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Anticholestatic Effect of Bardoxolone Methyl on Hepatic Ischemia-reperfusion Injury in Rats

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Background. Cholestasis is a sign of hepatic ischemia-reperfusion injury (IRI), which is caused by the dysfunction of hepatocyte membrane transporters (HMTs). As transcriptional regulation of HMTs during oxidative stress is mediated by nuclear factor erythroid 2-related factor 2, we hypothesized that bardoxolone methyl (BARD), a nuclear factor erythroid 2-related factor 2 activator, can mitigate cholestasis associated with hepatic IRI. **Methods.** BARD (2 mg/kg) or the vehicle was intravenously administered into rats immediately before sham surgery, 60 min of ischemia (IR60), or 90 min of ischemia (IR90); tissue and blood samples were collected after 24 h to determine the effect on key surrogate markers of bile metabolism and expression of HMT genes (Mrp (multidrug resistance-associated protein) 2, bile salt export pump, *Mrp3*, sodium-taurocholate cotransporter, and organic anion-transporting polypeptide 1). **Results.** Significantly decreased serum bile acids were detected upon BARD administration in the IR60 group but not in the IR90 group. Hepatic tissue analyses revealed that BARD administration increased mRNA levels of *Mrp2* and *Mrp3* in the IR60 group, and it decreased those of bile salt export pump in the IR90 group. Protein levels of multidrug resistance–associated protein 2, multidrug resistance–associated protein 3, and sodium-taurocholate cotransporter were higher in the IR90 group relative to those in the sham or IR60 groups, wherein the difference was notable only when BARD was administered. Immunohistochemical and morphometric analyses showed that the area of expression for multidrug resistance–associated protein 2 and for sodium-taurocholate cotransporter was larger in the viable tissues than in the necrotic area, and the area for multidrug resistance–associated protein 3 was smaller; these differences were notable upon BARD administration. **Conclusions.** BARD may have the potential to change HMT regulation to mitigate cholestasis in hepatic IRI.

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INTRODUCTION

Hepatic ischemia-reperfusion injury (IRI) is a mechanism underlying hepatic graft failure, which occurs in 2%–8% of liver transplantation cases.[1-3] However, it is difficult to predict the degree of organ damage in each individual case because different grafts exhibit variable vulnerability to hepatic IRI. Because of the lack of a reliable surrogate marker, organs with clinical risk factors (eg, old donor age, fatty change, or increased ischemia time) are often preemptively declined to prevent graft failure.[4] Considering the trend of worsening donor risk profiles, it has been estimated that utilization of the liver from deceased organ donors will fall from 78% to 44% by year 2030, if current practices remain unchanged.[5] As such, better understanding the molecular and cellular responses of the liver to IRI may provide an opportunity to identify a therapeutic target that can help decrease the rates of organ discard and graft failure.

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Cholestasis reflects the detrimental cellular activity of bile formation in hepatic injuries, and it is an important clinical sign of hepatic dysfunction caused by IRI.[6-9] Compromised bile metabolism in hepatic IRI is not only due to the loss of cell volume from necrosis as significant bile secretory dysfunction occurs in a mild form of hepatic IRI without necrosis.[10] This finding suggests that the dysfunction of bile metabolism in viable hepatocytes plays an important role in developing cholestasis. Bile formation is the process of the uptake of bile salts and other organic solutes from the basolateral membrane and excretion at the canalicular membrane of the hepatocyte; this occurs through the function of proteins that are collectively known as hepatocyte membrane transporters (HMTs).[6] Among the major subtypes of HMT, organic anion-transporting polypeptide 1 (OATP1), sodium-taurocholate cotransporter (NTCP), and multidrug resistance–associated protein 3 (MRP3) are on the basolateral membrane. In contrast, multidrug resistance–associated protein 2 (MRP2) and the bile salt export pump (BSEP) are on the canalicular membrane.[6,11] HMTs are highly regulated at transcriptional and posttranscriptional levels, and various physiological and pathological stimuli can induce or suppress them.[6] As such, the multilevel response of HMT genes to hepatic injury underlies the molecular mechanism of cholestasis.[12,13]

Hepatic IRI is known to significantly affect the transcription of HMT genes.[11,14] Among the factors regulating HMT genes, nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor that controls their expressions during oxidative stress via the antioxidant response element.[15-19] Recently, we have shown that a moderate degree (60 min) of hepatic ischemia induced the transcriptional activities of HMT genes, whereas a prolonged (90 min) hepatic ischemia suppressed them. Moreover, the induction of HMT mRNA levels by 60 min of ischemia was abolished by the *Nrf2* knockout status.[11] These observations suggest that IRI-triggered HMT gene expression may be dependent upon NRF2 and that NRF2 may be targeted to mitigate the cholestasis associated with hepatic IRI. Of note, although NRF2 is sequestered by Kelch-like ECH-associated protein 1 (KEAP1) in the cytosol under physiological conditions, oxidative stimuli can induce dissociation of NRF2 from KEAP1 and its subsequent nuclear translocation, triggering transcription of antioxidant response element–regulated genes.[11] The physical interaction of NRF2 and KEAP1 is a well-known target for the design of a therapeutic strategy. For example, bardoxolone methyl (BARD), a derivative of synthetic triterpenoids that binds to KEAP1, inhibits KEAP1-NRF2 interaction and induces release of NRF2 from the complex, eventually leading to activation of NRF2 as a transcription factor.[19] Indeed, beneficial effects of BARD mitigating tissue damages in mouse models of brain or hepatic IRI have been attributed to temporal activation of NRF2.[20,21] Given these observations and that NRF2 regulates HMT gene expression in mouse liver under normal conditions,[16] we hypothesized that pharmaceutical activation of NRF2 can improve bile metabolism in hepatic IRI via increased HMT gene expression. By extension, we predicted that intravenous BARD administration at the time of ischemia can mitigate cholestasis in hepatic IRI. In this study, we determined the effect of BARD on expression of HMT genes and the bile metabolism during hepatic IRI.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats at 7–8 wk old (median body weight of 246 g, interquartile range of 230–260 g, Charles River Laboratories) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility. The rats were fed Purina Lab Diet 5001 and reverse osmosis water ad libitum in Allentown ventilated caging with Sani-Chips course bedding (wood chips from PJ Murphy Forest Products, Ladysmith, WI). The animals were group housed, 2–4 per cage, in a 12-h light cycle (6 AM–6 PM) room. All procedures were performed during the 12 h of light of the cycle. All animals received humane care according to the 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. The experiments were approved by the Institutional Animal Care and Use Committee at The Medical College of Wisconsin (AUA00004857).

