GH-Releasing Hormone Induces Cardioprotection in Isolated Male Rat Heart via Activation of RISK and SAFE Pathways

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GHRH stimulates GH synthesis and release from the pituitary and exerts direct effects in extrapituitary tissues. We have previously shown that pretreatment with GHRH reduces cardiomyocyte apoptosis and improves heart function in isolated rat hearts subjected to ischemia/reperfusion (I/R). Here, we determined whether GHRH given at reperfusion reduces myocardial reperfusion injury and investigated the molecular mechanisms involved in GHRH effects. Isolated rat hearts subjected to I/R were treated at the onset of reperfusion with: 1) GHRH; 2) GHRH+GHRH antagonist JV-1-36; 3) GHRH+mitochondrial ATP-dependent potassium channel inhibitor 5-hydroxydecanoate; 4) GHRH+mitochondrial permeability transition pore opener atractyloside; 5) GHRH+ phosphoinositide 3-kinase/Akt inhibitor Wortmannin (WM); and 6) GHRH+signal transducer and activator of transcription-3 inhibitor tyrphostin-AG490 (AG490). GHRH reduced infarct size at the end of reperfusion and reverted contractility dysfunction in I/R hearts. These effects were inhibited by either JV-1-36, 5-hydroxydecanoate, atractylosid, WM, or AG490. Western blot analysis on left ventricles showed GHRH-induced phosphorylation of either the reperfusion injury salvage kinases (RISK), phosphoinositide 3-kinase/Akt, ERK1/2, and glycogen synthase kinase-3β or signal transducer and activator of transcription-3, as part of the survivor activating factor enhancement (SAFE) pathway. GHRH-induced activation of RISK and SAFE pathways was blocked by JV-1-36, WM, and AG490. Furthermore, GHRH increased the phosphorylation of endothelial nitric oxide synthase and AMP-activated protein kinase and preserved postischemic nicotinamide adenine dinucleotide (NAD^+) levels. These results suggest that GHRH protects the heart from I/R injury through receptor-mediated mechanisms, leading to activation of RISK and SAFE pathways, which converge on mitochondria and possibly on AMP-activated protein kinase. **(*Endocrinology* 154: 1624–1635, 2013)**

Several studies have suggested an important role for the GH/IGF-I axis in the regulation of cardiac growth and function, so that excess or deficit of these hormones is associated with deranged myocardial structure and performance (1, 2). Moreover, GH-releasing peptides, such as ghrelin and GH secretagogues (GHSs) (3–5), or ghrelin gene-derived products, such as obestatin (6), have been shown to exert protective effects against cell death and contractile dysfunction in hearts undergoing ischemia/reperfusion (I/R). These actions likely involve direct binding to cardiovascular-specific receptors and activation of signal transduction pathways conveying the cardioprotective signal from the sarcolemma to the mitochondria via enzymes of the so-called reperfusion injury salvage kinase (RISK) pathway (7, 8).

Abbreviations: AG490, tyrphostin-AG490; ATRA, atractyloside; AUC, area under the curve; AMPK, AMP-activated protein kinase; CPP, coronary perfusion pressure; dLVP, developed LVP; dP/dt_{max}, maximum rate of increase in LVP during systole; dP/dt_{min}, maximum rate of decrease in LVP during diastole; eNOS, endothelial nitric oxide synthase; GHRH-Post, GHRH-postconditioning; GHRH-R, GHRH receptor; GHS, GH secretagogue; GSK, glycogen synthase kinase; 5-HD, 5-hydroxydecanoate; I/R, ischemia/reperfusion; JAK, Janus kinase; LV, left ventricular; LVEDP, end-diastolic LVP; LVP, LV pressure; MI, myocardial infarction; mK_{ATP}, mitochondrial ATP-dependent potassium; mPTP, mitochondrial permeability transition pore; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PI3K, phosphoinositide 3-kinase; RISK, reperfusion injury salvage kinase; SAFE, survivor activating factor enhancement; STAT-3, signal transducer and activator of transcription-3; WM, Wortmannin.

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Until lately, however, the interest on the cardiovascular effects of the hypothalamic hormone GHRH has been very scant. We have recently shown that pretreatment with GHRH enhanced the postischemic recovery of left ventricular (LV) developed pressure and reduced the development of diastolic contracture and infarct size in isolated rat hearts subjected to I/R (9). The protective effect of GHRH was confirmed in adult rat ventricular myocytes and the cardiac cell line H9c2, on which GHRH prevented apoptosis induced by serum starvation or treatment with isoproterenol (9). In both cell types, as well as in isolated rat hearts, these effects involved GHRH receptor (GHRH-R) via activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway. In fact, GHRH-induced cardioprotection was abolished by the GHRH antagonist JV-1-36 and by a specific Akt inhibitor. ERK1/2 and adenylyl cyclase/cAMP/protein kinase A signaling were also involved (9). The direct action of GHRH, which appears to be without known side effects (10, 11), offers advantages in comparison with ghrelin and other GHS, whose pharmacologic potential is reduced by their pleiotropic actions (12), with a high risk of unexpected side effects. More recently, the cardioprotective effects of GHRH have been confirmed also in vivo. Indeed, a long-lasting treatment with the GHRH agonist JI-38 after both acute myocardial infarction (MI) (10) and chronic cardiac injury (13) improved cardiac performance and decreased infarct size, through GH/IGF-I-independent and GHRH-R-dependent mechanisms. However, the in vivo scenario does not allow an adequate evaluation of the actual GHRH target(s), because of "disturbing" events, such as endothelial/neutrophil interaction and neurohormonal responses to MI, which can interfere with its cardioprotective effects (14). Furthermore, the in vivo studies were not designed to reduce lethal reperfusion injury, because GHRH was administered 2 hours (10) or 1 month (13) after MI. This is far from the therapeutic window for reperfusion injury, which occurs in the very early reperfusion phase, ie, within the first minutes after reflow (15, 16). In fact, growth factors, such as IGF-I and vascular endothelial growth factor, were found to promote cardioprotection, when given at early reperfusion, through the RISK pathway (7, 17). It has been suggested that, in view of its clinical application, a treatment performed at the onset of myocardial reperfusion appears to be more feasible than pretreatment (18, 19). Based on the foregoing, we hypothesized that GHRH given at the onset of reperfusion would reduce myocardial reperfusion injury. Therefore, in the present study, we investigated the cardioprotective effects of GHRH, administered at early phase of reperfusion in isolated rat hearts. Infarct size and cardio-hemodynamic performances were assessed as end points of the myocardial damage. Involvement of GHRH-R and activation of cardioprotective signaling pathways were also investigated.

Cardioprotective pathways have been shown to converge on mitochondria via activation of mitochondrial ATP-dependent potassium (mK_{ATP}) channels, which generate small amounts of reactive oxygen species to prevent mitochondrial permeability transition pore (mPTP) opening (20). Therefore, mitochondria serve as both triggers and end-effectors of protection. In fact, either activation of mK_{ATP} or inhibition of mPTP opening prevent the release of apoptotic signals into the cytosol, contributing to cardiomyocyte survival (21). Moreover, mPTP opening has been shown to cause the release of mitochondrial nicotinamide adenine dinucleotide (NAD^+), and during postischemic reperfusion, mitochondrial NAD^+ is strongly reduced, in a process that is prevented by mPTP inhibitors (22). This suggests that the preservation of mitochondrial NAD^+ is associated with protection from myocyte death and that PTP plays a key role in this process (23, 24). Therefore, because NAD^+ is reduced after the mPTP opening, we also tested the hypothesis that treating isolated hearts with GHRH would lead to an increase in the NAD^+ to reduced nicotinamide adenine dinucleotide (NADH) ratio in the postischemic phase.

