HYDROGEN GAS INHALATION ATTENUATES ENDOTHELIAL GLYCOCALYX DAMAGE AND STABILIZES HEMODYNAMICS IN A RAT HEMORRHAGIC SHOCK MODEL

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ABSTRACT—**Background:** Hydrogen gas (H_2) inhalation during hemorrhage stabilizes post-resuscitation hemodynamics, improving short-term survival in a rat hemorrhagic shock and resuscitation (HS/R) model. However, the underlying molecular mechanism of H_2 in HS/R is unclear. Endothelial glycocalyx (EG) damage causes hemodynamic failure associated with HS/R. In this study, we tested the hypothesis that H_2 alleviates oxidative stress by suppressing xanthine oxidoreductase (XOR) and/or preventing tumor necrosis factor-alfa (TNF-α)-mediated syndecan-1 shedding during EG damage. **Methods:** HS/R was induced in rats by reducing mean arterial pressure (MAP) to 35 mm Hg for 60 min followed by resuscitation. Rats inhaled oxygen or H_2 + oxygen after achieving shock either in the presence or absence of an XOR inhibitor (XOR-I) for both the groups. In a second test, rats received oxygen alone or antitumor necrosis factor (TNF)-α monoclonal antibody with oxygen or H_2. Two hours after resuscitation, XOR activity, purine metabolites, cytokines, syndecan-1 were measured and survival rates were assessed 6 h after resuscitation. **Results:** H_2 and XOR-I both suppressed MAP reduction and improved survival rates. H_2 did not affect XOR activity and the therapeutic effects of XOR-I and H_2 were additive. H_2 suppressed plasma TNF-α and syndecan-1 expression; however, no additional H_2 therapeutic effect was observed in the presence of anti-TNF-α monoclonal antibody. **Conclusions:** H_2 inhalation after shock stabilized hemodynamics and improved survival rates in an HS/R model independent of XOR. The therapeutic action of H_2 was partially mediated by inhibition of TNF-α-dependent syndecan-1 shedding.

KEYWORDS—Fluid resuscitation, molecular hydrogen, syndecan-1, TNF-α, vascular permeability, xanthine oxidoreductase

INTRODUCTION

The dynamic equilibrium of the vascular system is profoundly disturbed by hemorrhagic shock (HS). Even after restoration of lost blood volume by fluid resuscitation, vascular dysfunction continues to progress depending on shock severity and the type of resuscitation fluids used (1). Endothelial dysfunction is known to be a major cause of hemodynamic

failure and secondary organ dysfunction, and so is a target for successful resuscitation after HS (2–6).

The endothelial glycocalyx (EG) is a network of membrane-bound proteoglycans and glycoproteins covering the endothelial lumen that maintains vascular homeostasis, including controlling vascular permeability, controlling microvascular tone, preventing microvascular thrombosis, and regulating leukocyte adhesion (7–10). Intravital microscopy has shown that EG thickness was reduced after hemorrhage in skeletal muscle and mesenteric venules (11) and degradation of EG is pertinent to increased HS and fluid resuscitation (HS/R) microvascular permeability (1, 4, 12). Fluid resuscitation further damages the EG via ischemia-reperfusion injury after initial HS-induced degradation (13). Syndecan-1, a transmembrane heparan sulfate proteoglycan and a primary component of the EG, is shed into the blood during HS/R (12, 14). Recent studies revealed that plasma syndecan-1 is a marker for glycocalyx shedding that is strongly correlated with both reduced EG thickness and increased microvascular permeability when different fluids are used for resuscitation in hemorrhaged rats (4). Therapeutics for maintaining vascular endothelial barrier function in the microcirculation by minimizing EG damage are unmet clinical needs.

The EG is degraded by metalloproteinases, heparanase, and hyaluronidase (15, 16). These sheddases are activated by

reactive oxygen species (ROS) and pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) (17, 18). One of the immediate responses in the complex pathophysiology of HS is the activation of the NFκB pathway, primarily through macrophages that detect damage-associated molecular patterns (19). Subsequently, TNF-α is released in the acute phase (20) and plays a pivotal role in the shedding of vascular EG, eventually increasing vascular permeability (21, 22).

Hydrogen (H_2) is a versatile medical gas that can be used effectively in various situations of ischemia-reperfusion (I/R) injury (23). We have previously reported that inhaled H_2 stabilizes hemodynamics and improves short-term survival after HS/R (24). However, several issues need to be addressed before clinical application. First, inhalation of H_2 was initiated at the beginning of hemorrhage, which is inconsistent with clinical settings. Second, mandatory mechanical ventilation at a fixed rate may have impaired the respiratory compensation during shock which left the criticism that effect of H_2 may have augmented. Third, resuscitation was performed with a saline infusion that was four times the shed blood volume. This large infusion of saline may provoke acidosis due to chlorine load. Fourth, the mechanisms and targets of H_2 have not been clarified. We therefore modified our previous protocol to better mimic clinical conditions to determine the efficacy of H_2, with spontaneous breathing after the induction of shock and resuscitation with balanced crystalloids. We hypothesize that H_2 stabilizes hemodynamics in rats after HS/R by targeting vascular EG.

H_2 has an antioxidative effect, but its mechanism has not been fully elucidated (23, 25). Xanthine oxidoreductase (XOR) is one of the major sources of ROS production after I/R injury (26, 27). XOR catalyzes the oxidation of hypoxanthine to xanthine, and then xanthine to uric acid. XOR exists in two interconvertible forms: xanthine dehydrogenase (XDH) and xanthine oxidase (XO). XDH is dominant in healthy tissue and uses NAD^+ as an electron acceptor. It is converted to the XO form either reversibly by the oxidation of sulfhydryl residues or irreversibly by proteolysis (28). Since XO uses oxygen (O_2) as a terminal electron acceptor to ROS, conversion from XDH to XO has been proposed to cause I/R injury. XOR is a homodimer with four molybdenum-containing molybdopterin cofactors per monomer. Metallic molybdenum is produced through reduction of molybdenum trioxide by using H_2 as a reducing agent. Thus, we hypothesize that H_2 suppresses ROS production by reducing the active center molybdenum thereby suppressing the activity of XOR.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (300 g–350 g) were used in all experiments (Sankyo Labo Service Corporation, Inc, Tokyo, Japan). Animals had food and water *ad libitum* at all times. Animals were housed at $22 \pm 1°C$ and $55 \pm 5\%$ humidity with a 12:12-h light:dark cycle. Rats were allowed to acclimatize for at least 5 days after arrival. The study was approved by the Institutional Animal Care and Use Committee (Keio University, No. 13002-3). All animal experiments are in concordance with ARRIVE guidelines (29).

