Upregulation and intrarenal redistribution of heat shock proteins 90 α and 90 β by low-sodium diet in the rat

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Abstract Two genes encoding isoforms heat shock protein (Hsp) 90 α and Hsp90 β constitute the Hsp90 subfamily. In addition to their role in regulating mineralocorticoid and glucocorticoid receptors, these proteins have been associated with nitric oxide production. However, little is known regarding Hsp90 isoform expression and regulation in kidney. In this study we characterized the expression and localization of Hsp90 isoforms and evaluated the influence of low-sodium intake on their expression and distribution in kidney by using reverse transcription–polymerase chain reaction, Western blot, and immunohistochemistry techniques. We found that Hsp90 α and Hsp90 β were expressed abundantly in both the renal cortex and the medulla; however, Hsp90 isoform expression was higher in the medulla than in the cortex. Immunohistochemistry of Hsp90 α and Hsp90 β showed intense staining in the apical membrane of proximal and distal tubules. In the outer cortex these proteins were localized intracytosolically, whereas in the inner renal medulla they were restricted mainly to the basolateral membrane. Expression of Hsp90 α and Hsp90 β was upregulated in the renal cortex during sodium restriction. In addition, both proteins exhibited redistribution from the cytoplasm to the basolateral side in thick ascending limb cells when rats were fed with a low-salt diet. Our results showed that Hsp90 α and Hsp90 β were expressed abundantly in renal tissue. Expression and localization patterns under normal and salt-restricted intake were different between the cortex and the medulla, suggesting that these proteins may be involved in different processes along the nephron. Hsp90 α and Hsp90 β upregulation induced by a low-sodium diet together with redistribution in thick ascending limb cells suggests that Hsp90 plays a role in the modulation of sodium reabsorption under these circumstances.

INTRODUCTION

The heat shock protein (Hsp) family is formed by several genes that encode for proteins with molecular mass between 10 and 170 kDa (Jäättelä 1999). In the majority of eukaryotic cells, 3 classes of Hsps—Hsp90, Hsp70, and small Hsp—are synthesized abundantly. Specifically, the 90-kDa subfamily is constituted of 2 genes known as

Hsp90 α and Hsp90 β in humans (Hsp86 and Hsp84 in mouse) (Rebbe et al 1987; Moore et al 1989). Hsp90 influences the activity and stability of a wide range of client proteins that function as key regulators in cellular growth, differentiation, and death pathways. Among the >100 known Hsp90 protein clients are found steroid receptors; transcription factors; tyrosine kinases; G-protein subunits; and neuronal, inducible, and endothelial nitric oxide synthases (García-Cardena et al 1998; Bender et al 1999; Yoshida and Xia 2003).

In the kidney the best-characterized Hsp90 function is the interaction of this protein with the mineralocorticoid receptor. Hsp90 together with other factors and cochaperones forms a heterocomplex that binds to the mineral-

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ocorticoid receptor (Couette et al 1996, 1998). The presence of Hsp90 in the complex seems to facilitate anchoring of unbound receptors to cytoskeleton and maintains the hormone-binding protein in a high-affinity conformation (Pratt 1993). In other words, association with Hsp90 stabilizes the mineralocorticoid receptor in its hormone-binding form. Then, binding of aldosterone to the receptor releases the Hsp heterocomplex, and the new receptor-aldosterone complex migrates to the nucleus and binds deoxyribonucleic acid (DNA), harboring the appropriated responsive elements in several genes.

One of the major regulators of Na⁺ excretion is aldosterone, and its effects are mediated by mineralocorticoid receptors. Transcriptional activation of these receptors in distal tubule epithelial cells (Tumlin 1997) triggers a series of events that stimulate Na⁺ retention by the kidney, including increased expression and activation of epithelial Na⁺ channel subunits (Masilamani et al 1999), which mediates apical Na⁺ entry across collecting duct principal cells and an abundance of thiazide-sensitive Na-Cl cotransporter in distal tubule (Velázquez et al 1996; Kim et al 1998). Because Hsp90 is required for proper conformation of the mineralocorticoid receptor before its binding to aldosterone, it is possible that it plays a role in regulating the effects of aldosterone. Moreover, it has been shown that some aldosterone effects in the collecting duct, such as activation of calcineurin, are mediated by the released Hsp heterocomplex rather than by the aldosterone-receptor complex (Tumlin et al 1997). In addition to the potential role of Hsp90 in aldosterone response, angiotensin II has been postulated as an inductor of Hsp70 and Hsp25, as well as of HO-1 expression, in the renal cortex (Aizawa et al 2000; Ishisaka et al 2002). However, no studies to date have addressed whether angiotensin II also modulates expression of Hsp90 isoforms.

