Angiotensin AT₁ Receptor Stimulates Heat Shock Protein 27 Phosphorylation In Vitro and In Vivo

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Abstract—The angiotensin type 1 receptor (AT₁) exerts a variety of its signaling and cellular actions through its effects on protein phosphorylation. Phosphoproteomic analysis of angiotensin (Ang) II–stimulated aortic smooth muscle cells revealed that heat shock protein 27 (HSP27) represents a major protein phosphorylation target of the AT₁ signaling pathway. Stimulation of cells with Ang II resulted in 1.7-fold (P<0.05) and 5.5-fold (P<0.001) increases in HSP27 phosphoisoforms at pl 5.7 and pl 5.4, respectively. This was accompanied by a 54% (P<0.01) decrease in the nonphosphorylated HSP27 isoform, located at pl 6.4. Treatment of samples with alkaline phosphatase reversed this redistribution of HSP27 phosphoisoforms. Ang II–stimulated HSP27 phosphorylation was completely blocked by pretreatment of cells with the AT₁ antagonist CV11974. Phosphoamino acid analysis demonstrated that Ang II–induced phosphorylation of both HSP27 phosphoisoforms occurred exclusively on serine. Protein kinase C inhibition completely blocked phorbol ester–induced HSP27 phosphorylation but did not impair Ang II–stimulated phosphorylation of HSP27, suggesting that AT₁ increased HSP27 phosphorylation by a protein kinase C–independent pathway. Intrajugular infusion of Ang II in rats increased HSP27 in aorta by 1.7-fold (P<0.02), and this response was inhibited by CV11974. These results suggest that Ang II–induced HSP27 phosphorylation is a physiologically relevant AT₁ signaling event. Because serine phosphorylation of HSP27 blocks its ability to cap F-actin, Ang II/AT₁–induced HSP27 phosphorylation may play a key role in actin filament remodeling required for smooth muscle cell migration and contraction. (Hypertension. 2001; 38:1260-1265.)

Key Words: angiotensin II ▪ aorta ▪ heat shock proteins ▪ phosphorylation ▪ protein kinases ▪ receptors, angiotensin II

Substantial experimental and clinical evidence suggests that the activation of the renin-angiotensin system contributes to macrovascular disease in part by promoting atherosclerosis and vascular smooth muscle cell (VSMC) remodeling.1–3 Angiotensin (Ang) II, the main effector peptide of the renin-angiotensin system, exerts a variety of effects on VSMCs, including stimulation of cellular contraction, migration, growth, and gene expression.4 The majority of these Ang II actions are mediated by the angiotensin type 1 (AT₁) receptor, which is coupled to the activation of a number of serine/threonine kinases including protein kinase C, S6 kinase, rho-associated kinase, and enzymes that mediate signaling through the mitogen-activated protein kinase (MAPK) pathways.5,6 Ang II also rapidly increases tyrosine phosphorylation by transactivation of the epidermal growth factor receptor7 and by activating a number of cytosolic tyrosine kinases such as calcium-dependent tyrosine kinase, PYK2, pp60(c-src) kinase, janus kinases (JAK2 and TYK2), and focal adhesion kinase.8 Activation of the Ang II/AT₁ signaling pathway has been shown to phosphorylate a limited number of effector and structural proteins including adapter and scaffolding proteins,9,10 intermediate filament proteins,11 and transcription factors.12

Although an array of Ang II–stimulated phosphoproteins have been identified, little is known regarding which proteins represent major phosphorylation targets of the AT₁ receptor signaling pathway(s). Although recent advances in proteomics may create new opportunities to characterize of Ang II signaling pathways and cardiovascular disease,13 the AT₁-stimulated phosphorylation targets detectable by this approach have not yet been identified. A two-dimensional gel electrophoresis (2-DE)–based proteomic analysis of total proteins from rat aortic smooth muscle cells (RASMCs) revealed that heat shock protein 27 (HSP27) represents the major low-molecular-weight phosphorylation target of the Ang II/AT₁ pathway. This finding suggests that kinase cascades that lead to HSP27 phosphorylation represent a major conduit of AT₁ receptor signaling. Ang II also increases HSP27 phosphorylation in aorta in vivo, suggesting that this response may have physiological significance. Because phosphorylation of HSP27 modulates actin filament assembly, migration, and contraction,14–16 HSP27 phosphorylation may provide an important link between AT₁ receptor activation and vascular remodeling.

