Heat Shock Protein 90 Transfection Reduces Ischemia-Reperfusion–Induced Myocardial Dysfunction via Reciprocal Endothelial NO Synthase Serine 1177 Phosphorylation and Threonine 495 Dephosphorylation

Christian Kupatt, Chantal Dessy, Rabea Hinkel, Philip Raake, Géraldine Daneau, Caroline Bouzin, Peter Boekstegers, Olivier Feron

Objectives—The interaction of the heat shock protein 90 (Hsp90) with the endothelial NO synthase (eNOS) has been shown to account for a sustained production of NO in vitro. Here, we examined whether overexpression of Hsp90 in a pig model of cardiac infarct could preserve the myocardium from the deleterious effects of ischemia–reperfusion.

Methods and Results—Percutaneous liposome-based gene transfer was performed by retroinfusion of the anterior interventricular vein before left anterior descending occlusion and reperfusion. We found that recombinant Hsp90 expression in the ischemic region of the heart led to a 33% reduction in infarct size and prevented the increase in postischemic left ventricular end diastolic pressure observed in mock-transfected animals. Regional myocardial function, assessed by subendocardial segment shortening in the infarct region, was increased in Hsp90-transfected animals at baseline and after pacing. All these effects were completely abrogated by administration of the NOS inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester. We further documented in vivo and in cultured endothelial cells that the cardioprotective effects of Hsp90 were associated to its capacity to act as an adaptor for both the kinase Akt and the phosphatase calcineurin, thereby promoting eNOS serine 1177 phosphorylation and threonine 495 dephosphorylation, respectively.

Conclusions—Hsp90 is a promising target to enhance NO formation in vivo, which may efficiently reduce myocardial reperfusion injury. (Arterioscler Thromb Vasc Biol. 2004;24:1435-1441.)

Key Words: Hsp90 ■ nitric oxide ■ eNOS ■ calcineurin ■ Akt

Myocardial ischemia caused by an occluded coronary artery is treated clinically by rapid re-establishment of perfusion, which is currently viewed as uniquely effective in preventing myocyte cell death. Nevertheless, a body of evidence documents that reperfusion also leads to organ dysfunction, including endothelial and microcirculatory disturbance, arrhythmias, and myocardial stunning. Among the multitude of strategies aiming to prevent ischemia-reperfusion (I/R)–induced myocardial detriments, overexpression of the endothelial NO synthase (eNOS) isoform in the myocyte or endothelial compartment has proven recently to be cardioprotective. The endothelial-targeted enhancement of eNOS activity appears particularly promising because it induces vasorelaxation, inhibits platelet aggregation as well as leukocyte recruitment, and antagonizes microcirculatory disturbances. Yet, increase in NO formation by endothelium can exert beneficial effects on the cardiomyocyte compartment, preserving myocyte function by preventing necrosis, apoptosis as well as reducing myocyte vulnerability via induction of hibernation.

However, the therapeutically desired increase in endothelial NO production does not necessarily require an increase in eNOS abundance because it may also evolve from specific post-translational modifications of endogenously expressed eNOS. Accordingly, the cardioprotective effects of insulin, corticosteroids, and vascular endothelial growth factor (VEGF) were recently found to stem from eNOS phosphorylation at the serine residue 1177 (Ser 1177; human sequence) on receptor-specific induction of the phosphatidylinositol 3-kinase (PI3K)/Akt–signaling pathway. We reported previously that this activation cascade implies assembly of a multiprotein complex in which heat shock protein 90 (Hsp90) acts as a scaffold, recruiting activated Akt in the eNOS vicinity. More recently, Hsp90 was also proposed to prevent eNOS uncoupling and detrimental production of superoxide anion by the NOS enzyme, particularly after chronic myocardial hypoxia. Furthermore, definitive credential to the existence of a dynamic Hsp90–eNOS interaction was granted recently by the mapping of respective binding sequences.
and extended to the neuronal NOS isoform. Finally, Hsp90 is not only acting as an adaptor molecule for assembly of the eNOS phosphorylation complex, but it also maintains the complex in its active form. Indeed, interaction with Akt prevents dephosphorylation of the kinase, thereby promoting its sustained activation and the associated antiapoptotic effects in endothelial cells (ECs). On the basis of this set of evidence, we hypothesized that endothelial overexpression of Hsp90 could exert cardioprotection in a pig model of myocardial I/R. Using targeted transfection of an Hsp90-encoding plasmid by a selective, pressure-regulated retroinfusion approach, we studied Hsp90 effects on the in vivo eNOS phosphorylation status and the indicators of I/R injury, such as infarct size, as well as regional and global myocardial dysfunction.