Although some studies have evaluated the intrarenal expression of Hsp family members (Hsp32, Hsp25, Hsp60, Hsp70/72, Hsp73, and α B-crystallin) (Muller et al 1996; Aizawa et al 2000; Smoyer et al 2000), little attention has been paid to Hsp90 α and Hsp90 β expression in renal tissue. Thus, in the present study we assessed and characterized the expression and localization of both Hsp90 isoforms in rat kidney. Because of the potential relevance of Hsp90 isoforms as mediators of aldosterone function, we also analyzed the expression and distribution of Hsp90 in kidney of rats treated with a low-salt diet as a physiologic approach to activate the renin-angiotensin-aldosterone system (RAAS).

We observed that Hsp90 α and Hsp90 β are abundantly expressed in the renal cortex and the medulla. Both isoforms are expressed along the nephron, showing specific cellular patterns depending on the nephron segment analyzed. In addition, a low-sodium diet (LSD) was associated with upregulation of Hsp90 isoform expression in

the renal cortex and redistribution of both isoforms within thick ascending limb cells.

MATERIALS AND METHODS

Male Wistar rats weighing 300–350 g were used in the study. All procedures followed were in accordance with our institutional guidelines. Two groups of 22 rats were studied. Rats maintained for 21 days on a normal diet served as the control group (N), and rats fed with an LSD (0.02%), as the experimental group. At the end of the study, rats were placed in metabolic cages for 24-hour urine collection to assess urine osmolality and sodium excretion. In addition, blood samples were taken to evaluate serum aldosterone levels. Aldosterone and sodium were determined by radioimmunoassay and with a NOVA4 electrolyte analyzer (NOVA Biomedical, Waltham, MA, USA), respectively.

Eighteen rats of each group were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg), and their kidneys were macroscopically divided into renal cortex and medulla, frozen in liquid nitrogen, and maintained at -80°C until use. Total ribonucleic acid (RNA) was isolated individually from the renal cortex and medulla from 6 rats of each group according to the guanidine isothiocyanate–cesium chloride method, as we have reported previously (Bobadilla et al 1997, 1998). Integrity of isolated total RNA was examined by 1% agarose gel electrophoresis, and RNA concentration was determined by ultraviolet (UV) light absorbance at 260 nm (Beckman DU640, Brea, CA, USA). To avoid genomic DNA contamination, all RNA samples were treated with RNase-free DNAase I.

Primer sequences to amplify Hsp90 α and Hsp90 β and glyceraldehyde phosphate dehydrogenate (GADPH) as a housekeeping gene were custom obtained (Invitrogen, Carlsbad, CA, USA) (Rocco et al 1992; Tang et al 1995). Sense primer for Hsp90 α was 5' ACA AGC ACA TAT GGC TGG ACA GCA 3', and antisense primer was 5' TTC AGT TAC AGC AGC ACT GGTATC 3', whereas sense primer for Hsp90 β was 5' ACC AGC ACC TAC GGC TGG ACA GCC 3', and antisense primer was 5' GAT CTC ATC AGG AAC TGC AGC ATT 3'. To verify Hsp90 isoform amplification, single bands of 320 base pairs (bp) for Hsp90 α and 318 bp for Hsp90 β were obtained and resolved in 1.5% agarose gels. Polymerase chain reaction (PCR) products were observed with ethidium bromide, gel was purified (Qiagen, Hilden, Germany), and the products were automatically sequenced (I PRISM 310, Perkin-Elmer, Wellesley, MA, USA).