Surgical Experiment

The experimental groups were divided into 3 based on the length of ischemia time: no ischemia (sham), moderate (60 min of ischemia, IR60), and prolonged (90 min of ischemia, IR90). The 3 surgical groups were further divided into vehicle versus BARD infusion groups. Therefore, 6 groups were allocated in this study (n = 5 each): vehicle-sham, BARD-sham, vehicle-IR60, BARD-IR60, vehicle-IR90, and BARD-IR90 (Figure 1). For the bardoxolone methyl groups, a dose of 2 mg/kg for BARD was chosen based on previous studies.[20,22] We prepared 100 µL of a 5 mg/mL BARD (CDDO Methyl Ester, SMB00376, Sigma-Aldrich, St Louis, MO) solution dissolved in dimethyl sulfoxide. The batch of solution was diluted with 0.9 mL of 0.2% Tween 80 (P1754, Sigma-Aldrich, St Louis, MO) in normal saline immediately before the infusion.

The experiment was performed under general anesthesia with isoflurane inhalation (1%–3%; oxygen flow 1 L/min). A transverse abdominal incision was made, and a total of 1 mL of the solution with 2 mg/kg of BARD was injected into the inferior vena cava (IVC, 1 mL/min) 5 min before the clamp application for hepatic ischemia. The infusion was performed using a 30-gauge needle (Exelint International Co., Los Angeles, CA) and an infusion pump (Genie Touch; Lucca Technologies, Harwinton, CT) under a surgical microscope (AmScope; United Scope LLC, Irvine, CA). No vascular repair was required at the needle puncture site. In the control group, 1 mL of the vehicle solution without BARD was injected into IVC in the same manner. The liver and vascular dissections were performed in the same manner in all animals, including the sham groups. The pedicle in the median and lateral lobes of the liver (approximately 70% of the volume of the liver) were temporarily occluded using a small vascular clamp. During hepatic ischemia, the abdomen was temporarily closed to prevent effects from temperature change and dehydration. After the experiment, the abdominal wall was closed in 2 layers and the animals were weaned from the anesthesia. In previous studies, changes of mRNA levels for HMT genes from hepatic IRI were most prominent at 24 h after reperfusion.[11,14,23] As such, all animals underwent sampling procedures for blood and liver tissue after 24 h of experiment under the same isoflurane general anesthesia. The blood samples were collected

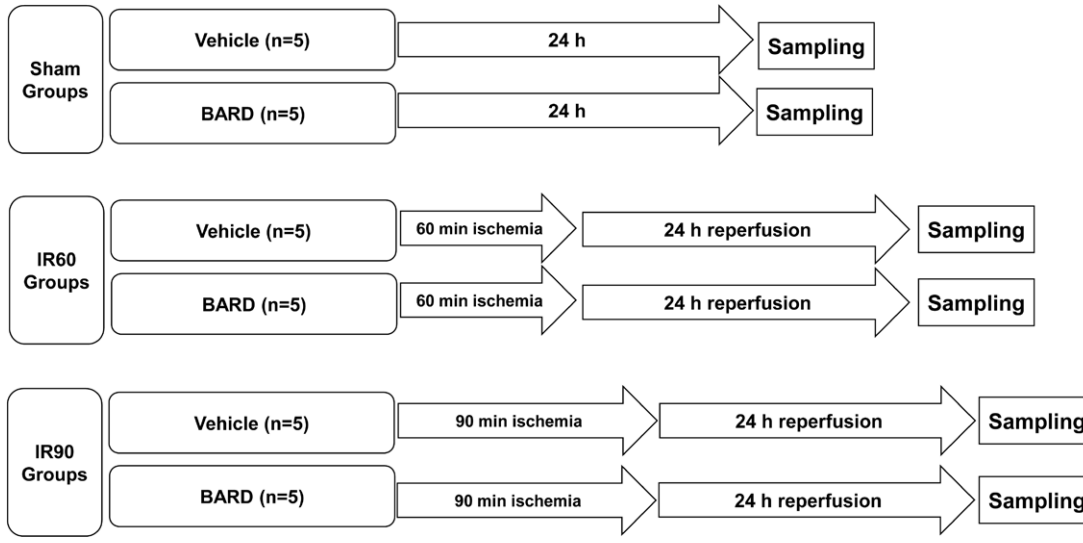

FIGURE 1. Experimental groups in this study. The rats in the sham, IR60, and IR90 groups underwent intravenous infusion (1 mL over 1 min into the IVC) of either a vehicle or a bardoxolone methyl solution (BARD, 2 mg/kg). In the sham group, samples were obtained 24 h after infusion. In the IR60 and IR90 groups, 70% partial hepatic ischemia was applied 5 min after the intravenous infusion of a vehicle or BARD. Hepatic inflow was restored after 60 min (IR60) or 90 min (IR90), and samples were obtained after 24 h. N = 5 in each group. BARD, bardoxolone methyl; IR60, 60 min of ischemia followed by 24 h of reperfusion; IR90, 90 min of ischemia followed by 24 h of reperfusion; IVC, inferior vena cava.

from IVC, and consistent sites of the median and left lobes of the liver were sampled for tissue analyses.

Histological Assessment

Liver samples, that were preserved in a 10% formalin solution (HT501320, Sigma-Aldrich, St Louis, MO), were stained using hematoxylin and eosin, and a pathologist blinded to the experimental allocation assessed the Suzuki score, which represents the degree of hepatic IRI. The Suzuki score was derived from the mean value of scores (0–4) for 7 criteria to assess the degree of hepatic IRI: inflammation, necrosis, overall degree of IRI, congestion, ballooning, steatosis, and cholestasis.[24]

Blood Tests

To assess the degree of hepatocyte injury, the levels of serum alanine transaminase (ALT) were measured. To assess bile metabolism, serum total bilirubin and bile acids levels were measured.[25] All blood tests were performed at a local veterinary laboratory (Marshfield Labs, WI).

Quantitative Polymerase Chain Reaction

The RNA abundance of the following HMT genes was analyzed: organic-anion-transporting polypeptide (*Oatp*), multidrug resistance–associated protein 2 (*Mrp2*), multidrug resistance–associated protein 3 (*Mrp3*), sodium-taurocholate cotransporting polypeptide (*Ntcp*), and BSEP (*Bsep*).[6] To determine the effect of BARD on proinflammatory markers, the mRNA levels of genes for tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured. A quantitative polymerase chain reaction (qPCR) was conducted using the following primers: *Mrp3* (Rn01452854_m1, ThermoFisher Scientific, Waltham, MA), *Mrp2* (Rn00563231_m1), *Ntcp* (Rn00566894_m1), *Oatp1* (Rn00755148_m1), *Bsep* (Rn01515444_m1), *Gapdh* (Rn01775763_g1), *Tnf* (Rn99999017_m1), and *Il6* (Rn01410330_m1). mRNA levels were presented by relative fold changes to the reference gene (glyceraldehyde-3-phosphate dehydrogenase, *Gapdh*). Details are available in the Supplementary Methods (SDC, http://links.lww.com/TXD/A256).

