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Concentration-dependent wrestling between detrimental and protective effects of H_2O_2 during myocardial ischemia/reperfusion

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Reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress are paradoxically implicated in myocardial ischemia/reperfusion (I/R) injury and cardioprotection. However, the precise interpretation for the dual roles of ROS and its relationship with the ER stress during I/R remain elusive. Here we investigated the concentration-dependent effects of hydrogen peroxide (H_2O_2) preconditioning (PC) and postconditioning (PoC) on the ER stress and prosurvival reperfusion injury salvage kinase (RISK) activation using an *ex vivo* rat myocardial I/R model. The effects of H_2O_2 PC and PoC showed three phases. At a low level (1 μM), H_2O_2 exacerbated I/R-induced left ventricular (LV) contractile dysfunction and ER stress, as indicated by enhanced phosphorylation of protein kinase-like ER kinase and expressions of glucose-regulated protein 78, X-box-binding protein 1 splicing variant, TNF receptor-associated factor 2, activating transcription factor-6 cleaved 50 kDa fragment, and caspase-12 cleavage, but the I/R-induced RISK activation including protein kinase B (PKB/Akt) and protein kinase Cε (PKCε) remained unchanged. Consistently, the postischemic LV performance in 1 μM H_2O_2 PC and PoC groups was improved by inhibiting ER stress with 4-phenyl butyric acid but not affected by the ER stress inducer, tunicamycin. At a moderate level (10–100 μM), H_2O_2 significantly improved postischemic LV performance and enhanced RISK activation, but it did no further alter the ER stress. The cardioprotection but not ER stress was abrogated with Akt or PKCε inhibitor wortmannin or εV1–2. At a high level (1 mM), H_2O_2 markedly aggravated the reperfusion injury and the oxidative stress but did not further enhance the RISK activation. In addition, 1 or 20 μM of H_2O_2 PC did not alter cardioprotective effects of ischemic PC in postischemic contractile performance and protein oxidation. Our data suggest that the differential effects of H_2O_2 are derived from a concentration-dependent wrestling between its detrimental stress and protective signaling.
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Reperfusion injury after acute myocardial ischemia is a complex phenomenon that consists of mechanical dysfunction and cell death.[1,2] Reactive oxidative species (ROS) are generated during the early phase of reflow and considered a major cause of reperfusion injury.[3,4] The mechanism may be related to the induction of calcium overload, membrane damage, and inflammation,[5–9] although these conclusions are still under debate.[10,11] Emerging evidence demonstrates that endoplasmic reticulum (ER) stress occurs during myocardial ischemia/reperfusion (I/R) and causes apoptosis via caspase-12.[12,13] Oxidative stress seems to be a direct activator of ER stress in the heart;[14] however, the precise interplay between ROS and ER stress during myocardial I/R injury has not yet been well established.

ROS also protect myocardium against I/R injury in preconditioning (PC) and postconditioning (PoC) scenarios via activating prosurvival reperfusion injury salvage kinase (RISK) pathways, especially through protein kinase B (PKB/Akt) and protein kinase Cε (PKCε),[15] with the downstream inhibition of glycogen synthase kinase-3β (GSK-3β).[16–18] However, the precise mechanisms for the dual roles of ROS have not yet been fully understood. Hydrogen peroxide (H_2O_2), as the most stable form of ROS, has been intensively used to simulate severe oxidative damage in cardiomyocytes, cardiac myoblasts, and hearts,[13,18–22] and it also provides protection against I/R injury under certain conditions.[8,11,13,20,21,23,24] Pretreatment of isolated perfused rat hearts with 20 μM of H_2O_2[8,20] or between 2 and 100 μM of

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Abbreviations: ROS, reactive oxygen species; ER, endoplasmic reticulum; I/R, ischemia/reperfusion; H_2O_2, hydrogen peroxide; PC, preconditioning; PoC, postconditioning; RISK, reperfusion injury salvage kinase; LV, left ventricular; GRP78, glucose-regulated protein 78; IRE-1, inositol-requiring enzyme-1; ATF6, activating transcription factor-6; Xbp-1s, X-box-binding protein 1 splicing variant; TRAF2, TNF receptor-associated factor 2; p50-ATF6, activating transcription factor-6 cleaved 50 kDa fragment; p-PERK, phospho-protein kinase-like endoplasmic reticulum kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 4-PBA, 4-phenyl butyric acid; TM, tunicamycin; PKB, protein kinase B; PKCε, protein kinase Cε; WM, wortmannin; GSK-3β, glycogen synthase kinase-3β; DNPH, 2, 4-dinitrophenylhydrazine; LVDP, left ventricle developed pressure; LVEDP, LV end-diastolic pressure; $+dp/dt$ max, maximum rate of pressure development over time; $-dp/dt$ max, maximum rate of pressure decay over time; IPC, ischemic precondition; Bal, balance

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H_2O_2[11] protects the heart against Ca^{2+} paradox injury or I/R-induced calpain-dependent proteolysis, whereas in other studies the cardioprotection only appears at 2 μM but not at 0.5, 1, 10 or 100 μM of H_2O_2 pretreatment.[21,23] These findings suggest that the concentration of ROS determines their different roles,[25,26] and ROS are cardioprotective at a low level but detrimental at a high level.[27–29] However, several questions remain unanswered: why do a number of animal and most clinical studies with antioxidants fail to convey cardioprotection;[17,30] why does the massive ROS burst that occurs at reperfusion not protect the I/R heart, though the ROS signaling in early reperfusion is crucial to PC- and PoC-induced cardioprotection;[4,16,17,31] and finally, why does the action of exogenous H_2O_2 change drastically within lower concentrations? We recently found that the higher level of ROS production than the one elevated during early reperfusion following 30-min *ex vivo* ischemia is essential to intermittent hypobaric hypoxia- (a cardioprotective model[32]) induced cardioprotection. This occurs by efficiently activating the downstream prosurvival signaling pathways, and such effects are mimicked by 20 μM H_2O_2 PoC during the first 5 min of reperfusion.[16] Through these observations, we hypothesized that a moderate ROS level, higher than a level that initiates injurious insult in the early reperfusion stage, is required to trigger effectively cardioprotective signaling, whereas the endogenous ROS generated during early reperfusion following a short period of ischemia does not seem to reach the threshold to trigger efficient cardioprotection.

To address this hypothesis, our present study examined the concentration-dependent responses of ER stress and prosurvival kinases to H_2O_2 PC and H_2O_2 PoC, as well as their relationship. Our data demonstrate that a sufficient amount of H_2O_2 is required to confer cardioprotection against I/R injury via the efficient activation of prosurvival kinases, whereas a low level of H_2O_2 is insufficient to activate RISK pathways and deleterious through its induction of ER stress during myocardial I/R. These findings provide new insights into understanding the controversial roles of ROS in myocardial I/R injury and cardioprotection.