GHRH effects were also assessed on phosphorylation of AMP-activated protein kinase (AMPK), which plays a central role in the metabolic response of the heart to stress and in the heart increases ischemic glucose uptake and limits myocardial injury and apoptosis (25–27). Finally, because transcription factor signal transducer and activator of transcription-3 (STAT-3) is part of the survivor activating factor enhancement (SAFE) pathway, which also converges on mitochondria during protection (28–30), STAT-3 activation by GHRH was also investigated.

Materials and Methods

Reagents

The chemicals and inhibitors were purchased from Sigma-Aldrich (Milano, Italy). Rat GHRH (GHRF, 1-44), was from Phoenix Peptides (Karlsruhe, Germany), and reagents to assess MI were from Merck (Milano, Italy). JV-1-36 was from Bachem (Bubendorf, Switzerland). P-ERK1/2, P-Akt (Ser473), Phospho (P)-endothelial nitric oxide synthase (eNOS) (Ser1177), P-glycogen synthase kinase (GSK)-3β (Ser9), P-STAT-3 (Tyr705), P-AMPKα (Thr172), and total antibodies were from Cell Signaling Technology (Euroclone, Milano, Italy).

Animals

Male Wistar rats (body weight, 450–550 g) provided by Janvier (Le Genest Saint Isle, France) received human care in compliance with the Italian law (DL-116, January 27, 1992) and in accordance with the Guide for the Care and Use of Laboratory

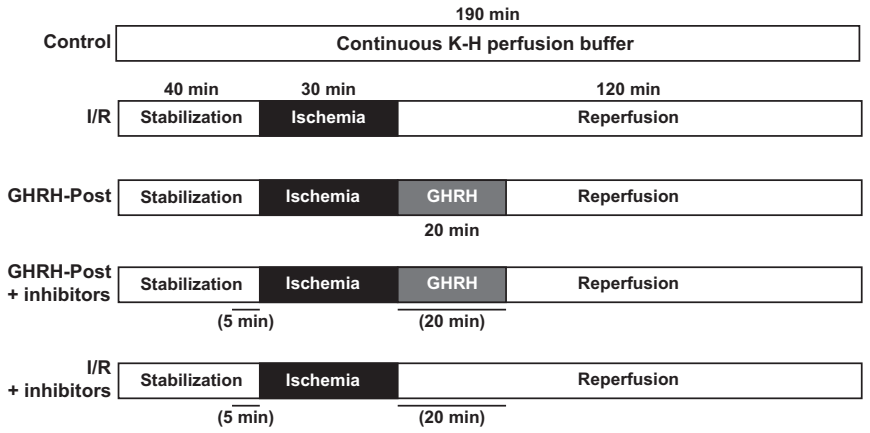

Figure 1. Experimental protocols. Before ischemia, hearts were randomly allocated to 1 of the experimental groups. Control hearts were buffer perfused for a total of 190 minutes. All other experimental hearts underwent 40 minutes of stabilization, 30 minutes of ischemia, and 120 minutes of reperfusion. Although GHRH was infused during the initial 20 minutes of reperfusion only, inhibitors were infused during the final 5 minutes of stabilization, as indicated by the number within round brackets under the bars, and during the initial 20 minutes of reperfusion. K-H, Krebs-Henseleit buffer.

Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). The scientific project has been supervised and approved by the Italian Ministry of Health (Rome, Italy) and by the Ethical Committee of the University of Torino.

Isolated heart perfusion

The methods were similar to those previously described (31, 32). Animals were anesthetized with urethane (1 g/kg ip); 10 minutes after heparin treatment, they were decapitated, the chest was opened, and the heart was rapidly excised. Isolated rat hearts were weighed, attached to the perfusion apparatus, and retrogradely perfused with oxygenated Krebs-Henseleit (K-H) buffer (127mM NaCl, 17.7mM $NaHCO_3$, 5.1mM KCl, 1.5mM $CaCl_2$, 1.26mM $MgCl_2$, and 11mM D-glucose), gassed with 95% O_2 and 5% CO_2. A constant flow was adjusted with a proper pump to obtain a typical coronary perfusion pressure (CPP) of 80–85 mm Hg during the initial part of stabilization. Thereafter, the same flow level (9 ± 1 mL/min·g) was maintained throughout the experiment. A small hole in the LV wall was performed to allow drainage of the thebesian flow, and a polyvinyl-chloride balloon was placed into the left ventricle and connected to an electromanometer for recording of LV pressure (LVP) (developed LVP [dLVP]). The balloon was filled with saline to achieve an end-diastolic LVP (LVEDP) of 5 mm Hg. CPP, coronary flow, and LVP were continuously monitored to assess the conditions of the preparation. The hearts were electrically paced at 280 bpm and kept into a temperature-controlled chamber (37°C).

I/R induction on isolated hearts

Each heart was allowed to stabilize for 40 minutes, at which time baseline parameters were recorded. After the stabilization period, hearts were subjected to a specific protocol, which included in all groups 30 minutes of global no-flow ischemia. In all groups, 30 minutes of ischemia were followed by a period of 120 minutes of reperfusion (see below). Pacing was discontinued on initiation of ischemia and restarted after the third minute of reperfusion (31, 32). Experimental protocols are described in Figure 1 and in the Results section.

Assessment of myocardial injury

Infarct areas were assessed as previously described (31, 32). In brief, the hearts were removed from perfusion apparatus at the end of reperfusion, and the left ventricles were dissected into 2- to 3-mm circumferential slices. After 20 minutes of incubation at 37°C in 0.1% nitro-blue tetrazolium in phosphate buffer solution, unstained necrotic tissue was carefully separated from stained viable tissue by an independent observer. The necrotic mass was expressed as a percentage of total LV mass. Although in this model the whole heart underwent normothermic ischemia, only the left ventricles had a fixed volume and preload; therefore, only the LV mass was considered as risk area.

Assessment of mPTP opening

mPTP remains closed during ischemia and opens in the first minutes of reperfusion, triggering cardiomyocyte cell death (21). Mitochondria possess more than 90% of the total tissue content of NAD^+. NAD^+ is lost during reperfusion, being released during mPTP opening. Therefore, low NAD^+ to NADH ratio due to reduction of NAD^+ content in cardiac tissue is an indicator of mPTP opening (23). Hearts ($n = 4$ for each group) were subjected to either 90 minutes perfusion only (Sham), to I/R only, or to GHRH-Post. Quantification of NAD^+ to NADH ratio was assessed at 20 minutes after the beginning of reperfusion according to the manufacturer's instructions using BioVision kits (BioVision, San Francisco, California). In brief, rat hearts were homogenized, cell debris was pelleted, and the resulting supernatant used for the quantification of the NAD^+ to NADH ratio using a spectrophotometer (Asys UVM 340) at 450-nm OD.

Western blotting

Immunoblot analyses were performed as described (9). LV samples were collected after 90 minutes of buffer perfusion (Sham) and at the end of treatment during reperfusion (after 20 min) for all other groups. All tissues were snap frozen in liquid nitrogen before being stored at −80°C until protein extraction. Nuclear and cytosolic proteins were extracted from the hearts by homogenization of the tissue in a lysis buffer as previously described (28). Briefly, 50 μg of total proteins for P-ERK, P-Akt, P-eNOS, P-GSK-3β, and P-AMPKα were resolved in 12% SDS-PAGE and incubated with the specific antibody. Nuclear extracts (50 μg) were used for P-STAT-3 detection. Blots were reprobed with antibodies against total ERK1/2, Akt, eNOS, GSK-3β, STAT-3, and AMPK for normalization.

