

Original Paper

Heat Shock Protein 70 and CHIP Promote Nox4 Ubiquitination and Degradation within the Losartan Antioxidative Effect in Proximal Tubule Cells

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Key Words

Proximal tubule cells • Angiotensin II • Losartan • Nox4/Hsp70/CHIP • Ubiquitination

Abstract

Background: Angiotensin II/Angiotensin II type 1 receptor (AT₁R) effects are dependent on ROS production stimulated by NADPH oxidase activation. Hsp70 regulates a diverse set of signaling pathways through their interactions with proteins. CHIP is a E3 ubiquitin ligase that targets proteins for polyubiquitination and degradation. **Aim:** We study whether Hsp70/CHIP contribute to the negative regulation of Nox4 after AT₁R blockage. **Methods/Results:** Primary culture of proximal tubule epithelial cells (PTCs) from SHR and WKY were stimulated with Angiotensin II (AII) or treated with Losartan (L) or Losartan plus Angiotensin II (L+AII). Losartan decreased AT₁R and Nox4 while enhancing caveolin-1 and Hsp70 protein expression in SHR PTCs. Immunoprecipitation and immunofluorescence proved interaction and colocalization of increased Hsp70/CHIP with decreased Nox4 in SHR PTCs (L) vs (AII). Hsp72 knockdown resulted in enhanced Nox4 protein levels, NADPH oxidase activity and ROS generation in (L+AII) revealing that Losartan was unable to abrogate AII effects on Nox4 expression and oxidative activity. Moreover, MG132 exposed PTCs (L) demonstrated blocked ubiquitinated Nox4 degradation and increased colocalization of Nox4/Ubiquitin by immunofluorescence. Conversely, Hsp72 depletion reduced Nox4/Ubiquitin colocalization causing Nox4 upregulation due to proteasomal degradation inhibition, although Losartan treatment. **Conclusion:** Our study demonstrates that Hsp70 and CHIP mediates the ubiquitination and proteasomal degradation of Nox4 as part of the antioxidative effect of Losartan in SHR.

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Introduction

The effects of the Angiotensin II/Angiotensin II type 1 receptor (AT₁R) are dependent on reactive oxygen species (ROS) production, which are stimulated by NADPH oxidase activation. ROS act as signalling molecules that mediate Angiotensin II-dependent signal transduction pathways [1, 2]. Data from *in vivo* and *in vitro* studies prove that locally generated Angiotensin II in proximal tubules (PT) induces renal injury through NADPH oxidase-dependent ROS production [3-6].

A role for the renin-angiotensin system in the development and/or maintenance of hypertension has been demonstrated in spontaneously hypertensive rats (SHR) [7]. Increased oxidative stress in SHR was found to contribute to proapoptotic and profibrotic mechanisms which are critical for renal injury [8].

Cloned from the kidney, Nox4 belongs to the Nox family of NADPH oxidases [9, 10]. Importantly, Nox4 is associated with mesangial cell, tubular cell or renal interstitial fibroblast dysfunction when responding to the factors that promote renal injury [11]. The mechanisms by which Nox4 is regulated in the kidney have not been determined. Caveolin-1 plays an important role as a platform for the compartmentalization of redox signaling events through NADPH oxidase-dependent production of ROS [12]. Thus, caveolin-1 is involved in Angiotensin II-induced NADPH activation in the kidney. Previously, we studied how caveolin-1 and Hsp70 possibly contribute to Nox4 expression regulation in SHR PT. We found that Losartan significantly increases Hsp70 membrane translocation and colocalizes with caveolin-1. The translocation of Hsp70 to PT membranes in Losartan treated SHR might exert a cytoprotective effect through Nox4 downregulation [13].

In SHR vascular smooth muscle cells (VSMCs), we reported on how a functional association of Hsp70 with Nox4/p22 phox provides a mechanism, which explains an antioxidative effect of Losartan along with subsequent cytoskeletal integrity modulation [14].

Hsp70 regulates a diverse set of signaling pathways through its interaction with proteins [15]. Carboxy terminus of Hsp70 interacting protein (CHIP), is a cytoplasmic protein that interacts with Hsp70 through its tetratricopeptide repeat domain (TPR), whereas its U-box domain contains its E3 ubiquitin ligase activity. CHIP regulates the Hsp70 chaperone function in part by driving the molecular triage decision, which determines whether proteins enter the productive folding pathway or result in client substrate ubiquitination and proteasomal degradation [16].

Growing from our previous findings, and for the reason that the mechanism that induces Losartan down regulation of Nox4 levels is not clearly understood, here we analyze Hsp70 and CHIP involvement.

We identify that indeed Hsp70 and CHIP are Nox4-interacting proteins, which promote its ubiquitination and proteasomal degradation within Losartan effect on SHR PTCs.

Materials and Methods

Reagents

Antibodies against Nox4, Hsp70, CHIP and β -actin were purchased Sigma-Aldrich (St. Louis, MO, USA). Antibodies against Megalin, caveolin-1 and AT₁R were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA) and against Ubiquitin by Millipore (California).

Animals

Spontaneously Hypertensive Rats (SHR) and Wistar-Kyoto (WKY) male rats (8-10 weeks old) were used. Indirect systolic blood pressure was measured by the tail cuff method prior to experimentation using a pulse sensor photoelectric CCP model in a Grass polygraph model 7 equipped with a 7P8 pre-amplified (Grass Medical Instruments). The average of 3 pressure readings was recorded. Body weight and systolic blood pressure are included in Table 1.

Table 1. Body weight and blood pressure in WKY and SHR rats

	WKY	SHR	<i>p</i>
Body Weight (g ± SEM)	153.75 ± 7.50	105.71 ± 6.20	<0.001
Blood Pressure (mmHg ± SEM)	116.10 ± 1.60	160.20 ± 1.33	<0.001

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the EAEC (Ethical Committee of Animal Experimentation of Argentina). All the experimental procedures of this study were previously approved by the Laboratory Animal Ethical Committee of the School of Medicine, Cuyo University, Mendoza (32/95 C.D.).

All surgery proceeding were performed under ketamine/xylazine (50 mg/kg and 2 mg/kg respectively) anesthesia, and all efforts were made to minimize animal suffering.

Cell Culture

The isolation of PTCs from SHR and WKY rats was performed as previously described [17, 18] with some modifications. Briefly, kidneys were excised and the cortex was separated. Fragments of renal cortex were desegregated and placed in ice cold Krebs-Henseleit solution (KHS) pH 7.4 and incubated with 95% O₂/5% CO₂ at 37 °C for 60 min. This suspension was centrifuged (60 g), the supernatant was discarded and the pellet re-suspended in ice cold KHS for thrice. Then, the pellet was re-suspended in 30 ml of ice-cold Percoll 50% solution in KHS and centrifuged for 30 min at 4 °C at 15.000 rpm on a Beckman Coulter centrifuge (Beckman Coulter, Inc, CA, USA). The fourth fraction was re-suspended in ice-cold KHS, washed 3 times as previously described and then re-suspended in DMEM F12 supplemented with 20% SFB. Cells were cultured with culture medium (DMEM F12 containing 1% penicillin/ streptomycin and 10% SFB), at 37 °C under 5% CO₂ in a humidified atmosphere. Cells were identified as PTCs by positive staining antibody against Megalin (Fig. 1A).

