the vasculature could hinder its beneficial effect in various diseases.

It should be noted that the present study has several limitations. Firstly, the authors have not included either an AM alone or AMBP-1 alone group in the current study. In a previous study using a rat model of hepatic IR injury, the authors demonstrated that the administration of a low dose of either human AM alone (12 μg/kg) or 40 μg/kg AMBP-1 alone did not show statistically significant changes in ALT, AST and LDH compared with the vehicle-treated animals [24]. Similarly, neither human AM alone at 48 μg/kg BW nor human AMBP-1 alone at 160 μg/kg BW prevented the significant increase in AST, ALT, lactate and creatinine in a rat model of hemorrhagic shock [38]. The above-mentioned doses are 2-fold the doses used in the present study. Since much of the work including additional combination doses of AM and AMBP-1, additional time points as well as AM alone or AMBP-1 alone were done in rodent models of sepsis, ischemia reperfusion injury and hemorrhagic shock [24, 38], we did not include in the current study separate groups of AM and AMBP-1 alone treatment groups. Not including either AM or AMBP-1 alone groups in the study is a potential problem for the study in interpreting the experimental results. Therefore, to confirm the physiological relevance of the combined treatment, additional studies including AM and AMBP-1 alone treatments are needed. Secondly, the present study only used a single dose of AM and/or AMBP-1. In a previous study using a rat model of hepatic I/R, the authors used 12 μg/kg human AM and 40 μg/kg human AMBP-1. AM at a dose of 12 μg/kg was used since it increases plasma AM levels that doubles the levels generally seen in sepsis (600–700 pg/ml). In the case of AM, it is a peptide with a very low concentration in the blood (about 50 ng/ml in humans) and it is possible that 12 μg/kg could elicit a physiological response. However, in the case of AMBP-1, it is a very abundant blood protein (about 500 μg/ml in humans). Therefore, it can be questioned how adding just 40 ug/kg would modify the physiopathology of the organism. To directly understand this relationship between AM and AMBP-1, the authors have previously conducted an experiment in a rodent model of sepsis. The thoracic aorta from rats subjected to polymicrobial sepsis by cecal ligation and puncture (CLP) was harvested at 20 h after CLP. The aortic levels of AMBP-1 were determined by Western blot analysis. Aortic level of AMBP-1 decreased significantly at 20 h after CLP. Aortic rings were then prepared and treated with 10^{-7} M AM alone or AM in combination with AMBP-1 at 2×10^{-9} M or 5×10^{-9} M. Vascular responsiveness to AM in vitro significantly decreased whereas treatment with AM in combination with AMBP-1 restored vascular relaxation in a dose related manner. These findings suggest that reduced AMBP-1 appears to be responsible for the vascular AM hyporesponsiveness observed during late or the hypodynamic phase of sepsis [34]. The role of AMBP-1 in maintaining vascular responsiveness to AM in vivo during late stage of sepsis was then examined. AMBP-1 at 40 μg/kg was infused intravenously at the beginning of sepsis for 20 min and AM at 12 μg/kg was continuously administered for the duration of 20 h using an Alzet mini-osmotic pump starting 3 h before the induction of sepsis. Cardiac output, systemic oxygen delivery, stroke volume, total peripheral resistance and organ blood flow were assessed at 20 h after CLP. Treatment with AM/AMBP-1 prevented the decrease in these measured systemic and regional hemodynamic