Methods

Animal Model and Retroinfusion

All pig experiments were conducted at the Institute for Surgical Research of the University of Munich. The experimental procedures were approved by the Bavarian Animal Care and Use Committee (AZ 211 to 2531-07/01). As described previously,19 the retroinfusion catheter was advanced into the anterior interventricular vein, draining the parenchyma perfused by the left anterior descending (LAD) coronary artery. After assessment of individual systolic occlusion pressure of the venous system, retroinfusion pressure was set 20 mm Hg above the latter. Continuous pressure-regulated retroinfusion of NaCl 0.9%–diluted liposomes containing the plasmid (1 mg) encoding for either green fluorescent protein (GFP; control group) or Hsp90 (original plasmid was a gift from Dr W.C. Sessa, Yale University, New Haven, Conn)19 was conducted for 2 × 10 minutes at day 0 (during LAD occlusion). Next, venous and arterial sheaths were withdrawn, the vessels ligated, and suture of the muscle and the skin performed.

Ischemia and Reperfusion

Ischemia and reperfusion of these animals were conducted at day 2, allowing for expression of the transfected gene product. A balloon was placed in the LAD distal to the bifurcation of the first diagonal branch and inflated. Correct localization of the coronary occlusion and patency of the first diagonal branch was ensured by contrast agent injection under fluoroscopy. Ischemia was maintained for 60 minutes, the point after which the balloon in the LAD was deflated, initiating reperfusion of the LAD perfusion area. After 24 hours of reperfusion, animals were brought back to the operating room, anesthetized, and instrumented to evaluate regional and global myocardial function and to determine infarct size, as described previously19 (also see http://atvb.ahajournals.org).

EC Culture and Transfection

Human umbilical vein ECs (HUVECs; Clonetics) and pig coronary ECs (PCECs) obtained by the explant procedure21,22 were cultured in endothelial basal medium (Clonetics) and used at early passages for transfection (http://atvb.ahajournals.org).

NO Production and cGMP Measurements

Determination of NO levels (eg, the accumulation of nitrites in the cell-bathing [serum-deprived] medium) was performed using the Nitric Oxide Colorimetric Assay (Roche Diagnostics). cGMP determination was performed with the radioimmunoassay kit from Bio-trak (Amersham).

Cardiac Tissue Processing, Immunoprecipitation, and Immunoblotting

The hearts were collected after in vivo methylene blue exclusion (negative staining of the area at risk [AAR]) and in situ tetrazolium red staining (infarct staining) so that selected cardiac tissue samples (control, infarcted, at risk) were isolated macroscopically and snap-frozen in a mixture of acetone and dry ice; this method was validated previously to be particularly efficient to preserve protein phosphorylation.23 Pig heart pieces (as well as serum-starved ECs) were then homogenized in a buffer containing phosphatase and protease inhibitors, and whole extracts were processed for immunoblotting (IB) or immunoprecipitation (IP) as described previously10,11 (also see http://atvb.ahajournals.org).

Statistical Methods

The results are given as mean ±SEM; for IB experiments, data were normalized for protein amounts in the dish. Statistical analysis of results between experimental groups was performed with 1-way or 2-way ANOVA or t test (for the comparison of preischemic and posts ischemic left ventricular end diastolic pressure [LVEDP]).

Results

Functional Evaluation of Hsp90 Transfection Effects on Postischemic Myocardium

To evaluate the functional impact of Hsp90 overexpression in postischemic myocardium, pigs were transfected 48 hours before ischemia with cDNA plasmids, encoding either Hsp90 or the reporter GFP. As detailed in Methods, we used a selective pressure-regulated retroinfusion device, an approach providing effective transgene expression in the area at risk of infarcted heart.10,24 GFP detection (Figure 1A) confirmed our previous observations that this retroinfusion method led mostly to the transfection of cardiac microvascular ECs and small vessels (versus myocytes). As shown in Figure 1B, the infarct area in mock-transfected animals amounted to 62 ± 5% of the AAR, as measured after 24 hours of reperfusion. Transfection with the Hsp90 plasmid decreased the infarct size to 42 ± 4% (P < 0.05). Importantly, this effect was NO dependent because the administration of Nω-nitro-L-arginine methyl ester (L-NAME; details available online at http://atvb.ahajournals.org) abrogated the protection provided by Hsp90 transfection (Figure 1B). Of note, Hsp90 transfection or L-NAME treatment did not significantly affect AAR size (Figure 1C).