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Methods

Cell Culture and Metabolic Labeling With Ortho ATP

RASMCs were harvested and cultured as described previously. Cells were incubated in phosphate-free minimum essential medium/0.1% (wt/vol) BSA containing 0.5 mCi/mL of 32P-orthophosphate (DuPont-New England Nuclear) for 3 hours at 37°C. Cells were stimulated with Ang II (Sigma) as indicated and harvested in Tris-urea lysis buffer containing 8 mmol/L urea, 4% 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate, 40 mmol/L Tris, 200 mmol/L phenylmethyl sulfonylfluoride, 1 μg/mL aprotinin, 1 mmol/L ortho-phenylenediamine, and 50 mmol/L dithiothreitol.

Sample Preparation

Samples were lysed for 1 hour at 22°C followed by protein precipitation in 20% (vol/vol) trichloroacetic acid (TCA) on ice for 30 minutes. Protein pellets were washed with ethanol and redissolved in a Tris-free urea-lysis sample buffer. Total protein amount was determined with the use of Protein Assay Kit (Bio-Rad).

Protein Denaturation

A subset of samples were precipitated with TCA, washed with ethanol, and resuspended in a buffer with 60 mmol/L Tris-HCl, pH 7.5, 1 mmol/L DTT, 1 μg/mL aprotinin, and 100 mmol/L NaCl. Samples were treated with 100 U of alkaline phosphatase (New England Biolabs) for 120 minutes at 30°C.

Two-Dimensional Gel Electrophoresis

First-dimension protein separation was performed by isoelectric focusing of 100 μg protein on immobilized pH gradient gel strips (pH 3 to 10, nonlinear; Amersham Pharmacia). Strips were equilibrated in 6 mol/L urea; 50 mmol/L Tris-HCl, pH 8.8; 30% (vol/vol) glycerol; 2% SDS; and 10 mg/mL DTT followed by separation in the second dimension by SDS–polyacrylamide gel electrophoresis on gels. Samples were then transferred to polyvinylidene difluoride (PVDF) or nitrocellulose membrane (Novex).

Immunoblotting and Image Analysis

HSP27 isoforms were separated by 2-DE and detected by immunoblot analysis. Membranes were incubated with polyclonal HSP-27 antibody (Santa Cruz Biotechnology) followed by visualization with peroxidase-conjugated anti-goat immunoglobulins and enhanced chemiluminescence (Amersham Pharmacia). Detection and quantification of the immunoblotted membrane was determined with the use of a PhosphorImager and the Imagequant software (Molecular Dynamics). Levels of individual HSP27 isoforms were quantified and expressed relative to total HSP27 immunoreactivity to normalize for protein recovery, as described previously. Acute Ang II stimulation did not affect total HSP27 levels measured by this method.

Phosphoamino Acid Analysis

Phosphoproteins from control and Ang II–stimulated cells were separated by 2-DE. The areas of nitrocellulose membrane containing HSP27 phosphoisoforms were excised, eluted, and hydrolyzed, and phosphoamino acid analysis was performed by cellulose thin-layer electrophoresis as described previously.

Intrajugular Injections of Ang II

Anesthetized male Sprague-Dawley rats (weight, 250 g) received a bolus 0.5 mL injection of 40 μmol/L Ang II in the absence or presence of a 5-fold molar excess of candesartan through an intrajugular catheter. Control rats were similarly injected with saline. After 15 minutes, aortic tissue was harvested and frozen in liquid nitrogen. Samples were homogenized in urea buffer, and TCA-precipitated protein fraction was separated by 2-DE and immunoblotted for HSP27 as described above.