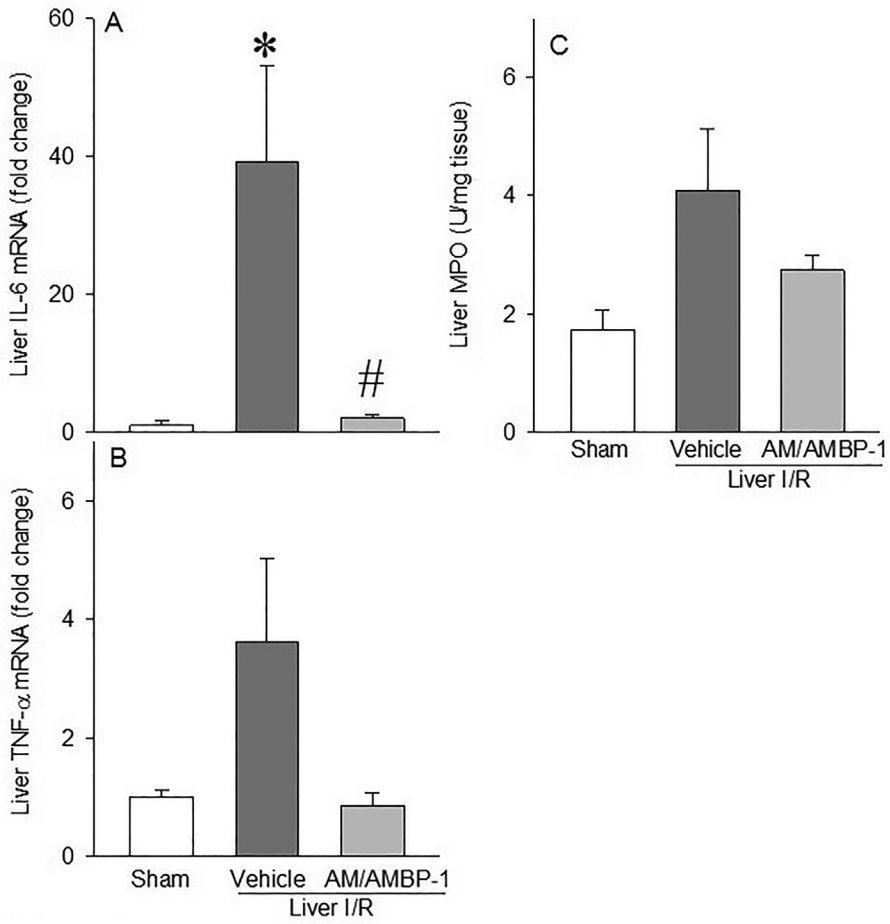

Figure 3. Hepatic I/R-induced liver inflammation is reduced following treatment with human AM/AMBP-1. Liver (A) IL-6 and (B) TNF-α mRNA expression in the ischemic liver determined by RT-qPCR. (C) Neutrophil infiltration assessed by measuring MPO activity. Data are presented as the means ± SEM [Sham (n = 3), Vehicle (n = 6) and AM/AMBP-1 (n = 6)] and compared by one-way ANOVA and the SNK test. *P < 0.05 vs. Sham; #P < 0.05 vs. Vehicle. I/R, ischemia/reperfusion; AM, adrenomedullin; AMBP-1, AM-binding protein 1; IL-6, interleukin 6; TNF-α, tumor necrosis factor α.

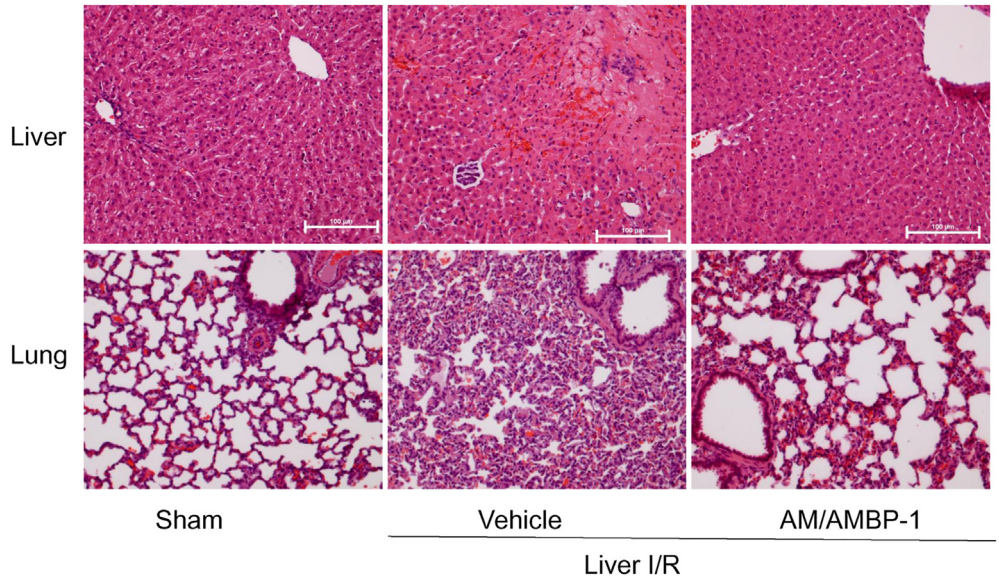

Figure 4. Hepatic I/R-induced tissue histology of the ischemic liver is improved following treatment with human AM/AMBP-1. Representative photomicrograph of H&E slides of ischemic liver tissues (top panel) and the lungs (bottom panel) from the Sham, Vehicle or human AM/AMBP-1 treated rabbits. I/R, ischemia/reperfusion; AM, adrenomedullin; AMBP-1, AM-binding protein 1.

parameters indicating that the combined treatment maintained cardiovascular response. Neither AM nor AMBP-1 alone was sufficient in maintaining cardiovascular stability at late sepsis [23]. In a subsequent study, the administration of human AM (24 μg/kg) and human AMBP-1 (80 μg/kg) at 10 h following the initiation of sepsis attenuated tissue injury and markedly improved the survival rate [39]. Treatment with AM/AMBP-1 not only restored cardiovascular response but also attenuated hepatic damage and improved survival in sepsis. Therefore, in the

Table 1. Suzuki scores for liver damage assessment following hepatic I/R injury.

Parameter	Sham	Vehicle	AM/AMBP-1
Congestion	0	4.0 (2.7–4.0)	2.5 (2.0–2.7)
Vacuolization	0.0 (0.0–0.7)	3.0 (2.2–3.2)	1.2 (1.2–1.4)
Necrosis	0.0 (0.0–0.7)	3.5 (2.4–3.7)	1.0 (1.0–1.4)
WBC infiltration	0	2.2 (1.3–2.6)	0.5 (0.5–0.7)

Data are presented as the median and interquartile range, n = 3. AM, adrenomedullin; AMBP-1, AM-binding protein 1; WBC, white blood cell.