Anesthesia, analgesia, and surgical procedures

Rats were anesthetized by intraperitoneal injection of medetomidine, midazolam, and butorphanol (0.15 mg/kg, 2.0 mg/kg, and 2.5 mg/kg, respectively). An additional third of the initial dose was injected upon limb extraction response to pain stimulation. Temperature was measured using a rectal probe (BAT-10, Physitemp Instruments Inc, Clifton, NJ) and maintained at 37°C with a heating pad. Polyethylene catheters (PE50, Natsume, Tokyo, Japan) were placed into the left femoral artery and vein for blood withdrawal/sampling and fluid administration, respectively. A Millar transducer catheter (SPR-320; AD Instruments, Bella Vista, NSW, Australia) was placed into the abdominal aorta from the right femoral artery for blood pressure monitoring. Electrocardiograms, heart rate, arterial blood pressure, and body temperature were continuously recorded using a polygraph (Powerlab System, LabChart; AD Instruments, Bella Vista, NSW, Australia).

HS/R protocol

HS was induced using the fixed-pressure model as previously described with minor modifications (24). Briefly, blood was withdrawn over 15 min to achieve a mean arterial pressure (MAP) of 35 ± 2 mm Hg to induce HS (Fig. 1). MAP was maintained at this level until 60 min after initial induction of HS by further blood withdrawal or reinfusion of shed blood. After the shock phase, rats were resuscitated with an infusion of three times the volume of shed blood of lactated-Ringer solution over 30 min. Maintenance fluid of lactated-Ringer at 1 mL/kg/h was administered during the observation phase. Rats were sacrificed by blood withdrawal under anesthesia at various time points. Sham-operated rats received all procedures except blood withdrawal.

Experimental groups

In the XOR inhibition experiment, rats were randomly sorted into four groups: 26% O_2, 74% N_2 gas (control); H_2 gas (H_2); oral XOR-I and 26% O_2, 74% N_2 gas (XOR-I); and oral XOR-I and H_2 gas (H_2+XOR-I) (Fig. 1A). In the experiment with anti-TNF-α monoclonal antibody (mAb), rats were randomized into three groups: 26% O_2, 74% N_2 gas (control); anti-TNF-α and 26% O_2, 74% N_2 gas (mAb); and anti-TNF-α and H_2 gas (H_2+mAb) (Fig. 1B). Given the nature of the protocol, it was impossible to blind the investigator to the treatments.

Gas inhalation

Control and H_2 (1.3% H_2, 26% O_2, 72.7% N_2) gases were manufactured at Taiyo Nippon Sanso Corporation (Tokyo, Japan). Control gas inhalation was initiated at the beginning of the experiment. H_2 gas inhalation began 30 min after induction of HS and continued until the end of the experiment (Fig. 1). Gas was provided through a nose cone to allow spontaneous respiratory compensation during HS/R.

Xanthine oxidase inhibition

The XOR-I Topiroxostat (Sanwa Kagaku Kenkyusho Co, Ltd, Aichi, Japan) was suspended in 0.5% methylcellulose and 10 mg/kg was orally administered 60 min before the HS induction. Control and H_2 groups were administered equivalent amounts of methylcellulose.

Anti-TNF-α antibody administration

TNF-α was neutralized by systemic administration of anti-TNF-α mAb as previously described with minor modifications (30). Anti-TNF-α (MAB510-100, R&D Systems, Minneapolis, Minn) was diluted with phosphate-buffered saline and 1 μg/kg was administered intravenously 60 min before the induction of HS. Control rats received an equivalent amount of phosphate-buffered saline as vehicle.