PTCs from SHR and WKY rats were stimulated with Angiotensin II (100 nM) for 15 min (AII), or treated with Losartan (10 μM) for 90 min (L) or with Losartan 90 min plus Angiotensin II for the last 15 min in the same concentrations (L+AII). Non-treated cells were used as control (C).

Flow Cytometry

Flow cytometry analysis was performed to determine PTCs primary cell culture purity. PTCs were resuspended fixation/permeabilization solution (BD Cytofix/Cytoperm, Bioscience) and incubated with Aqp-2 and Megalin antibodies, then incubated with the secondary antibody conjugated with FITC and APC and analyzed by flow cytometry. For each assay, at least 10000 cells were analyzed. FC analysis was performed with a FACSAria III cytometer (BD Bioscience). Data were analyzed with the FACSDIVA software (BD Bioscience). Controls included isotype-matched unspecific MoAbs used as negative controls. Flow cytometry analysis showed a 71.3% of PTCs expressing Megalin and 0.23% of Aqp-2 positive cells. The purity of the kidney PTC primary cell culture is reflected from the percentage of the cell population Megalin positive (Fig. 1B).

Protein Purification and Immunoblot Analysis

Western blot analysis was performed in the plasma membrane and the cytosolic fractions. Fractions were obtained as described Briones et al [19]. Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis. After protein transfer to nitrocellulose membranes, membranes were blocked, blotted with primary antibodies and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. A SuperSignal West Pico chemiluminescent substrate kit (Pierce/Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to visualize protein bands. Band densities were determined using the Image J software (Rasband WS, NIH, USA).

Reverse transcription-polymerase chain reaction analysis

The RT-PCR assay was performed as it has been previously described [13]. The cDNA for Nox4 NADPH oxidase subunit (primer sequences: TCAACTGCAGCCTGATCCTTT and TCTGTGATCCGCGAAGGTAAG) and β-actin (primer sequences: TGGAGAAGAGCTATGAGCTGCCTG and GTGCCACCAGACAGCACTGTGTTG) was

amplified. Densitometric analysis was performed by using Image J 1.6 software. The Nox4 signals were standardized against β -actin signal for each sample and results were expressed as a ratio.

Co-immunoprecipitation assay

Co-immunoprecipitation assays were performed as described by Ramirez et al [20]. Briefly, the anti-Nox4 was dissolved in a 0.1 M borate buffer pH 9.5, added to the Dynabeads M-280 Tosylactivated (DynaL Biotech). Normal Rabbit IgG (Sigma) was used as negative control.

Kidney microdissected proximal tubule membrane fractions were used as positive controls for Nox4 antibody (4). Following 48 h of incubation, the coated beads were washed with PBS containing 0.1% BSA and then with 0.2 M Tris pH 8.5 with 0.1% BSA and added to membrane samples containing equal quantities of protein. Incubated for 1 h, the samples were placed in the magnet and the supernatants were discarded. The beads were washed using a 0.1 M Na-phosphate and were suspended in sample buffer. Immunoprecipitated samples were separated on SDS-PAGE and immunoblotting for Nox4, Ubiquitin, Hsp70 and CHIP was performed as described above. The level of each protein was tested against the level of Nox4, and the results were expressed as the ratio.

Confocal Immunofluorescence Microscopy

The PTCs were plated on coverslips and treated as described previously. The cells were fixed in 4% paraformaldehyde, washed with PBS and blocked with 50 mM NH_4Cl , permeabilized with 0.05% saponin/0.5% BSA in PBS and incubated over night with primary antibodies as below, washed and incubated for 1 h with secondary antibodies fluorophores conjugated. Whole nuclei were visualized using Hoechst. The primary antibodies against Megalin, Hsp70, Nox4, CHIP and ubiquitin were used in a 1:100 dilution. The secondary antibodies used were Cy3-conjugated anti-mouse and Alexa 488-conjugated anti-rabbit or FITC-conjugated anti-goat. Images were obtained with an Olympus FV1000, (Olympus, Tokyo, Japan). The specificity of the immunostaining was evaluated by omission of the primary antibody. Co-localization coefficients were determined with JACoP Plugin Mac Biophotonics Image J using the Pearson overlap coefficient. The resulting coefficient value was the average of select image areas containing predominantly co-localization from at least 50 cells were examined in each group in three independent experiments.

Cell Transfection

Cell transfection assays were performed as described by Gil et al [14]. Briefly, for knockdown of Hsp72 expression, transient transfections were done with 2 $\mu\text{g}/\text{ml}$ shHsp72-pSIREN-RetroQ vector and/or pSIREN-RetroQ empty vector (ev) (Mock-transfection control) for 72 h using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. The shHsp72-pSIREN-RetroQ vector was generously provided by Dr. MY Sherman (Boston University Medical School, Boston, MA, USA). The vector contained the sequence of human Hsp72 as target for RNA interference: shHsp72 GAAGGACGAGTTTGAGCACAA (start 1961). No differences were shown between transfected empty vector cells and non-transfected cells. The transfection efficiency was evaluated in each experiment using pSIREN-DNR-DsRed-Express. After 72 h of transfection, cells were treated with AII or Losartan as previously described. The efficiency of silencing of Hsp72 was analyzed by western blot and immunofluorescence (Fig. 4A).

Measurement of NADPH Oxidase Activity

PTCs were treated with AII or Losartan as previously described. The lucigenin derived chemiluminescence assay was performed to determine NADPH oxidase activity in membrane fractions [21]. The luminescence was measured every 15 s for 3 min in a luminometer (Ascent Fluoroscan, Georg-Simon-OHM, Germany). Basal lectures in absence of NADPH was subtracted from each reading. Activity was expressed as percentage of arbitrary lights units. All assays were performed in duplicate.

Measurement of ROS in intact cells

Cells were loaded with CM-H2DCFDA 6 mM, dissolved in DMSO and incubated 45 min at RT. Fluorescence was measured continuously for 20 min on a microplate fluorometer (Fluoroskan Ascent, Labsystems) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Apocynin 50 mM was used as a negative control. ROS production was expressed in arbitrary fluorescence units. All assays were performed in duplicate.

Statistical Analysis

Data are presented as means \pm SEM from at least 3 independent experiments. Groups were compared using one-way analysis of variance (ANOVA). Bonferroni post hoc test was used to compensate for multiple testing procedures at as $p < 0.05$ significant level, using Prism 5 program (GraphPad Software Inc., La Jolla, CA, USA).

