Regulation and Localization of HSP70 and HSP25 in the Kidney of Rats Undergoing Long-Term Administration of Angiotensin II

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Abstract—Various renal insults result in induction of heat shock protein (HSP) expression within the kidney. Some of the HSPs induced in that manner are postulated to have renoprotective effects via either chaperoning actions or antioxidative properties. We have previously reported that long-term angiotensin (Ang) II administration induces the expression of renal HSP32, also known as heme oxygenase-1 (HO-1). Here, we investigated the regulation of expression and localization of other HSPs, including HSP70, HSP25, and αB-crystallin, in the kidney of rats undergoing long-term administration of Ang II (0.7 mg · kg⁻¹ · d⁻¹). Immunoblot analysis demonstrated that Ang II increased renal expression of HSP70 and HSP25, as well as HO-1, but that expression of αB-crystallin was unaffected by this treatment. The Ang II–induced increase in renal HSP70 and HSP25 was dependent on the angiotensin type 1 receptor activation but not on hypertension per se. Immunohistochemistry revealed that HSP70 and HSP25 were expressed in the medullar regions and in the renal arterial wall in the kidney of control rats. After Ang II infusion, signals for HSP70, HSP25, and HO-1 proteins increased in intensity in the endothelium and medial smooth muscle of the renal artery. In addition, all of these HSPs were induced in proximal renal tubular epithelial cells from the same segments, suggesting that similar mechanisms are responsible for upregulating these HSPs. Our data show that Ang II infusion induces renal HSP70 and HSP25, as well as HO-1, and that Ang II can induce expression of these HSPs in renal cells in a pressor-independent manner. (Hypertension. 2002;39:122-128.)

Key Words: angiotensin ■ AT₁ receptor ■ hypertension ■ heat shock protein ■ kidney

The heat shock response is a conserved, immediate, and transient cellular response to environmental stresses such as high temperatures,¹ oxidants,² and ATP depletion.³ It involves the induction of the so-called heat shock proteins (HSPs).⁴ Previous studies have demonstrated that several HSPs, including HSP32 (also known as heme oxygenase-1, HO-1),⁵ HSP25, HSP60, HSP70/HSP72, HSP73,⁶ and αB-crystallin,⁷ are present in the kidney.

HO-1/HSP32, an inducible form of HO, catalyzes the rate-limiting step in the degradation of heme. Induction of renal HO-1 has been shown to ameliorate renal injury induced by rhabdomyolysis.⁸ Gene ablation of HO-1 exacerbated cisplatin-induced renal tubular injury.⁹ Thus, the intrarenal HO system is thought to play a role in protecting renal function against various renal insults. The inducible form of HSP70 is also shown to be regulated in the kidney in response to stimuli such as ischemia² and hyperthermia.¹⁰ Overexpression of transfected HSP70 in the renal cells was found to have a protective action against cisplatin toxicity, oxidative injury,¹¹ and hyperthermia.¹² HSP25 and αB-crystallin are both members of the low-molecular-weight HSP family. They can be phosphorylatable at various amino acid positions, although their phosphorylation may not be essential for their chaperoning properties.¹³ Renal expression of HSP25 and αB-crystallin is known to be regulated,⁷,¹⁴ but their precise biological role in the kidney remains unclear.

We have recently shown that HO-1 is induced in tubular epithelial cells in response to administration of angiotensin (Ang) II and that the increased levels of HO-1 may play a role in ameliorating Ang II–induced renal dysfunction by suppressing Ang II–induced renal cell proliferation and apoptosis.⁵,¹⁵ Here, we will show the regulation and localization of HSP70, HSP25, and αB-crystallin in the kidney of Ang II–induced hypertensive rats.