Blood analysis

Arterial blood samples were collected at baseline (0 min; beginning of the shock phase), 60 min (end of the shock phase), and 150 min (1 h from the end of the resuscitation phase). The pH, oxygen tension, carbon dioxide tension, bicarbonate, base excess, hemoglobin, and potassium concentrations were assessed by arterial blood gas analyses (ABG) (iSTAT, Abbott Point Care Inc, Princeton, NJ). Lactate levels were measured using a Lactate Pro2 meter (ARKRAY, Kyoto, Japan). Blood samples were centrifuged at 1,500 × g for 10 min at 4°C, and plasma was stored at −80°C. Plasma concentrations of IL-1β
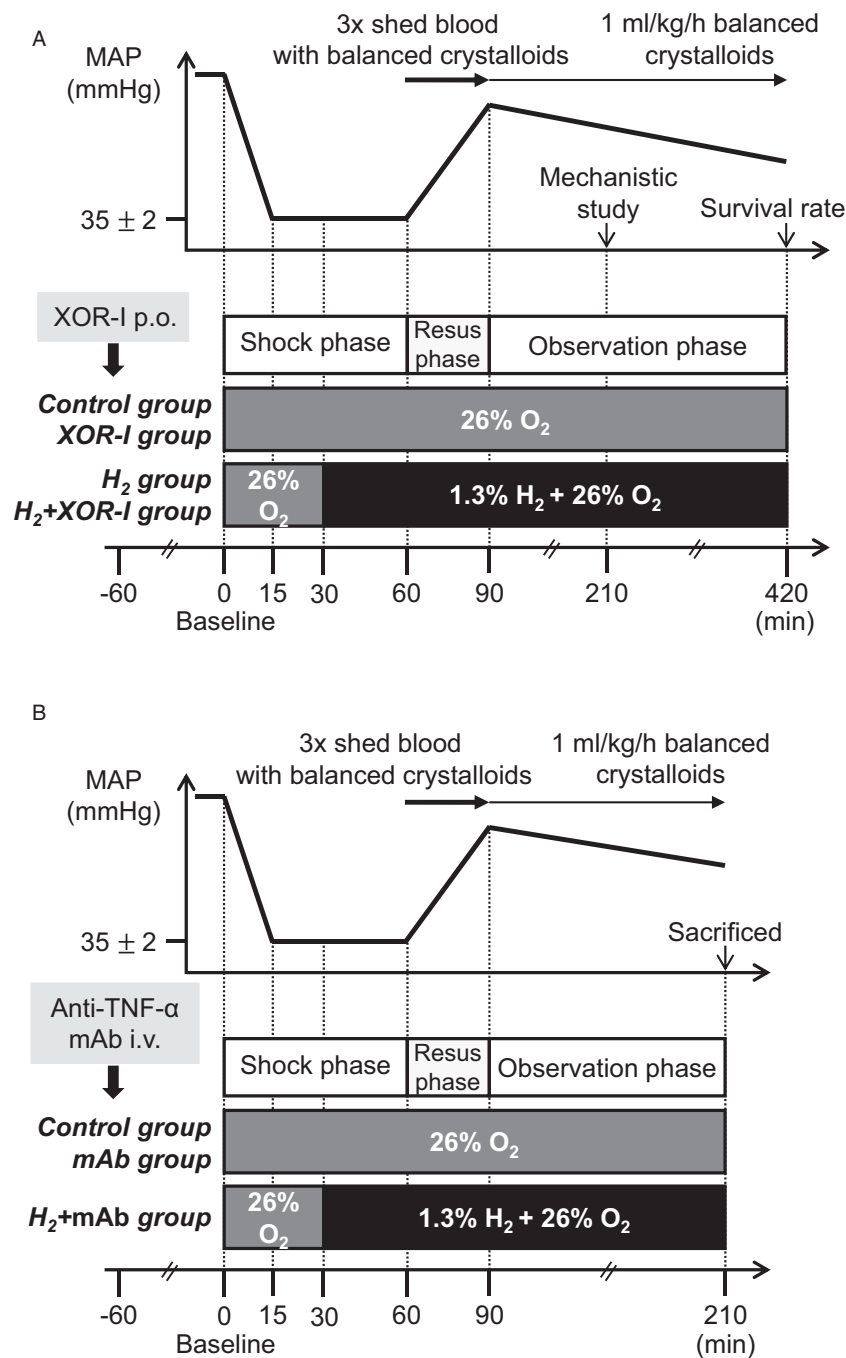
Fig. 1. **Experimental protocol for hemorrhagic shock and resuscitation.** Hemorrhagic shock was induced using the fixed-pressure model. MAP of 35 ± 2 mm Hg was achieved over 15 min by blood withdrawal and maintained until 60 min from the induction of hemorrhagic shock. After the shock phase, rats were resuscitated with an infusion of three times the volume of the shed blood of balanced crystalloids over 30 min. Maintenance fluid was administered during the observation phase. A, Rats inhaled oxygen or H$_2$ + oxygen after achieving shock either in the presence or absence of an XOR-I for both groups. B, Rats received oxygen alone or anti-TNF-α monoclonal antibody with oxygen or H$_2$ + oxygen. H$_2$ indicates hydrogen gas; i.v., intravenous injection; MAP, mean arterial pressure; P.O., per Os; Resus, resuscitation; TNF-α, tumor necrosis factor-alfa; XOR-I, xanthine oxidoreductase inhibitor.

(Invitrogen, Calif), TNF-α (Invitrogen), and syndecan-1 (Signalway Antibody LLC, Md) were measured with ELISA kits according to the manufacturer's instructions.

Bronchoalveolar lavage

A tracheostomy was performed after rats were sacrificed 2 h after resuscitation. Bronchoalveolar lavage was performed twice with 8 mL cold phosphate-buffered saline. Numbers of red blood cells (RBC) in bronchoalveolar lavage fluid (BALF) were manually counted.

XOR activity and purine metabolite measurement

XOR activity and purine metabolite concentrations, hypoxanthine, xanthine, and uric acid were measured in plasma, liver, kidney, and lung as previously described (31, 32).

Statistical analysis

Data are expressed as the means $\pm$ standard error mean. Differences between survival rates were tested using the Kaplan–Meier analysis. A log-rank test was

used to compare survival rates. One-way analysis of variance followed by a Tukey–Kramer multiple comparison test was used to compare the variables among groups. Hemodynamic data were analyzed using a mixed-effects model for repeated measures as previously described (24). Spearman rank correlation coefficient was used for evaluation of correlations between variables. *P* values under 0.05 were considered significant. All data were analyzed using SPSS 25.0 statistical software (SPSS Inc, Chicago, Ill).

RESULTS

H_2 and XOR-I improve MAP and survival after HS/R

We tested the hypothesis that H_2 suppresses enzyme activity of XOR to control I/R injury by comparing the therapeutic effects of H_2 and XOR-I alone or in combination after HS/R. ABG variables at baseline were comparable among all groups (See Table, Supplemental Digital Content 1, which demonstrates the ABG at baseline, http://links.lww.com/SHK/A956). Shed blood volume was not different between groups (Table 1). At the end of the shock phase, hemorrhaged rats developed severe lactic acidosis with respiratory compensation (Table 2). XOR-I and H_2+XOR-I administration appeared to suppress the accumulation of lactate during hypovolemic shock, but the

differences in lactate levels between these groups and the control were not statistically significant (Table 2).

Lactic acidosis improved after fluid resuscitation; however, lactate accumulation 60 min after completion of resuscitation was reduced in the single-treatment groups and this reduction was additive in the combination treatment group (Table 3).

