Kappa-Opioid Agonist U50,488H-Mediated Protection Against Heart Failure Following Myocardial Ischemia/Reperfusion: Dual Roles of Heme Oxygenase-1

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Key Words
Myocardial ischemia/reperfusion • Heart failure • κ-opioid • Heme oxygenase-1

Abstract
Backgrounds/Aims: The selective κ-opioid agonist U50,488H protects heart from myocardial ischemia-reperfusion (MI/R) injury. We examined whether U50,488H is also beneficial for MI/R induced heart failure. Methods: Anesthetized male Sprague-Dawley rats were subjected to 30 min of myocardial ischemia via left anterior descending coronary artery (LAD) occlusion, followed by 4 weeks of reperfusion. Infarct size was examined by Evans blue/triphenyl tetrazolium chloride (TTC) staining. Cardiac function and remodeling were examined by echocardiography and histology. HO-1 gene transcription and expression were measured by RT-PCR and western blot. Results: Compared to vehicle-treated MI/R rats, rats administered a single dose of U50,488H at the beginning of reperfusion exhibited reduced myocardial infarct size, oxidative stress, hypertrophy, and fibrosis, improved mechanical function, and greater neovascularization. U50,488H also increased myocardial heme oxygenase (HO)-1 gene transcription and expression, while pharmacological HO-1 inhibition reversed all protective effects of U50,488H. Furthermore, U50,488H protected control cultured cardiomyocytes against simulated I/R-induced apoptosis but not cultures subjected to shRNA-mediated HO-1 knockdown. Inhibition of HO-1 in the subacute phase of reperfusion reversed the U50,488H-
induced increase in neovascularization and suppression of oxidative stress. Finally, U50,488H increased Akt phosphorylation and nuclear translocation of Nrf2, a key HO-1 transcription activator, while inhibition of PI3K–Akt signaling abolished U50,488H-induced Nrf2 nuclear translocation, HO-1 upregulation, and cardioprotection. **Conclusion:** Activation of HO-1 expression through the PI3K–Akt–Nrf2 pathway may mediate the acute and long-term protective effects of U50,488H against heart failure by enhancing cardiomyocyte survival and neoangiogenesis and by reducing oxidative stress.

**Introduction**

Despite timely reperfusion, 25% of acute myocardial infarct survivors develop chronic heart failure. In fact, reperfusion injury is a major determinant of long-term outcome in myocardial ischemia/reperfusion (MI/R) patients [1, 2]. Expression of the κ-opioid receptor is altered in hamsters with hypertensive cardiomyopathy [3], and the selective κ-opioid agonist U50,488H [4] is protective against MI/R injury in acute MI/R models [5, 6], an effect abolished by the selective κ-opioid antagonist Nor-BNI [7]. Animal studies using permanent coronary artery ligation suggest that long-term outcome can be dramatically influenced by interventions during the subacute and chronic stages after myocardial infarct [8, 9], but the efficacy of κ-opioid agonists have not been examined.

The aim of the present study was to investigate for the first time the potential protective effects of U50,488H against MI/R-induced heart failure and the potential molecular mechanisms involved. Heme oxygenase-1 (HO-1) is beneficial in chronic models of heart failure [10-13] and it is also a key mediator of opioid effects [14]. Nevertheless, whether HO-1 contributes to the cardioprotective effect of U50,488H has not been established.

**Materials and Methods**

**Animal Preparation**

Male Sprague-Dawley (SD) rats used in the present study were purchased from the research animal facility of the Fourth Military Medical University and received humane care in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health; NIH publication no. 85-23, revised 1996). Experimental protocols were approved by the Fourth Military Medical University Committee on Animal Care. To produce MI/R injury, the left anterior descending coronary artery (LAD) was encircled by a 6-0 prolene suture 1 to 2 mm below the tip of the left atrial appendage and its ends were threaded through polyethylene tubing (PE-50) to form a snare for reversible coronary artery occlusion. After 30 min of ischemia, the snare was released to allow for reperfusion. A sham group was subjected to suture placement but the LAD was not occluded.