Methods

Animal Models
The rat Ang II hypertension model was induced in Sprague-Dawley rats (Nippon Bio-Supply Center) by subcutaneous implantation of an
osmotic minipump (Alzet model 2001, Alza Pharmaceutical) as described previously. In some experiments, the selective AT1 receptor antagonist losartan (25 mg·kg\(^{-1}\)·d\(^{-1}\); a kind gift from Dupont/Merck) or the nonspecific vasodilator hydralazine (15 mg·kg\(^{-1}\)·d\(^{-1}\); Sigma Chemical Co) was given in the drinking water beginning 2 days before pump implantation and throughout Ang II infusion. In a second model of hypertension, norepinephrine (NE) was infused at a rate of 2.8 mg·kg\(^{-1}\)·d\(^{-1}\) with the same system. Some rats were also given daily intraperitoneal injections of the HO-1 inducer hemin (50 µmol·kg\(^{-1}\)·d\(^{-1}\); Sigma), which was started 2 days before pump implantation and continued until the rats were killed.

**Protein Purification and Western Blot Analysis**

Protein purification and SDS PAGE were performed as described previously. Polyclonal antibodies against rat HO-1 (SPA895), HSP70 (SPA760), HSP25 (SPA801), and αB-crystallin (SPA223) and a monoclonal antibody against HSP70 (SPA810, StressGen) were used at a 1/2000 dilution; horseradish peroxidase–conjugated secondary antibody (Jackson ImmunoResearch) was used at a 1/2000 dilution. The ECL Western blotting system (Amersham Life Sci-)

**Isoelectric Focusing Electrophoresis for HSP25 and αB-Crystallin**

Protein samples from rat kidney were mixed with an equal volume of 8 mol/L urea, 4% Bio-lyte 3/10 (Bio-Rad), and 1% Triton X-100. For HSP25, samples were separated by isoelectric focusing (IEF) electrophoresis on 5% polyacrylamide gels containing 4 mol/L urea, 0.2% CHAPS, 5% glycerol, and 2% Bio-lyte 3/10. For αB-crystallin, samples were separated by IEF electrophoresis on 5% polyacrylamide gels containing 5.5 mol/L urea, 1.5% Bio-lyte 3/10, 0.5% Bio-lyte 5/7 (Bio-Rad), 5% glycerol, 5% acrylamide, and 0.2% N,N'-methylene-bis-acrylamide. After blotting onto the PVDF membrane, the membrane was hybridized with antibodies, and bands were visualized as described above.

**Immunohistochemistry of HSPs**

Immunohistochemistry was performed as described previously. Briefly, after the sections were incubated with the polyclonal antibody against HO-1, HSP70, or HSP25, used at a 1/200 dilution for 1 hour, slides were washed and incubated with biotinylated secondary antibody. After treating the slides with the Elite ABC kit (Vector Laboratories), antigens were visualized with the 3,3'-diaminobenzidine tetrahydrochloride (DAKO) system.

**Culture of Transformed Rat Proximal Tubular Cells**

Culture of established rat proximal tubular cells (IRPTCs) was performed as described previously. Cells were cultured in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum. Cultures were supplemented with 3.8 mg/mL NaHCO\(_3\), 25 mmol/L HEPES buffer (pH 7.5), 0.1 mmol/L sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.01 mmol/L nonessential amino acids.

**Statistical Analysis**

Data were expressed as mean±SEM. We used ANOVA followed by a multiple comparison test for comparisons on initial data before expressing results as a percentage of the control value. A value of \(P<0.05\) was considered statistically significant.

**Results**

**Effect of Ang II on the Expression of HSP70, HSP25, αB-crystallin, and HO-1**

Ang II infusion significantly increased expression of renal HSP70, HSP25, and HO-1 (Figure 1A and 1B). HSP25 appeared to reach maximal induction earlier than HSP70 and HO-1. Expression of αB-crystallin was unaffected by Ang II administration.