MAP recovered in response to fluid resuscitation; however, in the control group, it decreased to less than 65 mm Hg immediately after the completion of resuscitation. In contrast, MAP was significantly maintained in the H_2, XOR-I, and H_2+XOR-I groups (Fig. 2A). MAP was higher in the H_2 group than in the XOR-I group 60 min after completing fluid resuscitation; however, this difference subsequently resolved over time. With the combination therapy, MAP was maintained at a higher level than either therapy alone. Time course changes in heart rate were comparable among the four groups (Fig. 2B).

Survival 6 h after initiation of fluid resuscitation was 20% in the control group, and 83%, 80%, and 100% in the XOR-I, H_2, and H_2+XOR-I groups, respectively (Fig. 2C). These survival rates paralleled the hemodynamic changes after HS/R.

TABLE 1. **Blood withdrawal and resuscitation fluid variables**

Variables	Control n = 10	H_2 n = 12	XOR-I n = 11	H_2+XOR-I n = 11	P value
Body weight (g)	318.8 ± 6.3	328.8 ± 3.6	328.3 ± 3.1	327.5 ± 3.9	0.35
Shed blood volume (mL)	9.4 ± 0.2	9.9 ± 0.2	9.1 ± 0.2	9.3 ± 0.2	0.08
Shed blood volume (mL/kg)	30.0 ± 0.8	29.9 ± 0.4	27.7 ± 0.6	28.5 ± 0.7	0.07
Resuscitation fluid volume (mL)	28.3 ± 0.6	30.0 ± 0.7	27.3 ± 0.5	28.0 ± 0.7	0.07
Resuscitation fluid volume (mL/kg)	89.0 ± 2.5	89.8 ± 1.3	83.2 ± 1.9	85.5 ± 2.0	0.07

H_2 indicates hydrogen gas; XOR-I, xanthine oxidoreductase inhibitor.

TABLE 2. **Arterial gas analysis at 60 min after induction of shock**

Variables	Control n = 10	H_2 n = 12	XOR-I n = 11	H_2+XOR-I n = 11	P value
pH	7.313 ± 0.002	7.333 ± 0.02	7.327 ± 0.008	7.326 ± 0.008	0.71
pCO$_2$ (mm Hg)	26.1 ± 2.2	29.7 ± 2.0	29.5 ± 1.3	29.0 ± 1.5	0.50
pO$_2$ (mm Hg)	104.6 ± 2.2*	104.7 ± 2.7*	114.7 ± 3.3	120.9 ± 2.6	<0.001
HCO$_3^-$ (mmol/L)	13.5 ± 1.5	15.7 ± 1.0	15.5 ± 0.7	15.1 ± 0.8	0.45
B.E. (mEq/L)	−12.7 ± 1.7	−10.2 ± 1.1	10.5 ± 0.8	−9.5 ± 2.0	0.46
Potassium (mEq/L)	5.34 ± 0.2	5.98 ± 0.3	5.91 ± 0.3	5.45 ± 0.2	0.20
Lactate (mmol/L)	10.43 ± 1.3	9.32 ± 1.2	8.79 ± 0.5	7.13 ± 0.5	0.13
Hemoglobin (g/dL)	8.5 ± 0.2	9.1 ± 0.5	9.1 ± 0.2	9.3 ± 0.3	0.48

*$P < 0.05$, compared with the H_2+XOR-I group.
B.E. indicates base excess; H_2, hydrogen gas; XOR-I, xanthine oxidoreductase inhibitor.

TABLE 3. **Arterial gas analysis at 150 min after induction of shock**

Variables	Control n = 10	H_2 n = 12	XOR-I n = 11	H_2+XOR-I n = 11	P value
pH	7.417 ± 0.009	7.412 ± 0.01	7.406 ± 0.009	7.402 ± 0.009	0.70
pCO$_2$ (mm Hg)	32.6 ± 1.2	36.3 ± 1.2	34.8 ± 1.9	38.1 ± 1.1	0.07
pO$_2$ (mm Hg)	96.9 ± 1.6	99.6 ± 3.7	108.2 ± 4.2	109.4 ± 3.2*	0.03
HCO$_3^-$ (mmol/L)	21.0 ± 0.8	23.1 ± 0.8	21.8 ± 1.0	23.5 ± 0.8	0.17
B.E. (mEq/L)	−3.4 ± 0.9	−1.4 ± 0.9	−2.8.0 ± 1.0	−1.0 ± 0.9	0.24
Potassium (mEq/L)	4.66 ± 0.2	4.73 ± 0.1	4.67 ± 0.2	4.46 ± 0.1	0.63
Lactate (mmol/L)	4.68 ± 0.5	2.83 ± 0.2*	2.96 ± 0.3*	2.20 ± 0.2*	<0.001
Hemoglobin (g/dL)	6.8 ± 0.4	7.1 ± 0.4	7.5 ± 0.5	7.7 ± 0.3	0.25