**Experiment Protocols**

Two protocols were used. In the first protocol, SD rats were randomly assigned to the following groups: 1) I/R control group receiving ischemia for 30 min by LAD ligation followed by saline administration 5 min before reperfusion, 2) MI/R plus U50,488H (I/R+U50) group, treated as in group 1 but receiving 2 mg/kg U50,488H IV instead of saline 5 min before reperfusion, 3) I/R+U50+Nor-BNI group, treated as in group 2 but also receiving 2 mg/kg IV Nor-BNI (Tocris, USA) 5 min before U50,488H, 4) I/R + U50,488H + LY294002 group (I/R+U50+LY) receiving the specific PI3K inhibitor LY294002 (Sigma, St. Louis, US, 0.3 mg/kg IV) 5 min before U50,488H, 5) I/R + U50,488H + zinc protoporphyrin-IX group (I/R+U50+ZnPP-IX) receiving in addition to U50,488H the HO-1 inhibitor zinc protoporphyrin-IXr [15] (ZnPP-IX; 10 µmol/kg, IP) twice at 16 and 3 h prior to ischemia as previously described [16] and then twice daily for 6 days post-MI/R to achieve continuous HO-1 inhibition, and 6) a sham operation group. Evans Blue and triphenyl tetrazolium chloride (TTC) dual staining was performed after 24 h of reperfusion to compare infarct sizes among treatment groups. Expression of HO-1 was examined after 3, 24, 48, and 96 h of reperfusion by western blot. Cardiac function was monitored by echocardiography at baseline and after 24 h, 1 week, 3
weeks, and 4 weeks (day 1, 1 wk, 2 wk, and 4 wk, respectively) of reperfusion. Protein-bound malondialdehyde level, an index of oxidative stress, was measured after 4 days of reperfusion. Animals were sacrificed after 4 weeks of reperfusion for histological examinations of neovascularization and remodeling.

In the second protocol, SD rats were first subjected to MI/R or MI/R + U50,488H (I/R+U50) as described in protocol 1. After 24 h of reperfusion, rats from the I/R+U50 group were further assigned to the following two groups: 1) U50,488H + zinc protoporphyrin-IX co-treated (I/R+U50+ZnPP-IX P2) group injected with zinc protoporphyrin-IX (ZnPP; 10 µmol/kg, IP) twice daily from day 2 to day 6 post-ischemia, and 2) U50,488H (I/R+U50) group receiving normal saline instead of ZnPP-IX. Rats were sacrificed on day 7 and indices of neovascularization and oxidative stress were compared between groups.

Echocardiography

Transthoracic echocardiography was performed before (day 0, baseline) and after MI/R injury (day 1, 1 wk, 2 wk, and 4 wk) under light anesthesia induced by inhalation of a 1% isoflurane / 99% oxygen mixture through a nose cone. Heart rate was maintained at 200–250 beats per minute. Both two-dimensional and M-mode echocardiographs were obtained with a 15-MHz linear transducer (VisualSonics Vevo 2100, Toronto, ON, Canada). Left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), and posterior wall thickness at diastole (PWTd) were measured in short axis view at the papillary muscle level by M-mode tracing. All measurements were performed by investigators blinded to treatment group and represent the mean of 5 consecutive cardiac cycles. For every group, 8–16 rats were used for the echocardiography examination. Cardiac contractile function as represented by left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (FS) were calculated by computer algorithms.

Western Blot Analysis

Protein extracted from cardiac tissue or primary cardiomyocytes was separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against HO-1 (1:500) (Santa Cruz, USA), Akt (1:1000), phosphorylated Akt (Ser-473) (1:1000) (Cell Signaling, USA), or a combination. Immunolabeling was detected by secondary antibodies conjugated to IRDyeTM800 (1:20,000 dilution; Rockland Inc., IL, US) using an Odyssey infrared imaging system (LI-COR Inc., Lincoln, NE, US). In another set of samples, nuclear and cytoplasm proteins were isolated separately from heart tissue using an isolation kit (Pierce, USA) according to the manufacturer’s instructions and probed with a primary antibody against Nrf2 (1:500) (Santa Cruz, USA) for detection of Nrf2 nuclear translocation.