**Effects of Vasodilators, NE, and Hemin on the Expression of Renal HSPs**

To examine pressor dependency, either hydralazine or losartan was given to rats. Both vasodilators effectively normalized Ang II–induced elevation of blood pressure. Losartan completely blocked Ang II–induced upregulation of HSP70 and HSP25, but hydralazine did not affect Ang II–induced upregulation of these HSPs. Both antihypertensive drugs blocked Ang II–induced upregulation of HO-1 as reported previously. Although long-term NE administration resulted in an elevation of blood pressure similar to that caused by Ang II, it did not lead to altered expression of HSP70, HSP25, or HO-1 (Figure 2A and 2B). Although hematin administration markedly increased renal HO-1 expression as we have previously reported, it did not affect renal expression of HSP70 (114±29% of control; \(n=4\); \(P=NS\) versus control) or HSP25 (118±27% of control; \(n=4\); \(P=NS\) versus control) (Figure 2C).

**Phosphorylation of HSP25 and αB-Crystallin**

IEF electrophoresis revealed that both nonphosphorylated and phosphorylated forms of HSP25 were barely detectable in samples from control kidneys; however, both of these forms were readily detectable in the kidneys from Ang II–infused rats. The ratio of the phosphorylated form to the
nonphosphorylated form of \( \alpha B \)-crystallin was not significantly altered after Ang II infusion (Figure 3).

**Immunohistochemistry of HSPs**

In the normotensive rat kidney, weak staining of HSP70, HSP25, and HO-1 was seen mainly in proximal and distal tubular epithelial cells (Figure 4A through 4C). After Ang II administration, staining of HSP70, HSP25, and HO-1 in these regions became more intense (Figure 4D through 4F). Staining of the serial sections revealed that the same proximal tubular epithelial cells were positive for all 3 HSPs after Ang II infusion (Figure 4G through 4I). Staining of HSP70, HSP25, and HO-1 was seen in the medullary regions of the control rat (Figure 4J through L). In these regions, staining of HSP70 and HSP25 was apparently not altered (Figures 4M and 4N) but staining of HO-1 seemed to be increased (Figure 4O) after Ang II administration. In the renal pelvis, positive HSP70 staining was seen (Figure 5A). Intense staining of HSP25 was also seen in the cells in this region, in particular in dome-shaped cells (Figure 5B), although HO-1 staining was only weak in this region (Figure 5C). After Ang II administration, intensity of staining of HSP70 and HO-1 in the renal pelvis was slightly increased, but intensity of HSP25 staining in this region did not seem to be altered visibly after Ang II administration (Figure 5D through F). Positive staining of 3 HSPs was also seen in renal arteries in the control rats (Figure 5G through I). Staining of all 3 HSPs became more intense in the endothelium and medial smooth muscle of the renal artery and cells surrounding the artery, presumably fibroblasts and/or myofibroblasts (Figure 5J through L). Intense staining of HSP25 but not HSP70 or HO-1 was seen in the cells of peripheral nerves in control rats (Figure 5M through O). Intensity of staining of these 3 HSPs in the peripheral nerve did not seem to be altered visibly after Ang II administration (data not shown). In the heart, staining of HSP70, HSP25, and HO-1 was positive (data not shown), and after Ang II administration, intensity of staining of these HSPs was slightly increased (Figure 5P through S).

**Regulation of HSPs in Cultured Renal Tubular Cells**

In a final set of experiments, we investigated whether expression of HSP70 and HSP25 is regulated in IRPTCs. Cells were stimulated with an HO inducer, hemin (10 or 100 \( \mu \)mol/L), hydrogen peroxide (10 or 100 \( \mu \)mol/L), or Ang II (100 nmol/L) for 4 hours. We selected this dose of Ang II because stimulation of IRPTCs with Ang II at a dose of 100 nmol/L for 4 hours has been shown to upregulate HO-1 expression.\(^5\) As shown in Figure 6, expression of HSP70 and HSP25 was regulated in IRPTCs. Cells were stimulated with an HO inducer, hemin (10 or 100 \( \mu \)mol/L), hydrogen peroxide (10 or 100 \( \mu \)mol/L), or Ang II (100 nmol/L) for 4 hours. We selected this dose of Ang II because stimulation of IRPTCs with Ang II at a dose of 100 nmol/L for 4 hours has been shown to upregulate HO-1 expression.\(^5\) As shown in Figure 6, expression of HSP70 and HSP25, as well as HO-1, was increased in response to each of these stimuli.