Relative quantitation of Hsp90 α and Hsp90 β

Relative messenger RNA (mRNA) levels of Hsp90 α and Hsp90 β were assessed in renal cortex and medulla by

semiquantitative reverse transcription (RT)-PCR, as we have described previously (Bobadilla et al 1997, 1998, 1999; Feria et al 2003). In brief, RT was carried out with Moloney murine leukemia virus reverse transcriptase (Invitrogen), using 2.5 µg of total RNA from each renal cortex and each renal medulla. Then, one-tenth of individual RT samples of each group were used for each amplification in 20-µL-final volume reactions containing 1× PCR buffer, 0.1 mM of each deoxynucleoside triphosphate, 0.2 µCi of [α^{32} P]-deoxycytidine triphosphate (~3000 Ci/mmol, 9.25 MBq, 250 µCi), 10 µM of each primer, and 1 unit of *Taq* DNA polymerase (Invitrogen). Complementary DNA (cDNA) amplification was carried out in a thermal cycler (M.J. Research, Watertown, MA, USA) with the following profile: denaturation for 1 minute at 94°C; annealing for 1 minute at 57°C, and a 1-minute extension step at 72°C. GAPDH was coamplified simultaneously in each reaction. Genomic DNA contamination was checked by running samples through PCR procedure without adding RT.

Amplification kinetics was performed according to our standard procedure (Bobadilla et al 1997, 1998, 1999; Feria et al 2003). To analyze PCR products, half of each reaction was electrophoresed in 5% acrylamide gel. Bands were ethidium bromide stained and observed under UV light, cut out, suspended in 1 mL of scintillation cocktail (Ecolume, ICN, Aurora, OH, USA), and counted by liquid scintillation (Beckman LS6500, Fullerton, CA, USA). The amount of radioactivity recovered from excised bands was plotted on a log scale against the number of cycles. To semiquantify each Hsp90 isoform, all reactions were performed individually from each cortex or medulla in duplicate during the exponential phase.

Protein extraction and Western blot analysis

Proteins were extracted into 2 separate pools per group. Each pool was obtained from 6 different cortices or 6 medullas by homogenization using a Kinematica polytron homogenizer (Switzerland) in 4 volumes of lysis buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM ethylenediamine-tetraacetic acid [pH 7.0], and 0.5 mM 3-(*N*-morpholino)propanesulfonic acid [pH 7.0], containing 5 mM benzamidine and 5 mM dithiothreitol). Homogenates were centrifuged at 4000 × *g* for 4 minutes at 4°C to remove tissue debris without precipitating plasma membrane fragments. Protein concentrations were assessed in duplicate using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA).

Protein samples containing 50 µg of total protein in 10 µL loading buffer (6% sodium dodecyl sulfate [SDS], 15% glycerol, 150 mM Tris, bromophenol blue 3%, β -mercaptoethanol 2%, pH 7.6) were denatured by boiling for 5 minutes and were electrophoresed by SDS-polyacryl-

amide gel electrophoresis (PAGE). Proteins were transferred onto polyvinyl difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 2 hours at 400 mA. Prestained rainbow markers (Amersham) were used as molecular mass standards. Nonspecific binding sites were blocked for 30 minutes at 37°C in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% nonfat dry milk. Thereafter, membrane was incubated with primary antibody anti-Hsp90 1:1000 (Transduction Laboratories, Lexington, KY, USA) for 30 minutes at 37°C. Membranes were washed for 60 minutes with TBS-T changed every 15 minutes. Membranes were incubated with secondary antibody horseradish peroxidase (HRP)-conjugated goat antimouse IgG 1:80 000 (Transduction Laboratories) for 30 minutes at 37°C and washed again. Proteins were detected by using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and autoradiography according to the manufacturer's recommendations. Bands were scanned for densitometric analysis.