Real-Time PCR

Total RNA was extracted from individual samples using TRIzol (Invitrogen) and reverse transcribed into cDNA using the RevertAidTM First-Strand cDNA synthesis kit (Fermentas, Lithuania) according to the manufacturer’s instructions. The levels of mRNA transcripts were determined by quantitative real time PCR using template cDNA, specific primers, and the standard SYBR Green RT-PCR kit (Takara, Japan) according to the manufacturer’s instructions. The PCR reactions (25 µl/tube, in triplicate) were performed first at 95 °C for 10 s for denaturation followed by 35 cycles of 95 °C for 5 s and 60 °C for 34 s using the specific HO-1 primers 5’-TCAGTCCCAAACGTCGCGGT-3’ (forward) and 5’-GCTGTGCAGGTGTTGAGCC-3’ (reverse). The relative levels of HO-1 mRNA transcripts were normalized to the control β-actin.

Neonatal Rat Cardiomyocyte Culture and Treatment with Lentiviral vector shRNA Targeting HO-1

Neonatal cardiomyocytes were isolated from 1- or 2-day-old SD rats and cultured as previously described [17]. Two or three days after the primary culture, cultures were transfected with a lentiviral vector encoding shRNA targeting the rat HO-1 gene (5’-CCGUGGCAGUGGGAAUUUAUGCCAU-3’) or a control vector. Lentiviral vectors were purchased from Gene-Pharma Company, Ltd. (Shanghai, China). A pilot study with the GFP-expressing control lentivirus demonstrated that 70%–80% of the cells were transfected as detected by fluorescence microscopy (Olympus, Tokyo, Japan; Fig. 9a). Successful silencing of HO-1 was confirmed by real-time PCR and western blot before simulated ischemia/ reperfusion (SI/R) (Fig. 9b,c).

In Vitro Simulated Ischemia/Reperfusion (SI/R)

Simulated ischemia/reperfusion (SI/R) was performed by exposing the cardiomyocytes to an ‘ischemic’ buffer containing (in mmol/l) NaCl 137, KCl 3.8, MgCl₂ 0.49, CaCl₂·2H₂O 0.9, and HEPES 4, supplemented
with deoxyglucose 10, sodium dithionate 0.75, KCl 12, and lactate 20 (pH 6.5) for 2 h in a humidified cell culture incubator (21% oxygen, 5% CO₂, 37 °C) as previously described [18]. After simulated ischemia, cardiomyocytes were transferred to DMEM in a humidified cell culture incubator (21% O₂ / 5% CO₂, 37 °C) to simulate reperfusion. During simulated reperfusion, cardiomyocytes were treated with U50,488H (30 μmol/L) or vehicle. Compared to cardiomyocytes treated with control virus, HO-1 silencing abolished the reduction in cardiomyocyte apoptosis by U50,488H treatment (Fig. 10).

**Evaluation of Oxidative Stress and Neovascularization**

Oxidative stress was evaluated by immunostaining for malondialdehyde (MDA)-adducted proteins [19]. Digital images were analyzed by MetaMorph 4.5 image quantitation software. Neovascularization was assessed by immunostaining heart sections for CD31 [20]. In brief, sections were incubated with rabbit anti-CD31 (1:200; Abcam, Cambridge, MA, USA) mixed with mouse anti-α-actin (skeletal) (1:250; Abcam). Immunolabeling was visualized using a mixture of Cy3-conjugated anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibodies (Sigma).

**Statistical Analysis**

Treatment group means were compared by one-way ANOVA followed by post hoc pair-wise comparisons with Bonferroni correction. For the fractional shortening vs. time data, two-way ANOVA was conducted. Data are expressed as mean ± standard error of the mean (SEM). Significance was accepted at p < .05. GraphPad Prism-5 statistic software (La Jolla, CA) was used for all data analysis.

**Results**

**HO-1 inhibition blocked the cardioprotective effects of U50,488H during the acute phase of reperfusion**

Rats treated with the κ-opioid agonist U50,488H prior to ischemia/reperfusion (I/R+U50 group) exhibited significantly a lower infarct size index (Fig. 1) compared to vehicle-treated I/R rats (I/R group). However, co-treatment with the HO-1 inhibitor ZnPP-IX (I/R+U50+ZnPP-IX group) abrogated this U50,488H-associated reduction in infarct size, suggesting that the cardioprotective effects of κ-opioid activation are mediated by enhanced HO-1 activity.