*$P < 0.05$, compared with the control group.
B.E. indicates base excess; H_2, hydrogen gas; XOR-I, xanthine oxidoreductase inhibitor.
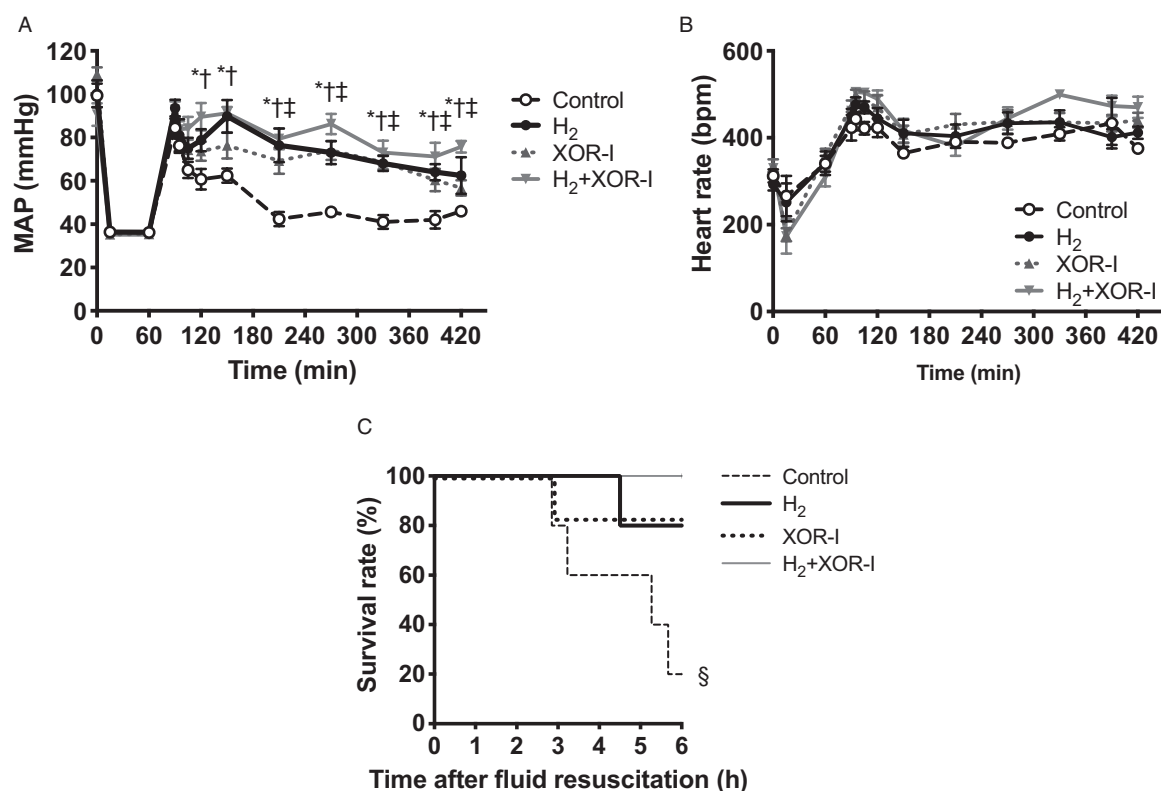
FIG. 2. **Mean arterial pressure and short-term survival was attenuated with H_2 and XOR-I.** A, MAP was significantly maintained in the H_2, XOR-I, and H_2+XOR-I groups. With the combination therapy, MAP was maintained at a higher level than either monotherapy. B, Time course changes in heart rate were comparable among the four groups. C, The survival rates 6 h after initiation of fluid resuscitation were 20% (1/5) in the control group, and were 83% (5/6), 80% (4/5), and 100% (5/5) in the XOR-I, H_2, and H_2+XOR-I treatment groups, respectively. Values are mean ± SEM; n ≥ 5 animals in each group. $^*P < 0.05$, H_2 compared with the control group; $^†P < 0.01$, H_2+XOR-I compared with the control group; $^‡P < 0.01$, XOR-I compared with the control group; $^§P = 0.03$, compared with the control group. H_2 indicates hydrogen gas; MAP, mean arterial pressure; XOR-I, xanthine oxidoreductase inhibitor.

XOR activity and purine metabolite levels are unaffected by H_2 treatment

XOR-I suppressed XOR activity as shown by increased hypoxanthine and decreased xanthine and uric acid before HS, during HS, and after resuscitation (see Figures, Supplemental Digital Content 2 and 3, which demonstrate XOR activity and purine metabolites before, during and after HS/R, http://links.lww.com/SHK/A957). However, XOR activity and purine metabolite levels in the H_2 group were similar to those in the control group.

Attenuation of glycocalyx shedding and vascular hyperpermeability by H_2

Inhaled H_2 attenuated symptoms including abdominal bloating and oliguria or anuria. The number of RBCs in the BALF, a marker for lung permeability, was significantly reduced by H_2 (Fig. 3A). Plasma syndecan-1 was not detected in sham rats but was increased by HS/R. H_2 treatment after HS/R reduced this increase in plasma syndecan-1 (Fig. 3B). In contrast, XOR-I did not lower plasma syndecan-1 levels. Moreover, a combination of XOR-I and H_2 showed no additive effect on plasma syndecan-1 shedding. Plasma syndecan-1 levels were negatively correlated with MAP measurements (Fig. 3C).

H_2 inhalation reduces inflammatory cytokine production

Two hours after the completion of fluid resuscitation, plasma TNF-α and IL-1β levels were elevated in hemorrhaged rats. H_2 reduced plasma TNF-α levels (Fig. 4A), as well as IL-1β, but this reduction was not significant (Fig. 4B). Plasma TNF-α levels were negatively correlated with MAP (Fig. 4C) but positively correlated with plasma syndecan-1 (Fig. 4D).

H_2 maintains hemodynamic stability after HS by suppressing TNF-α-mediated endothelial glycocalyx shedding

We investigated the effect of TNF-α on endothelial glycocalyx degradation, vascular hyperpermeability, and hemodynamic instability after hemorrhagic shock (See Tables, Supplemental Digital Content 4–7, which demonstrates HS/R variables and ABG, http://links.lww.com/SHK/A958). After treatment with anti-TNF-α antibody, we observed a reduction in plasma TNF-α (Fig. 5A), syndecan-1 shedding (Fig. 5B), and RBC leakage in the BALF (Fig. 5C), as well as stabilization of hemodynamics (Fig. 5D) after HS. The change in heart rate over time was comparable among all groups.