Immunohistochemistry

In 4 rats from each group, kidneys were perfused with phosphate buffer through a femoral catheter at pressure corresponding to the mean arterial pressure of each animal. After blanching of the kidney, the perfusate was replaced by 10% freshly prepared buffered formalin, and perfusion was continued until fixation was completed. Tissue samples were embedded in paraffin, and kidney sections were cut to 3-µm thickness. Antigen retrieval for Hsp90 α , Hsp90 β , and Na⁺K⁺-2Cl cotransporter detection was carried out by microwave heating in citrate buffer (0.01 M, pH 5.4) before adding HRP-conjugated reagents; endogenous peroxidase activity was blocked by 10 µg *d*-biotin/mL H₂O₂ solution. Then, slides were incubated for 1 hour with ready-to-use rabbit polyclonal antibodies against Hsp90 α or Hsp90 β (neomarkers distributed by Lab Vision Corporation, Fremont, CA, USA) and against renal-specific Na⁺K⁺-2Cl cotransporter (generously provided by Mark Knepper). Slides were washed and incubated with biotinylated secondary antibody, and staining was completed using 3'-3'-diaminobenzidine tetrahydrochloride as chromogen (DAKO Liquid DAB+, DAKO Corp., Carpinteria, CA, USA), followed by counterstaining with Meyer hematoxylin. Tissue slides of N and LSD groups were processed simultaneously under similar conditions.

Statistical analysis

Significance was defined as 2-tailed $P < 0.05$, and results are presented as mean $\pm$ standard error of mean. Hsp90 isoform expression is shown as the ratio between Hsp90

Table 1 Effect of low-sodium diet (LSD) on body weight (BW), urinary sodium excretion (UNaV), fractional excretion of sodium (FENa), urinary osmolarity (UOsm), and serum aldosterone

Group	BW (g)	UNaV (meq/24 h)	FENa (%)	UOsm (mM)	Aldosterone (pg/mL)
N (normal diet)	281.0 $\pm$ 11.7	0.61 $\pm$ 0.130	0.236 $\pm$ 0.05	622.0 $\pm$ 87.1	97.6 $\pm$ 25.7
LSD	299.4 $\pm$ 4.1	0.03 $\pm$ 0.002*	0.005 $\pm$ 0.002*	257.7 $\pm$ 47.2*	1592.5 $\pm$ 268*

* $P < 0.05$ vs rats fed with normal salt diet.

Fig 1. Polymerase chain reaction (PCR) amplification kinetics for Hsp90 α and Hsp90 β in renal tissue. (A,C) Hsp90 α PCR product during different number of cycles in renal cortex and medulla total ribonucleic acid (RNA), respectively. (B,D) Hsp90 β amplification kinetics from renal cortex and medulla total RNA, respectively. Plots are expressed as log scale of counts per minute from complementary deoxyribonucleic acid bands cut from acrylamide gels. Insets show autoradiography of the same PCR products stained with ethidium bromide.

and GAPDH PCR product. Differences among groups were analyzed by an unpaired *t*-test.

RESULTS

Physiologic parameters from animals studied are shown in Table 1. Rats fed with an LSD had similar body weight to rats fed with a normal diet. As expected, the LSD group presented a significant reduction of urinary excretion of sodium, fractional excretion of sodium, and osmolarity. This reduction in sodium excretion was accompanied by an important elevation of serum aldosterone, a result of the well-known activation of the RAAS during sodium restriction, as extensively reported by other authors (Tarjan et al 1980; Rubattu et al 1994; Jo et al 1996).

Expression and localization of Hsp90 α and Hsp90 β

A PCR fragment of both Hsp90 α and Hsp90 β was amplified from cortex and renal medulla total RNA using specific primers. Both PCR products were sequenced. The degree of identity between rat Hsp90 α and that of mouse and human was 95.1 and 91.5%, respectively, and for Hsp90 β it was 92.5 and 85.6%, respectively (GenBank accession Nos. AY027778 and AY027779). Thus, amplified

Fig 2. Messenger ribonucleic acid (mRNA) levels of Hsp90 α and Hsp90 β and Hsp90 protein expression. White bands represent results obtained in cortex, and hatched bands indicate results obtained in renal medulla. (A,B) mRNA levels of Hsp90 α and Hsp90 β , respectively, assessed by semiquantitative reverse transcription-polymerase chain reaction and expressed as the ratio of Hsp90 to glyceraldehyde phosphate dehydrogenase. (C) Western blot analyses showing Hsp90 protein expression in renal cortex and medulla from normal rats. Western blot was carried out using 2 different cortex and medulla pools. (D) Densitometric analysis of equal amounts of protein. * $P < 0.05$ vs renal cortex.

bands corresponded to rat orthologs of Hsp90 α and Hsp90 β .