Statistical analysis

Data are expressed as means ± SE. Two-way ANOVA and Tukey's honestly significant difference for post-ANOVA comparisons were used to evaluate the statistical significance of the

differences between groups. Postischemic recovery of pressure was evaluated comparing both the last point of the curves and the areas under the curve (AUCs) of the different groups. Significance was established when $P < .05$.

Results

GHRH-Post reduces infarct size

To determine whether GHRH given at reperfusion protects from I/R injury, we measured MI size in isolated perfused hearts. Experimental protocols (Figure 1): 1) I/R group (n = 11), hearts were stabilized and subjected to I/R only; 2) GHRH-Postconditioning (GHRH-Post) group (n = 7), GHRH (50nM) (9) was infused for 20 minutes immediately after ischemia; 3) GHRH with inhibitors, hearts were perfused with GHRH with either JV-1-36 (JV-1-36, 20nM, n = 6) (9), 5-hydroxydecanoate (5-HD) (100μM, n = 6) (33), atractyloside (ATRA) (20μM, n = 6) (34), Wortmannin (WM) (100nM; n = 6) (35), or tyrphostin-AG490 (AG490) (10μM, n = 6) (36) (perfusion with inhibitors started 5 minutes before ischemia and continued during the early 20 minutes of reperfusion in the presence of GHRH-Post [50nM]); and 4) in the groups treated with inhibitors only (n = 6, for each group), perfusion started 5 minutes before ischemia and continued during the early 20 minutes of reperfusion.

Cardiac weight (1396 ± 12 mg; range, 1121–1912 mg) and the risk area, ie, LV mass (838 ± 19 mg; range, 604-1320 mg) were similar in all groups. Total infarct size, expressed as a percentage of LV mass, was 61 ± 5% in the I/R group. GHRH-Post markedly reduced infarct size (26 ± 7%; $P < .05$ vs I/R group) (Figure 2A). The GHRH antagonist JV-1-36 (9) abolished GHRH-Post-induced cardioprotection (infarct size, 74 ± 7%), suggesting GHRH-R-mediated mechanisms. Similarly, GHRH-Post hearts treated with either the mK_{ATP} channel blocker, 5-HD (33), the selective opener of mPTP, ATRA (34), the PI3K/Akt inhibitor WM (35), or the Janus kinase (JAK)/STAT-3 inhibitor AG490 (36) showed reduced cardioprotection (infarct size: 59 ± 12, 50 ± 6, 61 ± 4, and 62 ± 6%, respectively) (Figure 2A), suggesting that GHRH-induced cardioprotection involves activation of the mK_{ATP} channels, PI3K/Akt, and JAK/STAT-3 pathways and attenuation of mPTP opening. The antagonists/inhibitors alone showed no effect on infarct size (Figure 2B).

GHRH-Post improves cardiac function

The hemodynamic parameters recorded at baseline conditions, after ischemia or at the end of reperfusion are reported in Table 1.

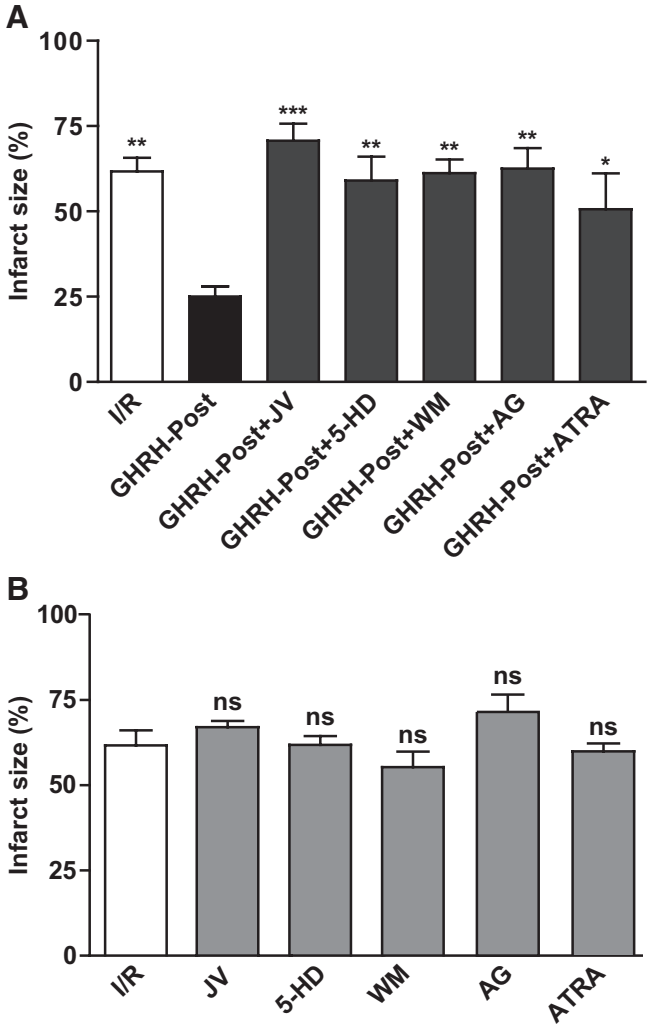

Figure 2. GHRH-Post reduces MI size in isolated rat hearts subjected to 30 minutes of ischemia and 120 minutes of reperfusion. (A) Hearts were treated with vehicle alone (I/R) or with GHRH (50nM) at the onset of reperfusion (GHRH-Post), in either absence or presence of the indicated antagonist/inhibitors. (B) Hearts treated with either vehicle (I/R) or antagonists/inhibitors alone. (A and B) JV-1-36 (JV), 20nM; 5-HD, 100μM; ATRA, 20μM; WM, 100nM; AG490 (AG), 10μM. Infarct size was determined by nitro-blue tetrazolium staining as detailed in Materials and Methods. Results are expressed as percent of ventricular mass and are the means ± SE (*$P < .05$, **$P < .01$, ***$P < .001$ vs GHRH-Post; ns, not significant vs I/R).

Coronary perfusion pressure

In all groups, CPP increased during reperfusion with respect to the baseline level. GHRH-Post limited this increase when given alone. This reduction was blunted by the antagonists (Figure 3A). Of note, JV-1-36 alone also limited perfusion pressure in reperfusion (Figure 3B). In fact, among groups with coinfusion of GHRH-Post+inibitors, GHRH-Post+JV-1-36 displayed lower values of CPP (Figure 3A). The other antagonists/inhibitors alone showed increase of CPP during reperfusion similar to I/R group. Postischemic recovery of CPP was evaluated comparing the last point of the curves, which

Table 1. Hemodynamic Parameters Recorded Before Ischemia (ie, at the End of Stabilization) and at the End of Reperfusion (ie, at the End of Experiments)