Results

Effects of H_2O_2 PC and PoC on postischemic recovery of myocardial contractile function. To determine the differential roles of H_2O_2 during myocardial I/R injury, we examined the concentration-dependent effects of H_2O_2 PC and PoC (Figure 1) on the postischemic recovery of myocardial contractile function in Langendorff-perfused rat hearts. H_2O_2 PC and PoC significantly improved the postischemic recovery of left ventricle developed pressure (LVDP), LV end-diastolic pressure (LVEDP), and maximum rates of pressure development or decay over time ($\pm dp/dt$ max) between the range of 10–100 μM of H_2O_2 with a peak around 20–30 μM, whereas this protective effect was lost at 300 μM and then completely suppressed at an excessive concentration (1 mM) of H_2O_2 (Figure 2). Interestingly, a lower concentration of H_2O_2 PC (1 μM) aggravated the I/R-induced suppression of LVDP and $\pm dp/dt$ max (Figures 2a, c, and d). These parameters also showed a

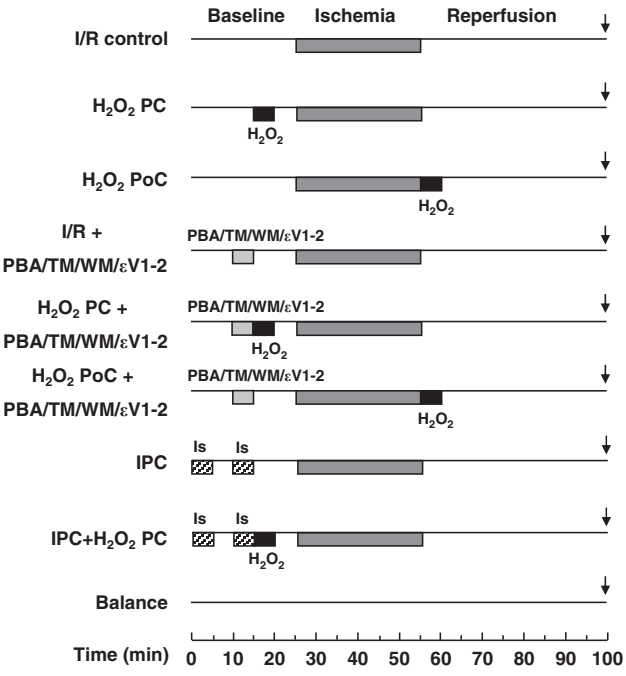

Figure 1 Experimental protocols. Isolated rat hearts were subjected to 30 min of ischemia followed by 45 min of reperfusion (I/R control). The hearts were randomly exposed to various concentrations of H_2O_2 (1, 3, 10, 20, 30, 100, 300, and 1000 μM) for 5 min followed by a 5-min washout before ischemia in PC groups or for first 5 min of reperfusion in PoC groups. A PI3K inhibitor WM (100 nM), ER stress inhibitor 4-PBA (1 mM), inducer TM (2.5 μg/ml), or PKCε inhibitor εV1–2 (10 μM) was perfused for 5 min with a 10-min washout before ischemia. IPC was induced by two cycles of 5-min ischemia (Is) before the onset of the index ischemia. At the end of the experiments, the hearts were used for western blot analysis. The equilibrium-perfused hearts with a corresponding period were collected as a balance control

suppression tendency at 1 μM of H_2O_2 PoC, but without statistical significance (Figures 2e, g, and h). These results suggest a quantitative threshold for H_2O_2 to trigger effective cardioprotection against I/R-induced contractile dysfunction and this threshold being higher than that initiates the injury. The PoC data also support our previous finding[16] that the endogenous ROS generated during early reperfusion is lower than the threshold to induce cardioprotection against I/R-induced contractile dysfunction.[16]

Concentration-dependent responses of prosurvival signaling pathways to H_2O_2. To understand why relatively high levels of H_2O_2 improve the recovery of myocardial function rather than aggravate the I/R injury, we examined the phosphorylation levels of Akt (Ser473), PKCε (Ser729), and their downstream target GSK-3β (Ser9), whose phosphorylation inhibits the opening of mitochondrial permeability transition pore, a crucial event in reperfusion injury[33] that contributes to cardioprotection.[34,35] The total expression levels of Akt, PKCε, and GSK-3β did not differ among groups, whereas their phosphorylation levels were significantly increased by I/R (Figure 3). The I/R-enhanced phosphorylation remained unchanged at lower concentrations (1–3 μM) of H_2O_2 but was further enhanced by H_2O_2 over 10 μM and reached a plateau around 30–100 μM (Figure 3). These results suggest that sufficient amounts of H_2O_2 are required