Amplification kinetics

Amplification kinetics for Hsp90 α and Hsp90 β cDNA from renal cortex and medulla total RNA are depicted in Figure 1. Two phases are clearly distinguished in each curve: the exponential and plateau phases. To evaluate the amount of expression of each Hsp90 isoform, we chose the midpoint of the exponential phase. As shown in Figure 1, amplification kinetics for Hsp90 α exhibited the exponential phase between 15 and 24 cycles in cortex and between 12 and 24 in renal medulla, whereas exponential phase for Hsp90 β was between 18 and 24 cycles in renal cortex and between 15 and 24 cycles in renal medulla. To routinely amplify and semiquantify Hsp90 isoforms, we chose 20 cycles, except for medullary Hsp90 α amplification, in which we used 18 cycles. Similar analysis was performed for GAPDH (data not shown).

Hsp90 α and Hsp90 β RT-PCR and Hsp90 Western blot analysis

Figure 2 depicts Hsp90 α and Hsp90 β mRNA levels expressed as the ratio between each isoform-amplified product over GAPDH from renal cortex and medulla total RNA. Semiquantitative RT-PCR analysis showed that Hsp90 α was more abundant in the renal medulla than in the renal cortex (1.21 ± 0.06 and 0.93 ± 0.04 , respectively; $P = 0.001$) (Fig 2A). In contrast, similar levels of Hsp90 β were observed between medulla and cortex (0.71 ± 0.05 and 0.75 ± 0.05 , respectively; $P =$ not significant) (Fig 2B). Greater expression of Hsp90 α in renal medulla was more apparent at the protein level (Fig 2C) because densitometric analysis from 2 different protein pools extracted from 6 normal cortices and 6 medullas revealed that Hsp90 band intensity in the medulla was higher than in the cortex (Fig 2D).

Hsp90 α and Hsp90 β immunohistochemical analysis

To evaluate whether Hsp90 α and Hsp90 β were expressed differently along the nephron, immunohistochemistry was performed to localize these proteins in renal tissue. In the glomeruli of normal rats, Hsp90 α and Hsp90 β staining was seen mainly in the glomerular capillaries, mesangial cells, and Bowman epithelia (as marked by the asterisk, arrowhead, and arrow, respectively, in Fig 3 B,C). In the proximal tubule, immunostaining for Hsp90 α and Hsp90 β was observed in the cytosol of the tubular epithelium (as marked by the asterisk in Fig 3 E,F, respectively). Interestingly, strong immunoreactivity was also detected in the proximal brush border membrane (indicated by short arrows), whereas no immunoreactivity was present in the basolateral side. In distal tubules a similar pattern was observed; the apical membrane showed more intense staining as compared with cytosol staining. In contrast, in cortical collecting ducts, Hsp90 isoforms principally exhibited intracytosolic distribution (figure not shown).

In the outer renal medulla, Hsp90 α and Hsp90 β were predominantly detected intracytosolically in the thick limb of Henle loop cells (Fig 3 H,I). Immunoreactivity for Hsp90 isoforms in the outer renal medulla was stronger than in the renal cortex and in the inner medulla sections. Interestingly, Hsp90 protein immunoreactivity observed in the outer medulla nearly disappeared from the cytosol of inner medullary tubular cells, to be localized predominantly in the basolateral epithelium (see arrows in Fig 3 K,L). In addition, strong positive staining of Hsp90 α and Hsp90 β was observed in the renal pelvis, in particular in dome-shaped and basal cells (marked with an asterisk in Fig 3 N,O). In contrast to the inner medulla, Hsp90 α and Hsp90 β were detected in the cytosol of collecting duct

cells of the papilla tip (as indicated by a double asterisk). In addition, Hsp90 α staining was also seen in interstitial cells and extracellular matrix (see arrowhead in Fig 3N), whereas faint Hsp90 β staining was detected in these regions (Fig 3O).

In addition, Hsp90 α staining was also seen in interstitial cells and extracellular matrix (see arrowhead in Fig 3N), whereas faint Hsp90 β staining was detected in these regions (Fig 3O).