Results

The effect of the AT₁ receptor antagonist Losartan on caveolin-1 and AT₁R protein levels

As to examine caveolin-1 and AT₁R protein levels, immunoblot analysis was performed on SHR PTCs. The purity of this cell population was determined by immunofluorescence and flow cytometry assays (Fig. 1A and B).

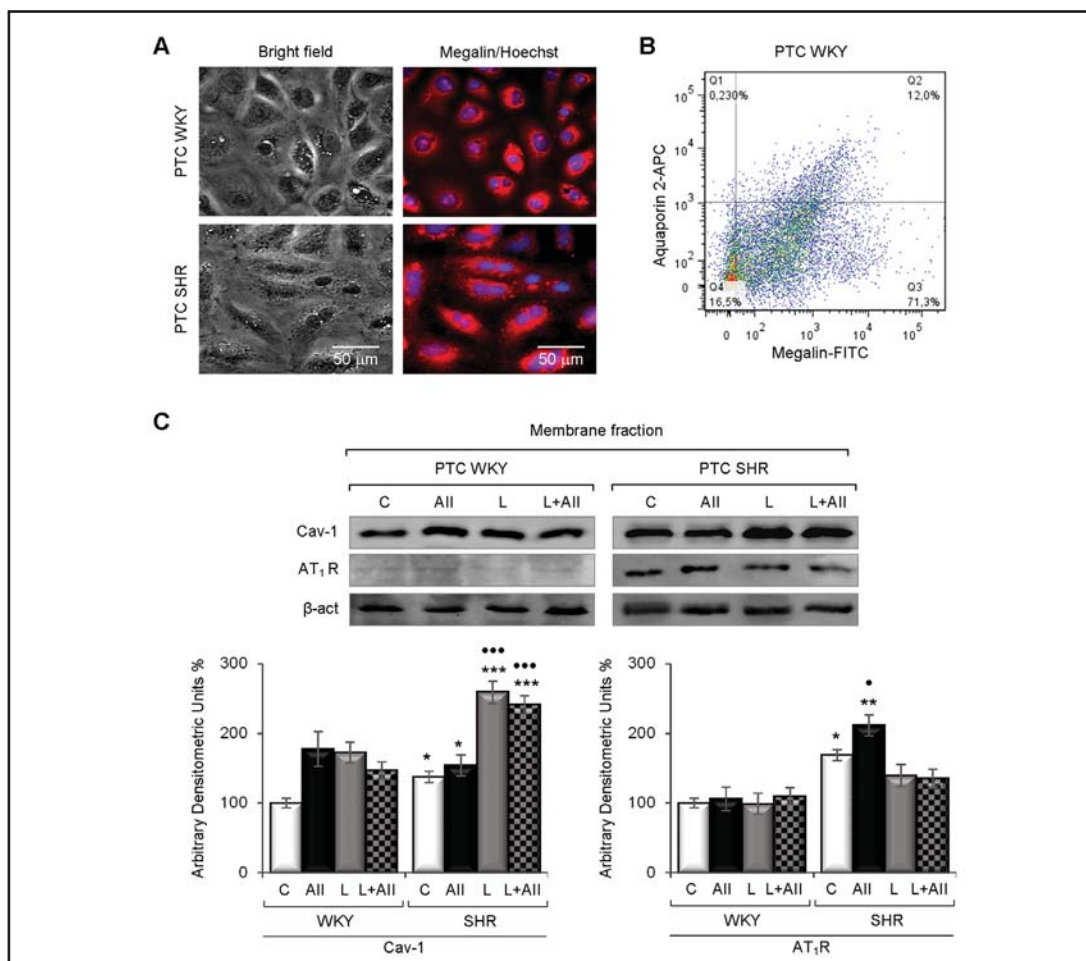


Fig. 1. Expression of caveolin-1 and AT₁R at protein levels in PTCs. Losartan effect. (A) Immunofluorescence of representative primary PTC culture from WKY (upper panel) and SHR (lower panel) stained with specific antibody for Megalin (red) and with Hoechst to visualize the nuclei (blue). Scale bar 50 μ m. (B) Flow Cytometry profile of PTC from WKY. Cells were stained with anti-megalin FITC and anti-Aqp2-APC. Flow cytometry analysis was performed using a FACS Aria III cytometer. (C) Immunoblot analysis of caveolin-1 and AT₁R from WKY and SHR PTC membrane and cytosol fractions. Cells were stimulated with Angiotensin II (100 nM) for 15 min (AII), treated with Losartan (10 μ M) for 90 min (L) or treated with Losartan for 90 min plus Angiotensin II for the last 15 min (L+AII). β -actin was used as a loading control. Band intensities were quantified by densitometric analysis. The ratio of caveolin-1/ β -actin (lower left panel) and the ratio AT₁R/ β -actin (lower right panel) were normalized to WKY control group PTC membranes. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs WKY PTCs control group (C). • $p < 0.05$; •• $p < 0.001$ vs SHR PTCs (C). Bars means \pm SEM, n=3.

Fig. 1C reveals higher AT₁R protein levels in SHR PTCs than in WKY PTCs. Focusing on SHR PTCs, after AII stimulation, we demonstrated higher levels of AT₁R associated with a slight decrease on caveolin-1 protein levels when compared with Losartan treatment. Likewise, losartan decreased AT₁R and increased caveolin-1 protein levels in VSMCs from SHR. The apparent association between the AT₁R and caveolin-1 turnover suggests that caveolin-1 and AT₁R may become physically associated with agonist stimulation.

Hsp70 translocation to the plasma membrane could be involved in the mechanism that is responsible for the Losartan effect on Nox4

Full AII expression signals a dependency on the ROS that is derivative of both: the NADPH oxidase and the functional relationship of AT₁R with caveolae. Nox4 is the major source of ROS in renal tubule cells [10]. Downstream redox regulation of cell functions includes the synthesis of HSPs in the stress response, such as for Hsp72 (an inducible fraction of Hsp70). CHIP, an Hsp70-associated E3 ubiquitin ligase, promotes protein degradation through the 26 S proteasome [16].

We hypothesized that the AT₁R blockage would mediate Hsp70 translocation, the chaperone involved in its antioxidative mechanism. We found that Losartan increased Hsp70 expression in SHR PTC membranes when compared with SHR PTC (C) and (AII) membranes.

The decreased Hsp70 in SHRPTC (L) vs (AII) in cytosolic fraction confirm that Hsp70 translocates to PTC membranes. Moreover, increased CHIP expression was shown in SHR PTC membranes after Losartan treatment when compared with PTC membranes stimulated with Angiotensin II (Fig. 2A). The lower Nox4 protein levels and the absence of differences in Nox4 mRNA (Fig. 2B) allow us to suggest that Hsp70/CHIP interaction may be involved in Nox4 proteasomal degradation after AT₁R blockage.

Hsp70/CHIP involvement in decreased Nox4 NADPH oxidase expression after Losartan treatment

In order to explore the potentially direct relationship between Nox4 and Hsp70/CHIP, we performed immunoprecipitation and immunofluorescence colocalization.