To verify the role of HO-1 in cardioprotection by U50,488H, we knocked down HO-1 expression in cultured cardiomyocytes by infection with a shRNA-encoding lentivirus vector as previously described [19]. After infection for 48 h (MOI: 50), both mRNA and protein levels of HO-1 were significantly downregulated (Fig. 9). While U50,488H reduced cardiomyocyte death in response to SI/R compared to vehicle-treated cultures, it failed to protect HO-1-silenced cardiomyocytes (Fig. 10). HO-1 silencing alone did not significantly influence cell apoptosis induced by SI/R.

**U50,488H Increased HO-1 Transcription and Expression**

After 3 h of reperfusion, U50,488H-treated rats (I/R+U50 group) showed significantly higher HO-1 mRNA expression in myocardium compared to the I/R group (Fig. 2a). After 24 h of reperfusion, I/R injury increased HO-1 expression in myocardium compared to sham controls (Fig. 11). HO-1 protein level was also significantly higher in I/R+U50 group myocardium compared to I/R group myocardium. After 6 days of reperfusion, HO-1 protein level in I/R+U50 group myocardium was still higher than in the I/R group (Fig. 2b, c). The HO-1 level in I/R+U50 group myocardium did not return to a level similar to that of the I/R group until day 9. Thus, U50,488H appears to potentiate HO-1 upregulation induced by I/R.

**U50,488H Improved Post-MI/R Cardiac Function and Suppressed Cardiac Remodeling**

Fractional shortening (FS) of the left ventricle, a functional index of cardiac output, declined with time after I/R (Fig. 3a, b). Rats treated with U50,488H exhibited greater preservation of baseline FS at day 1 post-MI/R compared to the I/R group. After 4 weeks, the
Fig. 1. HO-1 inhibition abolished the reduction in myocardial infarct (INF) size induced by U50,488H treatment during the acute phase of reperfusion. a, Representative illustrations of infarct sizes as determined by Evans Blue and TTC staining. b, Myocardial infarct (INF) size expressed as percentage of area at risk (AAR); c, The area at risk expressed as a percentage of left ventricle (LV) area. I/R, ischemia/reperfusion (30 min/24 h); U50, U50,488H; LY, LY294002; Znpp-IX, zinc protoporphyrin-IXr. Data are presented as mean ± SEM for eight rats in each group. *p < .05 vs. I/R; #p < .05 vs. I/R+U50.

Fig. 2. U50,488H increased HO-1 transcription and expression. a, Sample western blots showing HO-1 protein levels after 24 h, 3 days, 6 days, and 9 days of reperfusion. b, HO-1 mRNA level detected by quantitative real-time PCR after 3, 24, 48, and 96 h of reperfusion. c, Densitometric quantification of western blots showing changes in HO-1 protein expression. I/R, ischemia/reperfusion (A: 30 min/3 h; B: 30 min/24 h); U50, U50,488H; Data are presented as mean ± SEM for eight rats in each group. *p < .05 vs. I/R; **p < .01 vs. I/R.

I/R group showed a significant reduction in LVEF, an independent determinant of survival post-MIR [21], compared to the I/R+U50 group.
The I/R group also demonstrated significant LV dilation as evidenced by increased LVESD and LVEDD compared to the sham group at 4 weeks post-MI/R (Fig. 4a, b). Treatment with U50,488H (I/R+U50 group) alleviated LV dilation as indicated by reductions in both LVESD and LVEDD compared to the I/R group. Cardiac hypertrophy was observed in I/R group rats compared to sham group rats after 4 weeks of reperfusion as indicated by greater heart weight to body weight ratio (HW/BW) and posterior wall thickness at diastole (PWTd), while U50,488H treatment reversed these increases (Fig. 4c, d). Additionally, quantitative analysis of Masson’s trichrome staining for interstitial fibrosis within the ischemic border zone showed dramatically increased collagen deposition in I/R hearts, which again was reduced by U50,488H treatment (Fig. 4e, f).