Hsp90 α and Hsp90 β expression and localization with an LSD

Figure 4 A,B shows Hsp90 α and Hsp90 β mRNA levels in the renal cortex, respectively, from rats fed with an LSD as compared with normal rats. Hsp90 α mRNA levels were significantly more abundant in the LSD group than in the control group (2.6 ± 0.2 vs 1.7 ± 0.1 ; $P = 0.0005$) (Fig 4A). Similarly, Hsp90 β mRNA levels were greater in the LSD as compared with the N group (2.0 ± 0.2 vs 1.6 ± 0.1 ; $P = 0.017$) (Fig 4B). Upregulation of Hsp90 isoforms induced by low-sodium intake in the renal cortex was more apparent at the protein level. As shown in Figure 4C, Western blot analysis revealed that Hsp90 isoform densitometric value in the low-salt diet group was significantly higher than that observed in control rats (78.4 ± 1.2 AU vs 31.1 ± 3.5 AU; $P = 0.002$). Increase of Hsp90 α and Hsp90 β expression was observed only in the renal cortex.

In the renal medulla, Hsp90 α and Hsp90 β mRNA levels were not different between the LSD and N groups (Hsp90 α -GAPDH ratio was 0.7 ± 0.1 in N and 0.9 ± 0.1 in the LSD group, whereas the Hsp90 β -GAPDH ratio was 0.3 ± 0.03 in N and 0.5 ± 0.01 in the LSD group). Similarly, Hsp90 protein levels were not different in both groups studied (data not shown). However, although LSD induced no change in the amount of expression of Hsp90 proteins in the renal medulla, it was evident that sodium restriction was clearly associated with a change in Hsp90 α and Hsp90 β distribution of intracellular localization in thick ascending limb cells. As shown in Figure 5 A,B, Hsp90 α and Hsp90 β were localized in the cytosol of ascending limb cells of normal rats, whereas in LSD rats immunostaining was present mainly in the basolateral side of these cells (Fig 5 C,D). To corroborate basolateral Hsp90 redistribution in response to low-salt diet, nephron was stained with rabbit polyclonal antibody specific for Na⁺K⁺2Cl⁻ cotransporter (BSC1) because it has been extensively demonstrated that this cotransporter is exclusively expressed in the apical membrane of thick ascending limb (Kaplan et al 1996; Nielsen et al 1998; Mount et al 1999). Arrows in Figure 5E highlight immunostaining of apical membrane with anti-BSC1 antibody. A serial kidney section was used to colocalize

Fig 3. Immunohistochemistry of Hsp90 isoforms in renal cortex from rats fed with normal salt diet. (A,D,G,J,M) Negative immunostaining, (B,E,H,K,N) Hsp90 α immunoreactivity, (C,F,I,L,O) Hsp90 β immunoreactivity. (A–F) Renal cortex, (G–I) outer renal medulla, (J–L) inner renal medulla, (M–O) tip of papilla. In glomerulus, Hsp90 α and Hsp90 β immunoreactivity was observed in glomerular capillaries (see asterisk in B,C), mesangium (see arrowheads in B,C), and Bowman epithelium (see arrows in B,C). Under physiologic conditions, proximal and distal tubules were characterized by intense Hsp90 α and Hsp90 β staining in apical membrane (indicated by short arrows in E,F). Cytoplasm of proximal and distal tubules as well as cortical collecting ducts was diffusely stained (see asterisk in E,F). In outer medulla, staining of Hsp90 α and Hsp90 β was observed mainly in cytoplasm (H,I). In contrast, in inner medulla, reactivity was observed mainly in basolateral membrane (shown by arrows in K,L). In addition, immunostaining in outer medulla was more intense than in inner medulla and in renal cortex sections. In renal pelvis (N,O), intense Hsp90 α and Hsp90 β immunostaining was observed in epithelial lining of papilla (*) and in medullary collecting ducts (**). In addition, the arrow in (N) shows Hsp90 α reactivity in interstitial cells and extracellular matrix in tip of papilla, which was not observed for Hsp90 β . (A–L) Magnification 400 $\times$, (M–O) magnification 200 $\times$.