Statistics

Statistical significance using SigmaStat 2.03 was determined by 1-way ANOVA. Differences were designated to be significant at P<0.05.

Results

Identification of Ang II–Stimulated Low-Molecular-Weight Phosphoproteins as HSP27 Isoforms

Ang II–stimulated phosphorylation of proteins from ATP-labeled cells was analyzed by 2-DE. To disrupt macromolecular interactions that may contribute to selective protein loss, cell lysates were subjected to denaturing conditions of 8 mol/L urea and 20% TCA under reducing conditions before 2-DE analysis. This method revealed an array of phosphoproteins from control and Ang II–stimulated RASMCs (Figure 1, top panels). Among proteins <50 kDa, 2 highly expressed Ang II–stimulated phosphoproteins were identified with mobilities of ~27 kDa and isoelectric points at pI 5.7 and pI 5.4. Ang II stimulation increased incorporation of 32P by these phosphoproteins by 3-fold and 8-fold, respectively.

On the basis of the molecular weight and isoelectric points, a candidate immunoblot approach was used to determine if these phosphoproteins colocalized with HSP27. Lysates from ATP-labeled cells were separated by 2-DE, transferred to PVDF membranes, and immunoblotted with anti-HSP27 antibody. Visualization of HSP27 immunoreactivity by enhanced chemiluminescence revealed 4 isoforms, separated according to their pI (Figure 1, bottom panels). The 2 most acidic of these HSP27 isoforms, labeled phosphoprotein-1 (PP1) and PP2, colocalized with the ATP-labeled phosphoproteins visualized by autoradiography. The Ang II–induced increases in these HSP27 isoforms at pI 5.4 and pI 5.7 measured by immunoblotting were comparable with the increased 32P-incorporation into these spots. Two additional HSP27 isoforms at pI 6.4 and 5.8, which did not colocalize with ATP phosphobinding, were labeled as nonphosphoprotein-1 (NP1) and NP2, respectively.

Ang II Induces Phosphorylation of HSP27 in an Ang II Type 1 Receptor–Dependent Manner

To examine the role of the AT1 receptor in Ang II–induced HSP27 phosphorylation, cells were stimulated with Ang II in the absence or presence of the AT1 antagonist candesartan (CV11974, kindly provided by Dr Peter Morsing, Astra Hassle AB, Sweden). Stimulation of cells with Ang II (100 nmol/L) for 15 minutes decreased NP1 and NP2 levels by 55% and 31%, respectively (P<0.01 and <0.05, ANOVA; Figure 2). This Ang II response was associated with an increase in the relative amount of PP1 by 68% (P<0.05) and PP2 by 5-fold (P<0.001). Pretreatment of cells with 1 μmol/L candesartan completely blocked both Ang II–stimulated decreases in NP1 and NP2 levels and increased PP1 and PP2 levels (Figure 2). This Ang II–stimulated shift in HSP27 immunoreactivity was sustained at 30 minutes and occurred with an ED50 of 10 nmol/L Ang II (not shown). Treatment of lysates from Ang II–stimulated cells with alkaline phosphatase eliminated immunoreactivity of HSP27 at pI 5.4 and reduced immunoreactivity of HSP27 at pI 5.7 to control.
levels (Figure 2), demonstrating that phosphorylation is required for Ang II–induced acidic shift of HSP27 immunoreactivity. These results support the identification of the 32P-labeled proteins at pI 5.7 and 5.4 as phosphoisoforms of HSP27 and show that Ang II induces the redistribution of HSP27 from nonphosphorylated to phosphorylated isoforms.

Phosphoamino Acid Analysis of Ang II–Induced HSP27 Phosphorylation

Phosphoamino acid analysis was performed on PP1 and PP2 isoforms isolated from ortho 32P-labeled RASMCs, as shown in Figure 1. This analysis revealed that Ang II stimulation increased phosphoserine incorporation into both of these HSP27 isoforms (Figure 3). Phosphorylation of HSP27 on threonine and tyrosine was not detected under basal or Ang II–stimulated conditions.