	At the end of stabilization					At the end of experiments				
Group	CPP (mm Hg)	dLVP (mm Hg)	dP/dt_{max}	LVEDP (mm Hg)	dP/dt_{min}	CPP (mm Hg)	dLVP (mm Hg)	dP/dt_{max}	LVEDP (mm Hg)	dP/dt_{min}
Control	80 ± 2	79 ± 1	1710 ± 79	6 ± 1	−1578 ± 76	83 ± 2	77 ± 2	1698 ± 69	8 ± 1	−1569 ± 45
I/R	80 ± 3	81 ± 4	1594 ± 15	5 ± 1	−1568 ± 151	144 ± 13[a]	26 ± 3[b]	293 ± 61[b]	49 ± 2[b]	−482 ± 172[b]
GHRH-Post	79 ± 1	78 ± 3	1815 ± 91	4 ± 1	−1466 ± 63	101 ± 9[a]	60 ± 8[a]	1533 ± 303	18 ± 4[a]	−1343 ± 303
GHRH+JV-1-36	78 ± 2	75 ± 2	1353 ± 149	6 ± 2	−1403 ± 93	123 ± 12[a]	31 ± 9[b]	973 ± 211[a]	45 ± 19[b]	−835 ± 91[a]
GHRH+5-HD	77 ± 1	77 ± 1	1670 ± 226	5 ± 1	−1317 ± 235	143 ± 8[a]	31 ± 6[b]	851 ± 90[a]	43 ± 17[b]	−771 ± 73[a]
GHRH+ATRA	78 ± 3	79 ± 2	1573 ± 17	4 ± 1	−1316 ± 192	150 ± 15[b]	16 ± 5[b]	500 ± 80[b]	54 ± 29[b]	−595 ± 229[a]
GHRH+WM	77 ± 2	78 ± 1	1700 ± 98	6 ± 1	−1450 ± 69	166 ± 35[b]	14 ± 4[b]	391 ± 81[b]	38 ± 8[b]	−338 ± 44[b]
GHRH+AG490	79 ± 1	76 ± 2	1695 ± 95	5 ± 1	−1490 ± 89	173 ± 15[b]	15 ± 2[b]	429 ± 60[b]	42 ± 4[b]	−350 ± 19[b]
I/R+JV-1-36	79 ± 2	80 ± 3	1772 ± 96	6 ± 2	−1420 ± 78	93 ± 2[a]	26 ± 3[b]	592 ± 77[b]	52 ± 1[b]	−735 ± 17[b]
I/R+5-HD	81 ± 3	78 ± 2	1780 ± 77	5 ± 1	−1405 ± 92	134 ± 15[a]	25 ± 5[b]	310 ± 80[b]	57 ± 9[b]	−495 ± 229[a]
I/R+ATRA	81 ± 1	77 ± 1	1760 ± 97	6 ± 2	−1410 ± 70	147 ± 12[a]	28 ± 3[b]	297 ± 77[b]	50 ± 5[b]	−535 ± 17[b]
I/R+WM	80 ± 3	80 ± 3	1701 ± 89	4 ± 1	−1569 ± 89	175 ± 15[b]	15 ± 4[b]	511 ± 95[b]	45 ± 6[b]	−386 ± 69[b]
I/R+AG490	81 ± 4	79 ± 2	1698 ± 55	5 ± 1	−1685 ± 93	175 ± 8[b]	19 ± 1[b]	510 ± 35[b]	34 ± 2[b]	423 ± 32[b]

[a] $P < .05$ vs values before ischemia.

[b] $P < .01$ vs values before ischemia.

correspond to 120 minutes of reperfusion. These analyses revealed that GHRH-Post and JV-1-36+I/R groups present a significant reduction of CPP with respect to I/R group ($P < .05$ vs I/R group; not significant vs each other).

Diastolic and systolic function

Mechanical cardiac function was analyzed by measuring LVP. Temporal changes in LVEDP, as well as percent variation with respect to baseline of maximum rate of decrease in LVP during diastole (dP/dt_{min}), dLVP, and maximum rate of increase in LVP during systole (dP/dt_{max}), are reported in Figure 4, A–D, respectively. The insets of these panels show the AUC during postischemic recovery for all groups. I/R hearts showed a marked increase in LVEDP and a strong limitation of the recovery of dP/dt_{min}, dLVP, and dP/dt_{max} during reperfusion.

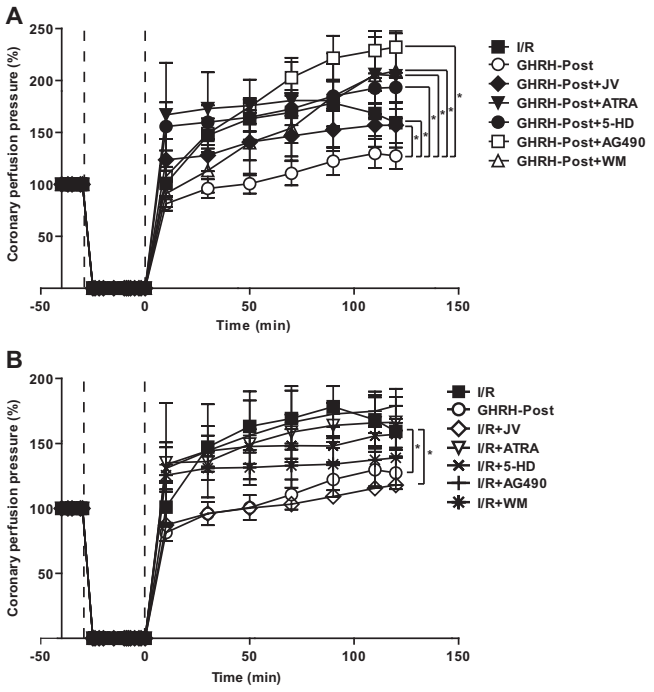

Figure 3. CPP during stabilization, ischemia, and reperfusion. Values are expressed as percent of baseline (preischemic value) and are the means ± SE. (A) I/R, GHRH-Post, and GHRH coinfused with inhibitors. (B) Inhibitors only. For comparative purpose are also reported I/R and GHRH-Post groups. (A) *$P < .05$ for GHRH-Post group vs all other groups; (B) *$P < .05$ for I/R vs GHRH-post and I/R+JV-1-36; P = not significant for I/R vs all other groups. Vertical dashed lines indicate the beginning and the end of the 30-minute global ischemia. JV, JV-1-36.

In GHRH-Post, LVEDP was strongly reduced when compared with the I/R hearts (25 ± 11 vs 49 ± 8 mm Hg, $P < .01$) (Figure 4A). In both GHRH-Post and I/R, the recovery of dP/dt_{min} during reperfusion was inversely related to the development of diastolic contracture. In fact, dP/dt_{min} values recorded from GHRH-Post hearts at the end of reperfusion were significantly increased with respect to those of I/R (119 ± 12 vs 40 ± 6%, $P < .01$) (Figure 4B), suggesting improved diastolic function.

Although in I/R hearts at the end of reperfusion dLVP was only 35 ± 11% of baseline, GHRH-Post markedly improved its recovery during reperfusion (dLVP was 92 ± 18% of baseline, $P < .01$ vs I/R) (Figure 4C). Figure 4D shows a similar trend for the recovery of dP/dt_{max} (I/R hearts, 44 ± 15; GHRH-Post, 105 ± 13%; $P < .01$).

All the antagonists/blockers reduced GHRH-induced protection on systolic and diastolic function. Indeed, LVEDP and dLVP values at the end of reperfusion in hearts treated with JV-1-36, 5-HD, ATRA, WM, and AG490 were comparable with those from I/R hearts (Figure 4, A and C, respectively). Similar effects were observed for dP/dt_{min} and dP/dt_{max} (Figure 4, B and D, respectively). The analysis of AUCs confirmed a significant improvement of diastolic (LVEDP and dP/dt_{min}) and systolic (dLVP and dP/dt_{max}) function in GHRH-Post group (Figure 4, insets in panels A–D). Moreover, the AUCs of LVEDP, dP/dt_{min}, dLVP, and