We then examined whether H_2 exerts additional therapeutic effects when TNF-α-mediated endothelial glycocalyx shedding was blocked in hemorrhaged rats. When hemorrhaged rats were pretreated with an anti-TNF-α antibody, no further decrease in plasma TNF-α was observed by the administration of H_2 (Fig. 5A). Under these conditions, no additive effect on plasma syndecan-1 shedding (Fig. 5B), RBC leakage in BALF (Fig. 5C), and hemodynamic improvement (Fig. 5D) was observed, indicating that the therapeutic effect of H_2 is exerted
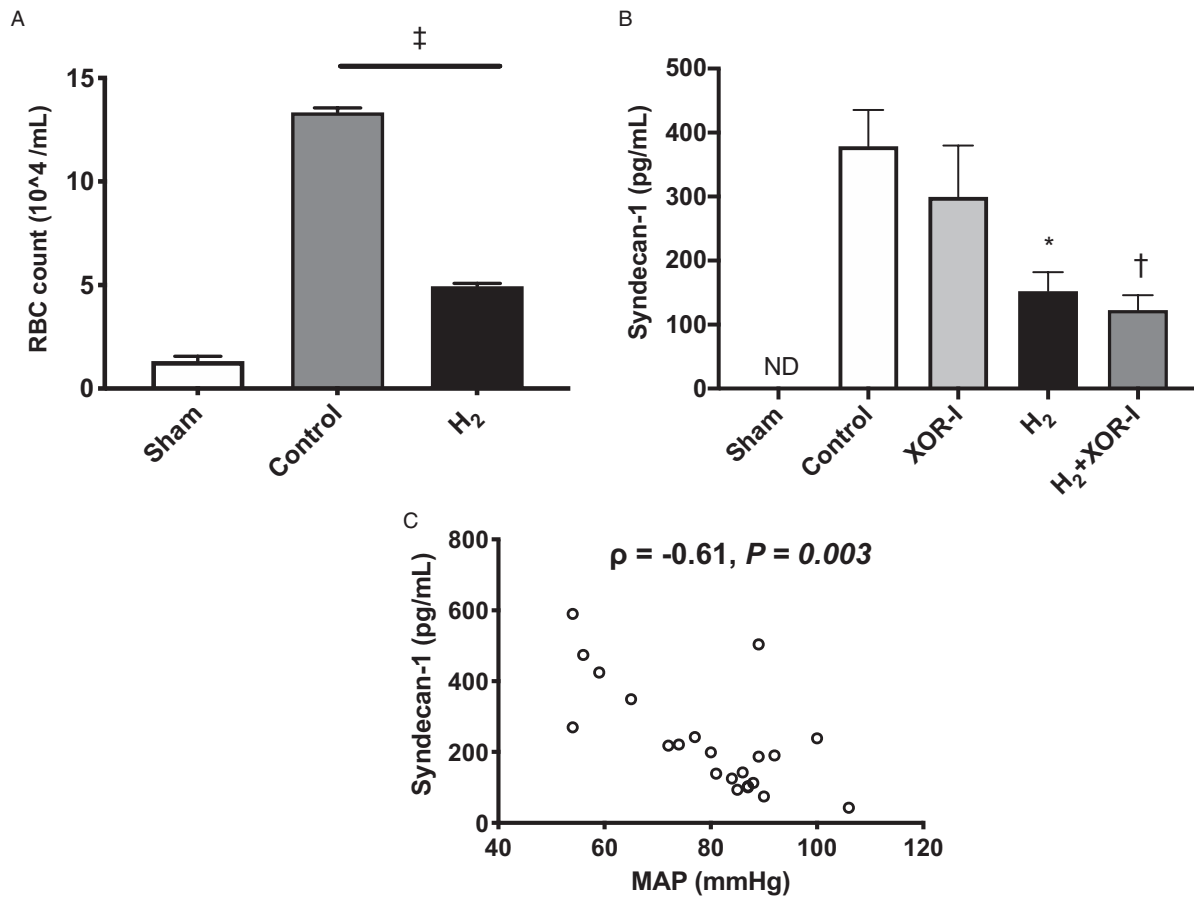
Fig. 3. **Glycocalyx shedding and vascular hyperpermeability was attenuated with H$_2$ inhalation.** A, RBC counts in bronchoalveolar lavage fluid were measured as a marker for vascular permeability. The RBC counts were elevated in the control group after HS/R. H$_2$ inhalation significantly attenuated RBC leakage into the bronchoalveolar lavage fluid compared with the control group. B, Plasma syndecan-1 was reduced with H$_2$ treatment. However, XOR-I did not lower plasma syndecan-1 level ($P = 0.70$). Moreover, a combination of XOR-I and H$_2$ did not show additive effect in attenuating plasma syndecan-1 levels. C, MAP and plasma syndecan-1 are negatively correlated. Values are mean ± SEM; N ≥ 10 animals in each group. $^*P = 0.02$, H$_2$ versus control group; $^\dagger P = 0.008$, H$_2$+XOR-I versus control group; $^\ddagger P < 0.001$. H$_2$ indicates hydrogen gas; MAP, mean arterial pressure; ND, not detected; RBC, red blood cell.

in part through the suppression of TNF-α-mediated EG shedding.

DISCUSSION

We have demonstrated that H$_2$ mitigated the harmful effects of protein-free fluid resuscitation therapy after shock. This effect of H$_2$ was at least in part mediated by the suppression of TNF-α-mediated EG degradation.

Previously, we reported that H$_2$ inhalation upon induction of HS enhanced tolerance to blood loss and hemodynamic stability after fluid resuscitation with four times the shed blood volume of normal saline (24). However, that experimental protocol was inconsistent with typical clinical scenarios and the high infusion volume was harmful (33, 34). Therefore, in this study H$_2$ inhalation was started after shock, mimicking H$_2$ inhalation in an ambulance. Rats maintained spontaneous respiration such that respiratory compensation for metabolic acidosis was not impaired. Furthermore, fluid resuscitation was performed with three times the amount of the shed blood with balanced crystalloids.

We asked if H$_2$ exerts its therapeutic effect by suppressing the activity of XOR and reducing molybdenum localized at the

active center of this enzyme. H$_2$ inhalation did not affect XOR activity, nor were hypoxanthine, xanthine, and urate concentrations changed during HS or 120 min after resuscitation. From these results, we conclude that H$_2$ exerts a therapeutic effect independent of XOR activity.

XOR-I had a hemodynamic stabilization effect equivalent to H$_2$ in the rat HS model after fluid resuscitation. However, as additive an effect of XOR-I and H$_2$ was observed, and XOR-I alone had no effect on syndecan-1 shedding, the mechanisms by which XOR-I and H$_2$ provided stabilization were different. XOR-I increased the pool of salvageable purines and increased ATP levels (35), while the inhibition of XOR increased the NAD/NADH ratio. The conversion of pyruvate to lactate is suppressed under conditions where the NAD/NADH ratio was increased (36, 37). Indeed, accumulation of lactate during shock was suppressed by the pre-administration of XOR-I, which may have beneficial effects related to reinforcement of ATP synthesis due to increased hypoxanthine levels.