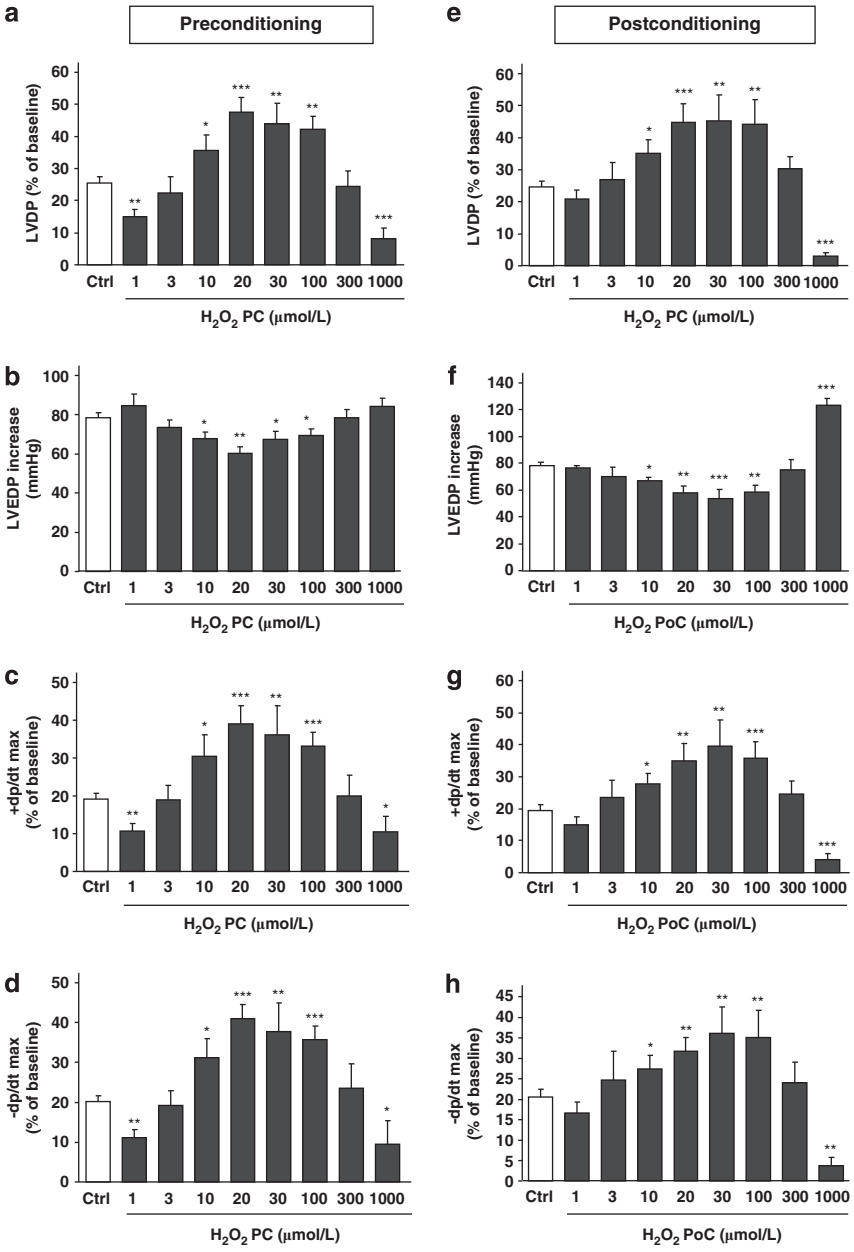

Figure 2 Concentration-dependent effects of H_2O_2 PC (left, **a**–**d**) and H_2O_2 PoC (right, **e**–**h**) on the postischemic recovery of LV contractile function. (**a** and **e**) LVDP. (**b** and **f**) LVEDP. (**c** and **g**) $+dp/dt$ max. (**d** and **h**) $-dp/dt$ max. $N=4$–7 each. Values are the mean $\pm$ S.E.M. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* control

to activate effectively the protective signaling, whereas the endogenous ROS generated during early reperfusion appear to be insufficient to trigger the RISK pathways.

Effects of H_2O_2 on the I/R-induced protein oxidation. To identify the effects of H_2O_2 on I/R-induced oxidative stress, we then examined the protein oxidation by measuring the 2, 4-dinitrophenylhydrazine (DNPH) derivatives of the common oxidation products protein carbonyls using western blot analysis. The content of myocardial protein carbonyls notably increased by I/R and was further enhanced by H_2O_2 PC and PoC in a concentration-dependent manner (Figure 4). The increases were statistically significant between 10–1000 μM of H_2O_2 in PC groups and 20–1000 μM in PoC groups, but the increase by 1000 μM of H_2O_2 at both PC and PoC was much higher than the 100 μM of H_2O_2 and the lower (Figure 4).

Concentration-dependent effects of H_2O_2 on the I/R-induced ER stress. ER stress has been shown to contribute to cardiomyopathy through three canonical pathways: inositol-requiring enzyme-1 (IRE-1), activating transcription factor-6 (ATF6), and phospho-protein kinase-like ER kinase (PERK).[36] To determine whether the low-level of ROS generated during early reperfusion is involved in myocardial I/R injury via the induction of ER stress, we analyzed the expression of ER stress markers predominant ER chaperone glucose-regulated protein 78 (GRP78),

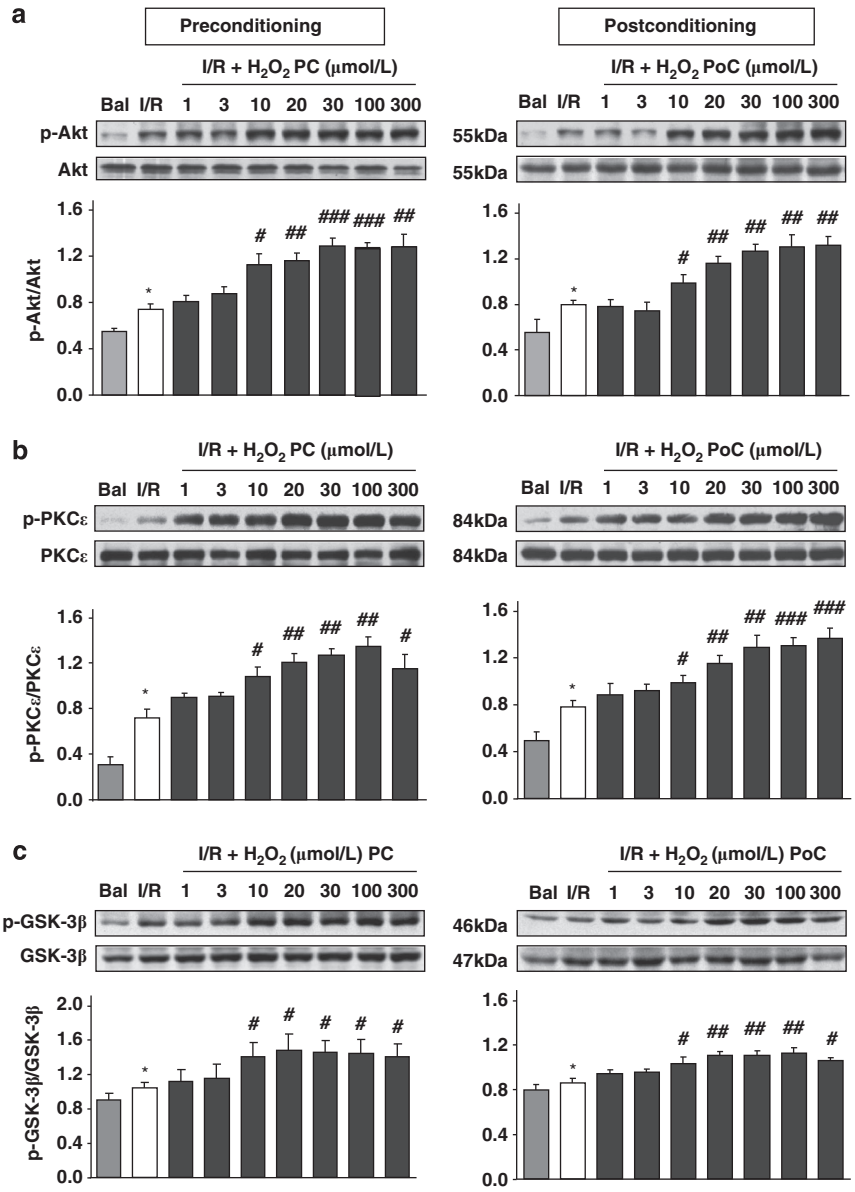

Figure 3 Concentration-dependent effects of H_2O_2 PC (left) and PoC (right) on the activation of prosurvival kinases in I/R LV. Representative immunoblots and averaged data for phosphorylations of PKB/Akt (**a**), PKCε (**b**), and GSK-3β, (**c**). Bal, balance. $N=4$ each. Values are the mean $\pm$S.E.M. $^{*}P<0.05$ *versus* balance; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *versus* I/R control