Western Analyses

Proteins were extracted from liver tissues that had been snap frozen. Protein levels were determined by a capillary electrophoresis immunoassay using the Simple Western system (Wes, ProteinSimple, San Jose, CA) following the manufacturer's instructions. The primary antibodies used are as follows: OATP1 (bs-0607R; Bioss Antibodies), NTCP (bs-1958R; Bioss Antibodies), BSEP (bs-1954R; Bioss Antibodies), MRP2 (ab203397; Abcam), MRP3 (250760; Abbiotec), and GAPDH (sc-376632, Santa Cruz Biotechnology for primary antibodies from a mouse host, or 2118, Cell Signaling Technology for antibodies from a rabbit host). Protein levels were determined by area under the chemiluminescence curve corrected to GAPDH levels using proprietary software (Compass for SW, Version 3.1.7, ProteinSimple, San Jose, CA). Details are available in the Supplementary Methods (SDC, http://links.lww.com/TXD/A256).

Immunohistochemistry

One representative animal per group that showed the median value of protein levels for each HMT was selected for immunohistochemistry (IHC). All slides were sectioned at 4 μm, mounted on Poly-L-Lysine coated slides, deparaffinized, antigen retrieved, and stained using the Leica Bond Rx automated staining platform. Target proteins were detected by IHC using the following primary antibodies: OATP1 (bs-0607R; Bioss Antibodies), NTCP (PA5-80001; ThermoFisher), BSEP (LSC490094/135382; LS Bio), MRP2 (AIX-801-037 Enzo), and MRP3 (250760; Abbiotec). To amplify the signals, the labeled streptavidin (horseradish peroxidase)-biotin method was used for NTCP and BSEP, and the polymer horseradish peroxidase–based method was used for MRP2, MRP3, and OATP1. Chromogen 3,3′Diaminobenzidine (DAB) was used for visualization. Details are available in the Supplementary Methods (SDC, http://links.lww.com/TXD/A256).

Digital Imaging Analyses

The IHC images were digitally captured by a high resolution, whole slide scanner (NanoZoomer HT 2.0, Hamamatsu, Japan) at 40× magnification, and the data were reviewed using NDPview (version 2.7.43, Hamamatsu, Japan) for virtual image exploration. Random regions of interest (ROIs) were captured separately in nonnecrotic and necrotic areas by a board-certified pathologist blinded to the grouping. Because of the limited area of viable portions in IR90, 4–10 ROIs were captured as available. The scanned images were imported in the software (VIS Image Analysis Software, Visiopharm, Hoersholm, Denmark) and analyzed at 20× magnification to quantify DAB expressions. The software was trained to separately capture DAB positive regions and the hepatic tissue area by a preset thresholding and the linear Bayesian classification. The area of DAB positive compared with that of the hepatic tissue area for each ROI was expressed as a percentage value.

Statistical Analyses

The data were expressed as the median with interquartile ranges. $P < 0.05$ was considered significant, and differences between 2 groups were measured by the Mann-Whitney test. Differences among 3 groups were measured by the Kruskal-Wallis test and the post hoc Mann-Whitney test with Bonferroni's correction. Statistical analyses were performed using Prism 7 for Windows (GraphPad Software, Inc., San Diego, CA).

RESULTS

BARD Administration Immediately Before Ischemia Can Attenuate Cholestasis After Reperfusion

An ischemia time of 60 min has been suggested as the limit of tolerable ischemia time in clinical and in experimental settings.[11,26-28] The degree of hepatic injury in IR60 and IR90 was demonstrated by increased Suzuki scores and increased serum levels for ALT, bile acids, and total bilirubin levels (Figure 2A–C). The hepatic tissue damage was also reflected by increased hepatic tissue mRNA levels for proinflammatory cytokines (Figure 2D). Importantly, the administration of BARD significantly decreased the levels of serum bile acids in the IR60 group, whereas similar effects were not observed in the IR90 group (Figure 2C). The effect of BARD administration on other hepatic injury markers, such as Suzuki score, serum ALT, and serum total bilirubin, did not reach statistical significance (Figure 2B and C). These data suggest that BARD administration at the time of ischemia may attenuate postreperfusion cholestasis if the duration of ischemia is not prolonged to an extreme level.

BARD Administration Decreases *Tnf* mRNA Levels After Reperfusion Only When Ischemia Time Is Prolonged

Proinflammatory cytokines such as TNF-α or IL-6 decrease HMT expressions, leading to cholestasis.[23,29] Specifically, TNF-α and IL-6 are known to suppress the transcription of HMT genes, such as *Ntcp*, *Oatp1*, *Mrp2*, and *Bsep*.[6,30] Moreover, BARD is known to inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in Kupffer cells, which are responsible for the production of TNF-α and IL-6 during hepatic IRI.[31,32] To assess the indirect effect of BARD on HMT genes through the proinflammatory cytokines, mRNA levels of *Tnf* and *Il6* were also measured. The BARD administration suppressed transcription activities for proinflammatory cytokines, and the effect was prominent only in IR90, not in IR60 (Figure 2D). Of note, the anticholestatic effect of BARD was significant only in IR60, as described above. As such, anticholestatic effect of BARD is not likely to be mediated through the proinflammatory cytokines.

BARD Administration Induces the Transcriptional Activities of HMT Genes, and the Effect Is Not Detected When the Ischemia Time Is Prolonged

The effects of BARD on transcriptional activities in HMT genes were assessed by a qPCR. Among the canalicular HMT genes, BARD administration significantly increased mRNA levels of *Mrp2* in IR60 but not in IR90 (Figure 3A). These findings are in concordance with the anticholestatic effect of BARD in IR60. In contrast, mRNA levels of *Bsep* were not affected in the sham or IR60 groups, but they were significantly decreased upon BARD administration in the IR90 group (Figure 3B). Canalicular bile salt concentrations are >1000-fold higher than those in the cytosol, and transport across the canalicular HMTs (eg, MRP2 and BSEP) is the rate-limiting step in overall hepatocellular bile salt excretion.[33] BARD affected mRNA abundance of both canalicular HMT genes: *Mrp2* in IR60 positively and *Bsep* in IR90 negatively.

The effect of BARD administration was also assessed for the basolateral HMTs. The levels of *Mrp3* mRNA were increased by BARD administration not only in IR60 but also in the sham group, whereas similar effects were not observed in the IR90 group (Figure 3C). In contrast, mRNA levels of *Ntcp* and *Oatp1* were not affected by BARD administration (Figure 3D and E).

