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Graft Reconditioning With Nitric Oxide Gas in Rat Liver Transplantation From Cardiac Death Donors

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Background. Liver transplant outcomes using grafts donated after cardiac death (DCD) remain poor.

Methods. We investigated the effects of ex vivo reconditioning of DCD grafts with venous systemic oxygen persufflation using nitric oxide gas (VSOP-NO) in rat liver transplants. Orthotopic liver transplants were performed in Lewis rats, using DCD grafts prepared using static cold storage alone (group-control) or reconditioning using VSOP-NO during cold storage (group-VSOP-NO). Experiment I: In a 30-min warm ischemia model, graft damage and hepatic expression of inflammatory cytokines, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and endothelin-1 (ET-1) were examined, and histologic analysis was performed 2, 6, 24, and 72 hr after transplantation. Experiment II: In a 60-min warm ischemia model, grafts were evaluated 2 hr after transplantation (6 rats/group), and survival was assessed (7 rats/group).

Results. Experiment I: Group-VSOP-NO had lower alanine aminotransferase (ALT) (P<0.001), hyaluronic acid (P<0.05), and malondialdehyde (MDA) (P<0.001), hepatic interleukin-6 expression (IL-6) (P<0.05), and hepatic tumor necrosis factor-alpha (TNF-α) expression (P<0.001). Hepatic eNOS expression (P<0.001) was upregulated, whereas hepatic iNOS (P<0.01) and ET-1 (P<0.001) expressions were downregulated. The damage of hepatocyte and sinusoidal endothelial cells (SECs) were lower in group-VSOP-NO.

Experiment II: VSOP-NO decreased ET-1 and 8-hydroxy-2′deoxyguanosine (8-OHdG) expression and improved survival after transplantation by 71.4% (P<0.01).

Conclusion. These results suggest that VSOP-NO effectively reconditions warm ischemia-damaged grafts, presumably by decreasing ET-1 upregulation and oxidative damage.

Keywords: Cardiac death donor, Organ preservation, Ischemia–reperfusion injury, Warm ischemia, Liver transplantation.

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The chronic and growing shortage of donor organs has led to the acceptance of extended criteria donor grafts, such as donations after cardiac death (DCD). Ex vivo graft reconditioning studies of marginal organs using hypothermic (1) and normothermic (2) liver perfusion systems with oxygenation have had promising results. A pilot study demonstrated the safety and feasibility of liver preservation using hypothermic machine perfusion (3); however, normothermic machine perfusion requires additional testing before clinical application. Another hypothermic method with a potential to recondition extended criteria donor grafts is venous systemic oxygen persufflation (VSOP), in which grafts are retrogradely perfused with gaseous oxygen through the venous system. VSOP during cold storage has a protective effect against apoptotic changes and improves graft quality, particularly in extended criteria grafts (4–8). Another advantage of VSOP is its ease of use because complex devices are not required, in contrast to machine perfusion systems (4). In VSOP, the use of oxygen alone has yielded inferior results for preserving ischemia-damaged grafts because of the generation of oxygen free radicals (5). Previous studies demonstrated promising results for resuscitating damaged grafts when VSOP was used in combination with antioxidants (9).

Nitric oxide (NO) is a highly diffusible free radical with a short half-life of 5–10 sec in biological systems. In the
liver, numerous clinical benefits of NO have been described, for example, positive effects on circulation, energy storage, and mitochondrial damage protection as well as negative effects on oxidative stress and leukocyte activation (10).

Decreased hepatic NO production immediately after portal reperfusion in liver transplantation was suggested to contribute to ischemia-reperfusion (I/R)–dependent hepatic injury (11). Some studies showed that administration of NO donors, such as S-nitrosothiols, diazeniumdiolates, sodium nitroprusside, FK409, liver-selective NO donors, and inhaled NO, protect against hepatic I/R injury (10). However, in vivo NO administration after liver transplantation may cause vascular hyporeactivity and decompensation (12). To avoid these, NO administration is limited to liver grafts during storage.