From the subjective improvements observed in symptoms including abdominal bloating and anuria, we hypothesized that H$_2$ targets vascular endothelial cells and suppresses vascular hyperpermeability, unregulated vasodilation, microvascular thrombosis, and augmented leukocyte adhesion. Normally,
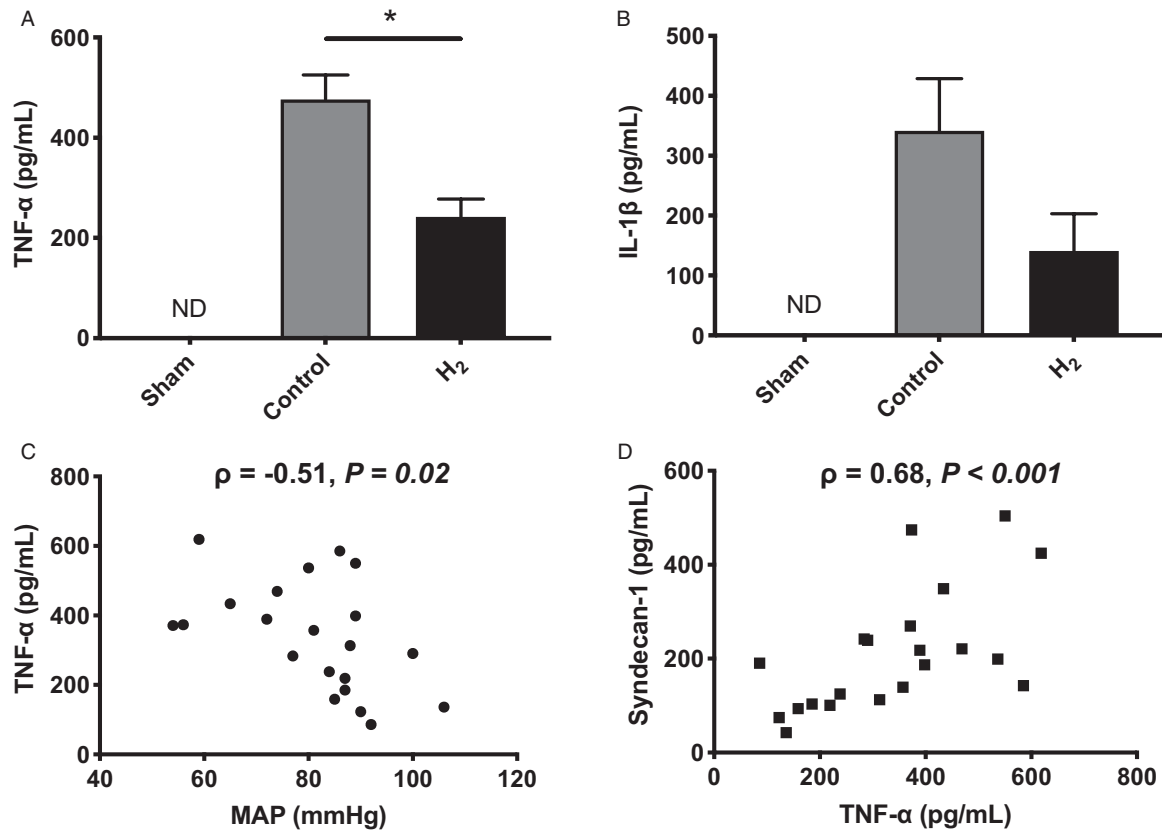
FIG. 4. **Inflammatory cytokines were attenuated with H₂ inhalation and negatively correlated with syndecan-1.** A, H₂ inhalation reduced plasma TNF-α. B, H₂ inhalation reduced IL-1β but this reduction was not significant ($P = 0.09$). C, MAP and plasma TNF-α are negatively correlated after HS/R. D, TNF-α and syndecan-1 are positively correlated after HS/R. *$P < 0.001$. Values are mean ± SEM; n ≥10 animals in each group. H₂ indicates hydrogen gas; IL-1β, interleukin-1 beta; MAP, mean arterial pressure; ND, not detected; TNF-α, tumor necrosis factor-alfa.

the EG provides a barrier against vascular permeability in part by serving as a negatively charged molecular sieve. This barrier limits transvascular movement of negatively charged and/or >70-kDa molecules (38). Moreover, the EG senses fluid-shear forces and transmits them to endothelial cells, initiating nitric oxide-mediated vasorelaxation. The EG has anticoagulant and anti-adhesive effects on the surface of endothelial cells, whose functions are significantly altered following hypovolemic shock by both ischemia and reperfusion. Activation of endothelial cells leads to release of vasoactive substances and inflammatory mediators, including TNF-α, which induces the production of several matrix metalloproteinases and heparanase in endothelial cells (15, 39). As a result, the EG is degraded and its function impaired, leading to enhanced microvascular permeability, edema caused by fluid leakage into surrounding tissue, augmented leukocyte adhesion, platelet aggregation, and dysregulated vasodilation.

Several studies indicate that TNF-α plays a pivotal role in vascular failure and end-organ damage during acute HS and subsequent resuscitation (20–22). In this study, we showed that systemic administration of anti-TNF-α antibody reduced circulating levels of syndecan-1 and improved hemodynamic stability, suggesting a substantial role for TNF-α in glycocalyx degradation. We found that H₂ suppresses the release of TNF-α and therefore suppresses degradation of the EG, preventing vascular endothelial dysfunction after HS/R.