BARD Administration Increases HMT Protein Levels More Prominently in IR90 Than in IR60

Protein levels were measured for canalicular and basolateral HMTs. Protein levels of MRP2 were higher in IR90 compared with those in the sham group or IR60, and the difference was significant only in the presence of BARD (Figure 4A). In contrast, protein levels of BSEP were not significantly different among groups (Figure 4B). Protein levels of MRP3 were not different among the vehicle groups, but those in IR90 were significantly higher than those in IR60, in the presence of BARD (Figure 4C). NTCP protein levels in IR90 were increased in both vehicle and BARD groups, but the difference between IR60 and IR90 was significant only in the presence of BARD (Figure 4D). In contrast, protein levels of OATP1 were not significantly different among groups (Figure 4E). In summary, the relative protein levels of MRP2, MRP3, and NTCP in IR90 were significantly increased in the presence of BARD. No correlation between qPCR results and protein levels was observed in this study. Considering the multilayered pretranscriptional and posttranscriptional regulation of HMTs, the lack of correlation between mRNA and the protein levels of HMTs has been described in the literature.[12,30,34,35] As such, qPCR and protein quantification have been suggested as complementary to the investigation of the regulatory mechanisms of HMTs.

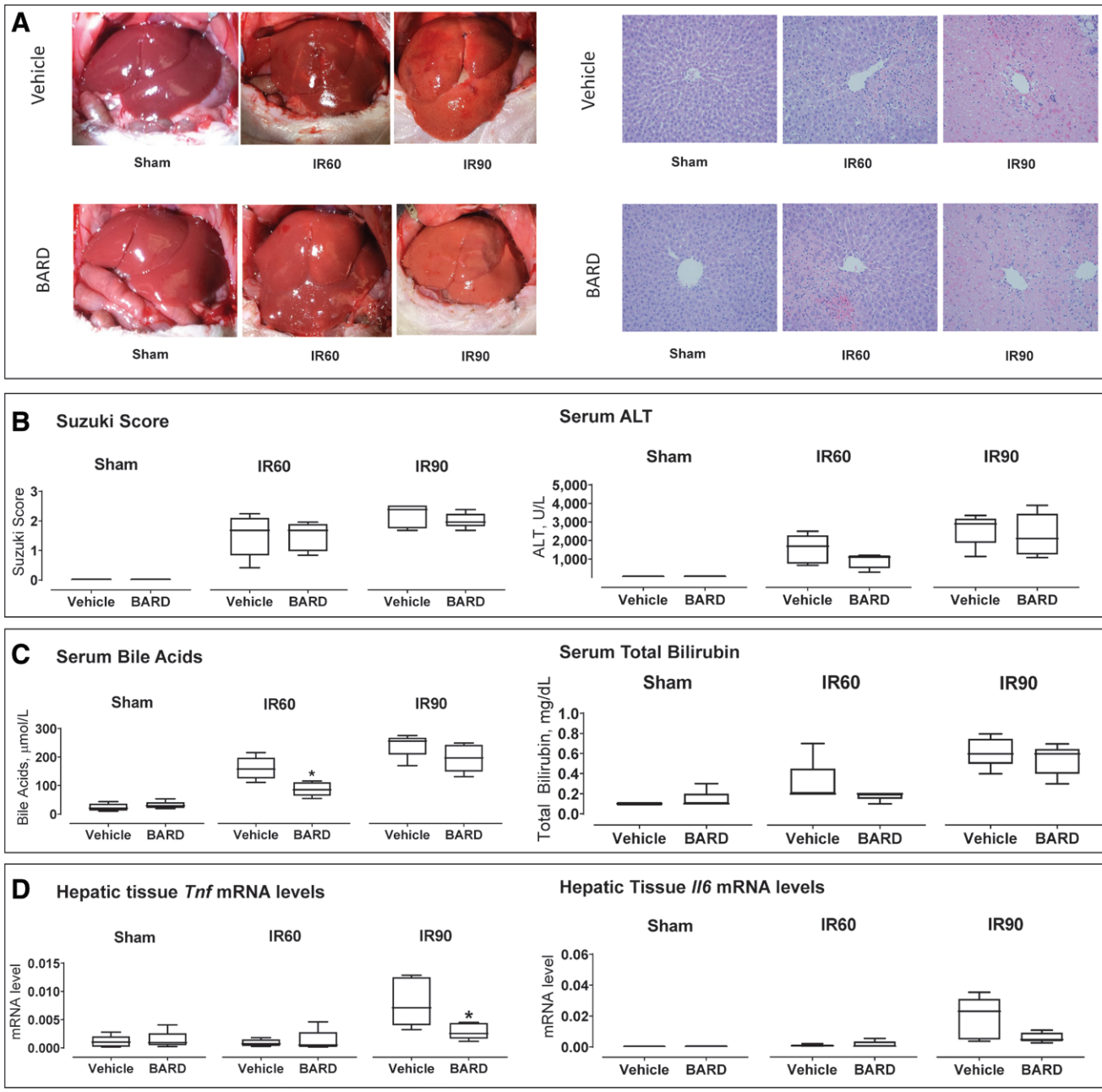

FIGURE 2. Hepatic IRI assessed by blood and hepatic tissue samples. (A) Representative photos of gross and histological findings at the time of sampling in groups with a vehicle or BARD administration are presented. (B) IRI caused increased Suzuki scores and serum ALT levels in IR60, and they were increased further in IR90. However, they were not significantly affected by BARD. (C) Cholestasis induced by hepatic IRI was depicted by serum levels of bile acids and total bilirubin. The serum bile acids' level was significantly decreased by BARD administration in IR60 but not in IR90. The change of serum bilirubin levels by BARD was not statistically significant. (D) The hepatic tissue mRNA levels of *Tnf* and *Il6* were higher in IR90 than those in sham or IR60. The hepatic tissue *Tnf* mRNA levels were not significantly changed by BARD in the sham and the IR60 groups, but they were significantly decreased in the IR90 group. The change of hepatic tissue *Il6* mRNA levels by BARD administration was not statistically significant. *$P<0.05$ by Mann-Whitney test. N = 5 in each group. ALT, alanine transaminase; BARD, bardoxolone methyl; IL6, interleukin 6; IR60, 60 min of ischemia followed by 24 h of reperfusion; IR90, 90 min of ischemia followed by 24 h of reperfusion; IRI, ischemia-reperfusion injury; Tnf, tumor necrosis factor.