Srinivasan et al. first demonstrated that VSOP using NO gas (VSOP-NO) could decrease portal venous pressure more effectively than VSOP alone in an in vitro study using DCD grafts (13). Yagi et al. confirmed the superiority of VSOP-NO over VSOP alone in in vivo liver transplantation of 30% small-for-size grafts because of improved microcirculation (14). In contrast to partial graft transplantation, DCD grafts are exposed to severe warm ischemia. Although the precise pathophysiology of warm ischemia–damaged grafts is unknown, the activation of Kupffer cells and CD4+ T cells, the production of oxidative stress, neutrophil-mediated inflammation, and hepatocellular injury are reportedly involved (15). NO is involved in many pathways that affect each other and also have synergistic effects. We hypothesized that the antioxidant effects of NO (16) could protect sinusoidal endothelial cells (SECs), which are involved in early sinusoidal repopulation, consequently improving microcirculation. Thus, we evaluated the effect of VSOP-NO on I/R injury, the recovery phase, and safety in an in vivo rat transplantation model.

RESULTS

Experiment I. 30-min Warm Ischemia

Chronologic Analysis of Serum Alanine Aminotransferase, Hyaluronic Acid, and Malondialdehyde

Alanine aminotransferase (ALT), a parameter for hepatocellular damage, was significantly lower in group-VSOP-NO over 72 hr (two-way analysis of variance [ANOVA]: \(P<0.001\)). Hyaluronic acid, a marker of endothelial damage (17), was significantly lower in group-VSOP-NO (\(P=0.039\)), and serum MDA, a marker of oxidative stress, was also lower in group-VSOP-NO (\(P<0.001\)) (Table 1).

Messenger RNA Expression in Liver Grafts

Group-VSOP-NO showed downregulated interleukin-6 (IL-6) expression (\(P=0.017\)). In addition, in the liver, relative mRNA expressions of tumor necrosis factor-alpha (TNF-\(\alpha\)) (\(P<0.001\)), endothelin-1 (ET-1) (\(P<0.001\)), and inducible nitric oxide synthase (iNOS) were lower in group-VSOP-NO (\(P=0.0057\)). Relative mRNA expression of endothelial nitric oxide synthase (eNOS) was significantly higher in group-VSOP-NO (\(P<0.001\)).

Histologic Analysis

Hematoxylin–eosin staining of livers 6 hr after transplantation (Fig. 1A, B) revealed vacuolization and congestion in group-control (Fig. 1A) but almost normal liver architecture in group-VSOP-NO (Fig. 1B). Transmission

| TABLE 1. Time course for serum and gene expression analyses |
|-------------|-------------|-------------|-----------------
|            | 2 hr        | 6 hr        | 1 day          | 3 days         |
| ALT (IU/mL)      |             |             |                 |                |
| Control       | 2770.0±151.3| 4084.2±1196.5| 1895.8±303.0    | 234.0±92.8     |
| VSOP-NO      | 2035.0±261.2| 1088.5±261.2c| 457.3±43.0      | 205.5±92.7     |
| Hyaluronic Acid (ng/mL) |         |             |                 |                |
| Control       | 294.5±90.8  | 281.6±58.8  | 1179.9±202.1    | 428.2±133.6    |
| VSOP-NO      | 197.2±12.5  | 117.2±19.5  | 695.1±174.7     | 118.6±18.3     |
| MDA (\(\mu\)M) |             |             |                 |                |
| Control       | 4.8±0.8     | 7.7±0.5     | 4.2±0.9         | 2.1±0.3        |
| VSOP-NO      | 4.0±0.7     | 2.8±0.5c    | 1.9±0.3a        | 2.6±0.3        |
| IL-6 (%\(\beta\)-actin) |         |             |                 |                |
| Control       | 2098.7±1996.7| 4227.4±960.0| 25.6±8.9        | 43.9±20.0      |
| VSOP-NO      | 85.2±31.6   | 753.9±185.7a| 1.8±0.3         | 29.4±11.8      |
| TNF\(\alpha\) (%\(\beta\)-actin) |         |             |                 |                |
| Control       | 40.6±12.2   | 115.1±21.8  | 31.0±6.1        | 18.4±2.3       |
| VSOP-NO      | 21.9±7.9    | 23.1±8.9f   | 18.5±5.7        | 22.4±6.2       |
| ET-1 (%\(\beta\)-actin) |         |             |                 |                |
| Control       | 16.0±5.6    | 21.0±2.9    | 8.0±1.0         | 1.7±0.3        |
| VSOP-NO      | 3.1±0.6b    | 6.1±0.9c    | 1.4±0.3         | 1.7±0.3        |
| iNOS (%\(\beta\)-actin) |         |             |                 |                |
| Control       | 7.1±2.4     | 4.4±1.0     | 2.7±1.4         | 3.2±0.8        |
| VSOP-NO      | 1.4±0.3b    | 2.4±0.7     | 2.3±0.8         | 1.7±0.5        |
| eNOS (%\(\beta\)-actin) |         |             |                 |                |
| Control       | 0.3±0.1     | 1.0±0.2     | 0.6±0.1         | 1.0±0.1        |
| VSOP-NO      | 1.0±0.2     | 1.5±0.5     | 4.6±0.4c        | 1.4±0.3        |