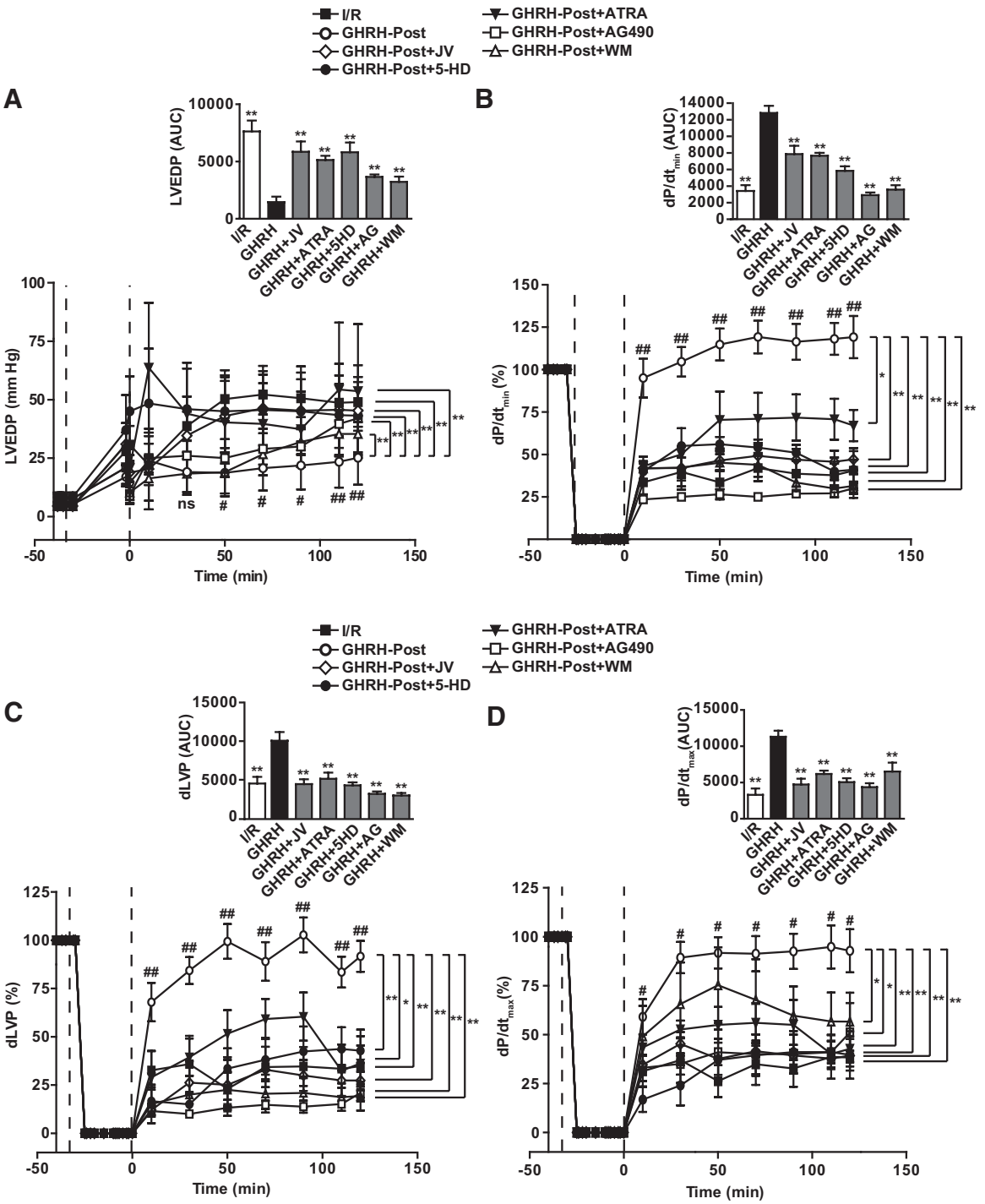

Figure 4. Effect of GHRH-Post on diastolic and systolic function. LVEDP (A) and dP/dt_{min} (B) during reperfusion in rat hearts undergoing 30 minutes of ischemia and 120 minutes of reperfusion. Results are expressed as absolute values (LVEDP) or as percent of baseline (preischemic value) (dP/dt_{min}). (A) $^{**}P < .01$ for GHRH-Post group vs I/R, GHRH-Post+JV-1-36, GHRH-Post+5-HD, GHRH-Post+ATRA, GHRH-Post+WM, and GHRH-Post+AG490 group. $\#P < .05$ and $\#\#P < .001$ for GHRH-Post vs I/R (single time points). (B) $^{*}P < .05$ for GHRH-Post group vs GHRH-Post+ATRA group; $^{**}P < .01$ for GHRH-Post group vs I/R and GHRH-Post+JV-1-36, GHRH-Post+5-HD, GHRH-Post+WM, and GHRH-Post+AG490 group. $\#\#P < .001$ for GHRH-Post vs I/R (single time points). (C and D) dLVP (C) and dP/dt_{max} (D). Values are expressed as percent of baseline (preischemic value). (C) $^{*}P < .05$ for GHRH-Post group vs GHRH-Post+ATRA group; $^{**}P < .01$ for GHRH-Post vs I/R, GHRH-Post+JV-1-36, GHRH-Post+5-HD, GHRH-Post+WM, and GHRH-Post+AG490 group. $\#\#P < .001$ for GHRH-Post vs I/R (single time points). (D) $^{*}P < .05$, for GHRH-Post group vs GHRH-Post+ATRA and GHRH-Post+JV-1-36 group. $^{**}P < .01$ for GHRH-Post vs I/R, GHRH-Post+5-HD, GHRH-Post+WM, and GHRH-Post+AG490 group. $\#P < .05$ for GHRH-Post vs I/R (single time points). Vertical dashed lines indicate the beginning and the end of the 30-minute global ischemia. Insets show the AUC ($^{**}P < .01$ vs GHRH-Post). JV, JV-1-36.