IHC Expressions of HMTs Are Different Depending on the Degree of IRI With Regard to Its Intensity and Localization

The HMT can be functional only when it is located alongside the cell membrane of a nonnecrotic, viable hepatocyte. A decrease of bile flow during hepatic IRI can be caused by the translocation of HMTs into the cytosolic space by endocytic internalization from the canalicular membrane without significant change in protein levels.[36-39] The subtle translocation can be observed by IHC as a focal dispersion and blurring of the staining, and this altered staining pattern has been observed in clinical specimens in liver transplantation.[40] We observed the distribution of HMTs using IHC: the staining for canalicular HMTs (ie, MRP2 and BSEP) demonstrated panlobular, strong expression along 1 side of each cell. The staining was crisp in viable areas, especially in the sham and IR60 groups (Figure 5A and B). In contrast, the staining for MRP2 and BSEP was dispersed and granular in areas of necrosis

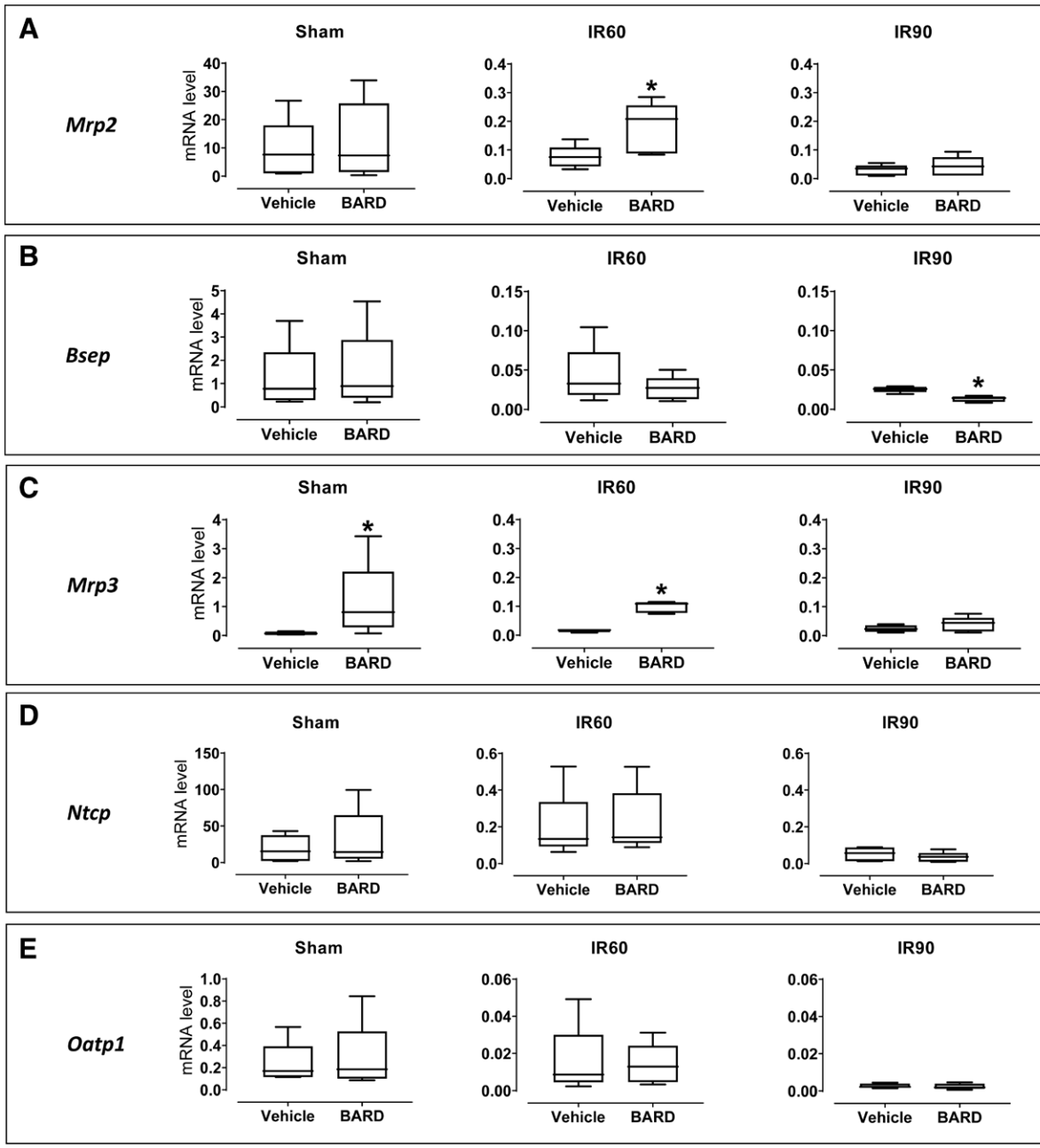

FIGURE 3. The effect of BARD administration on the mRNA levels of *HMT* genes in hepatic tissue. The mRNA levels were expressed as values relative to *Gapdh* levels. Significant decreases of mRNA levels of target genes after IRIs are shown by the diminished maximum ranges of the Y axis in each graph. (A) The mRNA levels of *Mrp2* were significantly increased by BARD administration in the IR60 group but not in the sham or IR90 groups. (B) The mRNA levels of *Bsep* were significantly decreased by BARD administration in the IR90 group but not in the sham or IR60 groups. (C) The mRNA levels of *Mrp3* were significantly increased by BARD administration in the sham and IR60 groups but not in the IR90 group. (D) The mRNA levels of *Ntcp* were not significantly changed by BARD administration in any group. (E) The mRNA levels of *Oatp1* were not significantly changed by BARD administration in any group. *$P<0.05$ by Mann-Whitney test. N=5 in each group. BARD, bardoxolone methyl; Bsep, bile salt export pump; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HMT, hepatocyte membrane transporter; IR60, 60 min of ischemia followed by 24 h of reperfusion; IR90, 90 min of ischemia followed by 24 h of reperfusion; IRI, ischemia-reperfusion injury; Mrp, multidrug resistance-associated protein; Ntcp, sodium-taurocholate; Oatp, organic anion-transporting polypeptide 1.

(Figure 6A and B). The IHC staining of basolateral HMTs (MRP3, NTCP, and OATP1) demonstrated more diffuse staining around the cell membrane (Figure 5C–E). MRP3 staining demonstrated weak (vehicle) to moderate (BARD) granular staining in viable areas of the samples from the sham group. However, samples from IR60 and IR90 showed significant staining only in areas of necrosis without significant staining in viable hepatocytes (Figure 5C). NTCP staining was noticeably strong in the sham and IR60 groups, especially with BARD administration (Figure 5D). The pattern of staining for NTCP was panlobular and diffuse in viable hepatocytes, but it was patchy and cytoplasmic in areas of necrosis (Figure 6D). Most of the IHC staining in this study demonstrated a panlobular pattern, but the OATP1 staining in IR60 and IR90 was more predominantly distributed in zone 1. The staining of OATP1 was granular overall, and it was stronger with BARD