Mean±standard error of the mean of serum value after transplantation. ALT, alanine aminotransferase; MDA, malondialdehyde; IL-6, interleukin-6; TNF\(\alpha\), tumor necrosis factor-alpha; ET-1, endothelin-1; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase.

Control graft stored in cold storage alone, VSOP-NO graft stored in venous systemic oxygen persufflation with nitric oxide gas. ALT, \(P=0.001\); hyaluronic acid, \(P=0.039\); MDA, \(P=0.001\); IL-6, \(P=0.017\); TNF-\(\alpha\), \(P=0.001\); ET-1, \(P=0.001\); iNOS, \(P=0.0057\); and eNOS, \(P=0.001\). Bonferroni's posttest: \(^aP<0.05\), \(^bP<0.01\), and \(^cP<0.001\) versus VSOP-NO.
electron microscopy 6 hr after transplantation revealed well-preserved SECs covering the space of Disse, which was filled with microvilli (Fig. 1D, F) in group-VSOP-NO and partially disrupted sinusoidal walls (Fig. 1C, E) in group-control.

Immunofluorescence of Rat Endothelial Cell Antigen-1

Fewer viable RECA-1-positive SECs were observed 6 hr after transplantation in both groups (Fig. 2B, F). Furthermore, 24 hr after transplantation, a few SECs tended to be stained with RECA-1 in group-VSOP-NO alone (Fig. 2C, G). Fluorescent SECs were also detected 72 hr after transplantation in group-control (Fig. 2D).

Experiment II-A. 60-min Warm Ischemia: Survival Study

In group-control, the 10-day survival rate was 0%, and all deaths occurred within 24 hr of transplantation (Fig. 3). In group-VSOP-NO, the 10-day survival rate was improved to 71.4% (P<0.009). Autopsies in group-control revealed multiple focal necroses (grade: 3.4±0.4) in the livers and hemorrhagic ascites. In group-VSOP-NO, there was almost no necrosis (grade: 1.0±0.0).

Experiment II-B. 60-min Warm Ischemia: Evaluation of Early Phase Injury

Relative hepatic ET-1 mRNA expression levels 2 hr after portal reperfusion were higher in group-control than
in group-VSOP-NO (P<0.005; Fig. 4A). Figure 4B and C shows the staining of SECs with 8-hydroxy-2′deoxyguanosine (8-OHdG) as a marker for oxidative DNA damage 2 hr after portal reperfusion. In group-control (Fig. 4B), broad sinusoidal cells and partial hepatocytes were stained, whereas in group-VSOP-NO, few sinusoidal cells were stained (Fig. 4C).

**DISCUSSION**

We report, for the first time, the beneficial effects of VSOP-NO in in vivo rat DCD liver transplantation. The changes in severe I/R injury associated with warm ischemia-damaged DCD grafts reportedly occur in two distinct phases, that is, the early phase less than 2 hr after portal reperfusion and the later injury phase 6 to 48 hr after portal reperfusion (16). To evaluate how hepatocytes and SECs are affected in DCD transplantation, we assessed inflammatory cytokines, oxidative stress, and indicators of hepatocyte and SEC function as well as parameters influenced by NO administration, including ET-1, iNOS, and eNOS. We chose 2 hr as the early phase, 6 to 24 hr as the late phase, and 72 hr as the early recovery period associated with I/R injury.