**Discussion**

In the present study, we have shown that long-term administration of Ang II increases expression of HSP70 and HSP25, as well as HSP32 (HO-1), in the rat kidney. Losartan completely blocked Ang II–induced upregulation of all 3 HSPs. On the other hand, hydralazine completely blocked Ang II–induced HO-1 upregulation but failed to block Ang II–induced upregulation of HSP70 or HSP25, which suggested that different mechanisms may regulate Ang II–induced HO-1 upregulation compared with renal HSP70 and HSP25 induction in the kidney. Interestingly, NE did not alter expression of these HSPs, suggesting that hypertension alone was not a sufficient stimulus to increase renal expression of these HSPs.

We found that HSP70 is upregulated in the rat kidney with long-term infusion of Ang II but not NE. Xu et al\(^{18}\) have previously reported that HSP70 was induced in the rat kidney after bolus vasopressin injection. They also showed that vasopressin-induced upregulation of renal HSP70 is a
pressor-independent process, because a vasopressor antago-
nist failed to block vasopressin-induced HSP70 upregulation.
Therefore, it seems that acute or chronic systemic hyperten-
sion per se is not a sufficient physiological stimulus for the
induction of renal HSP70. Xu et al\textsuperscript{18} also reported that bolus
injection of Ang II did not result in upregulation of renal
HSP70 expression. Because we continuously infused Ang II,
the difference in the duration of drug administration may
explain the different results after Ang II injection with respect
to HSP70 upregulation. It is important, however, to keep in
mind that our data did not conclude that there is no relation-
ship between hypertension per se and regulation of HSP70.
Hamet et al\textsuperscript{19} have reported heightened expression of renal
HSP70 in the genetic hypertensive animals only after heat
exposure of the whole body. Thus, it is possible that although
NE treatment did not increase renal HSP70 expression, it may
modulate HSP70 expression in response to some physiolog-
ical stimuli such as heat shock.

Although Ang II administration increases the expression of
HSP70, HSP25, and HO-1 with a similar time course,
hydralazine completely abrogated Ang II--induced HO-1
upregulation but did not affect Ang II--induced upregulation