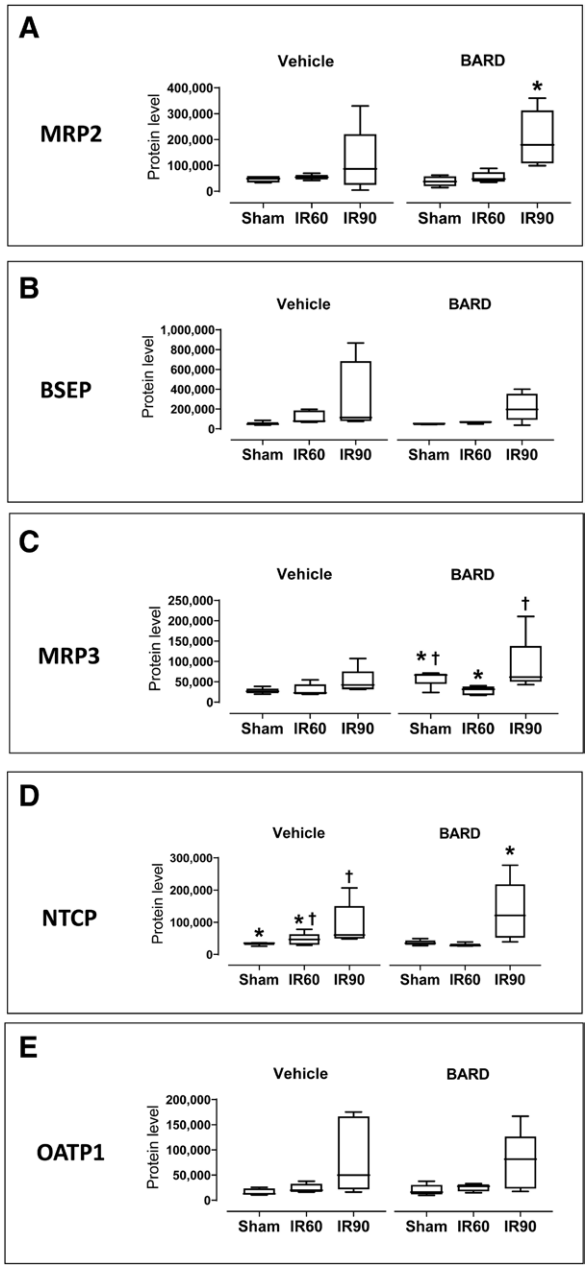

FIGURE 4. The effect of BARD administration on the HMT protein level. The protein levels were expressed as values relative to GAPDH level. (A) MRP2 levels were not significantly different among the groups where the vehicle was administered; however, MRP2 levels were significantly higher in the IR90 group among BARD groups. (B) BSEP levels were not significantly different among the vehicle or BARD groups. (C) MRP3 levels were not significantly different among the vehicle groups; however, MRP3 levels were significantly lower in the IR60 group and higher in the IR90 group among BARD groups. (D) Among the vehicle groups, NTCP levels of IR90 were significantly higher than those of the sham group. Among the BARD groups, NTCP levels of IR90 were significantly higher than those of both the sham and IR60 groups. (E) OATP1 levels were not significantly different among the vehicle or BARD groups. The different symbols on the bars (*, †) indicate significant differences between the groups by the post hoc Mann-Whitney test with Bonferroni's correction after the Kruskal-Wallis test. N = 5 in each group. BARD, bardoxolone methyl; BSEP, bile salt export pump; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMT, hepatocyte membrane transporters; IR60, 60 min of ischemia followed by 24 h of reperfusion; IR90, 90 min of ischemia followed by 24 h of reperfusion; IRI, ischemia-reperfusion injury; MRP2, multidrug resistance-associated protein 2; MRP3, multidrug resistance-associated protein 3; NTCP, sodium-taurocholate cotransporter; OATP1, organic anion-transporting polypeptide 1.

administration in the sham and IR60 groups but weaker with BARD in IR90 (Figure 5E). As such, BARD administration may affect the protein levels and localization, and they are also influenced by the duration of ischemia.

BARD Administration Affects the Expression of HMTs Determined by Tissue Necrosis

The effect of tissue necrosis on the intensity of IHC staining was measured using digital analyses in representative samples from IR90. The sizes of the DAB positive areas for MRP2 and BSEP were smaller in the area of necrosis (Figure 6A and B). Of note, the difference between viable and necrotic areas for MRP2 was significant only in the sample from animals with BARD administration (Figure 6A). The sizes of the DAB positive areas for MRP3 were larger in the area of necrosis, and the difference was significant only in the samples from animals with BARD administration (Figure 6C). The sizes of the DAB positive areas for NTCP were smaller in the area of necrosis, and the difference was significant only in the sample from animals with BARD administration (Figure 6D). The sizes of the DAB positive areas for OATP1 were larger in the area of necrosis (Figure 6E). In summary, the differences in the IHC staining from necrosis became more prominent with BARD in MRP2, MRP3, and NTCP.

DISCUSSION

Our data suggest that BARD administration at the time of ischemia can decrease serum bile acids in association with increased expression of certain HMT genes and altered HMT protein expression in hepatic tissues, support the feasibility of pharmaceutical intervention of postreperfusion cholestasis. It has been reported that 4 consecutive daily gavages of oltipraz, another compound of NRF2 activator, induced Mrp2, Mrp3, and Mrp4 in normal mouse liver without IRI.[16] In our study, the effects of a single dose of BARD were more prominent in livers with IRI than those without. A single dose of BARD for animals without IRI did not affect mRNA abundance of HMTs except that of *Mrp3*. In this study, BARD administration increased the mRNA abundance of HMT genes in IR60 (eg, *Mrp2* and *Mrp3*) and suppressed them in IR90 (eg, *Bsep*). Moreover, we have previously shown that the mRNA levels of HMT genes in IR90 were significantly depleted, whereas those in IR60 were preserved, and the preservation of HMT mRNA levels in IR60 was abolished by *Nrf2* knockout status.[11] Accordingly, we speculate that NRF2 may play a role in HMT gene expression during hepatic IRI. Nevertheless, there are limits in this conclusion, because the observed effects of BARD may also be attributed to other functions of BARD. For example, BARD can mediate not only NRF2 activation but also NF-κB inhibition.[32] NF-κB is also a transcription factor, and it can indirectly regulate HMT gene expression through its control on proinflammatory cytokines.[23,29,30,32] In this study, the effect of BARD on those cytokines was observed only in IR90. In contrast, the main beneficial effect of BARD (eg, decreased serum levels of bile acids) was observed in IR60, not in IR90. As such, the contributing effect of BARD on anticholestasis was presumed to be more prominent in IR60 than in IR90. In this regard, increased mRNA levels of *Mrp2* and *Mrp3* in IR60 could have been assumed to be important components of the pharmaceutical action of BARD. However, the lack of changes