In Experiment I, VSOP-NO decreased ET-1 and iNOS expression levels 2 hr after portal reperfusion and attenuated increases in ALT, inflammatory cytokines, eNOS upregulation, and MDA elevation 6 to 24 hr after portal reperfusion. The early phase of warm ischemia-induced reperfusion injury in DCD grafts is reportedly associated with activation of Kupffer cells and production of oxidative stress. Moreover, Klune et al. reported that the first step of the I/R injury cascade is the liberation of ET-1, which activates stellate cells and leads to constriction of hepatic sinusoids and accumulation of oxidative stress, activating Kupffer cells and initiating upregulation of inflammatory cytokines (18). In Experiment II-A, death occurred 2 hr after portal reperfusion, and autopsies of both groups suggested that the cause was graft failure without vascular or biliary complications or bleeding because of gaseous perfusion, which has been associated with VSOP. We concluded that differences at 2 hr after portal reperfusion influenced the survival rate in Experiment II-A.

According to the results of real-time polymerase chain reaction (RT-PCR) in Experiment I, the key parameters affecting survival are ET-1 and iNOS. However, exogenous NO may have suppressed iNOS in the current study; therefore, we focused on ET-1. In Experiment I, we used MDA, an end-product of lipid peroxidation, to assess whether oxidative stress plays a role in the effect of VSOP-NO on I/R injury.

**FIGURE 2.** Immunofluorescence analysis using rat endothelial cell antigen 1 (RECA-1) of hepatic expression 2, 6, 24, and 72 hr after portal reperfusion in control (A–D) and VSOP-NO (E–H) groups. CV, central venous area; PV, portal venous area; control grafts stored in cold storage alone; VSOP-NO grafts stored in venous systemic oxygen persufflation using gaseous NO.
injury. The spectrophotometric thiobarbituric acid-reactive substances assay used in the current study is extremely easy to use but is nonspecific; other substrates (e.g., aldehydes) can react with thiobarbituric acid (19). By contrast, 8-OHdG is a sensitive indicator of oxidative DNA damage. To determine if oxidative stress is an early factor in survival, 8-OHdG was used in Experiment II. We speculate that the critical benefit of VSOP-NO administration is the decrease in oxidative damage and ET-1 upregulation, resulting in inactivation of inflammatory responses and protection of SECs and hepatocytes.

In our study, VSOP-NO decreased the incidence of initial death because of long warm ischemia. Several studies have demonstrated the relationship between the duration of warm ischemia and posttransplant survival in pig (20) and rat (21) models. We chose 60 min as the critical time for prolonged warm ischemia because a 1-week survival rate of less than 10% is appropriate for primary nonfunction, in which we observed decreased oxidative damage and ET-1 upregulation, resulting in inactivation of inflammatory responses and protection of SECs and hepatocytes.

In the liver, iNOS is not expressed under normal circumstances; however, under certain conditions, iNOS has either a protective or toxic effect (22). In hepatic I/R injury, iNOS exacerbates liver injury (23). In addition, iNOS expression is known to be downregulated by steroids, tumor growth factor-β, the heat shock response, p53, and NO itself (24). In this study, exogenous NO decreased iNOS upregulation and suppressed the exacerbation of hepatic I/R injury. However, eNOS is constitutively expressed in SECs and is related to sinusoidal circulation (25). A previous report demonstrated that repopulation of SECs requires 12 to 48 hr in denuded sinusoids (26). Our results demonstrate eNOS upregulation 24 hr after transplantation. Shear stress has been reported as an inducer of eNOS production (27). In the current model, warm ischemia strongly injured SECs. In Experiment II-A, all parameters but one in group-control approached those in group-VSOP-NO by day 3, and no rats died. Our results demonstrate that 30-min warm ischemia is not crucial, and thus, hepatocyte damage could recover by 3 days. Hyaluronic acid, a parameter of sinusoidal endothelial injury, showed a different trend from the other parameters, increasing 6 to 24 hr after portal reperfusion but not recovering to the level in group-VSOP-NO within 72 hr. On the other hand, RECA-1 immunohistochemical analysis demonstrated that damaged SECs recovered within 24 hr of transplantation in group-VSOP-NO and within 72 hr in group-control. We hypothesize that VSOP-NO stimulated the early repopulation of the endothelial structure, resulting in eNOS upregulation 24 hr after portal reperfusion, but the recovery of hyaluronic acid indicates that the SECs require greater than 72 hr for adequate functional recovery.