phosphorylated PERK, spliced X-box-binding protein-1 (Xbp-1s), and TNF receptor-associated factor 2 (TRAF2) for IRE-1 pathway, cleaved 50 kDa fragment of ATF6 (p50-ATF6), and prolonged ER stress marker cleaved caspase-12.[6,7,12,36–38] Myocardial I/R caused severe ER stress, characterized by marked increases in the phosphorylation of PERK at Thr980 and expressions of Xbp-1s, p50-ATF6 (Figure 5), GRP 78, TRAF2, and caspase-12 cleavage (Supplementary Figure S1). These increases were further enhanced by the low level of H_2O_2 (1 μM) but reached a plateau between 3 and 300 μM of H_2O_2 in both PC and PoC groups (Figure 5 and Supplementary Figure S1). Thus, the ER stress is already induced by the low-level H_2O_2, at which cardioprotection has not yet been efficiently induced.

Low-level H_2O_2 aggragated myocardial I/R injury via inducing ER stress. We then examined the contribution of I/R- and low-level H_2O_2-induced ER stress to I/R injury by analyzing the postischemic myocardial contractile function with and without an ER stress inhibitor 4-phenyl butyric acid (4-PBA) and an ER stress inducer tunicamycin (TM; Figure 1). Inhibition of ER stress by 1 mM of 4-PBA significantly improved the postischemic recovery of LVDP, LVEDP, and $+d p/d t$ max in I/R control and 1 μM of H_2O_2 PC and PoC groups (Figures 6a–d). In addition, the H_2O_2 PoC groups had a better postischemic recovery of LVDP and $-d p/d t$ max, but made no difference compared with the I/R control when treated with 2.5 μg/ml of ER stress inducer TM (Figures 6a–d). The induction of ER stress by TM and

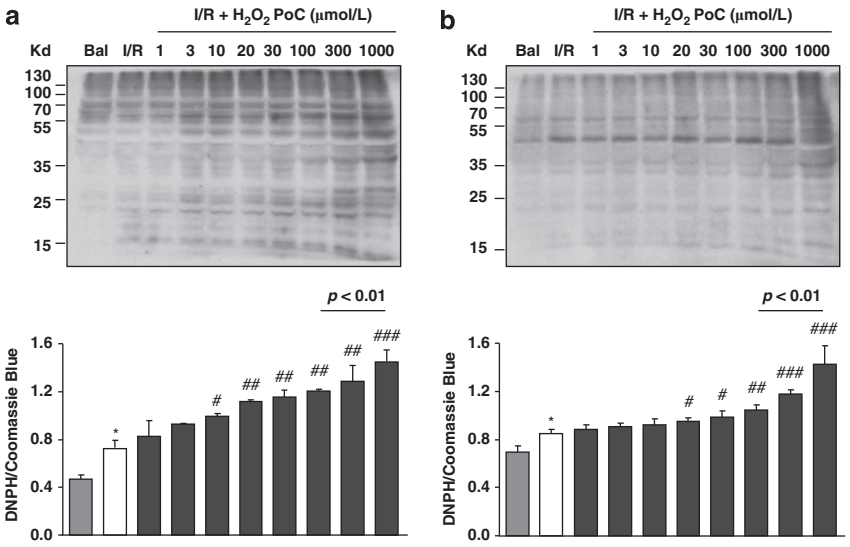

Figure 4 Concentration-dependent effects of H_2O_2 PC (left) and PoC (right) on myocardial protein oxidative stress during I/R injury. Representative immunoblots and averaged data of the DNPH derivatives of protein carbonyls, common products of protein oxidation, in LV tissues from H_2O_2 PC (**a**) and PoC (**b**) groups. Parallel gels were stained with Coomassie brilliant blue R250 dye as a control of protein loading. Bal, balance. $N = 4$ each. Values are the mean ± S.E.M. $^{*}P < 0.05$ *versus* balance; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *versus* I/R control, or as indicated on the figure

inhibition by 4-PBA were further confirmed by western blot analysis of the phosphorylation of PERK and the expressions of Xbp-1s, p50-ATF6 (Figure 6e), GRP78, TRAF2, and cleaved caspase-12 (Supplementary Figures S2a–d). The ER stress inhibitor 4-PBA but not inducer TM completely reversed I/R- or 1 μM of H_2O_2 PC- and PoC-induced increases in the expression of ER stress markers (Figure 6e and Supplementary Figures S2a–d).

Involvement of Akt and PKCε activation in the cardioprotection induced by H_2O_2. We next examined the contribution of Akt and PKCε pathways to the moderate H_2O_2-protected postischemic contractile function by inhibiting phosphoinositide 3-kinases (PI3Ks) (upstream activator of Akt) with wortmmanin (WM) and PKCε with εV1–2. WM (300 nM) and εV1–2 (10 μM) showed no effect on the postischemic recovery of LVDP, LVEDP, and $\pm$ dp/dt max in the I/R group, but they diminished the protective effects of 20 μM H_2O_2 PC and PoC (Figures 7a–d), suggesting that the cardioprotection induced by moderate H_2O_2 depends on the activation of Akt and PKCε pathways.

Because ER stress reached a plateau when moderate H_2O_2 efficiently activated the RISK pathways (Figures 3 and 5), we asked whether the activation of RISK pathways limits ER stress development. Both WM and εV1–2 treatment enhanced the expression of ER stress markers and caspase-12 cleavage in the I/R group, but they did not further enhance the ER stress level seen in 20 μM H_2O_2 PC and PoC group (Figure 7e and Supplementary Figures S2e–h). Thus, the I/R-activated RISK pathways contribute to the suppression of ER stress, but the ER stress seems to reach a maximal level under moderate H_2O_2.