Evaluation of the global myocardial function reflected these findings (Figure 2A). Indeed, postischemic LVEDP was significantly increased in control pigs, whereas such elevation was absent from Hsp90-transfected pigs. Again, this Hsp90-mediated protective effect was abolished by use of L-NAME (Figure 2A). We also assessed the regional myocardial function in the ischemic area by determining the subendocardial segment shortening (SES) at baseline and after pacing. As shown in Figure 2B, SES of the infarct area was dramatically higher in Hsp90-transfected animals than in controls at all beating rates observed. Furthermore, this local gain in function in the infarct zone was NO mediated because it was completely abrogated by L-NAME administration (Figure 2B). In the AAR, besides a slight reduction in SES in mock-transfected pigs, no significant difference was found between animal groups (Figure 2C).

Evaluation of Hsp90 Transfection Effects on Phosphorylation Status of eNOS in Postischemic Myocardium

To verify that Hsp90-mediated changes in eNOS activity accounted for the observed cardioprotection, we performed
biochemical experiments aimed at identifying changes in phosphorylation status of eNOS (see Introduction) from whole tissue extracts.

We first examined the relative abundance of Hsp90 in control and ischemic regions of the heart after transfection. As shown in Figure 3A (top), Hsp90 protein amounts were increased dramatically in the transfected myocardium (also see Figure 3B). Next, we blotted the membranes with antibodies against phospho-Ser 1177 (P-Ser 1177) and phospho-threonine 495 (P-Thr 495) eNOS. As suggested by previous studies, the extent of Ser 1177 phosphorylation was largely related to the level of Hsp90 expression (Figure 3A, second panel). Indeed, although the expression of eNOS was not different in Hsp90-transfected versus mock-transfected hearts (Figure 3A, bottom), the extent of phosphorylation on Ser 1177 was significantly increased in...
transfected animals. Levels of Ser 1177 phosphorylation were 2.1- and 2.7-fold higher in the AAR and in the infarct zone, respectively, than in the control zone of Hsp90-transfected hearts (Figure 3B). Interestingly, a mirror pattern was obtained for Thr 495 because the phosphorylation of eNOS on this residue was undetectable in the AAR and infarct regions of the Hsp90-transfected myocardium, whereas in the nonischemic region of the same hearts, Thr 495 was consistently found phosphorylated (Figure 3A, third panel and Figure 3B). In mock-transfected animals, the extent of Ser 1177 and Thr 495 phosphorylations were not altered regardless of heart regions (Figure 3A and 3B).

We then examined whether changes in Ser 1177 and Thr 495 phosphorylation could be related to the increased interaction of eNOS with Hsp90 and the consecutive recruitment of the kinase Akt and the phosphatase calcineurin (PP2B). Indeed, Akt has been reported previously to promote eNOS Ser 1177 phosphorylation in an Hsp90-dependent manner (see Introduction), whereas calcineurin was shown to dephosphorylate eNOS on Thr 495 and to interact with Hsp90 (although this latter finding was obtained in studies unrelated to the NO field).

We first showed that antibodies directed against eNOS could coimmunoprecipitate Hsp90 and, inversely, that Hsp90 antibodies could precipitate eNOS from infarct region extracts of Hsp90-transfected myocardium (Figure 3C), indicating that a robust interaction occurred between eNOS and Hsp90 in transfected animals. We further documented that significantly higher amounts of Akt and calcineurin were detected in eNOS IP from Hsp90-transfected hearts (454 ± 54% and 196 ± 17%, respectively, vs mock-transfected hearts; P < 0.01; n = 3; Figure 3C). Finally, we verified whether observed changes in eNOS phosphorylation after Hsp90 transfection were associated with increased NOS activity. Indeed, we found that the cGMP content was almost 2-fold higher (P < 0.05; n = 6) in Hsp90-
transfected hearts (versus mock-transfected hearts) and that this was strictly NO mediated because the cGMP increase was completely prevented in L-NAME–treated Hsp90-transfected pigs (Figure 3D).

