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ORIGINAL PAPER

Hsp70 regulation on Nox4/p22phox and cytoskeletal integrity as an effect of losartan in vascular smooth muscle cells

Andrea Fernanda Gil Lorenzo · Victoria Bocanegra · María Eugenia Benardon · Valeria Cacciamani · Patricia G. Vallés

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Abstract A series of signaling cascades are activated after angiotensin II binds to angiotensin II type I receptor (AT_1R), a peptide that is an important mediator of oxidative stress. Hsp70 regulates a diverse set of signaling pathways through interactions with proteins. Here, we tested the hypothesis of angiotensin II AT_1R inhibition effect on Hsp70 interaction with Nox4/p22phox complex and Hsp70 leading to actin cytoskeleton modulation in spontaneously hypertensive rats (SHR) vascular smooth muscle cells (VSMCs). SHR and Wistar–Kyotto rats (VSMCs from 8 to 10 weeks) were stimulated with angiotensin II (100 nmol/L) for 15 min (AII), treated with losartan (100 nmol/L) for 90 min (L), and with losartan for 90 min plus angiotensin in the last 15 min (L+AII). Whereas SHR VSMCs exposure to angiotensin II overexpressed AT_1R and Nox4 nicotinamide–adenine dinucleotide phosphate (NADPH) oxidase and slightly downregulated caveolin-1 expression, losartan decreased AT_1R protein levels and increased caveolin-1 and Hsp70 expression in SHR VSMC membranes. Immunoprecipitation and immunofluorescence confocal microscopy proved interaction and colocalization of membrane translocated Hsp70 and Nox4/p22phox. Increased levels of Hsp70 contrast with the decreased immunoprecipitation of Nox4/p22phox and RhoA in membranes from SHR VSMCs (L) vs SHR VSMCs (AII). Hsp72 depletion resulted in higher Nox4 expression and increased NADPH oxidase activity in VSMCs (L+AII) from SHR when contrasted with nontransfected VSMCs (L+AII). After Hsp72 knockdown in SHR VSMCs, losartan could not impair angiotensin II-enhanced stress fiber formation and focal adhesion assembly. In conclusion, our data showing a negative regulation of Hsp70 on Nox4/p22phox demonstrates a possible mechanism in explaining the antioxidative function joined to cytoskeletal integrity modulation within the effects of losartan in VSMCs from SHR.

Keywords Oxidative stress · Heat shock proteins · NADPH subunits · Spontaneously hypertensive rats · Stress fibers · Cytoskeleton integrity · Hypertension

Introduction

The G protein-coupled angiotensin II type I receptor (AT_1R) mediates the known physiological and pathological actions of angiotensin II and undergoes rapid desensitization and internalization after agonist stimulation (Touyz 2005). A series of signaling cascades are activated after angiotensin II binds to the AT_1R, the peptide being a potent mediator of oxidative stress. Reactive oxygen species (ROS) function as signaling molecules in vascular smooth muscle cells (VSMCs) and contribute to growth, hypertrophy, migration differentiation, and cytoskeletal remodeling (Valko et al. 2007). Full expression of angiotensin II signaling in VSMCs is dependent on ROS derived from nicotinamide–adenine dinucleotide phosphate (NADPH) oxidase and the dynamic association of the AT_1R with caveolae/lipid rafts (Ushio-Fukai et al. 1999; Hitomi et al. 2007). Therefore, caveolin plays an important role as a platform that provides a central driving force for the assembly of NADPH oxidase subunits and the recruitment of other related receptors and regulatory components. Downstream redox regulation of cell functions includes the synthesis of heat shock proteins (HSPs) in the stress response (Stephanou and Latchman 1999). HSPs may directly regulate specific stress-responsive signaling pathways. The cellular functions of

A. F. Gil Lorenzo · V. Bocanegra · P. G. Vallés
IMBECU-CONICET (National Council of Scientific and Technical Research of Argentina), Mendoza, Argentina

M. E. Benardon · V. Cacciamani · P. G. Vallés (✉)
Área de Fisiología Patológica, Departamento de Patología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Centro Universitario, 5500, Mendoza, Argentina
e-mail: pvalles@fcm.uncu.edu.ar

intracellular Hsp70 have been thoroughly studied and include limiting protein aggregation, facilitating protein refolding, and chaperoning proteins leading to improved cell survival (Hartl 1996; Nollen and Morimoto 2002). Moreover, certain HSPs (including Hsp70) provide cellular protection by decreasing oxidative stress linked with the upregulation of Hsp70 expression (Hartl 1996).