Previously treated membrane fractions from SHR and WKY PTCs were immunoprecipitated with anti-Nox4 antibody to assess the possibility that Hsp70/CHIP and Nox4 may become physically associated on AT₁R inhibition. The immunoprecipitates were then subjected to western blot analysis, with mAbs against Hsp70, CHIP and Nox4. As shown in Fig. 2C in SHR PTCs (L) and (L+AII) vs PTCs (C) higher protein levels of Hsp70 and CHIP interact with decreased Nox4 immunoprecipitation in the membrane fractions. Conversely, increased membrane Nox4 and lower Hsp70 and CHIP protein expression were shown in SHR PTCs (AII).

Furthermore, as shown by immunofluorescence, upregulated Hsp70 colocalizes with downregulated Nox4 exhibiting a punctate staining in a perinuclear distribution and at the plasma membrane in a pattern similar to the distribution of Nox4 in SHR PTCs (L) and PTCs (L+AII) (Fig. 3A). Nox4 labeling was also shown around the nucleus. A clear overlap between increased Nox4 and CHIP with punctuate staining in a perinuclear distribution was shown in Losartan treated SHR PTCs vs PTCs (C) (Fig. 3B). These results reveal that both Hsp70 and CHIP are linked to a decrease in Nox4 expression, which probably induces Nox4 ubiquitination when on route to its proteasomal degradation.

In order to further validate the association between Hsp70/CHIP and Nox4, SHR PTCs were transfected with shHsp72-pSIREN-RetroQ vector to transiently silence Hsp72 (inducible fraction of Hsp70) expression and then exposed to the different treatments. Hsp72 knockdown significantly decreased Hsp72 protein levels by 62% when related to ev SHR PTCs (Fig. 4A). Western blot analysis in SHR showed that Hsp72 knockdown led to higher Nox4 protein levels by 35% in PTCs (AII) and by 25% in (L+AII) treated PTCs with decreased CHIP protein levels by 35% in PTCs (L) when related to ev PTCs. Nox4 protein levels in transfected SHR PTCs (L) resembled those of the transfected PTCs at basal condition (Fig. 4B).

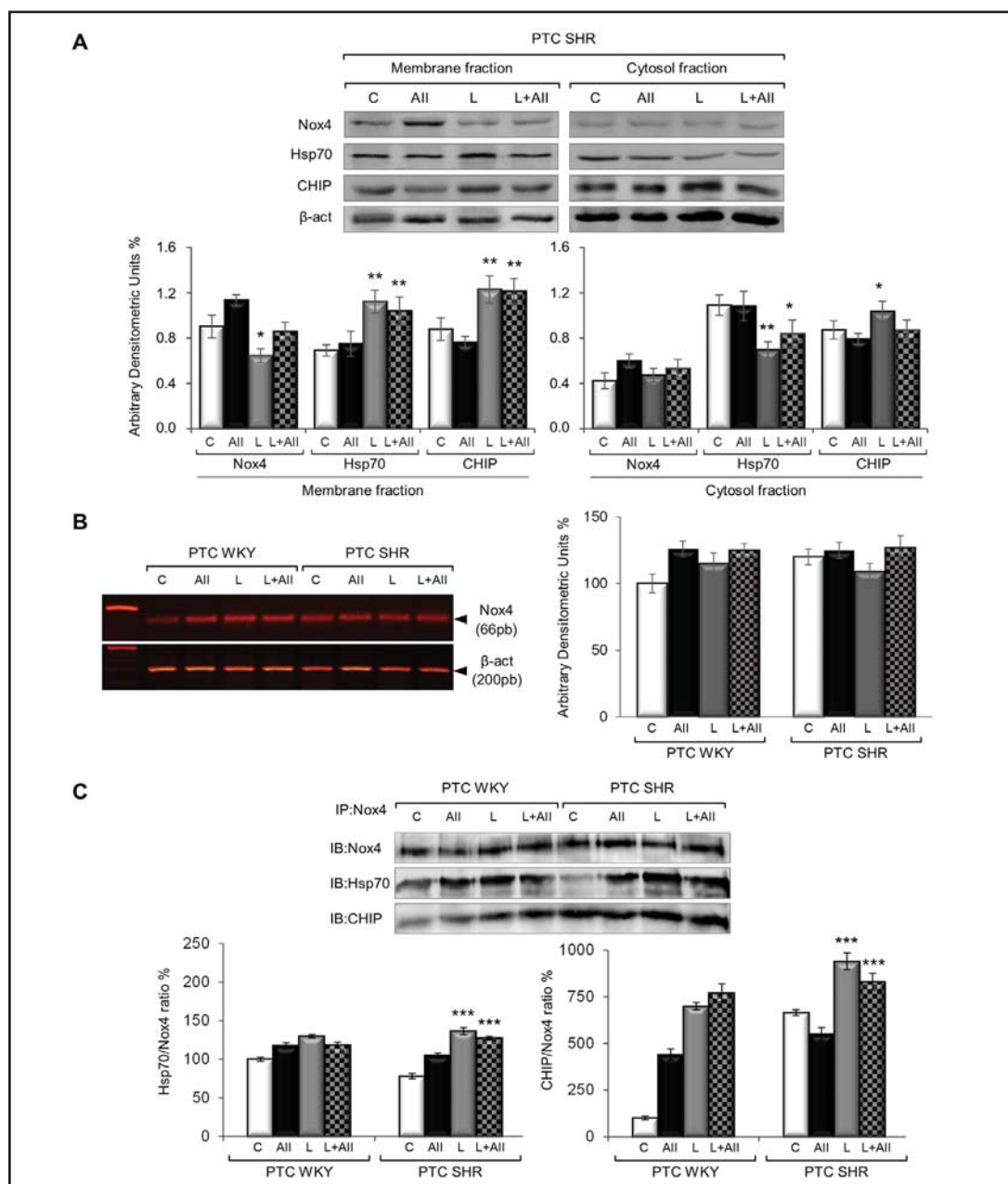


Fig. 2. Hsp70/CHIP involvement in decreased Nox4 NADPH oxidase subunit expression after Angiotensin II type 1 receptor antagonist treatment. WKY and SHR PTCs were stimulated with Angiotensin II (100 nM) for 15 min (All), treated with Losartan (10 μ M) for 90 min (L) or treated with Losartan for 90 min plus Angiotensin II for the last 15 min (L+All). (A) Immunoblot analysis of Nox4, Hsp70 and CHIP from PTC membrane and cytosol fractions of SHR. β -actin was employed as a loading control. Increased Hsp70 and CHIP and a lower expression of Nox4 were shown in PTCs (L) and (L+All) related to PTCs (All) membrane fractions. Band intensities were quantified by densitometric analysis. Bars means \pm SEM; n=3. *p<0.05, **p<0.01 vs PTCs (C). (B) Quantitative RT-PCR of Nox4 in WKY and SHR PTCs. Representative gels of Nox4 mRNA. The corresponding housekeeping β -actin is included in below. Densitometric analysis show the relative concentration of Nox4 mRNA to β -actin mRNA. Bars means \pm SEM; n=3. (C) Immunoprecipitation assay. WKY and SHR PTC membrane fractions were immunoprecipitated with anti-Nox4 antibody and immunoblotted with Hsp70 and CHIP antibodies. Band intensities were quantified by densitometric analysis. The ratio of Hsp70/Nox4 and the ratio CHIP/Nox4 are shown in WKY and SHR groups. ***p<0.001 significantly different from controls. Bars means \pm SEM; n=3.