Dissection of Hsp90-Mediated Reciprocal Regulation of eNOS Ser 1177 Phosphorylation and Thr 495 Dephosphorylation in ECs

The causal link between Hsp90–eNOS interaction and eNOS phosphorylation/dephosphorylation changes were evaluated in isolated ECs. We first used HUVECs that were transfected either with the same Hsp90-encoding construct as used in vivo or the empty vector and exposed, for half of the dishes, to geldanamycin, a drug known to alter eNOS–Hsp90 interaction. As expected, Hsp90 overexpression strongly promoted its association with eNOS, Akt, and calcineurin (Figure 4A, compare lanes 1 and 3). In parallel, we observed that Ser 1177 phosphorylation as well as Thr 495 dephosphorylation were stimulated in Hsp90-transfected HUVECs (Figure 4B, compare lanes 1 and 3). More importantly, we found that these eNOS phosphorylation changes as well as the Hsp90–eNOS interaction were exquisitely sensitive to the action of geldanamycin (Figure 4A and 4B, compare lanes 3 and 4); similar results were obtained when using radicicol, another Hsp90 inhibitor that does not redox cycle (data not shown). Importantly, geldanamycin had no effect on either Akt or calcineurin recruitment to the Hsp90 complex (Figure 4A, bottom), in agreement with the existence of numerous distinct binding domains within Hsp90. Figure 4C shows that, in agreement with IB data, geldanamycin prevented eNOS activation increase in Hsp90-overexpressing ECs.

In a second series of experiments, we used ECs derived directly from pig coronary arteries and examined Hsp90-induced eNOS phosphorylation status changes after short (10 minutes) and longer (60 minutes) VEGF exposure and also in basal conditions. We found that in unstimulated conditions, Hsp90 transfection led to Ser 1177 eNOS phosphorylation but failed to induce Thr 495 dephosphorylation (Figure 5A, compare lanes 1 and 4). When these experiments were repeated on ECs stimulated for 10 minutes with VEGF, the extent of eNOS associated with Hsp90 increased and, although the Ser 1177 eNOS phosphorylation also increased, Thr 495 eNOS phosphorylation was not further decreased; this was observed in sham- and Hsp90-transfected cells

Figure 4. Reciprocal regulation of eNOS Ser 1177 phosphorylation and Thr 495 dephosphorylation in ECs. HUVECs transfected with a plasmid encoding for Hsp90 or with the empty vector were incubated in the presence or absence of 1 μg/mL geldanamycin and then rapidly collected. A, Corresponding lysates were immunoblotted with antibodies directed against Hsp90, Akt, and calcineurin, either directly or after IP with eNOS or Hsp90 antibodies (indicated as IgG). B, Lysates were also immunoblotted with antibodies directed against eNOS and the indicated phosphorylated residues. These experiments were repeated 2× with similar results. C, Bar graph represents the amounts of nitrites accumulated for 6 hours in the medium bathing the indicated cell populations (n=4; **P<0.01 vs sham CTL group).

Figure 5. Effects of Hsp90 transfection on VEGF-induced regulation of eNOS phosphorylation in pig coronary ECs. PCECs transfected with a plasmid encoding for Hsp90 or with the empty vector were exposed to VEGF for the indicated time. A, Corresponding lysates were immunoblotted with antibodies directed against Hsp90, total or phosphorylated eNOS, either directly or after IP with eNOS antibodies. B, Bar graph shows the densitometric analyses of Hsp90 IB from eNOS IP and of P-Ser 1177 eNOS and P-Thr 495 eNOS immunoblots (*P<0.05; **P<0.01 vs corresponding basal [no VEGF] condition; n=3).
The dephosphorylation on Thr 495 occurred at later stages associated with a further increased recruitment of Hsp90 in the eNOS complex.