After NO administration, excess NO promotes its reaction with superoxide (28), resulting in the formation of peroxynitrite (29), a molecule extremely toxic to cells (30). We previously demonstrated NO concentration of 40 ppm was optimal to produce a significant effect (14). This study demonstrated the safety of NO administration during cold storage.

Remarkably, in Experiment II-A, VSOP-NO resulted in successful reconditioning of severely damaged DCD grafts. A few studies have reported reclamation of DCD grafts exposed to 60-min warm ischemia. Peaks et al. and Tolboom et al. successfully rescued almost all transplants that underwent 60-min warm ischemia by normothermic extracorporeal perfusion (2, 31). In terms of survival, after

![FIGURE 3. Kaplan-Meier survival curves of recipient rats after transplantation of 60-min warm ischemia-damaged livers stored in cold storage alone (control) and venous systemic oxygen persufflation using nitric oxide gas (VSOP-NO). Survival rates for VSOP-NO and control were 71.4% and 14.3%, respectively, at 12 hr and 71.4% and 0%, respectively, at 10 days; \( P=0.009 \) versus control using the log-rank test (n=6 per group).](www.transplantjournal.com)
60-min warm ischemia, VSOP-NO appeared to be inferior to machine perfusion. The insult in DCD grafts is multifactorial and depends on the duration of warm ischemia as well as recipient status, anhepatic time, I/R injury involving preservation solutions, duration of cold ischemia, and surgical techniques. Stegemann et al. previously reported the superiority of VSOP over machine perfusion in a model of long-term preservation of DCD grafts (32). Further studies are needed to compare normothermic machine perfusion and VSOP-NO. Machine perfusion requires a pump, plasma, erythrocytes, and a dialyzer, whereas the VSOP system requires only gas cylinders and ice. Despite efforts to simplify and decrease expenses associated with machine perfusion, the current VSOP system remains more convenient and economical.

In the current study, we applied 3-hr cold ischemia, which is shorter than that for DCD transplantation in the typical clinical setting. DCD grafts exposed to prolonged warm ischemia poorly tolerated longer durations of cold ischemia (33).

Other methods to recover liver grafts, such as hypothermic or normothermic machine perfusion and VSOP, have been shown to be effective for longer periods of storage, but not yet in combination with longer warm ischemia and following longer cold ischemia. In future studies, we plan to evaluate a prolonged cold ischemia group to demonstrate the efficacy of VSOP-NO for DCD grafts. Tolboom et al. reported that normothermic oxygenated perfusion results in less I/R injury (2). Thus, we speculate that subnormothermic retrograde perfusion preservation with gaseous NO could provide improved graft survival.

Other clinically relevant long-term effects of warm ischemia, such as ischemic cholangiopathy, were not investigated in this study. Cholangiography and quantitative comparisons require long-term observations (34); thus, further studies involving long-term observations of DCD in a large animal model are needed.

In conclusion, retrograde administration of gaseous oxygen and NO during cold storage is an easy and safe method to recondition marginal DCD liver grafts. Our results will benefit future applications of this method; however, we recommend that our technique should be applied cautiously to the clinical setting. Specifically, further experiments with large animals and longer observation periods are necessary before applying this novel organ preservation technique to clinically marginal grafts.
MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the Animal Research Committee of Kyoto University, and all animals received humane care according to criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No 86-23, revised in 1985). Male LEW/CrlJrj rats weighting 180 to 220 g (Charles River Laboratories Japan, Inc., Yokohama, Japan) were used as liver donors/ recipients. The animals were housed under specific pathogen-free conditions in a temperature- and humidity-controlled environment with a 12-hr light/dark cycle with access to tap water and standard chow pellets ad libitum.