Effects of H_2O_2 PC in the cardioprotection of classical IPC. Ischemic precondition (IPC) is a well-established cardioprotective model where ROS has a crucial role mediating the activation of prosurvival pathways.[39,40] To further determine the contribution of H_2O_2 in the cardioprotective effects of IPC, we performed 1 μM or 20 μM of H_2O_2 PC together with IPC (Figure 1) and compared their cardioprotective effects on each other. As seen in Figure 1, I/R-induced contractile dysfunction was aggravated by 1 μM of H_2O_2 PC but improved by 20 μM of H_2O_2 PC at a level comparable with those conferred by IPC (Supplementary Figures S3 a–d). Neither 1 nor 20 μM of H_2O_2 PC had additive effects on the improved contractile function by IPC, although the IPC plus 1 μM of H_2O_2 PC reversed the inhibition of 1 μM of H_2O_2 PC on the postischemic contractile function (Supplementary Figures S3 a–d). Moreover, IPC showed a comparable level of protein oxidation with the 20 μM of H_2O_2 PC and the IPC plus 1 or 20 μM of H_2O_2 PC, whereas the I/R-increased protein oxidation remained unchanged in 1 μM of H_2O_2 PC group (Figure 4 and Supplementary Figure S3e). These results suggest that 20 μM of H_2O_2 PC may share similar cardioprotective mechanisms with IPC and may have a comparable level of ROS release during the induction phase of cardioprotection.

Discussion

In this study, we described quantity-dependent and three-phase differential effects of H_2O_2 in myocardial I/R injury and protection. We demonstrated that H_2O_2 at the lower concentration aggravates I/R injury via the enhancement of I/R-induced ER stress before the sufficient activation of RISK pathways. H_2O_2 PC and PoC at moderate concentrations, higher than a level that aggravated reperfusion injury, markedly induce cardioprotection via efficient activation of downstream prosurvival signaling pathways, whereas they induce a comparable level of ER stress as the lower concentration of H_2O_2 PC or PoC. Furthermore, excessive

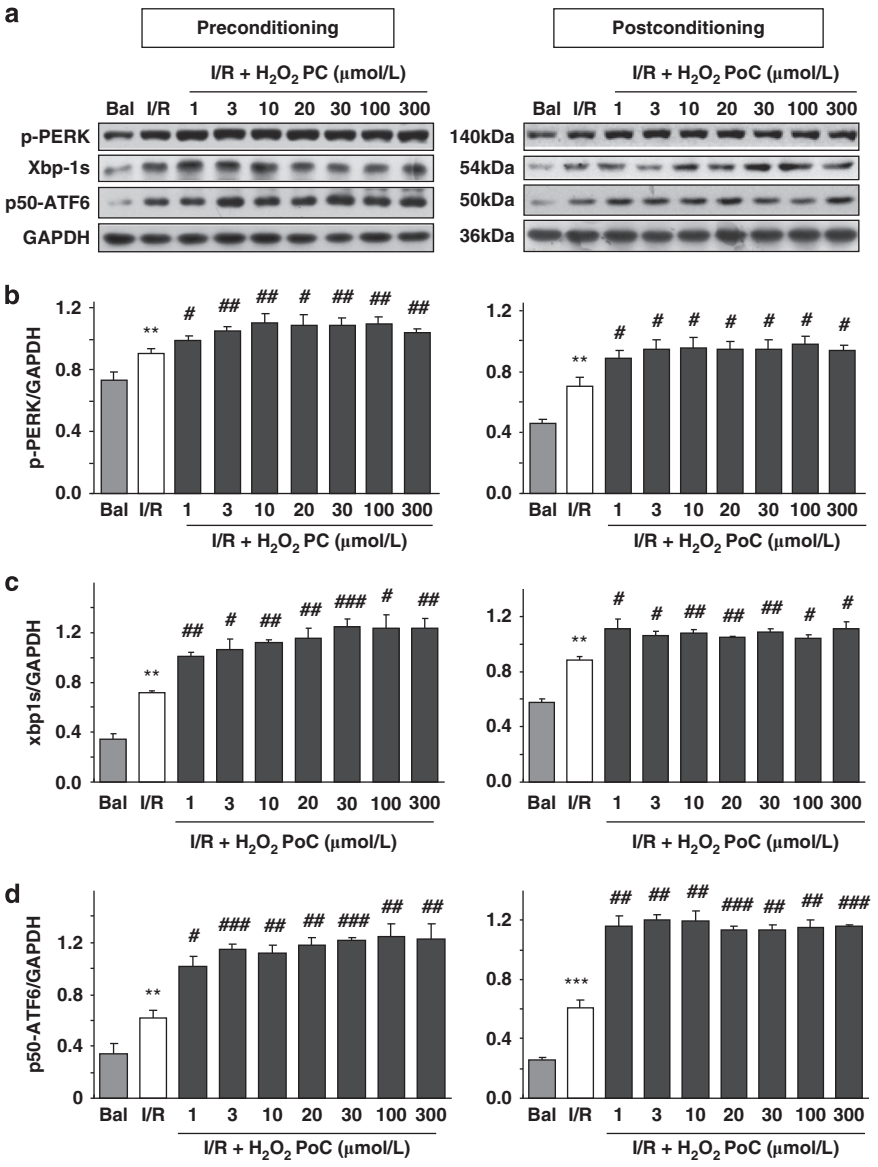

Figure 5 Concentration-dependent effects of H_2O_2 PC (left) and PoC (right) on ER stress in I/R LV. Representative immunoblots (**a**) and averaged data for the expression of ER stress markers, including (**b**) PERK phosphorylation, (**c**) Xbp-1s, and (**d**) p50-ATF6. GAPDH was used as an internal control. Bal, balance. $N = 4$ each. Values are the mean ± S.E.M. $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* balance; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *versus* I/R control

H_2O_2 exacerbates I/R injury when the activation of protective mechanisms reaches a plateau and fails to counteract severe nonspecific oxidative stress, but does not further increase ER stress. In addition, 20 μM H_2O_2 PC-conferred cardioprotective effects are comparable with the IPC in postischemic contractile performance and protein oxidation. These results confirm and extend previous findings that H_2O_2 PC and PoC can function as either injurious or protective stimuli determined by the wrestling between the detrimental and protective signaling roles triggered by different amounts of H_2O_2. Our data also indicate that the endogenous ROS generated during I/R is insufficient to trigger efficient cardioprotection. These findings provide a new angle to interpret the controversial roles of ROS in myocardial injury and protection.