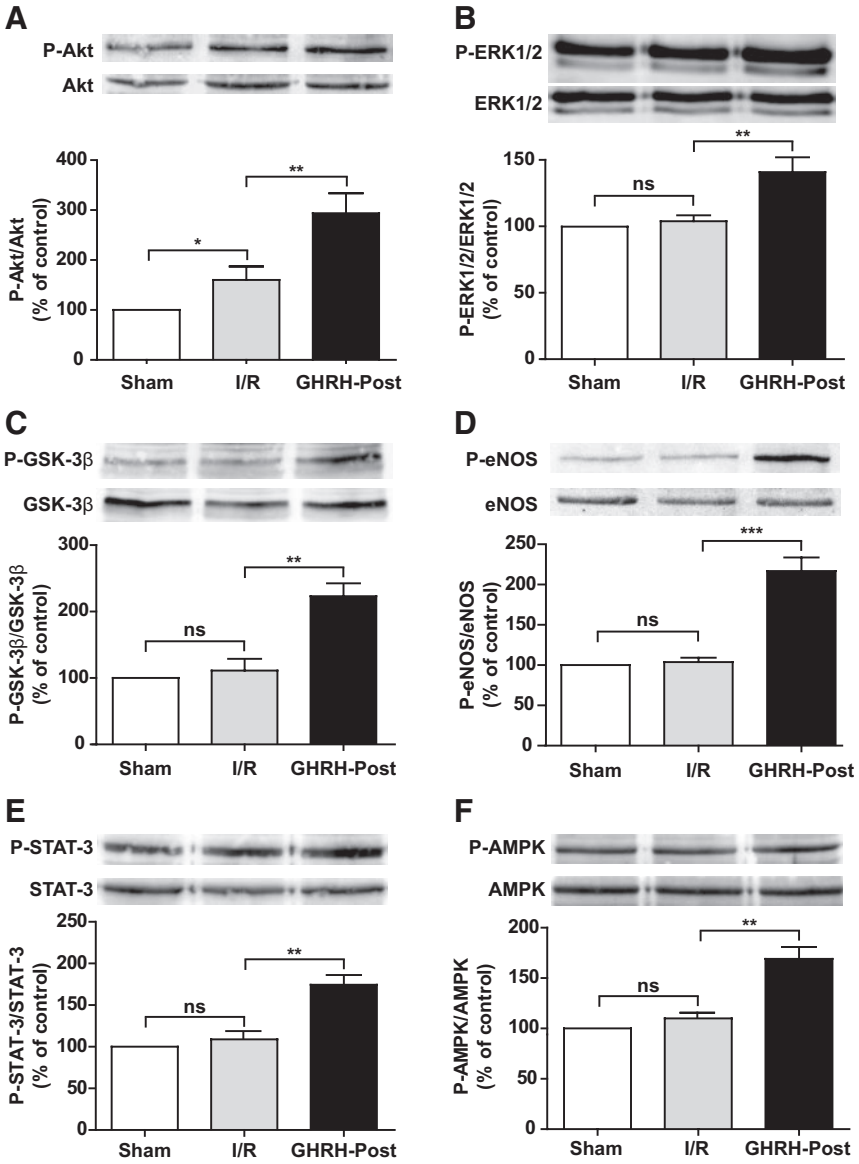

Figure 5. Effect of GHRH-Post on activation of RISK and SAFE pathways. Western blot analysis was performed on lysates from LVs collected during reperfusion (after 20 min) of either Sham (no I/R), I/R, or ischemic hearts, which were reperfused in the presence of GHRH (GHRH-Post). (Upper panels) Phosphorylation of Akt (A), ERK1/2 (B), GSK-3β (C), eNOS (D), STAT-3 (E), and AMPK (F). Equal protein loading was determined by reprobing with antibodies to the respective total proteins (lower panels). Blots are representative of at least three independent experiments. Graphs represent the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as percent of Sham. $*P < .05$, $**P < .01$, $***P < .001$; ns, not significant.

dP/dt_{max} observed in GHRH-Post plus antagonists were similar to those observed in I/R group.

Of note, in the hearts where the inhibitors/antagonists were used alone, the recovery of diastolic and systolic function was similar to that of I/R group (Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

GHRH-Post induces activation of RISK and SAFE pathways

The RISK pathway confers powerful cardioprotection and is recruited by both ischemic preconditioning and postconditioning (24, 37). Therefore, the effects of GHRH-Post were investigated on activation of main components of the RISK pathway, such as Akt, ERK1/2, and their downstream targets eNOS and GSK-3β (7, 8). Akt phosphorylation on Ser473 in I/R hearts, which showed a small but significant increase as compared with Sham, was further enhanced by GHRH-Post (Figure 5A). GHRH-Post also increased the activation of ERK1/2 as compared with I/R alone, whereas I/R per se had no effect with respect to Sham (Figure 5B). Similarly, GHRH-Post increased phosphorylation and thereby inhibition of GSK-3β on Ser9, with respect to I/R hearts. Conversely, phospho-GSK-3β was unchanged in I/R hearts as compared with Sham (Figure 5C). Phospho-eNOS (Ser1177) levels were unchanged in I/R hearts as compared with Sham, whereas they were strongly increased by GHRH-Post (Figure 5D).

STAT-3 is a key component of the SAFE pathway, which, apart from or in addition to RISK, confers protection in both ischemic preconditioning and postconditioning (24, 28). GHRH-Post increased nuclear STAT-3 phosphorylation on Tyr705. Phospho-STAT-3 was unchanged in I/R hearts, as compared with Sham (Figure 5E).

Next, the effect of GHRH-Post was assessed on activation of AMPK, which has been shown to regulate myocardial metabolism during I/R and to reduce ischemic injury during postischemic reperfusion (26). GHRH-Post increased phospho-AMPK, whereas AMPK phosphorylation was unchanged in I/R vs Sham (Figure 5F).

GHRH antagonist JV-1-36 inhibits GHRH-Post-induced activation of RISK and SAFE pathways

Based on the finding that JV-1-36 inhibited GHRH-Post-induced cardioprotection, we determined whether JV-1-36 would also reduce GHRH-Post effect on activation of RISK and SAFE pathways. GHRH-Post-in-

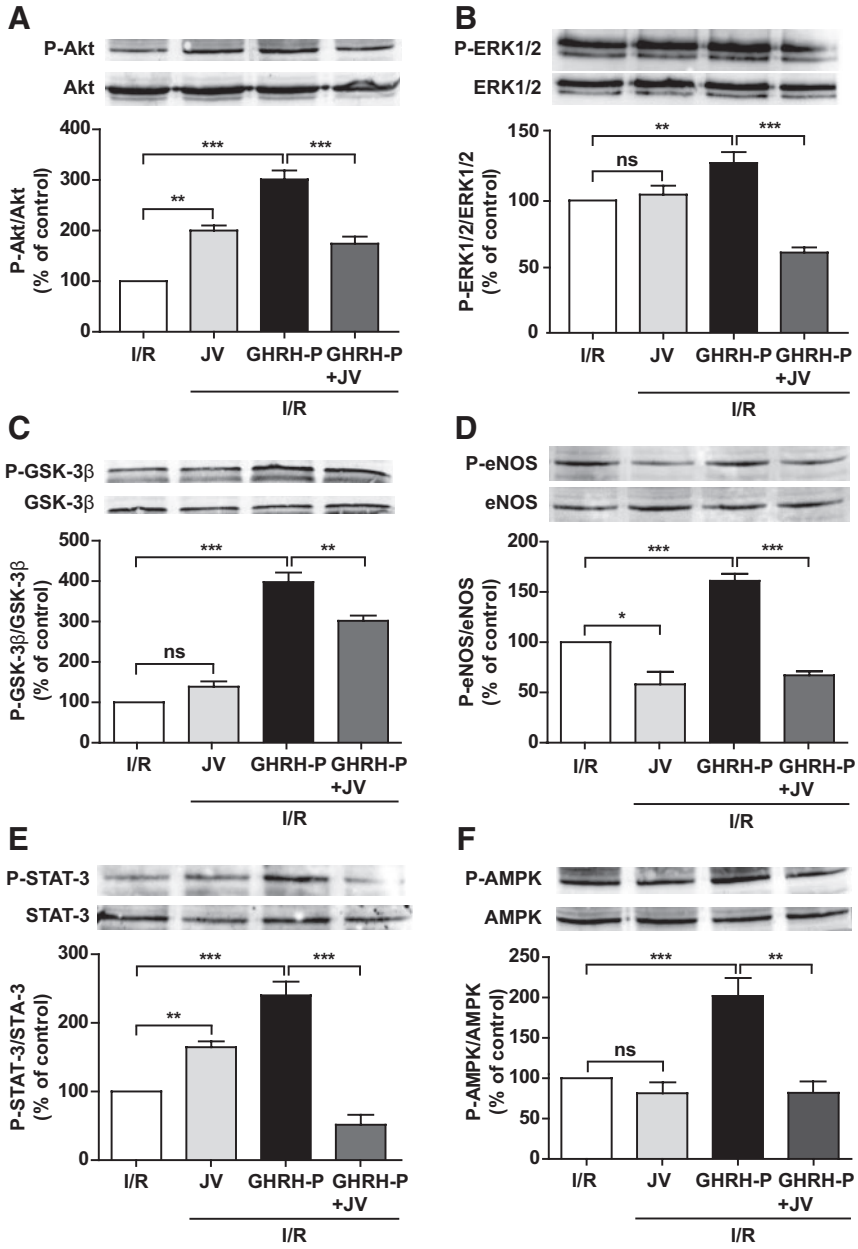

Figure 6. JV-1-36 inhibits GHRH-Post-induced activation of RISK and SAFE pathways. Western blot analysis was performed on lysates from LVs of I/R hearts during reperfusion (after 20 min), which were either untreated or treated with JV-1-36 (JV), GHRH-Post (GHRH-P), or GHRH-Post and JV-1-36. (A–F) (upper panels) Phosphorylation of the indicated pathways. Equal protein loading was determined by reprobing with antibodies to the respective total proteins (lower panels). Graphs represent the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as percent of I/R. n = 3. $*P < .05$, $**P < .01$, $***P < .001$; ns, not significant.

duced phosphorylation of Akt, ERK1/2, GSK-3β, eNOS, STAT-3, and AMPK in I/R hearts was strongly inhibited by JV-1-36 (Figure 6), suggesting that GHRH-Post-induced cardioprotection involves GHRH-R signaling and activation of downstream survival kinases. JV-1-36 alone increased Akt and STAT-3 phosphorylation in I/R hearts, reduced that of eNOS, and showed no effect on ERK1/2, GSK-3β, and AMPK.