**HO-1 Inhibition Abolished the Benefits of U50,488H for Cardiac Function and Remodeling**

The possible role of HO-1 in U50,488H-induced improvements in post-I/R cardiac function and remodeling was then examined. Inhibition of HO-1 activity by ZnPP-IX treatment abolished the preservation of FS by U50,488H as demonstrated by echocardiography at multiple time points (Fig. 3a, b). In addition, ZnPP-IX significantly reduced LVEF preservation by U50,488H after 4 weeks of reperfusion (Fig. 3c).

After 4 weeks of reperfusion, LVESD and LVEDD of the U50,488H+ZnPP-IX group were similar to the I/R group and significantly higher compared to the I/R+U50 group (Fig. 4a, b),
**Fig. 4.** Improved cardiac remodeling by U50,488H treatment and reversal by HO-1 inhibition. Left ventricular (LV) enlargement, cardiac hypertrophy, and fibrosis 28 days following MI/R in the different treatment groups. a. Left ventricular end systolic diameter (LVESD) b. Left ventricular end diastolic diameter (LVEDD). c. Heart weight/ body weight (HW/BW). d. Posterior wall thickness at diastole (PWTd). e,f. Representative sections and analysis with Masson's trichrome staining (fibrosis stained in blue) within the ischemic border zone. Data presented as mean ± SEM for eight rats in each group. *p < .05 vs. I/R; #p < .05 vs. I/R+U50.
suggesting that the reduction in LV enlargement by U50,488H was also dependent on HO-1 upregulation. Inhibition of HO-1 abolished the reductions in HW/BW and PTWd (Fig. 4c, d) observed in I/R+U50 group rats. Finally, HO-1 inhibition with ZnPP-IX (I/R+U50+ZnPP-IX group) reversed the diminution of fibrosis observed in I/R+U50 group rats (Fig. 4e, f).

**U50,488H Attenuated Oxidative Stress and Promoted Neovascularization in a HO-1 Dependent Manner**

To evaluate myocardial oxidative stress, protein-bound malondialdehyde level was examined. As shown in Figure 5a, protein-bound malondialdehyde level was significantly increased in the I/R group compared to the sham group after 4 days of reperfusion. Treatment with U50,488H reduced the I/R-induced increase in protein-bound malondialdehyde, and
Tong et al.: HO-1 Mediates U50,488H Protection Against I/R Induced Heart Failure

Fig. 7. U50,488H increased HO-1 transcription and expression via activation of PI3K-Akt signaling and ensuing Nrf2 nuclear translocation. a,b. Nuclear Nrf2 levels in myocardium. c,d Akt phosphorylation in myocardium. e,f Relative HO-1 mRNA and protein expression levels in myocardium. I/R, ischemia/reperfusion (30 min/ 3 h); U50, U50,488H; LY, LY294002. Data presented as mean ± SEM for eight rats in each group. **p < .05 vs. I/R; ##p < .05 vs. I/R+U50; #p < .01 vs. I/R+U50.

Fig. 8. Proposed cardioprotective signaling pathway by U50,488H.

this reduction was abolished by HO-1 inhibition (I/R+U50 vs. I/R+U50+ZnPP-IX group; Fig. 5a). Angiogenesis was evaluated by CD31 staining. As shown in Figure 5b, CD31 staining revealed markedly increased capillary density in the marginal area of the infarct in I/R+U50 group rats compared to the I/R group rats, indicating that U50,488H promoted angiogenesis post-ischemia. The enhanced LV capillary density observed in the I/R+U50 group was significantly reduced by ZnPP-IX co-treatment (I/R+U50+ZnPP-IX group), indicating that the pro-angiogenic effects of U50,488H are dependent on HO-1.

These results strongly suggest that cardioprotection and preservation of cardiac function by U50,488H are mediated by HO-1 upregulation. However, the HO-1 inhibitor
that reversed all U50,488H effects was applied both prior to and during the first 6 days of reperfusion (i.e., during both acute and subacute phases), so it remained unclear if κ-opioid activation can also protect against cardiac injury through HO-1 upregulation if delivered following I/R. In experimental protocol 2, animals treated with U50,488H were all subjected to 30 min of ischemia and 24 h of reperfusion before receiving vehicle or the HO-1 inhibitor. Despite the same level of injury during the acute reperfusion phase, HO-1 inhibition in the subacute phase (day2 to day6) reversed the oxidative stress and neovascularization reduction induced by U50,488H (Fig. 6). This suggests that increased HO-1 expression in the subacute phase of reperfusion can have long-term benefits on cardiac function via enhanced neovascularization and oxidative stress alleviation.