Figure 4. Immunohistochemistry of HSPs
in the renal cortex and medullary regions.
A, D, G, J, and M, HSP70 staining. B, E,
H, K, and N, HSP25 staining. C, F, I, L,
and O, HO-1 staining. A through C, D
through F, G through I, J through L, and
M through O are from serial sections. A
through C, HSP staining in the cortex of
the control kidney. Staining of HSP70 (A),
HSP25 (B), and HO-1 (C) was minimal in
the cortex of the control kidney. D
through I, HSP staining in the cortex of
the kidney of rats infused with Ang II for
7 days. After Ang II stimulation, the stain-
ing of HSP70 (D), HSP25 (E), and HO-1
(F) was more intense in the cortex, and
higher-magnification views revealed that
the same tubular epithelial cells were
positive for HSP70 (G), HSP25 (H), and
HO-1 (I). J through L, HSP staining in the
medullar region of the control kidney.
HSP70 (J) and HSP25 (K) but not HO-1
(L) stained positively in the medullar
region in the control kidney. M through O,
HSP staining in the medullar region of the
kidney after Ang II infusion. After Ang II
infusion, the staining intensity of HSP70
(M) and HSP25 (N) did not alter visibly. In
contrast, the intensity of HO-1 staining
increased in these regions (O). Magnifica-
tion, \( \times 500 \) (A through F), \( \times 900 \) (G
through I), and \( \times 250 \) (J through O).
of HSP70 or HSP25. These results indicate that the mechanisms of induction of HO-1 and of HSP70 or HSP25 may differ at least in part. The pathways by which HSPs are upregulated by Ang II in vivo need to be addressed in future studies. Because Ang II increases the production of reactive oxygen species in renal cells in vivo and in vitro,\(^{20,21}\) it seems possible that the Ang II–induced upregulation of renal HSP resulted from either increased levels of intrarenal Ang II or reactive oxygen species. This concept would be further supported by the findings that stimulation of IRPTCs by either Ang II or hydrogen peroxide leads to an increase in the expression of these HSPs. We have previously demonstrated that renal HO-1 may have a role in renal protection against Ang II–induced injury. Because HSP70 and HSP25 are also induced by Ang II in the kidney, these HSPs may also play a role in renoprotection. We do not know which molecule has the predominant effect in renoprotection. However, because hemin administration specifically induced renal HO-1, the protective effect of hemin treatment shown in the previous study\(^5\) may reflect the protective action of induced HO-1 against Ang II–induced nephrotoxicity. The possible role of induced of HSP70 and HSP25 in the kidney was not examined in the present study.
Several previous studies have suggested the possible physiological role(s) of these HSPs in vivo. Hamet et al. have reported that HSP70 polymorphism is associated with a blood pressure difference in recombinant inbred strains. In addition, the same group has reported that the HSP25 polymorphism is also significantly associated with blood pressure. These data suggest that regulation of HSP70 and HSP25 expression may have a role in the modulation of blood pressure. On the other hand, it has recently been shown that induction of HSP70 and HSP25 may not suppress cellular proliferation and apoptosis induced by heat shock or by serum deprivation in vascular smooth muscle cells. Whether HSP70 and HSP25 may act against cellular proliferation and apoptosis in the kidney should be clarified in future studies.

Immunohistochemical analysis showed that expression of 3 HSPs was minimal in any tubular epithelial cells. HSP70 and HSP25 were expressed in the medullary regions. These patterns of HSP70 or HSP25 expression are consistent with previous findings. The strong HSP25 immunoreactivity observed in the peripheral nerves is also consistent with the finding that HSP25 could be observed in peripheral nerves in other organs (e.g., intrinsic cardiac neurons). After Ang II infusion, increased expression of these HSPs was seen in the arterial wall. Previously, HSP70 and HO-1 were found to be upregulated in the aortas of hypertensive animals, which suggests that increased hemodynamic stress may have a role in the increase in HSP70 or HO-1 observed in the renal arterial wall of Ang II-induced hypertensive rats. Another possibility is that HSP70 and HO-1 are induced in the vascular cells in an Ang II-specific manner. This latter possibility is supported by the finding that HSP70 was induced by Ang II in vascular smooth muscle cells, although HO-1 was downregulated by Ang II in these cells.

The physiological relevance of the HSP upregulation in the arterial wall remains unclear; however, increased phosphorylated HSP25 may participate in the regulation of vascular tone by improving the viability of vascular cells against various stimuli. We also found here that these HSPs were increased in endothelial cells of the renal artery. Increased expression of HSP70 and HSP25 may play a role in ameliorating vascular damage provoked by hemodynamic stress, partially through the overproduction of reactive oxygen species.

In summary, expression of HSP70 and HSP25, as well as HO-1, was upregulated in the kidney of the rat with long-term Ang II administration. Although regulation of HO-1 and regulation of HSP70 or HSP25 have different pressor dependencies, the staining of serial renal sections clearly demonstrates that these HSPs are induced in the same tubular epithelial cells, indicating that a similar mechanism may be responsible for their upregulation. Physiological roles of Ang II-induced HSP70 and HSP25 should be clarified in future studies.

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References


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