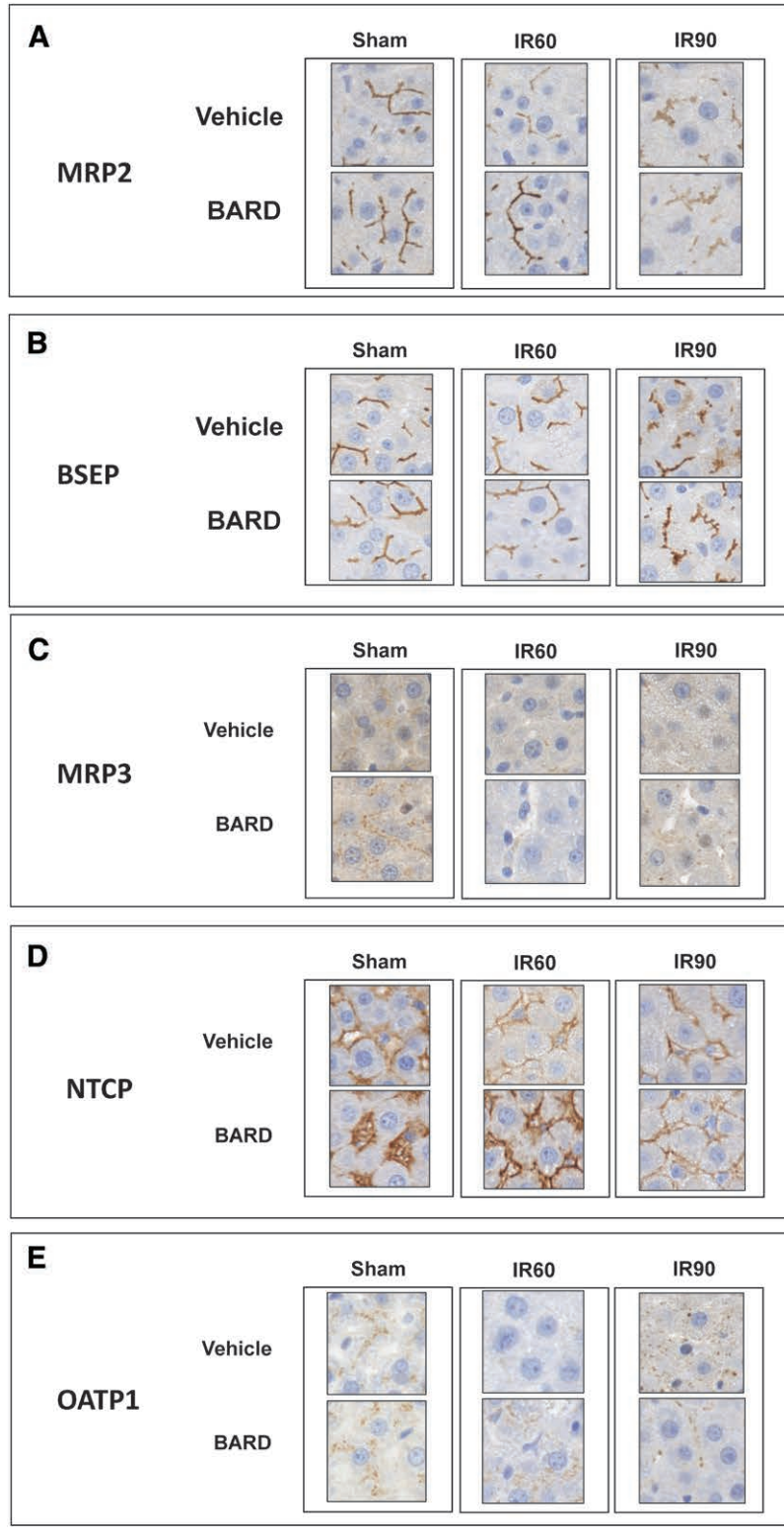

FIGURE 5. Representative photos of IHC findings in viable area. Staining for canalicular HMTs (MRP2 and BSEP) demonstrated a panlobular, strong expression along 1 side of each cell, and staining for basolateral HMTs (MRP3, NTCP, and OATP1) demonstrated more diffuse staining around the cell membrane. (A) IHC staining for MRP2 was crisp along the canalicular membrane in sham and IR60, and it was dispersed into the cytoplasm in IR90. (B) IHC staining for BSEP also showed crisp canalicular staining in sham and IR60 and dispersed staining into the cytosol was observed in IR90. (C) MRP3 staining demonstrated weak (vehicle) to moderate (BARD) granular staining in viable areas of the samples from the sham group. However, samples from IR60 and IR90 did not show significant staining in the viable hepatocytes. (D) NTCP staining was noticeably strong in the sham and IR60 groups, especially with BARD administration. (E) The staining of OATP1 was granular overall, and it was stronger with BARD administration in the sham and IR60 groups but weaker with BARD in IR90. BARD, bardoxolone methyl; BSEP, bile salt export pump; HMT, hepatocyte membrane transporter; IHC, immunohistochemistry; IR60, 60 min of ischemia followed by 24 h of reperfusion; IR90, 90 min of ischemia followed by 24 h of reperfusion; multidrug resistance-associated protein 2; MRP3, multidrug resistance-associated protein 3; NTCP, sodium-taurocholate cotransporter; OATP1, organic anion-transporting polypeptide 1.