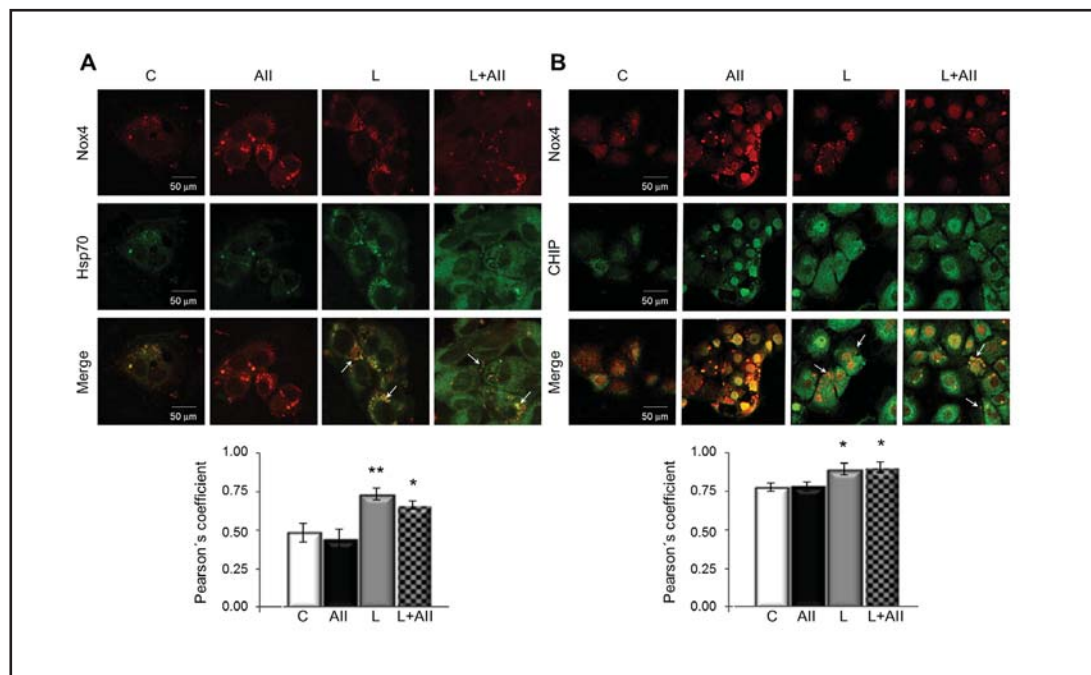


Fig. 3. Losartan treated effect on colocalization of Hsp70 and CHIP with Nox4 in PTCs from SHR. (A) PTCs were double labeled with Hsp70 (green) and Nox4 (red) antibodies. Arrows indicate areas of colocalization (yellow) in the merge. Colocalization of Nox4 with Hsp70 was determined using the Pearson's coefficient. * $p < 0.05$; ** $p < 0.01$ vs PTCs at basal conditions. (B) PTCs were double labeled with CHIP (green) and Nox4 (red) antibodies. Arrows indicate areas of colocalization (yellow) in the merge. Colocalization of Nox4 or with CHIP was determined using the Pearson's coefficient * $p < 0.05$ vs PTCs at basal conditions. Data are expressed as mean \pm SEM; 50 cells were analyzed for each treatment in three independent experiments. Scale bar 50 μ m.

After Hsp72 silencing, we next explored CHIP and Nox4 association by immunofluorescence. Nox4 staining was observed in a perinuclear homogeneous pattern of distribution in (L) and (L+AII) treated PTCs from SHR with a significant decrease in Nox4/CHIP colocalization when related to ev PTCs. Nox4 intense staining in a dotted pattern was shown in transfected SHR PTCs (AII).

For these results, after Hsp72 silencing of SHR PTCs, Losartan could not prevent an Angiotensin II enhanced Nox4 expression.

Hsp70 knockdown on NADPH oxidase activity and ROS generation by Nox4. The Losartan effect

To determine whether Hsp70 could be involved in the regulation of PTCs oxidase functioning, we measured NADPH oxidase activity and ROS production in Hsp72 knockdown PTCs.

Transfected PTCs with the plasmid to silence Hsp72 showed a higher NADPH oxidase activity and ROS production at basal conditions.

Ang II stimulation resulted in a significant increase in fluorescence levels. This response was sustained for up 900ms after Ang II addition. Apocynin, a selective inhibitor of NADPH oxidase, completely abolished the Ang II-stimulated response indicating that NADPH oxidase is the source of intracellular ROS (data not shown). The pre-exposure of SHR PTCs to Losartan, (L+AII), significantly reduced Ang II-mediated ROS generation.

In SHR PTCs (L) and (L+AII), the Hsp72 silencing caused a near two-fold (L) and three-fold (L+AII) increase in NADPH oxidase activity. There was also a significant increment of time-dependent intracellular ROS generation when compared with ev PTCs and non silenced Losartan treated PTCs (Fig. 5A and B).