While our results provide new information relevant to the treatment of HS, we did not directly observe a change in microvascular glycocalyx thickness. We used lactated-Ringer solution for fluid resuscitation and therefore cannot comment on how the use of H₂ in HS/R may work in conjunction with the use of plasma in clinical resuscitation, which is reported to cause less shedding of endothelial glycocalyx than protein-free solutions (14, 40, 41). Therefore, further studies are needed to evaluate the therapeutic effects of H₂ during plasma resuscitation. The mechanism of action for H₂ in HS/R is not only the suppression of systemic release of TNF-α; it is also necessary to understand the effects of H₂ on the nervous, endocrine, and metabolic systems. Although we focused on hyperpermeability after HS/R in this study, TNF-α also decreases vascular reactivity through decreased endothelial nitric oxide synthase (NOS) expression and elevated expression of inducible NOS. Future *ex-vivo* studies will clarify the positive effects of H₂ on reduced vascular reactivity after HS/R. Despite these questions, H₂ is a promising treatment option for HS/R that can protect EG and be used in addition to traditional treatments before definitive hemostasis is attained in both field and clinical settings.

In conclusion, we report for the first time that inhalation of H₂ after HS stabilized hemodynamics and short-term survival after fluid resuscitation with balanced crystalloids. This effect was independent of XOR activity and reduced EG degradation in part through the attenuation of TNF-α production. Thus, we
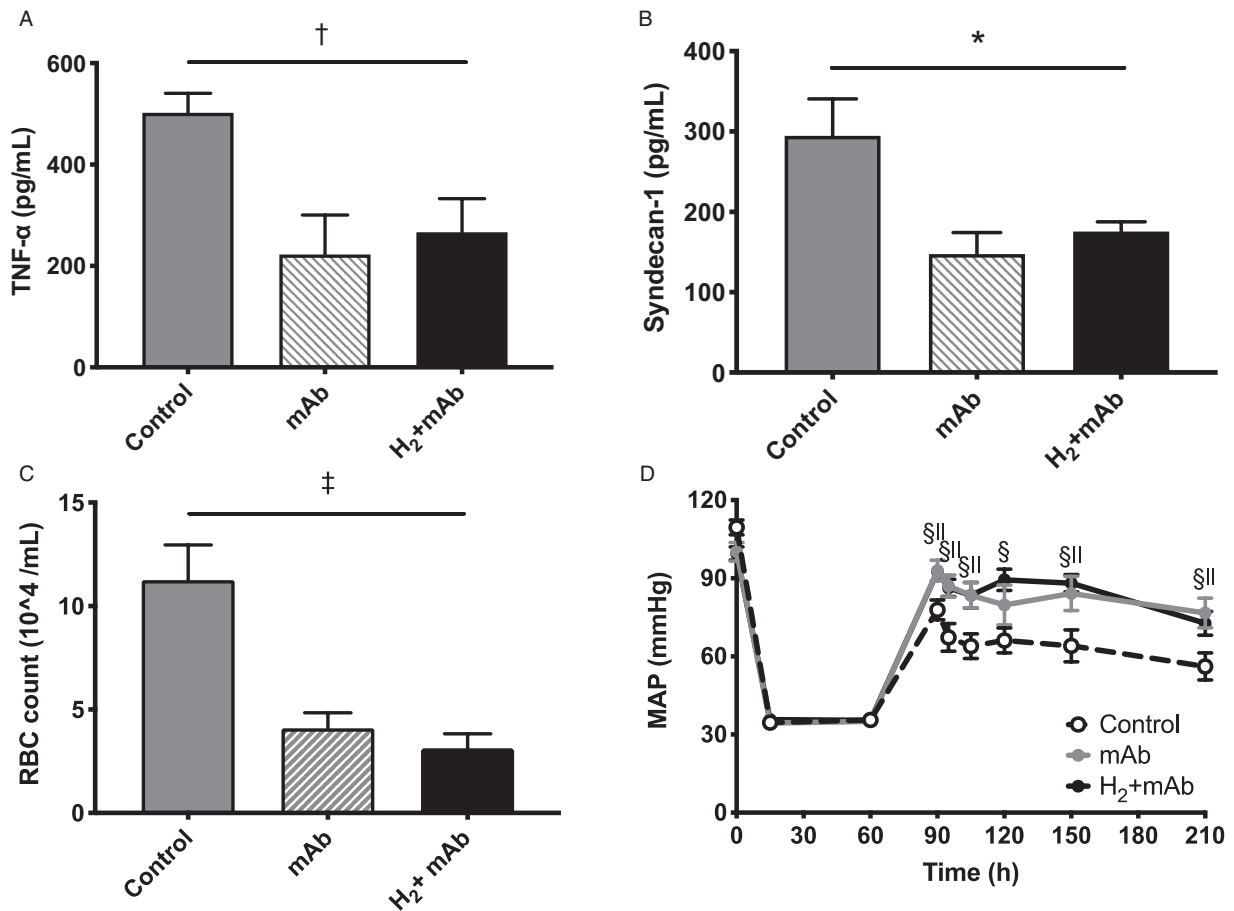
FIG. 5. **Systemic administration of anti-TNF-α antibody attenuated elevation of plasma TNF-α, improved MAP and endothelial glycocalyx degradation.** Systemic administration of an anti-TNF-α monoclonal antibody: (A) attenuated plasma TNF-α concentration and addition of H_2 did not confer an additive effect on plasma TNF-α concentration; (B) attenuated plasma syndecan-1concentration, but there was no observable additive effect of H_2; (C) significantly reduced the number of leaked RBCs in the bronchoalveolar lavage fluid, but did not have an additive effect in combination with H_2 inhalation; (D) attenuated MAP, but an additive effect was not observed with the concomitant treatment of H_2. *$P = 0.04$, ANOVA; †$P = 0.004$, ANOVA; ‡$P < 0.001$, ANOVA; §$P < 0.01$ mAb versus control group; ||$P < 0.01$, H_2+mAb versus control group. Values are mean ± SEM; $n \geq 8$ animals in each group. H_2 indicates hydrogen gas; mAb, anti-TNF-α monoclonal antibody; MAP, mean arterial pressure; RBC, red blood cell; TNF-α, tumor necrosis factor-alfa.

propose that H_2 may be useful for reducing inflammatory cytokines and vascular endothelial dysfunction that further exacerbate organ dysfunction associated with HS/R.

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