Effect of Protein Kinase C Inhibition on Ang II–Stimulated and Phorbol Ester–Stimulated HSP27 Phosphorylation

Because Ang II activates protein kinase C (PKC) in VSMCs and direct activation of PKC has been shown to increase the phosphorylation of small heat shock proteins, the role of PKC in Ang II–stimulated phosphorylation was examined.
Cells were pretreated with the PKC inhibitor GF 109203X (GFX, 5 μmol/L) for 15 minutes followed by stimulation with either Ang II or phorbol 12-myristate 13-acetate (PMA) for 15 minutes. Levels of HSP27 phosphoisoform PP2 normalized to total HSP27 content were quantified by immunoblotting, as described in Figure 2. GFX did not affect baseline HSP27 phosphorylation and appeared to enhance Ang II–stimulated HSP27 phosphorylation (Figure 4). Direct activation of PKC by treatment of cells with phorbol ester (PMA) also increased HSP27 phosphorylation, and this response was completely blocked by GFX, confirming the efficacy of this inhibitor.

Effect of Ang II on Aortic HSP27 Phosphorylation In Vivo

To determine whether Ang II affects vascular HSP27 phosphorylation in vivo, the distribution of HSP27 isoforms was examined in aortic tissue isolated from Sprague-Dawley rats. Anesthetized rats received an intrajugular 500-μL bolus injection of saline in the absence or presence of 40 μmol/L Ang II or 200 μmol/L candesartan. Fifteen minutes after injection, aortic tissue was harvested and HSP27 isoform distribution was examined by immunoblotting, as described in Figure 2. Ang II decreased the nonphosphorylated HSP27 NP1 isoform by 25% (P=0.05) relative to saline controls and by 35% (P<0.02) compared with candesartan-treated controls (Figure 5). In addition, Ang II infusion increased levels of PP2 by 70% (P<0.02) relative to saline controls and by 3-fold compared with candesartan-treated controls. The Ang II–induced changes in HSP27 isoforms at NP1 and PP2 levels in vivo were consistent with those observed in cultured RASMCs (Figure 2). Coinfusion of candesartan blocked these Ang II responses, indicating that the AT₁ receptor mediates the effects of Ang II on HSP27 in vivo. Ang II or candesartan did not affect the levels of NP2 and PP1 in aorta.

Discussion

This report provides the first evidence that HSP27 represents a major low-molecular-weight phosphorylation target of the Ang II/AT₁ receptor pathway in RASMCs and that its phosphorylation also occurs in aorta in vivo. Analysis of 32P-labeled cellular phosphoproteins from RASMCs by 2-DE identified 2 separate isoforms of HSP27, located at pl 5.4 and pl 5.7, as the major phosphoproteins in Ang II–stimulated cells. The Ang II–stimulated phosphorylation of HSP27 occurred with an ED₅₀ of 10 nmol/L and was completely blocked by candesartan, showing that this response occurs at physiologically relevant Ang II concentrations and is mediated by the AT₁ receptor subtype. Treatment of lysates from Ang II–stimulated cells with alkaline phosphatase reduced immunoreactivity of HSP27 isoforms at pl 5.4 and pl 5.7 to control levels, demonstrating that phosphorylation is required for Ang II–induced acidic shift of HSP27 immunoreactivity.

Previous reports have shown that the p38 MAPK pathway, through the activation of downstream kinases MAPK activated protein (MAPKAP) and p38-regulated/activated protein kinase, mediates the phosphorylation of HSP27. Recently, it has been shown that a reactive oxygen species–induced stimulation of p38 MAPK in response to Ang II resulted in an activation of MAPKAP kinase-2 and an increase in immunoprecipitable phosphorylated HSP27. The latter, however, has neither been quantified nor normalized and was limited to cultured cells. Because HSP27 exists both in monomeric and polymeric forms and associates with actin filaments, which may affect its solubility and antigen accessibility, it is important to quantify the effects of Ang II on HSP27 phosphorylation relative to total HSP27 protein. The quantification of HSP27 isoforms according to isoelectric focusing has been described previously. 2-DE analysis demonstrated that Ang II not only increases levels of highly phosphorylated HSP27, which migrate with an acidic pl 5.4, but also decreases levels of the nonphosphorylated HSP27 isoform NP1. This nonphosphorylated form of HSP27 is the biologically active isoform that binds to F-actin.