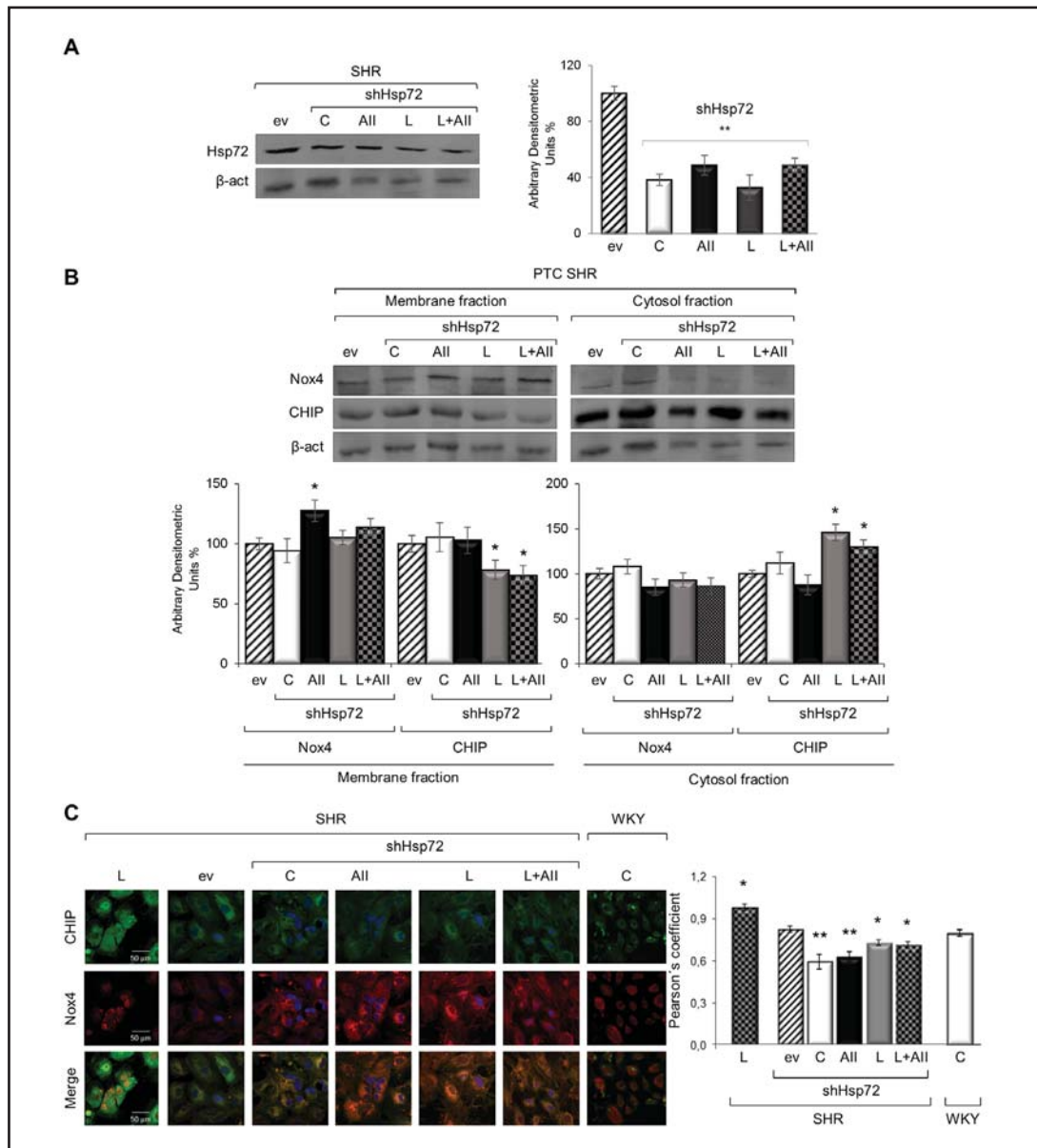


Fig. 4. Hsp72 knockdown effect on Nox4 NADPH oxidase and CHIP protein levels in SHR PTCs following Losartan treatment. SHR PTCs transfected with shHsp72-pSIREN-RetroQ vector to transiently silence Hsp72 expression and empty vector (ev). PTCs were stimulated with Angiotensin II (100 nM) for 15 min (All), treated with Losartan (10 μM) for 90 min (L) or treated with Losartan for 90 min plus Angiotensin II for the last 15 min (L+All). (A) The efficiency of silencing of Hsp72 from PTCs of SHR was analyzed by western blot $**p < 0.01$ vs Nt PTCs. Bars are means \pm SEM; $n = 2$. (B) Immunoblot analysis of Nox4 and CHIP from PTC membrane and cytosol fractions of SHR. β -actin was employed as a loading control. Band intensities were quantified by densitometric analysis. The ratio of Nox4/ β -actin and CHIP/ β -actin from membrane fractions (lower left panel) and from cytosol fractions (lower right panel) are shown. $*p < 0.05$ vs Nt PTCs. (C) Immunofluorescence co-localization of CHIP with Nox4 in PTCs from SHR and WKY rats. Immunofluorescence images of SHR empty vector PTCs (L) (left), PTCs transfected with shHsp72-pSIREN-RetroQ plasmid vector (shHsp72) and treated or not with Losartan (central) and empty vector WKY PTCs (right) were double labeled with anti-Nox4 (red) and CHIP (green), antibodies. Nuclei were labeled with Hoechst (blue). Colocalization of Nox4 with CHIP was determined using the Pearson's coefficient. $*p < 0.05$ and $**p < 0.01$ vs Nt PTCs. Data are expressed as mean \pm SEM; 50 cells were analyzed for each treatment in three independent experiments. Scale bar 50 μm.

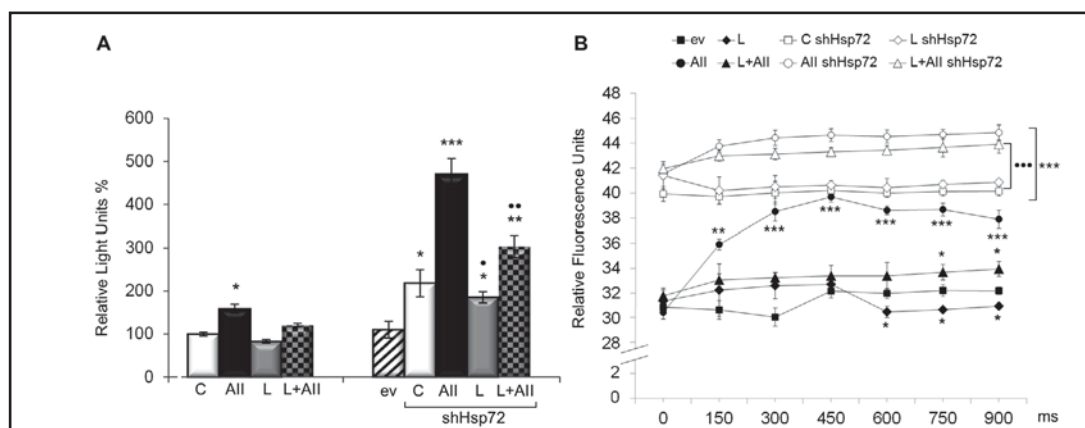


Fig. 5. Hsp72 knockdown effect on NADPH oxidase activity and intracellular ROS generation in SHR PTCs after Angiotensin II type 1 receptor antagonist treatment. (A) Lucigenin-enhanced chemiluminescence was used to measure NADPH oxidase activity in membrane fractions from empty vector PTCs and from knockdown Hsp72 PTCs exposed to Angiotensin II stimulation or Losartan treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with Nt PTCs. • $p < 0.05$; •• $p < 0.01$ vs Losartan treated PTCs. Bars are means \pm SEM; $n = 2$. (B) The generation of intracellular ROS as assessed by 5-(and-6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence in empty vector PTCs and from Hsp72 knockdown PTCs exposed to Angiotensin II stimulation or Losartan treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs Nt PTCs. ••• $p < 0.001$ vs Losartan treated PTCs. Values are presented as means \pm SEM; $n = 2$.