Hsp90 α in the same area. As shown in Figure 5F, immunostaining with anti-Hsp90 α antibody is positive in the opposite membrane to BSC1 immunoreactivity, indicating that Hsp90 α reactivity is located in the basolateral membrane. Thus, these observations suggest that with a low-salt diet, Hsp90 proteins in thick ascending limbs migrate from the cytosol to the basolateral membrane.

DISCUSSION

In the present study we assessed Hsp90 α and Hsp90 β expression in normal rat kidney and the effect of low-salt diet during 21 days. We observed that expression of both Hsp90 isoforms was higher in medulla than in renal cortex in normal rats. Immunohistochemical analysis revealed that Hsp90 α and Hsp90 β were similarly distrib-

Fig 4. Upregulation of Hsp90 induced by low-sodium intake in renal cortex. (A,B) Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of Hsp90 α and Hsp90 β and (C) Hsp90 Western blot analysis. (A,B) Upper autoradiographies represent Hsp90 α and Hsp90 β and lower glyceraldehyde phosphate dehydrogenase (GADPH) RT-PCR products obtained from individual cortices from N (white bars) and LSD groups (black bars). Graphs depict average and standard error of Hsp90 α -GADPH and Hsp90 β -GADPH ratios from each group. (C) Densitometric analysis of Western blot showing Hsp90 protein expression in renal cortex. Insets show immunoblots carried out using 2 different cortex pools. * $P < 0.05$ vs control group.

uted along the nephron, from glomerulus to papilla. Expression of Hsp90 isoforms, however, was upregulated by low-salt diet in renal cortex. In addition, sodium restriction resulted in the redistribution of both proteins within thick limb cells.

In renal cortex, Hsp90 α and Hsp90 β were expressed in glomerular capillaries, mesangial cells, and Bowman epithelia. Staining of Hsp90 α and Hsp90 β was also de-

Fig 5. Redistribution of Hsp90 α and Hsp90 β in outer medulla in rats fed with an LSD. (A,C) Outer medulla Hsp90 α immunostaining in N and LSD groups, respectively. (B,D) Outer medulla Hsp90 β immunoreactivity in N and LSD, respectively. Magnification 400 $\times$. Sodium restriction during 21 days induced redistribution of both Hsp90 proteins in thick limb of Henle loop. Hsp90 proteins nearly disappeared from cytoplasm to be redistributed in basolateral side. (E,F) Serial kidney sections of rat fed with an LSD staining with Hsp90 α and Na⁺K⁺2Cl⁻ cotransporter antibodies. Arrows in (E) show apical localization of Na⁺K⁺2Cl⁻ cotransporter in ascending limb cells, whereas arrows in (F) show basolateral localization of Hsp90 α in these cells.

tectable in cytosol of proximal and distal epithelial cells, with strong immunoreactivity in apical membrane. Aufrecht et al (1998) and Schober et al (1998) reported a similar pattern of expression for Hsp25 in proximal tubule, ie, predominant expression in apical side with less staining in cytoplasm or nucleus. In this study we also found that in outer medulla, Hsp90 α and Hsp90 β are mainly and abundantly located in the cytoplasm of thick limb cells, whereas in inner renal medulla, proteins were detected in the basolateral membrane of thin limb cells and collecting ducts. In addition, when collecting duct was near the renal pelvis, reactivity became more intense and was observed mainly in the cytoplasm of these cells.