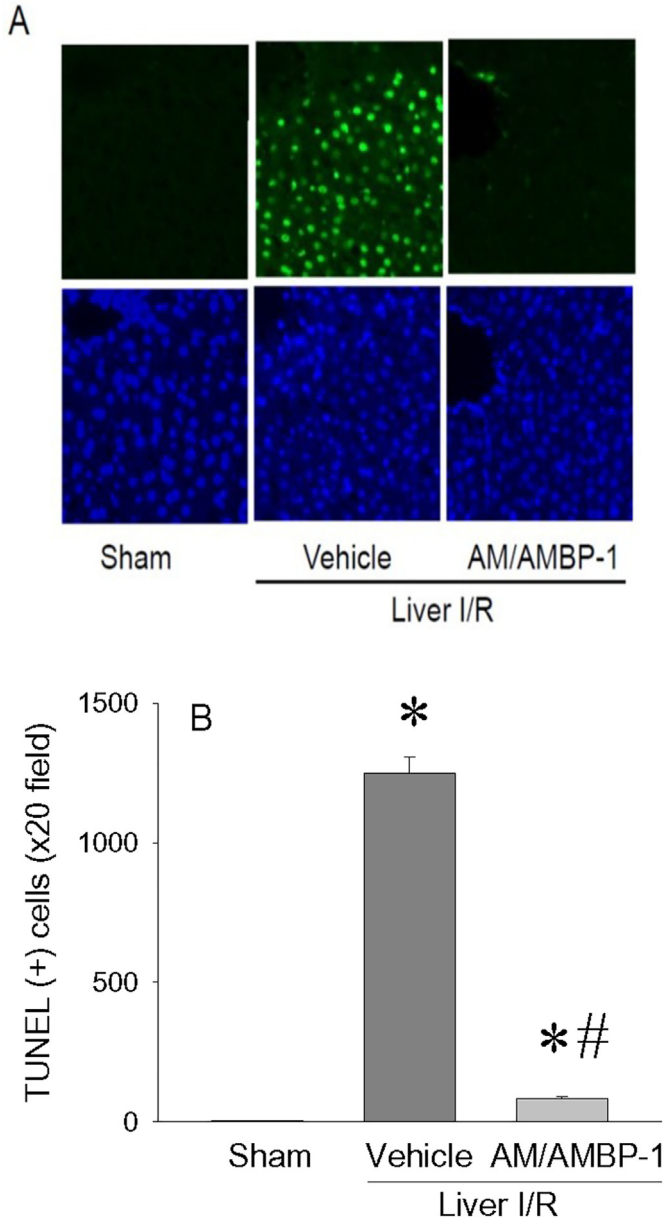

Figure 5. *Hepatic I/R-induced apoptosis in the ischemic liver is reduced following treatment with human AM/AMBP-1.* (A) Representative photomicrographs of TUNEL staining in ischemic liver tissues. B) TUNEL-positive cells per x20 field (x200 magnification) calculated from (A). Data are presented as the means ± SEM (n = 3/group) and compared by one-way ANOVA and the SNK test. *P < 0.05 vs. Sham; #P < 0.05 vs. Vehicle. I/R, ischemia/reperfusion; AM, adrenomedullin; AMBP-1, AM-binding protein 1.

present study of hepatic IR in rabbits, the authors chose to use human AM at 24 ug/kg and human AMBP-1 at 80 ug/kg. Similarly, treatment with similar dose of AM/AMBP-1 in a rodent model of hemorrhagic shock, prevented the decrease in cardiac output and blood flow to the liver, gut and the kidneys. Treatment with AM/AMBP-1 also increased mean arterial pressure from vehicle-treated hemorrhaged animals but still remained lower than the sham operated animals [40]. These prior findings clearly showed the significant role AM/AMBP-1 play in the physiopathology of the organism. Therefore, it can be hypothesized that AM/AMBP-1 treatment can restore cardiac function following hepatic IR in rabbits. However, future studies are warranted in order to confirm such conclusion. Fourthly, the present study only examined one time point (i.e., 20 h following reperfusion). Future studies are thus required to address the optimal doses and the time course for treatment.

In conclusion, additional experiments with AM and AMBP-1 alone treatments are needed in this non-rodent species of hepatic I/R injury to confirm the physiological relevance of the combined treatment. Therefore, the present study suggests that treatment with low-dose human AM, which does not induce hypotension, in combination with human AMBP-1, could be developed as a novel therapeutic for hepatic I/R injury.

Declarations

Author contribution statement

Asha Jacob: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Zhimin Wang: Performed the experiments; Analyzed and interpreted the data.

Hao Ting Yen: Performed the experiments.

Ping Wang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare the following conflict of interests: Ping Wang has been issued Patents (US 6,864,237 B2; US 6,884,781 B2; WO/2005/097172) for the use of AM/AMBP-1 for shock and ischemia/reperfusion treatments. The other authors declare that they have no competing interests.

Additional information

No additional information is available for this paper.

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