Losartan did not impair either NADPH oxidase activity or ROS generation in Hsp72 knockdown SHR PTCs. In sum, our data show that Hsp70 is involved in the Losartan antioxidative effect.

Hsp70 Promotes the Proteasomal Degradation of Nox4

In order to investigate the mechanism underlying Hsp70-mediated Nox4 downregulation, we studied the effect of Hsp70 on Nox4 ubiquitination and proteasomal degradation. SHR PTCs stimulated with AII or treated with Losartan, were exposed to the proteasome inhibitor MG132 to block degradation of ubiquitinated proteins. Immunoprecipitated Nox4 in SHR was western blotted for Hsp70, CHIP and Ubiquitin (Fig. 6A). The physical interaction among these proteins, in PTCs (L) exposed to MG132 treatment demonstrates an inhibited Nox4 protein degradation. Thus, the increased ratio of Nox4/CHIP reveals Nox4 ubiquitination without proteasomal degradation in the presence of Hsp70 (Fig. 6A). Furthermore, through immunofluorescence and colocalization analysis, (L) and (L+AII) SHR PTCs exposed to MG132 showed higher ubiquitinated Nox4 immunostaining with a perinuclear distribution related to non MG132 treated PTCs (Fig. 6B). Conversely, Hsp72 knockdown PTCs reveal decreased Nox4/Ubiquitin colocalization resulting in Nox4 upregulation because of the ubiquitination reduction, even with Losartan treatment (Fig. 6B lower panel).

In conjunction with these observations, Hsp70/CHIP promotes ubiquitination and degradation of Nox4 through a proteasome-dependent pathway.

Discussion

We identify a novel role for Hsp70 as a negative regulator of Nox4. Hsp70/CHIP mediates the ubiquitination and proteasomal degradation of Nox4 thereby inhibiting Nox4-dependent protein expression, which is included within the Losartan antioxidative effect on SHR PTCs.

AT₁R activation in PT by locally produced AII plays an important role in the pathogenesis of hypertension and renal injury.

AII stimulation promotes AT₁R interaction with caveolin-1 as well as the trafficking of the receptor from the membrane fractions into caveolin-1-enriched lipid rafts. This interaction

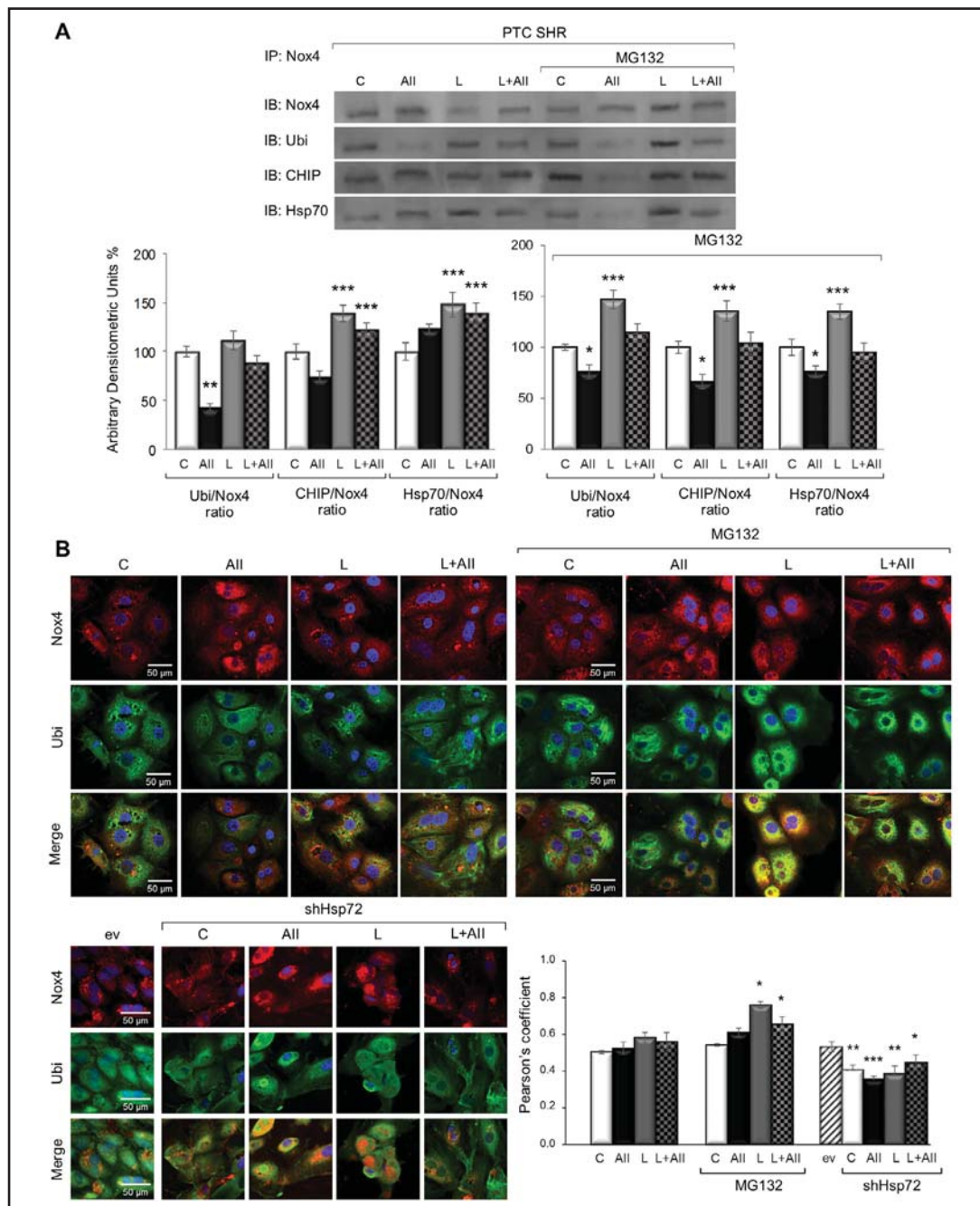


Fig. 6. Losartan effect on proteasomal degradation of Nox4 in SHR PTCs. PTCs were stimulated with Angiotensin II (100 nM) for 15 min (All), treated with Losartan (10 μ M) for 90 min (L) or with Losartan for 90 min plus Angiotensin II for the last 15 min (L+All) in presence and absence of 15 μ M MG 132. (A) Immunoprecipitation with anti-Nox4 antibody and immunoblotted with Nox4, Ubiquitin, Hsp70 and CHIP antibodies. Band intensities were quantified by densitometric analysis. The ratio of Ubiquitin/Nox4, CHIP/Nox4 and Hsp70/Nox4 of PTCs with or without MG132 treatment are shown. Bars means \pm SEM; n=3. *p<0.05, **p<0.01, ***p<0.001 vs PTCs (C). (B) Immunofluorescence of SHR PTCs treated with 15 μ M MG132 (upper right panel), non-MG132 treated PTCs (upper left panel) and knockdown Hsp72 PTCs (lower left panel) were stained with specific antibodies for Nox4 (red), Ubiquitin (green) and with Hoechst (blue) to visualize the nuclei. Colocalization of Nox4 with Ubiquitin was determined using the Pearson's coefficient. *p<0.05 vs PTCs (C). Data are expressed as mean \pm SEM; 50 cells were analyzed for each treatment in three independent experiments. Scale bar 50 μ m.