It has been observed that Hsp90 expression in the rat proximal tubule is upregulated by nephrotoxic agents such as gentamicin or cisplatin (Beck et al 2000; Borkan and Gullans 2002). In addition, in human proximal tubule cells heat and arsenite induce expression of Hsp90 α and Hsp90 β , indicating that induction of Hsp90 may reduce renal damage during stress insults (Somji et al 2002). However, the physiologic role of constitutively expressed Hsp90 α and Hsp90 β proteins in kidney has never been addressed. Their intense expression pattern along the nephron suggests that these proteins are involved in several specific processes. Organized interactions between low-affinity mineralocorticoid receptor and Hsp90 heterocomplex are required to shape the mature receptor characterized by high hormone affinity (Borkan and Gullans 2002). In other words, in the absence of Hsp90 the affinity of mineralocorticoid receptor for aldosterone is very low. After binding of aldosterone to the high-affinity receptor, Hsp90 complex dissociates. Subsequently, aldosterone-receptor complex is translocated into the nucleus and binds to DNA steroid-responsive elements (Couette et al 1996, 1998). Thus, activation of mineralocorticoid receptor initiates a series of events that include sodium retention. In addition, it has been suggested that some aldosterone effects are mediated by the liberated Hsp90 heterocomplex rather than by the aldosterone-receptor complex (Tumlin et al 1997). Thus, a very interesting potential role for Hsp90 isoforms in renal physiology is modulation of the effects of aldosterone in kidney. In the present study we evaluated activation of the RAAS system induced by LSD on expression and localization of Hsp90 α and Hsp90 β in kidney. We observed that sodium restriction induced upregulation of Hsp90 α and Hsp90 β mRNA levels in the renal cortex, an effect more apparent at the protein level (Fig 4). It is possible, then, that increased Hsp90 expression serves to enhance response to aldosterone by switching more mineralocorticoid receptors to their high-affinity conformation. To test this hypothesis, it will be necessary to prevent association between Hsp90 and the mineralocorticoid receptor. However, compounds that possess this effect—such

as geldanamycin—also prevent interaction of Hsp90 with other proteins such as nitric oxide synthases, resulting in modification of renal hemodynamics, which by itself alters sodium reabsorption rates.

Aizawa et al (2000) and Ishizaka et al (2002) reported that angiotensin II was able to induce Hsp70, Hsp25, and HO-1 expression, particularly in the renal cortex. These studies suggest that angiotensin II could also be responsible for increasing expression of Hsp90 isoforms with a low-salt diet. Another possibility, however, is that aldosterone itself could induce expression of these proteins. Further studies will be necessary to determine the mechanism by which an LSD induces cortical Hsp90 isoform upregulation.

Another potential role of the Hsp90 subfamily is regulation of renal vascular tone and sodium excretion by the well-known positive effect of Hsp90 on nitric oxide generated by endothelial nitric oxide synthase (eNOS). García-Cardena et al (1998) observed that interaction between Hsp90 and eNOS in human umbilical vein and bovine lung endothelial cells enhanced production of nitric oxide by eNOS. In addition, Shah et al (1999) showed that interaction between Hsp90 and eNOS participated in regulating vasomotor function in mesenteric vessels; in addition, they demonstrated that excessive nitric oxide production in experimental portal hypertension was mediated in part by enhanced Hsp90-eNOS interaction. On the other hand, recent evidence shows that uncoupling of eNOS and Hsp90 results in increased eNOS-dependent superoxide generation (Pritchard et al 2001). In kidney, preliminary data (Carmines et al 2002) showed that uncoupling between Hsp90 and eNOS with geldanamycin reduced renal afferent arteriolar diameter, suggesting that interaction between Hsp90 and eNOS is required to maintain appropriate afferent tone.

Ortiz et al (2001) showed that nitric oxide production by eNOS in thick ascending limb resulted in reduction of salt reabsorption due to inhibition of Na-K-2Cl cotransporter. They also observed that increased tubular flow in isolated and perfused thick ascending limbs was associated with trafficking of eNOS to the apical membrane and that eNOS migrated together with Hsp90 (Ortiz et al 2002), suggesting that to reduce Na-K-2Cl cotransporter function, the eNOS-Hsp90 complex migrated to the apical membrane. In this regard, one of the most intriguing observations in the present study is that LSD was associated with redistribution of Hsp90 isoforms from the cytoplasm to the basolateral membrane in the thick limb of Henle loop. Thus, in a condition under which salt reabsorption in the thick ascending limb is enhanced, Hsp90 migrates to the basolateral membrane. Because a recent study revealed that Hsp90 stabilizes Na⁺-K⁺-ATPase within cells (Bidmon et al 2002), it is possible that migration of Hsp90 to the basolateral membrane during low-

salt diet not only resulted in preventing Hsp90-eNOS inhibition of Na-K-2Cl cotransporter but also in increasing the number or activity of basolateral Na⁺-K⁺-ATPase by Hsp90 chaperone activity. Additional studies will be necessary to provide insight into the role of Hsp90 in regulating the activity of renal cotransporters.

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