To support this statement, interaction of Hsp70 with NQO1, a potent antioxidant that catalyzes two-electron reduction of various quinones, with NADH or NADPH as an electron donor, was previously found in coimmunoprecipitation; interaction of Hsp70 could only be observed with wild-type NQO11 but not with mutant NQO12 protein (Anwar et al. 2002). Consistent with this notion, we previously studied caveolin-1 and Hsp70 contributions to the regulation of Nox4 expression in the proximal tubules of spontaneously hypertensive rats SHR, (Bocanegra et al. 2010).

As a multi-subunit enzyme complex, NADPH oxidases have the unusual composition of catalytic transmembrane-spanning subunits (NOX), as well as several structural and regulatory proteins localized in both the membrane and the cytosol (Bedard and Krause 2007). Among the membrane components of NADPH oxidase, Nox4 directly interacts with membrane-bound p22phox and does not require cytosolic subunits; it is capable of activation in the absence of p47phox or p67phox (Martyn et al. 2006). Nox4 has been identified in the endoplasmic reticulum, mitochondria, and the nucleus of vascular cells (Montezano and Touyz 2012). In addition, it appears to be localized at focal adhesions in VSMCs, a highly organized site at which the actin cytoskeleton attaches indirectly, through the proteins vinculin, paxillin, and talin, to transmembrane heterodimeric α- and β-integrins (Hilenski et al. 2004). Initial steps in integrin signaling for the assembly of focal adhesions are modulated by RhoA, which also plays a prominent role in regulating cytoskeleton organization by promoting the assembly of focal adhesions and actin stress fibers (Ridley 2001). The assembly of macromolecular complexes at membrane adhesion sites during the contractile stimulation of smooth muscle tissues is necessary for the initiation of actin filament polymerization and to regulate the formation of the adhesion complexes that link actin filaments to the extracellular matrix (Gunst and Zhang 2008). The molecular mechanisms by which G protein-coupled receptor activation links to signaling events that regulate actin polymerization in smooth muscle cells have not been fully delineated. The association of Nox4 with the cytoskeleton may have functional consequences in addition to providing compartmentalization of ROS production (Hilenski et al. 2004). The role of Hsp70 on cytoskeletal modulation and integral membrane protein interactions remains to be determined (Riordan et al. 2004).

In the genetic model of rat hypertension, SHR, a role for the renin–angiotensin system in the development and/or maintenance of hypertension has been demonstrated (Yoneda et al. 2005). Increased oxidative stress has also been described in human and in different models of hypertension, including SHR (Cruzado et al. 2005). In this study, we tested the hypothesis of Hsp70 regulation on Nox4/p22phox complex and cytoskeleton modulation included within losartan effects on VSMCs from SHR.

Methods

Materials

The antibodies Hsp70 (H5147), α-actin (A5228), vinculin (SAB42000080, 010M4785), GAPDH (G9545) were purchased from Sigma (St. Louis, MO, USA). Nox4 H-300 (sc-30141), p22phox (sc-20781), AT_1R (sc 570363), caveolin-1 (sc 894), and RhoA (sc-179) were provided by Santa Cruz Biotechnology.

Animals

SHR and Wistar–Kyotto (WKY) male rats (8–10 weeks old) were used. Indirect systolic blood pressure was measured by the tail cuff method 3–4 days before the start of the experiments using a pulse sensor photoelectric CCP model in a Grass polygraph model 7 equipped with a 7P8 preamplifier (Grass Medical Instruments). The average of three pressure readings was recorded. SHR systolic pressure was significantly higher than that in WKY (160.47±0.94 vs 107.14±1.81 mmHg, $p<0.0001$ respectively). WKY body weight was significantly higher than that of SHR rats (225.54±4.94 vs 178.48±4.07 g, $p<0.0001$, respectively).

Cell culture

VSMCs derived from mesenteric resistance arteries were isolated following the technique described previously (Touyz et al. 1994). Cells between the second and ninth passages identified as VSMCs by positive antibody staining against α-actin were used for these experiments. The cultures were not subjected to quiescence. In subconfluent cultures (50–90 %), the culture medium was replaced with a serum-free medium before experimental treatments. For each group of WKY or SHR, four different conditions were performed: control (C), angiotensin II (AII; 100 nmol/L), losartan (L; 100 nmol/L), and losartan plus angiotensin II (L+AII) in the same concentrations. VSMCs were treated with angiotensin II for 15 min with losartan for 90 min alone or exposed to losartan for 90 min adding angiotensin II for the last

15 min. VSMCs from SHR were stimulated with angiotensin II for this short period (15 min) in order to study the early intracellular signaling mechanisms.