PI3K/Akt and STAT-3 inhibitors reduce GHRH-induced activation of RISK and SAFE pathways

Based on the finding that the RISK and SAFE inhibitors reduced GHRH cardioprotective effects, we investigated whether they would also prevent GHRH-Post-induced activation of RISK and SAFE signaling pathways. Phosphorylation of Akt and GSK-3β, which are both part of the RISK pathway, was reduced in GHRH-Post hearts treated with the Akt inhibitor WM (Figure 7, A and B). Moreover, AG490, the inhibitor of STAT-3, which is involved in the SAFE pathway, reduced not only GHRH-Post-induced phosphorylation of STAT-3 but also that of Akt (Figure 7, C and D). These results strengthen the assumption that the RISK and SAFE pathways are involved in GHRH cardioprotective effects.

Assessment of mPTP opening

The NAD^+ to NADH ratio was found similar in the Sham and GHRH-Post groups (0.31 ± 0.06 and 0.33 ± 0.07, respectively, P = not significant). However, it was significantly reduced in the I/R group (0.18 ± 0.03, $P < .05$) (Figure 8), due to a decrease in NAD^+ content. This result suggests that GHRH-Post prevents NAD^+ to NADH ratio decrease upon reperfusion by inhibiting mPTP opening.

Discussion

This study shows that GHRH, given at the onset of reperfusion, induces cardioprotection through the phosphorylation/modulation of kinases included in the RISK (Akt, ERK1/2, and GSK-3β) and SAFE (STAT-3) pathways and inhibition of mPTP opening. Accordingly, GHRH preserved postischemic NAD^+ levels; moreover, it induced the phosphorylation of eNOS, a downstream target of Akt (38, 39), and of AMPK, a metabolic regulator against oxidative stress (25–27). Finally, our findings sug-

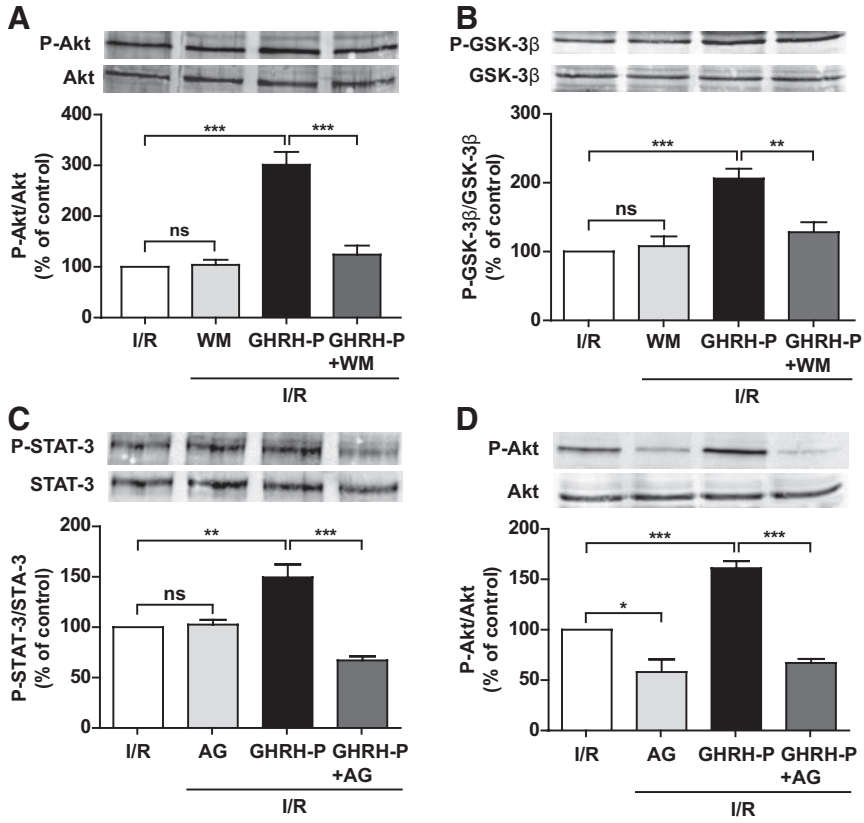

Figure 7. GHRH-induced activation of RISK and SAFE is reduced by PI3K/Akt and STAT-3 inhibitors. Western blot analysis was performed on lysates from LVs of I/R hearts during reperfusion (after 20 min), which were either untreated or treated with WM, AG490 (AG), GHRH-Post (GHRH-P), GHRH-Post and WM, or GHRH-Post and AG. (A and B) Akt (A) and GSK-3β (B) phosphorylation (upper panels), in the presence of WM (100nM). (C and D) STAT-3 (C) and Akt (D) phosphorylation (upper panels) in the presence of AG. Equal protein loading was determined by reprobing with antibodies to the respective total proteins (lower panels). Graphs represent the densitometric analysis of phosphorylated proteins normalized to total proteins and reported as percent of I/R. n = 3. $^{**}P < .01$, $^{***}P < .001$; ns, not significant.

gest that GHRH-R is required for the GHRH cardioprotective action and for activation of the downstream signaling pathways.

Small synthetic peptidyl or nonpeptidyl GHSs, as well as ghrelin gene-derived peptides, have been shown to exert protective effects against cell death and contractile dysfunction in I/R hearts, akin to ischemic preconditioning (4–6, 40). We have recently reported similar activity for GHRH, when administered before the ischemic period (9). Although preconditioning has significant cardioprotective effects, its clinical application is limited, because it must be initiated before myocardial ischemia. Conversely, postconditioning has highlighted the importance of intervening at the onset of myocardial reperfusion to protect the ischemic heart, a clinically more relevant time point for intervention in patients with acute MI and undergoing angioplasty (16, 18, 19). However, 2 recent studies using an established ischemic PostC algorithm found no protection against infarct size (41, 42), suggesting that ischemic postconditioning may not become broadly applicable in clinical routine. Therefore, the translation to clinical use of pharmacological intervention may be more appealing. This assumption led us to investigate whether GHRH exerts comparable cardioprotective effects also when applied at the beginning of reperfusion. Our present study confirms that, like other peptide mediators (17, 31), cytokines (28), or pharmacological agents (27), GHRH induces cardioprotection when administered during the first phases of reperfusion. This effect was clear-cut, in particular, for improvement of LV+dP/dt_{max} and reduction of diastolic contracture, which has been suggested as very good indicator of I/R injury in isolated rat hearts subjected to global ischemia (43). The concentration at which this peptide exhibits a protective effect is comparable with that already shown for GHSs, ghrelin (5, 40) or obestatin (6), or GHRH itself when given before reperfusion (9).