We hypothesized that a moderate and sufficient increase of ROS during early reperfusion is required to activate effectively the downstream protective signaling pathways, whereas the endogenous ROS generated during early reperfusion does not reach the threshold for efficient cardioprotection.[16] This hypothesis is supported by the observation that the higher level of ROS production, than that elevated by I/R during early reperfusion, is required for the IHH-induced cardioprotection by efficient activation of Akt and PKCε pathways.[16] The hypothesis is further supported by the data that I/R induces a noticeable activation of Akt and PKCε (Figure 3) but not enough to reach the threshold for efficient cardioprotection (Figures 7a–d). Moreover, the phosphorylation levels of Akt/PKB, PKCε, and GSK-3β are much higher in 10–300 μM of H_2O_2 PC and PoC than those in I/R alone or in 1–3 μM of H_2O_2 PC and PoC (Figure 3), whereas the inhibition of either Akt/PKB or PKCε activity completely reverses the middle concentration of H_2O_2 PC- and PoC-afforded cardioprotection

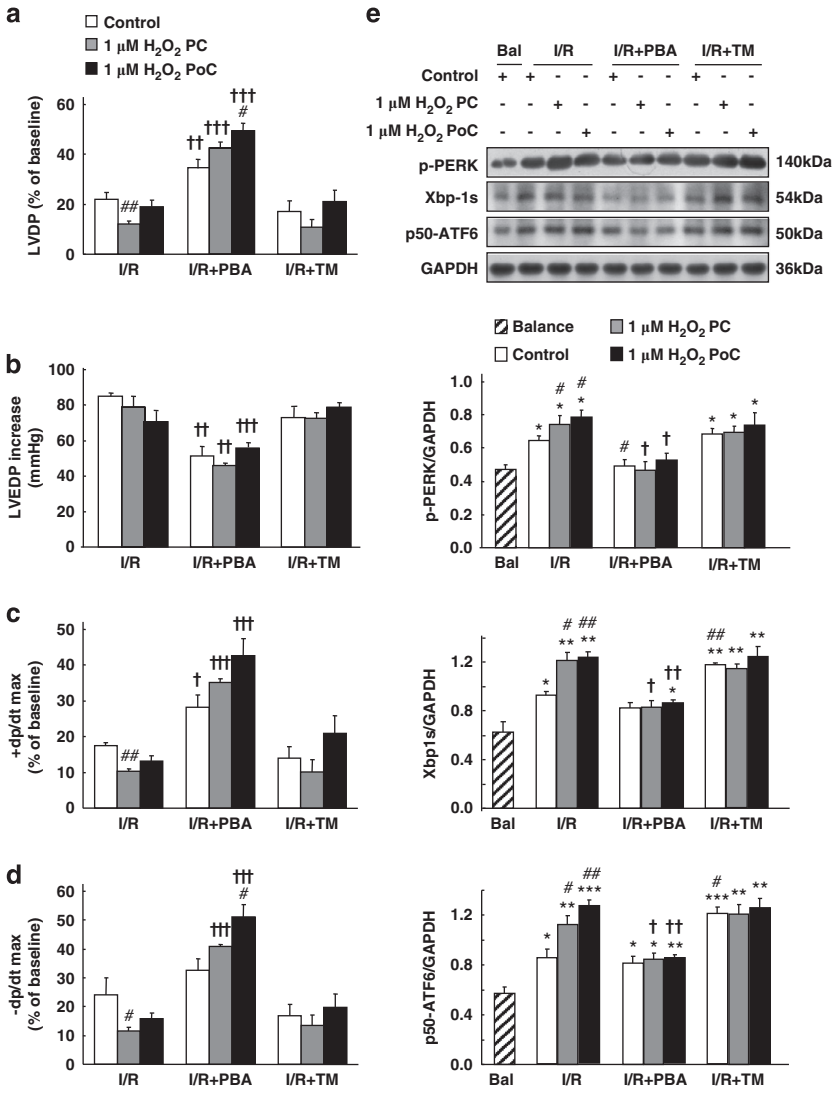

Figure 6 Involvement of ER stress in I/R-altered postischemic recovery of contractile function with and without the lower concentration of H_2O_2 PC and PoC. Effects of ER stress inhibitor 4-PBA (1 mM) and inducer TM (2.5 μg/ml) on the postischemic recovery of LVDP (**a**), LVEDP (**b**), $+$dp/dt max (**c**), $-$dp/dt max (**d**), and the ER stress markers (**e**). (**e**) Top panels: representative immunoblots; lower panels: averaged immunoblots data for PERK phosphorylation, Xbp-1s, and p50-ATF6. Bal, balance. $N = 4$–7 each. Values are the mean ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* balance; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *versus* I/R control; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ *versus* corresponding H_2O_2 groups

(Figures 7a–d). Similar phenomena of moderate but not lower or higher concentrations of H_2O_2 being cardioprotective were observed previously, although the concentrations vary with the treatment times and duration.[21,23] Thus, there is a quantitative threshold for H_2O_2 to induce efficiently protective signaling pathways and thereby trigger sufficient cardioprotection against I/R injury. Furthermore, the enhancement of redox signaling by the addition of 10–100 μM H_2O_2 during the first 5 min of reperfusion, a phase that has been thought to generate excessive ROS,[4,16,41,42] induces a significant activation of the RISK pathway and cardioprotection (Figure 2). This is consistent with our previous observation of 20 μM of H_2O_2 PoC treated during the first 5 min of reperfusion[16] along with the report from Ytrehus *et al.*,[25] who treats with 1 μM of H_2O_2 during the first 30 min of reperfusion. Although a lower concentration of H_2O_2 is used in the latter, its protective effect may be due to a much longer treatment time as the activation of protective signaling by ROS accumulates with time.[42] Therefore, the endogenous ROS generated during early reperfusion in I/R hearts are insufficient to induce cardioprotection owing to the lower than threshold activated prosurvival signaling to trigger efficient cardioprotection. Higher levels of ROS are required for the efficient activation of protective signaling pathways, which results in cardioprotection. The data also explains why differential roles of exogenous H_2O_2 within lower concentrations (0.5–100 μM) are seen in ischemic hearts,[8,11,20–22] as endogenous ROS levels vary with the extent and duration of myocardial I/R.