**The PI3K–Akt–Nrf2 Pathway Mediated U50488H-induced HO-1 Transcription and Expression**

In our previous report, PI3K/Akt was shown to mediate cardioprotection by κ-opioid activation. In the present study, we demonstrate that HO-1 upregulation is necessary for the cardioprotective effects of U50,488H. Nrf2 is a key activator of HO-1 gene transcription and its nuclear translocation is controlled by kinases such as PI3K/Akt. We found that Nrf2 nuclear translocation in myocardium was far greater in the I/R group than the sham group after 24 h of reperfusion (Fig. 11), and translocation was further increased in the I/R+U50 group (Fig. 7a, b). Akt phosphorylation was reduced by I/R compared to sham controls (Fig. 11). The I/R+U50 group exhibited higher Akt phosphorylation compared to the I/R group and this effect was abolished by the PI3K inhibitor LY294002 (I/R+U50+LY group vs. I/R+U50 group). Nuclear translocation of Nrf2 was also abolished by LY294002, suggesting that U50,488H amplifies I/R-induced activation of the PI3K–Akt–Nrf2 pathway. Inhibition
Tong et al.: HO-1 Mediates U50,488H Protection Against I/R Induced Heart Failure

Fig. 10. Silencing of HO-1 abolished the reduction in apoptosis induced by U50,488H in cultured neonatal rat cardiomyocytes subjected to simulated ischemia/reperfusion (SI/R). a. Representative images of TUNEL-stained cultured cardiomyocytes. Top row: TUNEL-positive cardiomyocytes. Bottom row: total nuclei determined by DAPI staining. b. Cardiomyocyte apoptotic index expressed as the percentage of TUNEL-positive myocytes over total nuclei. c. Cardiomyocyte caspase-3 activity. SI/R, simulated ischemia/reperfusion (2 h/4 h). Data presented as mean ± SEM of 5 independent experiments in each group. *p < .05 vs. SI/R+Control virus; #p < .05 vs. SI/R+U50+Control virus. Virus, lentiviral-vectored shRNA. Scale bar = 50 µm.

Discussion

The cytoprotective efficacy of U50,488H has been confirmed in multiple studies [6, 19]. However, previous investigations focused on the acute effects. In the present study, we provide the first demonstration that U50,488H provides not only protection against acute myocardial cell injury, but also potent long-term benefits against I/R-induced heart dysfunction by promoting neovascularization and by suppressing fibrosis.

U50,488H is a highly selective κ-opioid agonist with few other non-target effects, among which is low-potency sodium channel blockade [22]. However, U50,488H most likely exerted cardioprotection in the present study via κ-opioid receptor activation as all measured effects were abolished by Nor-BNI, a selective κ-opioid antagonist that can sustain receptor blockade for at least 4 days in mice [7]. Further, the affinity of Nor-BNI for the κ-opioid receptor is 169-fold higher than at μ receptors and 153-fold higher than at δ receptors [23]. The κ-opioid receptor would thus be selectively inhibited by Nor-BNI at the administered dosage (2 mg/kg, IV). In male rats, the half-life of U50,488H is only about 2 hours [24], while long-term benefits were observed up to 4 weeks post-ischemia in our
Fig. 11. I/R injury modulates HO-1 expression, Akt phosphorylation, and Nrf-2 nuclear translocation. a, c. I/R injury induces HO-1 expression. a, d. I/R injury reduces Akt phosphorylation. b, e. I/R injury increases Nrf-2 nuclear translocation. I/R, ischemia/reperfusion. Data presented as mean ± SEM of six rats in each group. **p < .01 vs. Sham; *p < .05 vs. Sham.