Interestingly, the cardioprotective effect of GHRH has been recently confirmed by Kanashiro-Takeuchi et al (10) in vivo, where the long-lasting treatment with the GHRH agonist JI-38 activated myocardial repair after both acute MI and chronic ischemic heart disease (10, 13). These studies suggested that GHRH effects involve cell-cycle reentry, angiogenesis, and likely cardiac stem cell activation; however, the signaling pathways triggered by GHRH were not investigated. Kanashiro-Takeuchi et al (10) also showed GHRH-R expression in rat heart and sarcolemmal membrane of cardiomyocytes, in agreement with our previous findings (9). Moreover, GHRH-induced cardioprotection involved activation of GHRH-R, as demonstrated by use of a selective receptor antagonist (13). Accordingly, we have recently demonstrated that the GHRH antagonist JV-1-36 inhibits GHRH-induced survival in adult rat ventricular myocytes and prevents GHRH-induced cardioprotection before the ischemic period in isolated rat heart (9). Here, JV-1-36 reduced GHRH-Post cardioprotection and hampered the activation of signal transduction cascades, further indicating a key role for GHRH-R in GHRH-Post cardioprotective effects.

Administration of GHRH to HeLA human cervical cancer cells transfected with the GHRH-R has been pre-

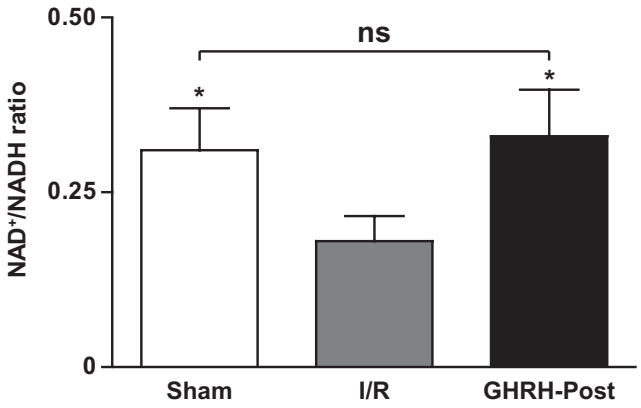

Figure 8. NAD^+ to NADH ratio in Sham, I/R, and GHRH-Post groups. The ratio of NAD^+ to NADH is used as an index of mPTP opening. The lower is the ratio the higher is the open probability of the pore. Results are the mean ± SE (n = 4 for each group). *$P < .05$ for Sham and GHRH-Post vs I/R; ns, not significant.

viously shown to activate JAK2/STAT-3 pathway (44). Importantly, GHRH antagonists are based on the sequence of the native GHRH and may therefore occasionally display some agonistic effects. This could explain why in the present study JV-1-36 alone slightly increased STAT-3 phosphorylation in I/R hearts. In turn, the increase of PI3K/Akt by JV-1-36 may result from activation of survival mechanisms by the cells, to counteract the increase of harmful signaling pathways, such as STAT-3.

The effects of both mK_{ATP} inhibition by 5-HD and mPTP opening by ATRA in various models of infarct size and postischemic mechanical recovery have already been studied (32, 45). Our present observation that 5-HD and ATRA abolish the protective effect of GHRH suggests that both activation of mK_{ATP} and mPTP closure play a relevant role in GHRH-induced cardioprotection. In addition, the preserved myocardial NAD^+ to NADH ratio in GHRH-Post hearts further supports an involvement of mPTP closure in GHRH-induced cardioprotection. Several effectors of the RISK and SAFE pathways are involved in the cardioprotection elicited by ischemic PostC, which converge on mitochondria, and specifically on mPTP (24, 28, 34). Indeed, inhibition of mPTP at the onset of reperfusion has been found to strongly reduce MI size in both laboratory and clinical settings (34, 46). Activation of mK_{ATP} also plays an important role in cardioprotection (20). K_{ATP} channels (both on sarcolemmal and mitochondrial membranes) are biosensors that enable detection of metabolic distress signals and operate adjusting membrane potential-dependent functions to match energetic demands of the working heart (20, 47). Energy demand in the heart is also linked to AMPK, which is activated in response to stressors, such as ischemia (25). AMPK has been shown to protect from ischemic injury, to reduce infarct size, and to limit the development of cardiomyocyte hypertrophy, through stimulation of glucose uptake, as well as mitochondrial fatty acid uptake and oxidation (25–27). AMPK activity in the heart is modulated by hormones, such as adiponectin, leptin, and ghrelin, or cytokines, like TNF-α (26). Furthermore, AMPK increases glucose uptake in skeletal muscle (48), suggesting beneficial effects against insulin resistance. Here, we firstly show that GHRH may not only open mK_{ATP} channels but also activates AMPK during reperfusion, which further supports its cardioprotective action and suggests a role in heart metabolism. GHRH-Post also increased the phosphorylation of eNOS, a well-known AMPK target in cardioprotection (49, 50). In addition, besides being a downstream target of the RISK pathway, NO has the potential to inhibit mPTP opening, with the possible involvement of mK_{ATP} (7). Interestingly, because GHRH induces vasodilation, as inferred by GHRH-induced CPP reduction, it is likely that NO is mainly produced within the endothelium. In fact, NO is principally a paracrine effector, being synthesized by 1 cell type to diffuse and modify the biology of neighboring cells (eg, smooth muscle and cardiomyocytes). In particular, expression of eNOS within endothelial cells exceeds that in cardiomyocytes by more than 4:1 (51), suggesting that a substantial degree of physiological eNOS signaling in the cardiovascular tissues is paracrine.

Akt, which is also activated here by GHRH, has been previously shown to phosphorylate eNOS (8, 39), further contributing to the inhibition of the mPTP opening and myocardium protection. Both Akt and ERK1/2 play important protective roles against reperfusion injury and are part of the prosurvival RISK pathway. Their function in postconditioning has been confirmed in both nondiseased animal hearts and in postinfarct remodeling (7, 24). Moreover, the cardioprotective role of the RISK pathway has been confirmed in ex vivo studies in human atrial trabeculae subjected to I/R injury (52).

We found here that GHRH also promoted phosphorylation/inactivation of GSK-3β. GSK-3β is a substrate of prosurvival protein kinases, including Akt, ERK1/2, and AMPK, and is a step to which multiple protective signaling pathways converge (53). Furthermore, mPTP opening has been shown to be limited by phosphorylation/inhibition of GSK-3β (54); however, this effect has not been fully understood, and the role of GSK-3β in postconditioning is controversial (24). Inhibition of PI3K/Akt signaling by WM was found here to abrogate the cardioprotective effects of GHRH-Post and also to prevent GHRH-Post-induced phosphorylation of PI3K/Akt and GSK-3β. These findings provide further evidence that GHRH displays its cardioprotective activities through the RISK pathway.

GHRH also induced the phosphorylation of STAT-3, which is included in the so-called SAFE pathway (28–30).

Indeed, STAT-3 may also play a pivotal role in the interaction between RISK and SAFE in PostC signaling (30). STAT-3 promotes cardioprotection via the phosphorylation and inactivation of the proapoptotic factors Bad and Bax (28). Moreover, pharmacological inhibition of STAT-3 was found to abrogate both the PostC-induced phosphorylation of STAT-3 and Akt, suggesting that Akt may be downstream of STAT-3 (29). Accordingly, we found here that inhibition of STAT-3 by AG490 reduced both GHRH-Post cardioprotective effects, as well as GHRH-Post-induced Akt phosphorylation, suggesting close interaction between STAT3 and Akt in the cardioprotective signaling pathways activated by GHRH-Post. Recent data suggest that the SAFE pathway may also target the mPTP, initially described as an end-effector of the RISK pathway (30).

In conclusion, the present study shows that administration of nanomolar concentrations of GHRH in early phase of reperfusion induces cardioprotection. GHRH cardioprotective effect involves receptor-mediated activation of both RISK and SAFE pathways, as well as important modulators of mitochondrial function and metabolism. Interestingly, similarly to GHRH-R, GHRH mRNA and protein have been found in peripheral tissues, including the heart (9, 55). Therefore, local intracardiac GHRH may also play a role in protecting the myocardium from prolonged and excessive stress, suggesting potentially important implications in clinical settings.

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