Interestingly, the induction of ER stress antecedes the activation of RISK pathways by H_2O_2 in I/R hearts in a

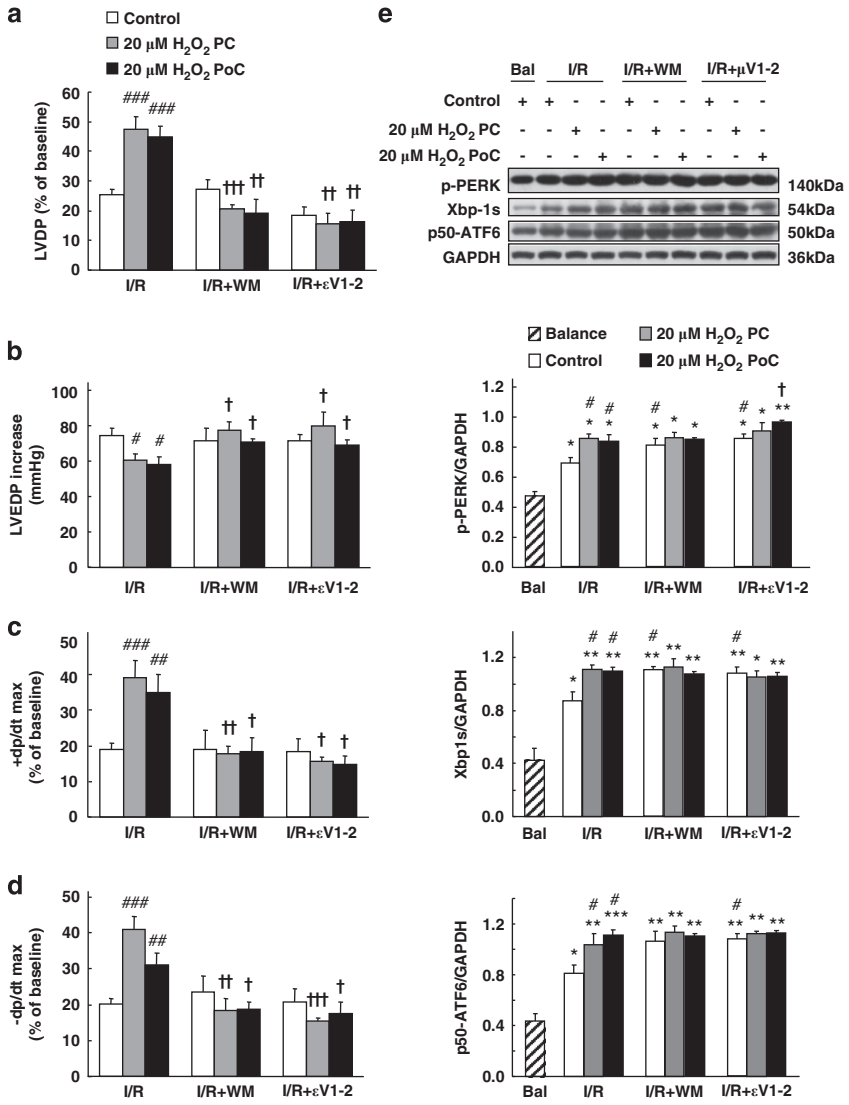

Figure 7 Activation of Akt and PKCε contributes to moderate concentration of H_2O_2 PC- and PoC-protected postischemic recovery of contractile function but does not affect the ER stress. (**a**–**e**) Effects of PI3K (upstream activator of Akt) and PKCε inhibitors WM (300 nM) and εV1–2 (10 μM) on the postischemic recovery of LVDP (**a**), LVEDP (**b**), $+$ d*p*/d*t* max (**c**), $-$ d*p*/d*t* max (**d**), and (**e**) the expression of ER stress markers. (**e**) Top panels: representative immunoblots; lower panels: averaged immunoblot data for PERK phosphorylation, Xbp-1s, and p50-ATF6. $N=4$–7 each. Bal, balance. Values are the mean $\pm$ S.E.M. $^{*}P<0.05$, $^{**}P<0.01<0.001$ *versus* balance; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *versus* I/R control; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ *versus* corresponding H_2O_2 groups

concentration-dependent manner (Figures 3 and 5 and Supplementary Figure S1). The I/R-induced myocardial contractile dysfunction is aggravated by the low level (1 μM) of H_2O_2 PC via ER stress activation but is reversed by ER stress inhibition (Figures 2 and 6). This is consistent with previous observations of the mediation of myocardial I/R injury[6,7,12] by ER stress and of the hypoxic or ischemic conditioning-induced cardioprotection via the attenuation of ER stress.[21,43,44] In addition, the I/R-induced ER stress is further enhanced by the low level (1 μM) of H_2O_2, and it remains stable under higher levels of H_2O_2 PC and PoC between 3 and 300 μM (Figure 5 and Supplementary Figure S1). This stability may be caused by the counteractive effect of cardioprotective signaling pathways triggered by higher levels of H_2O_2. Our data indicate that I/R-activated PI3K and PKCε inhibit the ER stress during I/R as both WM and εV1–2 treatment enhance the ER stress in the I/R group but not in the moderate H_2O_2 PC and PoC groups (Figure 7e and Supplementary Figures S2e–h). The latter effect suggests that the induction of ER stress by 20 μM H_2O_2 may already reach the maximal limit. This is supported by the plateaus of ER stress seen between 3 and 300 μM H_2O_2 in both PC and PoC groups (Figure 5 and Supplementary Figure S1). The other possibility is that the protection against ER stress is mainly due to JAK2/STAT3 activation rather than that of PI3K/AKT.[45] The RISK pathway-mediated cardioprotection may be through a mechanism other than the salvage of an ER stress-induced injury and requires further investigation.

Another interesting finding is that the I/R-induced myocardial protein oxidation is significantly increased in a concentration-dependent manner when H_2O_2 is between 10 or 20 and 1000 μM H_2O_2 either at PC or PoC (Figure 4), along

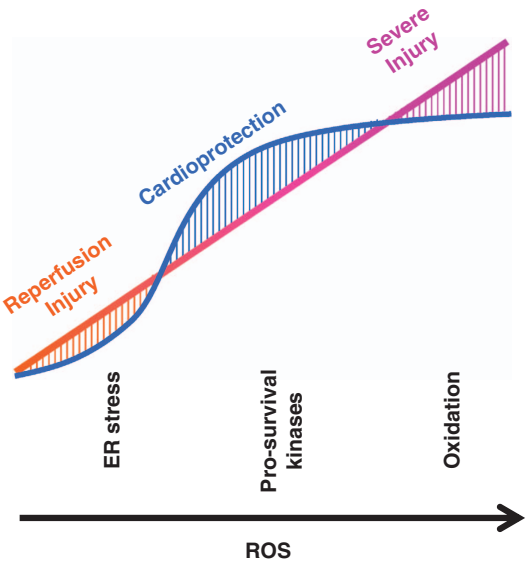

Figure 8 Schematic model showing the mechanisms underlying dual roles of H_2O_2 in myocardial I/R. The eventual outcome is a wrestling between the detrimental (straight line) and protective signaling (S-curve) roles of H_2O_2

with an efficient activation of the PI3K/AKT pathway that reaches a peak around 100 μM H_2O_2 (Figure 3). H_2O_2 PC at 100 μM has been shown to counteract oxidative stress through an activated PI3K/AKT pathway;[21,46] however, this level surpasses the maximum for the RISK pathway activation and fails to offset further increased severe nonspecific oxidative stress.