represents an important target for the dynamic control of receptor signalling [22, 23]. Addressing SHR PTCs, we showed that higher AT₁R protein levels were induced by the AII association with a slight reduction in caveolin-1 protein levels whereas Losartan treatment decreased AT₁R and enhanced caveolin-1 protein levels. It is possible that AII stimulation results in caveolae internalization and the translocation to intracellular compartments in which the protein is degraded [22, 24]. Signal-transducing molecules bind to caveolin-1, functioning as redox signaling events through NADPH oxidase-dependent production of ROS [25, 26]. Nox4, which is abundantly expressed in kidney PT cells [10], is functionally linked to oxygen sensing, is implicated as a major source of renal ROS [27, 28]. Notwithstanding, the mechanism whereby Nox4 activity is regulated remains unclear.

Here, we investigate the role of Hsp70 in the mechanism responsible for the effect of Losartan on ROS through its negative regulation of Nox4. Hsp70 plays a critical role in the recovery of cells from stress [29]. In doing so, Hsp70 facilitates competing pathways of protein folding and degradation [30, 31], which provides cellular protection by decreased oxidative stress [15].

As support to this statement, the association of Hsp70 with NADPH, quinone oxidoreductase 1, was demonstrated to play an important role in the stability and functionality of the protein [32]. We provided evidence for the translocated Hsp70 and Nox4 downregulation interaction in SHR microdissected proximal tubule membranes after AT₁R blockage [13].

It is known that Hsp70 promotes the proteasomal degradation of proteins by recruiting the ubiquitin ligase CHIP, a key component of the chaperone-dependent quality control mechanism [33, 34]. Client proteins are efficiently targeted by CHIP, particularly when they are partially or frankly misfolded, as is the case for most proteins binding to Hsp70 through exposed hydrophobic residues [16, 35]. In this way, Nox4 is roughly divided in two large domains: an N-terminal cluster of hydrophobic membrane-spanning sequences, and a C-terminal protein domain [36].

Our results demonstrate that Losartan induces the physical association between the higher amounts of Hsp70/CHIP that co-precipitated with lower Nox4 as an effect of Hsp70/CHIP interaction with Nox4 in SHR PTC membrane fractions. Nox4 protein downregulation and the unchanging Nox4 gene expression in SHR PTCs (L) suggest a chaperone involvement in Nox4 degradation. For this reason, immunofluorescence colocalization of Hsp70/CHIP with Nox4 was shown in (L) and (L+AII)-treated SHR PTCs. The enhanced Hsp70 and CHIP expression contrasts with the lower Nox4 labelling occurring at the PTCs (L). Conversely, Hsp72 knockdown impaired the decay of Nox4 protein levels and decreased CHIP expression despite Losartan treatment. This shows that Hsp70 protein directly interacts with Nox4 and mediates selective Nox4 degradation by its association with CHIP. Hsp72 silencing in SHR PTCs (L) lead to similar values of NADPH oxidase activity and ROS generation as it does for transfected SHR PTCs (C). Furthermore, we can infer that Losartan was unable to impair the SHR increased oxidative activity in Hsp72 absence due to the ability of the plasmid vector to transiently silence PTCs.

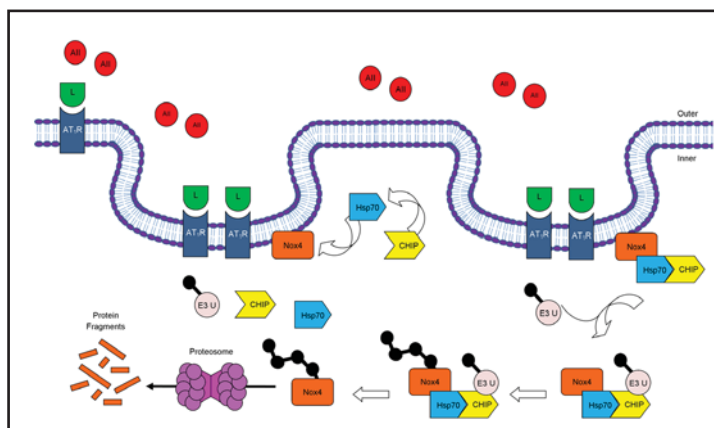
Taken together, these loss-of-function data indicate that Hsp70 is directly involved in Nox4 regulation through the physical and functional interactions that come under the antioxidative effect of Losartan.

Of note, CHIP acts to tilt the folding-refolding machinery toward a degradative pathway and it serves as a link between the chaperone and proteasome system; and, a recent study provided evidence on the Hsp70 interaction with HIF-1 leading to its degradation through recruitment the E3 ubiquitin ligase CHIP, as a molecular mechanism in prolonged hypoxia [37].

The carboxy terminus of Hsp70-interacting protein (CHIP), a member of E3 ubiquitin ligase, functions as a link between Hsp70 and the proteasome systems, playing a vital role in maintaining the protein homeostasis in the cytoplasm [35].

Our results show that Hsp70 and E3 ubiquitin ligase CHIP interact with Nox4, by inducing its ubiquitination and subsequent proteasomal degradation in the presence of Losartan.

Fig. 7. Proposed mechanism by which Hsp70/CHIP interaction mediates the negative regulation of Nox4 by its ubiquitination and degradation within the antioxidative effect of Losartan in PTCs from SHR.



In this regard, PTCs exposed to the proteasome inhibitor MG132 blocked degradation of ubiquitinated Nox4, even with Losartan treatment. Hence, the higher Nox4/CHIP ratio in SHR PTC (L) exposed to the proteasome inhibitor MG132 confirmed the enhanced ubiquitination of Nox4 without degradation. Moreover, immunofluorescence analysis shows that the increased colocalization of Nox4 with Ubiquitin following MG132 exposure was significantly decreased in Hsp72 knockdown PTCs (L), which lead to increased Nox4 levels due to its ubiquitination reduction, regardless of Losartan treatment. These results indicate that Hsp70 and CHIP selectively regulate the protein stability of Nox4 in the presence of Losartan as proposed in Fig. 7.

In conclusion, our data demonstrate that after AT₁R blockage, Hsp70 interacts and cooperates with CHIP to regulate Nox4 ubiquitination and degradation providing a possible explanation for the mechanism that drives the Losartan antioxidative effect.

This study provides new insights into the mechanisms responsible for Losartan therapeutic intervention against oxidative stress-mediated renal tubular injury.

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Disclosure Statement

We declare that have no conflicts of interest.

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