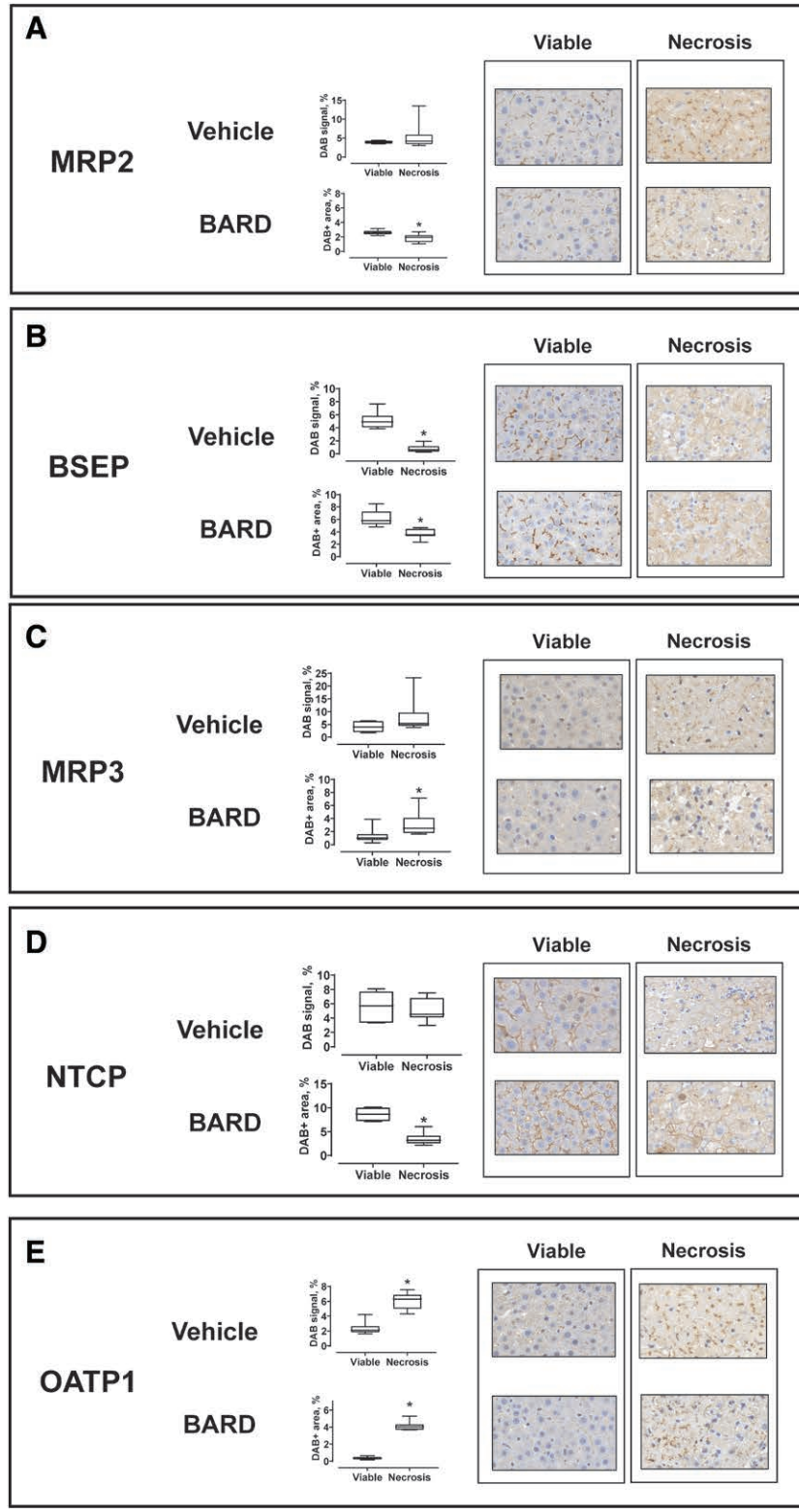

FIGURE 6. Digital analyses for the DAB positive area to compare the size of the IHC staining for HMTs in viable and necrotic areas from the same sample. Four to 10 regions of interest were selected in the representative samples from IR90. The staining was generally crisp in viable areas, whereas it was dispersed and granular in areas of necrosis. (A) The sizes of the DAB positive area for MRP2 were smaller in the area of necrosis, and the difference was significant only in the samples from animals with BARD administration. (B) The sizes of the DAB positive areas for BSEP were smaller in the area of necrosis. (C) The sizes of the DAB positive areas for MRP3 were larger in the area of necrosis, and the difference was significant only in the sample from animals with BARD administration. (D) The sizes of the DAB positive areas for NTCP were smaller in the area of necrosis, and the difference was significant only in the sample from animals with BARD administration. (E) The sizes of the DAB positive areas for OATP1 were larger in the area of necrosis. *$P < 0.05$ based on the Mann-Whitney test. BARD, bardoxolone methyl; BSEP, bile salt export pump; DAB, 3,3'Diaminobenzidine; HMT, hepatocyte membrane transporters; IHC, immunohistochemistry; IR60, 60 min of ischemia followed by 24 h of reperfusion; IR90, 90 min of ischemia followed by 24 h of reperfusion; MRP2, multidrug resistance-associated protein 2; MRP3, multidrug resistance-associated protein 3; NTCP, sodium-taurocholate cotransporter; OATP1, organic anion-transporting polypeptide 1.

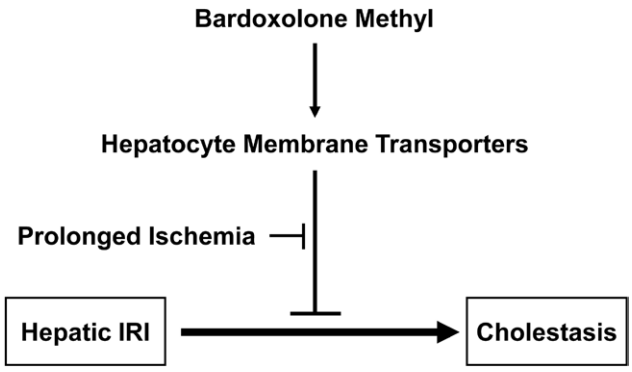

FIGURE 7. Schematic presentation of the working model used in this study. BARD may mediate anticholestatic effects under IRI conditions by regulating HMTs and the duration of ischemia time can affect the biologic responses of the liver to BARD. BARD, bardoxolone methyl; HMT, hepatocyte membrane transporter; IRI, ischemia-reperfusion injury.

on corresponding HMT protein levels in this study suggests another regulatory mechanism.

HMTs exist in a recycling pool for rapid mobilization and insertion between the submembrane vesicle and the plasma membrane.[6,37,41] This has been suggested as a mechanism of cholestasis in oxidative stress because prooxidant compound induced the internalization of HMTs, and it could be reverted by glutathione, an antioxidant.[41] Moreover, it has been shown that alteration of bile flow in hepatic IRI was coincided by MRP2 translocation.[42] IRI-induced translocation of canalicular HMTs (eg, cytosolic staining pattern) was observed in this study, especially in IR90. NRF2 is a master regulator of oxidative stress control and activates >250 genes during acute cellular stress.[19] As such, the administration of BARD might have affected other targets relevant to the regulation of IRI-induced translocation of HMTs.

Furthermore, we also found that the area of severe injury with necrosis demonstrated increased or decreased sizes of HMT staining area, depending on the type of target HMTs, and the findings were variable depending on the presence of BARD. In addition to the HMT translocation, epigenetic control, intracellular energy metabolism, tight junction permeability, enterohepatic circulation, and factors of protein degradation may also account for the regulation of HMT activity and bile metabolism in cells upon BARD treatment. A major limitation of this preliminary study is the small sample size and limited number of sampling time points to fully demonstrate effects of BARD on bile metabolism and serum cholestatic markers. In addition, the effect of BARD on posttranscriptional control of HMTs was inconclusive in this study because of a lack of quantitative analysis to assess the proportion of HMT translocation in hepatocytes. As such, these intricate mechanisms need to be interrogated in our future studies to better address the potentially beneficial effects of BARD on metabolism in hepatic IRI.

In summary, BARD administration at the time of ischemia showed the potential to mitigate cholestasis in hepatic IRI in our animal model (Figure 7). Our preliminary data provided initial evidence supporting the potential of BARD to prevent cholestasis in hepatic IRI. It is warranted to further determine whether BARD has the potential as a pharmaceutical intervention to improve clinical outcomes of liver transplantation.

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