Protein purification and immunoblot analysis

Western blot analysis for Nox4, p22phox, Hsp70, RhoA, vinculin, α-actin, caveolin-1, and AT_1R was performed in the cytosolic and/or in the plasma membrane fractions. Fractions were obtained as described (Briones et al. 2011). Cell suspension was subjected to three cycles of freezing and thawing. Nuclei and nonlysed cells were pelleted at 1000×*g* for 10 min at 4 °C. The supernatant was centrifuged at 100,000×*g* for 60 min at 4 °C. The membrane fraction contained in the pellet was resuspended in lysis buffer. The supernatant was designated cytosolic fraction. Extracted protein was quantified by the Bradford assay. Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes and treated as previously described. NHE1 was used as a specific control of the membrane fraction and tubulin as a control of cytosol fraction. GAPDH was used as a loading control. The images were analyzed using the McBiophotonic ImageJ software.

Immunocoprecipitation assay

VSMCs (10^7) were lysed as described in protein purification and immunoblot analysis sections. Immunocoprecipitation assays were developed according to previously described protocols (Ramirez-Sanchez et al. 2007). Briefly, the procedure was carried out using Dynabeads M-280 Tosylactivated (Dynal Biotech). Anti-Nox4 antibody was dissolved in 0.1 mol/L borate buffer pH 9.5, added to the Dynabeads, and then vortexed for 1 min. Following 48-h incubation and rotating at 4 °C, samples were placed in the magnet and the supernatants were removed and discarded. The coated beads were washed with a buffer containing PBS pH 7.4 with 0.1 % BSA and then with 0.2 mol/L Tris pH 8.5 with 0.1 % BSA. Subsequently, equal volumes of membrane and cytosol samples were adjusted to contain equal quantities of protein and added to the coated beads. Following 1-h incubation at 2–8 °C, the samples were placed in the magnet and the supernatants were removed and discarded. The beads were washed using a 0.1 mol/L Na-phosphate pH 7.4 and were suspended in sample buffer, and boiled for 3 min. The supernatant was removed and stored at −70 °C. Immunoprecipitated samples were separated on SDS-PAGE and immunoblotting for Nox4, Hsp70, p22phox, and RhoA was performed as described above. Kidney microdissected proximal tubule membrane fractions were used as positive controls for Nox4 antibody (Bocanegra et al. 2010). The level of each protein was tested against Nox4 protein levels and the results were expressed as the ratio.

Measurement of NADPH oxidase activity

VSMCs were treated with angiotensin II (100 nmol/L) alone for 15 min, losartan (100 nmol/L) for 90 min alone, or exposed to losartan for 90 min plus angiotensin II for the last 15 min. Cells were fractioned as previously described. The lucigenin-derived chemiluminescence assay was used to determine NADPH oxidase activity in membrane fractions (Callera et al. 2005). The luminescence was measured every 15 s for 3 min in a luminometer (Ascent Fluoroscan, Georg-Simon-OHM, Germany). Basal lectures in the absence of NADPH were subtracted from each reading. Activity was expressed as arbitrary light units per milligram protein.

Cell transfection

Primary culture of VSMCs was routinely cultured as described previously. For the silencing technique, subconfluent cultures were used. For the knockdown of Hsp72 expression, transient transfections were done with 2 μg/ml shHsp72-pSIREN-RetroQ vector and pSIREN-RetroQ empty vector (mock transfection control, empty vector (EV)) for 72 h using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the 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). The transfection efficiency was evaluated in each experiment using pSIREN-DNR-DsRed-Express (a gift from Dr. MY Sherman). After 72 h of transfection, the medium was removed and the cells were treated with angiotensin II and losartan as previously described. For the western blot analysis, a lysated cell was used. Immunofluorescence and confocal microscopy were performed on fixed cells. The efficiency of Hsp72 silencing was analyzed by immunofluorescence and immunoblotting in protein total fraction. VSMCs without transfection and VSMCs transfected only with the empty vector were considered as negative controls.