Donor Procedure and Graft Storage

Experiment I. 30-min Warm Ischemia

Rat livers were retrieved after 30-min warm ischemia induced by phrenotomy 5 min after injection of 300 units of heparin sodium, as described previously (21). In the control group, grafts were preserved in University of Wisconsin solution (ViaSpan, Astellas Pharma Inc., Tokyo, Japan) at 4°C. In the VSOP-NO group, grafts were treated using VSOP using gaseous NO at 40 ppm, as previously reported (14). Briefly, catheter was inserted into the thoracic caval vein, and both the portal and intrahepatic caval veins were clamped with atraumatic clips. Small pinpricks were made, which allowed the gaseous oxygen and NO from the catheter to escape during cold storage. After 3 hr of cold storage, orthotopic liver transplantation was performed using the DCD grafts and a modified Kamada’s method (35). Anhepatic time was 14.0 ± 0.3 min in the VSOP-NO group and 14.3 ± 0.2 min in the control group (P=0.38).

Subsequently, 2, 6, 24, and 72 hr after portal reperfusion (n=6 at each time point for each group), serum and liver graft tissue samples were collected prior to euthanasia by exsanguination.

Experiment II-A. 60-min Warm Ischemia: Survival Study

The lethality of a longer-duration (60-min) warm ischemia model and the effect of VSOP-NO against lethal stress were verified in a series of experiments. Rats were divided into the control group and the VSOP-NO group (n=7 each). The 60-min warm ischemia-damaged grafts were transplanted into recipients similar to Experiment I. Survival rate was investigated for 10 days after transplantation. To determine histological alterations, necrotic lesions were graded on a 4-point scale as follows: 1=no changes or negligible lesions, affecting 0%–10% of field, 2=mild, affecting 10%–40%, 3=moderate, affecting 40%–70%, and 4=severe, affecting >70%.

Experiment II-B. 60-min Warm Ischemia: Evaluation of Early Injury

The relationship between the results of Experiments I and II-A was confirmed in another series of experiments. Control and VSOP-NO (n=6 each) rats underwent the same surgery as animals in Experiment II-A. Liver graft tissue samples were collected 2 hr after portal reperfusion.

Measurement of Liver Enzymes

Serum ALT and hyaluronic acid levels were measured using a standard spectrophotometric method using an automated clinical analyzer (I.CA-BM9030, JEOL Ltd., Tokyo, Japan).

Malondialdehyde

To estimate the levels of lipid peroxidation products, serum MDA levels were measured using the thiobarbituric acid assay according to the manufacturer’s protocol (NWLSL malondialdehyde assay, Northwest Life Science Specialties, LLC, WA, USA).

Real-Time Polymerase Chain Reaction

For gene expression analysis, RT-PCR was performed using TaqMan technology, as previously reported (36). The probe and primers for TNF-α (assay ID Rn01525859_g1), IL-6 (assay ID Rn01410330_m1), iNOS (assay ID Rn00561646_m1), eNOS (assay ID Rn2132634_s1), ET-1 (assay ID Rn00561129_m1), and beta-actin (B-actin) (assay ID Rn00667869_m1) were obtained for TaqMan gene expression assays from Applied Biosystems, Life Technologies Japan Ltd., Japan.

Immunohistochemistry

To assess the damage and recovery of SECs, immunofluorescence was assessed using rat endothelial cell antigen 1 (RECA-1: MCA-970R, Serotec, Oxford, UK). Alexa Fluor 594 goat antimouse IgG (A-10005; Invitrogen, Life Technologies Japan Ltd., Japan) was used as the secondary antibody. To evaluate oxidative DNA damage, 8-OHdG (N45.1, Japan Institute for the Control of Aging, Nikken Seil Co., Ltd, Shizuoka, Japan) and hematoxylin–eosin were used according to the manufacturer’s protocol.

Histologic Assessment of Tissue Injury

Liver samples were fixed with 4% paraformaldehyde, embedded in paraffin wax, and sectioned (4-μm thickness). Slides were stained with hematoxylin–eosin.

Electron Microscopy

Fixation of liver samples was performed as previously reported (37). Sections were observed by transmission electron microscope (H-7650; Hitachi, Ltd., Tokyo, Japan).

Statistical Analyses

Results are expressed as mean±standard error for each group. Statistical analysis of the groups at each time point was performed with two-way ANOVA and Bonferroni’s post hoc test. For the survival study, a Mantel–Cox log-rank analysis was performed. The Mann–Whitney U test was used to analyze hepatic ET-1 expression in Experiment II-B. P<0.05 was considered statistically significant. Calculations were performed with Prism Software Version 5.0c (GraphPad Software, Inc. CA, USA).

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