IPC has been shown to stimulate the ROS release before I/R and prevented reperfusion injury via triggering the activation of prosurvival pathways in IPC.[39,40] This is further supported by our observations of no additive cardioprotective effects of 20 μM H_2O_2 PC to the IPC (Supplementary Figures S3a–d) and 20 μM H_2O_2 PoC to intermittent hypoxia.[16] These results also imply that IPC and intermittent hypoxia might release comparable levels of H_2O_2 during the induction phase of cardioprotection with the moderate range of H_2O_2 used here. This is supported by similar levels of protein oxidative responses between 20 μM of H_2O_2 PC and IPC (Supplementary Figure S3e) and comparable activation levels of Akt and PKCε by 20 μM of H_2O_2 PoC and intermittent hypoxia-enhanced ROS production during early reperfusion.[16] Further studies need to be performed to confirm this possibility by providing direct evidence.

Based on the observations above, we propose a schematic model to explain the dual roles of ROS in myocardial I/R. As shown in Figure 8, the quantity of ROS determines their eventual effects through a wrestling between the detrimental (straight line) and protective signaling (S-curve) roles. Normally, ROS are generated at a low level and act as signaling molecules implicated in the physiologic control of the cell function in cardiomyocytes.[35] Myocardial I/R injury increases ROS production during early reperfusion, which is insufficient to trigger cardioprotection but already injurious through ER stress. Moderate amounts of ROS reach the threshold to trigger cardioprotection via the efficient activation of prosurvival kinases, and thereby overwhelm the detrimental effects of ROS. When the heart is exposed to excessive ROS, the activation of protective mechanisms reaches a plateau and fails to counteract severe nonspecific oxidative stress.

In conclusion, our data demonstrate that the differential effects of H_2O_2 are derived from a quantity-dependent wrestling between its detrimental and signaling roles. A sufficient amount of H_2O_2 is required to confer cardioprotection against I/R injury via the efficient activation of prosurvival kinases, whereas a low level of H_2O_2 is insufficient to trigger cardioprotection and deleterious through its induction of ER stress. The precise role of endogenous ROS in cardioprotection needs further investigation.

Materials and Methods

Animal care. Animals used in this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (Publication 85-23; National Institutes of Health, Bethesda, MD, USA), and all procedures were approved by the Institutional Review Board of Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and School of Medicine, Shanghai Jiao Tong University (Shanghai, China).

I/R injury model in Langendorff-perfused rat hearts. After rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), the hearts were rapidly excised and perfused with Krebs–Henseleit solution at 37 °C using a Langendorff apparatus at a constant pressure of 80 mm Hg as described previously.[16,47,48] A water-filled latex balloon connected to a pressure transducer (Gould P23Db; AD Instrument, Sydney, NSW, Australia) was inserted into the LV cavity to achieve a stable LVEDP of 5–10 mm Hg during initial equilibration. After equilibration perfusion, the heart was subjected to 30 min of global no-flow ischemia followed by 45 min of reperfusion. LVDP and $\pm dp/dt$ max were evaluated with PowerLab system (AD Instrument). IPC was induced by two cycles of 5-min ischemia before the onset of the index ischemia (30 min; Figure 1) as reported previously.[49,50]

Experimental protocols. Isolated hearts were randomly exposed to different concentrations of H_2O_2 (1, 3, 10, 20, 30, 100, 300, and 1000 μM) for 5 min, followed by a 5-min washout before ischemia in H_2O_2 PC groups or for the first 5 min of reperfusion in H_2O_2 PoC groups. PI3K inhibitor WM (300 nM; Millipore, Temecula, CA, USA),[51] PKCε inhibitor εV1–2 (10 μM; Anaspec, Fremont, CA, USA),[52] ER stress inhibitor 4-PBA (1 mM; Sigma-Aldrich, St. Louis, MO, USA),[53] and ER stress inducer TM (2.5 μg/ml; Sigma-Aldrich)[54] were perfused for 5 min with a 5-min washout before ischemia; hearts undergoing time-matched normal perfusion were used as controls, indicated as balance (Figure 1). At the end of the experiments, the hearts were rapidly removed and frozen in liquid nitrogen for western blot analysis.

Western blot analysis. Proteins were prepared as described previously.[16] Freeze-clamped LV tissues (200–300 mg) were homogenized briefly in 10 volumes of lysis buffer containing (in mM) 20 Tris-HCl (pH, 7.4), 150 NaCl, 2.5 EDTA, 50 NaF, 0.1 $Na_4P_2O_7$, 1 Na_3VO_4, 1 PMSF, 1 DTT, 0.02% (v/v) protease cocktail (Sigma-Aldrich), 1% (v/v) Triton X-100, and 10% (v/v) glycerol. The homogenates were centrifuged two times at 20 000 $\times$ *g* at 4 °C for 15 min, and the supernatants were saved as total proteins. Protein concentrations were determined by the BCA method. Equal amounts of proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Western blot analysis was performed under standard conditions with specific antibodies, including anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-PKCε (Ser729), anti-PKCε, anti-phospho-GSK-3β (Ser9), anti-GSK-3β, anti-PERK, anti-Xbp-1s, anti-p50-ATF6 (Abcam, London, UK), anti-TRAF2, anti-GRP78, anti-caspase-12 (Abcam), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Antibodies were purchased from Cell Signaling (Danvers, MA, USA), unless noted otherwise. The immunoreaction was visualized using an enhanced chemiluminescent detection kit (Amersham, London, UK), exposed to X-ray film, and quantified by densitometry with a video documentation system (Gel Doc 2000; Bio-Rad).

Protein oxidation analysis. To evaluate the effects of H_2O_2 conditioning on myocardial oxidative stress during I/R, we measured protein carbonyls, common products of protein oxidation,[10] in LV tissues homogenated in protein

lysis buffer without DTT. Protein carbonyls were measured using an immunoblot kit to detect the DNPH derivatization of protein carbonyls, as per the manufacturer's instruction (Cell Biolabs, San Diego, CA, USA).

Statistical analysis. Data were expressed as means ± S.E.M. Statistical significance was determined using ANOVA or repeated-measures ANOVA for multiple comparisons or repeated measurements. Significant differences between two mean values were estimated using Student's *t*-test. A P-value < 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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