Confocal immunofluorescence microscopy

VSMCs cells were fixed with a 4 % paraformaldehyde solution in PBS for 20 min at 37 °C, washed with PBS and blocked with 50 mM NH_4Cl in PBS, permeabilized with 0.05 % saponin in PBS containing 0.5 % BSA, and incubated with the following primary antibodies in a 1:100 dilution: mouse monoclonal against Hsp70, rabbit

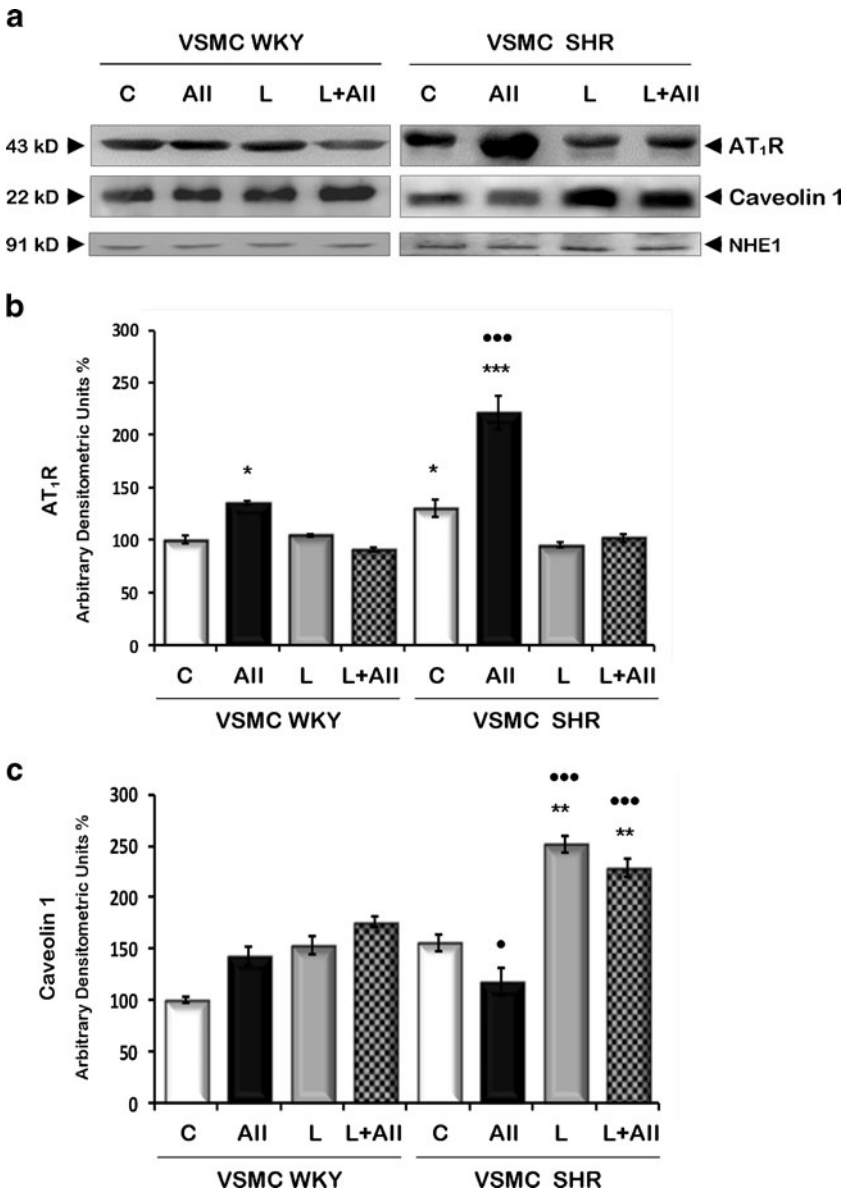
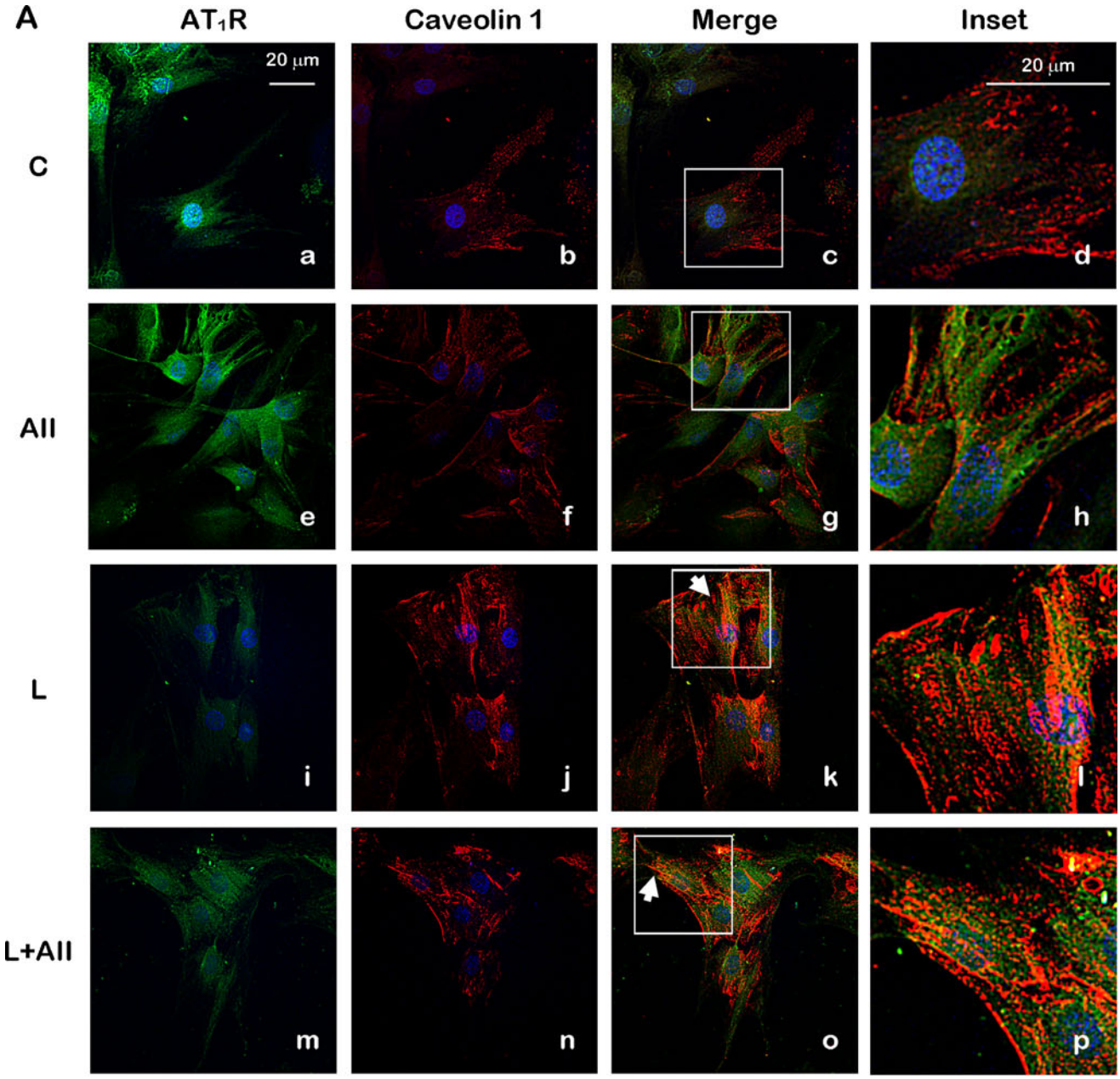