Identification of Hsp90 as a key regulator of the eNOS phosphorylation status, taken together with previous studies documenting the interaction between Hsp90 and various phosphatase(s), further elucidates the mechanism of enzymatic eNOS activation. Besides its scaffolding effects, Hsp90 is also known to protect Akt from dephosphorylation (ie, deactivation) by PP2A but also to promote activation of PP2B. Altogether, these findings indicate that in vivo activation of eNOS by Hsp90 overexpression is mainly regulated by Akt-dependent Ser 1177 phosphorylation and also involves calcineurin-mediated Thr 495 eNOS dephosphorylation. However, further studies are required to evaluate whether the latter influences the net increase in bioactive NO and how this is influenced by the time or absolute abundance of Hsp90. Nevertheless, in our experimental conditions, the scaffold capacity of Hsp90 appears particularly suited to sustain eNOS activation, such as that required for cardioprotection against I/R injury; this might extend to mediation of acute effects of preconditioning, which have been attributed to postischemically enhanced eNOS activity. In fact, eNOS phosphorylation on Ser 1177 has been documented to be associated with the cardioprotection provided by VEGF, statins, corticosteroids, insulin, and even cyclosporine.

For each of these strategies, Hsp90 has been either identified directly as the effector allowing activation of the prosurvival PI3K/Akt pathway or at least as an obligatory chaperone for involved receptors (eg, hormone-bound glucocorticoid receptors). Therefore, Hsp90 appears as a common, primary target for various therapeutic stimuli involving Akt activation and NO production.

In summary, we demonstrated that Hsp90 overexpression in the ischemic region of infarcted hearts can preserve the myocardium from the I/R deleterious effects through an exquisite stimulation of the endothelial NO pathway. Our data further indicate that the mechanism involved in this feature of Hsp90 comprised eNOS Ser 1177 phosphorylation by Akt as well as Thr 495 dephosphorylation by calcineurin. This reciprocal regulation emphasizes the relevance of strategies aiming to interfere with this upstream key activator of NO-mediated pathways, particularly in pathophysiological situations in which myocardial protection is desirable.

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References
2. Brunner F, Maier R, Andrew P, Wolkart G, Zechner R, Mayer B. Mitochondrial eNOS dephosphorylation (Figure 5A, lanes 3 and 6). This was associated with an increased interaction between eNOS and Hsp90 and a small decrease in Ser 1177 eNOS phosphorylation when compared with the 10-minute VEGF exposure (Figure 5A and 5B).

Discussion
In the present study, we found that targeted overexpression of Hsp90 reduced 2 important facets of myocardial reperfusion injury: infarct size and myocardial dysfunction (Figures 1 and 2). The NOS inhibitor L-NAME entirely abolished these effects, indicating that NO accounted for most of the Hsp90-mediated cardioprotection.

Importantly, we also provided mechanistic insights into how Hsp90 can stimulate eNOS activity from in vivo and in vitro experiments. Although the stimulatory effect of eNOS phosphorylation on Ser 1177 by Akt has already been largely documented, this report is the first to demonstrate the occurrence of the dephosphorylation of eNOS on Thr 495 in animal studies. More interesting is identification of the role of Hsp90 as a key determinant of the intimate relationship between the phosphorylation on one site (ie, Ser 1177) and the dephosphorylation on the other (ie, Thr 495). Stimulatory effects of each eNOS activity post-translational modification have been reported by different groups. Accordingly, Ser 1177 phosphorylation is proposed to improve the electron flux through the enzyme and to increase its affinity for calmodulin, whereas dephosphorylation of Thr 495 is thought to suppress the steric inhibition for calmodulin association to its binding site.

Recent data suggest that in intact cells, eNOS dephosphorylation on Thr 495 is associated with an increased superoxide anion production. This phenomenon, known as eNOS uncoupling, was documented to be overcome by the eNOS concomitant phosphorylation on Ser 1177. Therefore, the major role of Thr 495 eNOS phosphorylation could consist of anion production. This phenomenon, known as eNOS uncoupling) by PP2A but also to promote activation of PP2B. Altogether, these findings indicate that in vivo activation of eNOS by Hsp90 overexpression is mainly regulated by Akt-dependent Ser 1177 phosphorylation and also involves calcineurin-mediated Thr 495 eNOS dephosphorylation. However, further studies are required to evaluate whether the latter influences the net increase in bioactive NO and how this is influenced by the time or absolute abundance of Hsp90. Nevertheless, in our experimental conditions, the scaffold capacity of Hsp90 appears particularly suited to sustain eNOS activation, such as that required for cardioprotection against I/R injury; this might extend to mediation of acute effects of preconditioning, which have been attributed to postischemically enhanced eNOS activity. In fact, eNOS phosphorylation on Ser 1177 has been documented to be associated with the cardioprotection provided by VEGF, statins, corticosteroids, insulin, and even cyclosporine.

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