Activation of phorbol ester–sensitive PKC isoforms (α, β, γ, δ, ε, θ, η) increased HSP27 phosphorylation, and this response was blocked with GFX, which inhibits the common

Figure 3. Phosphoamino acid analysis of HSP27 isoforms PP1 and PP2. Cells were labeled with 32P and stimulated with Ang II (100 nmol/L, 15 minutes). Cells were then lysed and separated by 2-DE as described in Figure 1. 32P-labeled PP1 and PP2 spots were excised and subjected to phosphoamino acid analysis. Locations of P-Ser, P-Thr, and P-Tyr standard stained with ninhydrin are indicated.

Figure 4. Effect of PKC inhibition on Ang II–stimulated and PMA-stimulated HSP27 phosphorylation. Cells were pretreated for 15 minutes with either 5 mmol/L GFX as indicated or DMSO and stimulated with 100 nmol/L Ang II or PMA. HSP27 isoforms were quantified by immunoblotting, as shown in Figure 2. Bar graph represents levels of HSP27 phosphoisoform PP2 normalized to total HSP27. *Statistically significant differences (P<0.05).

Figure 5. Effect of PKC inhibition on Ang II–stimulated and PMA-stimulated HSP27 phosphorylation. Cells were pretreated for 15 minutes with either 5 mmol/L GFX as indicated or DMSO and stimulated with 100 nmol/L Ang II or PMA. HSP27 isoforms were quantified by immunoblotting, as shown in Figure 2.
II–stimulated serine phosphorylation of HSP27 occurs at significant differences, *P<0.05 and **P<0.02.

Precipitated protein fraction was separated by 2-DE, and HSP27 isoform distribution was visualized by immunoblot analysis. Representative blots and bar graph quantification (n=4 to 7 per group) are shown. Significant differences, *P<0.05 and **P<0.02.

Because the 2 previously identified phosphorylation sites on human HSP27 and murine HSP25 are conserved at Ser15 and Ser86, in the isoform located at pI 5.7 and diphosphorylation of these sites. Based on the phosphorylation-dependent acidic shift of HSP27, the presence of 2 phosphoisofoms would be consistent with the monophosphorylation of HSP27, at either Ser15 or Ser86, in the isoform at pI 5.7 and diphosphorylation of these isoforms at pI 5.4, as described in References 15 and 16. Although Ang II increases phosphorylation of isoforms at both pI 5.7 and 5.4 in cultured RASMCs, Ang II selectively increased levels of the pI 5.4 isoform in vivo. These results suggest that the physiologically significant Ang II effects on HSP27 are reduced levels of the nonphosphorylated HSP27 isoform at pI 6.4 and the increased phosphorylation of the (diphosphorylated) PP2 isoform.

Phosphorylation of HSP27 plays an important role in modulating its structure and functions. Nonphosphorylated monomeric HSP27 binds to F-actin as a capping protein and thereby blocks actin polymerization, whereas phosphorylated HSP27 does not affect actin polymerization. Thus, the Ang II–induced decrease in levels of the nonphosphorylated isoform of HSP27, observed both in vitro and in vivo, would be expected to increase the dynamics of actin assembly, which is essential for VSMC migration. In summary, HSP27 was identified as the major low-molecular-weight target of Ang II/AT1 stimulated phosphorylation in RASMCs by a proteomic approach. In vivo studies demonstrated that Ang II phosphorylates HSP27 in aorta. The Ang II–induced redistribution of HSP27 phosphoisofoms would be expected to enhance actin remodeling, which is essential for VSMC migration, and impair the chaperone activity of HSP27, which recovers proteins partially denatured by cellular stress. Thus, HSP27 phosphorylation may contribute to the AT1 receptor effects on cell migration, contraction, and the recovery of denatured proteins.

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