Animal model of MI/R. One possible mechanism by which U50,488H produces long-term protection against MI/R injury is through a reduction in cardiomyocyte death during the acute phase of reperfusion [4, 25, 26]. On the other hand, long-term effects may be independent of agonist presence. For instance, opioid receptor pathways have been shown to be chronically activated by exercise [27, 28]. In addition, interventions beyond the acute reperfusion phase, such as remote ischemic post-conditioning applied every 1 or 3 days over 28 days of reperfusion, also provided cardioprotection [29]. In our present study, U50,488H promoted neovascularization in the marginal region of the infarcted myocardium and limited oxidative stress, myocardial hypertrophy, and interstitial fibrosis, reactions known to degrade long-term cardiac function [30], even when applied after a delay. Thus, it appears that U50,488H has benefits independent of acute cytoprotection, and further evidence strongly suggests that these distinct temporal effects are mediated, at least in part, by HO-1 induction.

HO-1 is the inducible member of the heme oxygenase family, rate-limiting enzymes catalyzing the breakdown of heme into free ferrous iron, carbon monoxide, and biliverdin [30, 31]. HO-1 is a well documented cytoprotective enzyme [10, 32], and myocyte-restricted HO-1 overexpression was shown to improve survival and LV remodeling in post-MI failing hearts [33]. In the present study, U50,488H amplified and prolonged the increase in HO-1 transcription and expression in post-I/R heart. Inhibition of HO-1 during the acute and subacute phases of reperfusion (1–6 days post-ischemia) reversed the infarct size reduction induced by U50,488H. In cultured neonatal rat cardiomyocytes subjected to SI/R, reduced HO-1 expression by shRNA knockdown did not increase apoptosis compared to controls, but did abolished the anti-apoptotic effect of U50,488H. In the subacute phase of reperfusion, increased neoangiogenesis and reduced oxidative stress were observed in U50,488H-treated hearts, while inhibition of HO-1 from day 2 to day 6 reversed these effects. Collectively, these results indicate that HO-1 activation is a key mediator of U50,488H-induced cardioprotection.
and that HO-1 possesses dual efficacy, reducing myocardial cell death and ensuing infarct formation in the acute phase of reperfusion while promoting neoangiogenesis and reducing remodeling (fibrosis) in the subacute phase.

The control of HO-1 expression occurs primarily at the transcriptional level [34, 35] and Nrf2 is one of the most potent transcriptional activators of HO-1. Induction requires Nrf2 nuclear translocation and binding to the antioxidant response element (ARE) controlling expression of HO-1 as well as other phase II antioxidant and stress-inducible genes [36]. In the present study, we demonstrate that U50,488H treatment increases Nrf2 in cardiomyocyte nuclei, suggesting Nrf2 nuclear translocation. Phosphorylation by PI3K–Akt signaling is known to enhance Nrf2 dissociation from Keap1, its cytoplasmic repressor, allowing subsequent nuclear translocation [37]. In the present study, U50,488H significantly enhanced PI3K–Akt signaling activity concomitant with Nrf2 nuclear translocation. Inhibition of Akt using LY294002 abolished U50,488H-induced Nrf2 nuclear translocation, HO-1 upregulation, and cardioprotection. Collectively, these results indicate that PI3K–Akt–Nrf2 signaling links κ-opioid activation with upregulated HO-1 transcription and expression.

This study has several limitations. We did not test whether continuous κ-opioid inhibition has any impact on the outcome in our chronic model, although a previous study found that the selective κ-opioid antagonist Nor-BNI alone did not worsen outcome in an acute I/R model [38]. In the present study, only a single dose of U50,488H was administered at the beginning of reperfusion and the extent of κ-opioid activation was not directly determined. In our future studies, continuous κ-opioid inhibition with pharmacological or genetic methods will be employed to clarify the role of κ-opioid receptor signaling following ischemia, especially in the subacute and chronic phases.

In summary, a single dose of U50,488H administered at the beginning of reperfusion reduced the severity of subsequent heart failure in our rat model of MI/R injury. We further demonstrate that HO-1 upregulation is critical for prevention of cardiomyocyte death and improved functional recovery by U50,488H. The PI3K–Akt–Nrf2 signaling pathway mediates the U50,488H-induced increase in HO-1 transcription and expression (Fig. 8).

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Disclosure Statement

The authors declare no conflicts of interest.

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