Fig. 1 Expression of caveolin-1 and angiotensin II AT_1R at protein levels in VSMCs. Losartan effect. VSMCs from WKY and SHR rats were stimulated with angiotensin II (100 nmol/L) for 15 min (*AII*), treated with losartan (100 nmol/L) for 90 min (*L*), and treated with losartan for 90 min plus angiotensin II for the last 15 min (*L+AII*). Membrane fractions were subjected to western blot analysis with anti-AT_1R and anti caveolin-1 antibodies. NHE1 was used as a membrane fraction control. (**a**) Band intensities were quantified by densitometry. The ratio of AT_1R/NHE1 (**b**) and the ratio of caveolin-1/NHE1 (**c**) were normalized to membrane WKY control group. *$p<0.05$; **$p<0.01$; ***$p<0.001$ vs WKY control group. •••$p<0.001$ vs SHR VSMCs at basal conditions. *Bars* means±SEM, $n=4$

monoclonal against vinculin, rabbit polyclonal against p22phox, and against Nox4. Cy3-conjugated mouse monoclonal antibodies against α-SM actin were used. The cells were then incubated for 1 h at room temperature with a mixture of the two or three secondary antibodies conjugated with fluorescent compounds Cy2, Cy3, Cy5, or fluorescein isothiocyanate according to the requirement. Hoechst was used for cell nuclei stain. The protein distribution and colocalization were analyzed by immunofluorescence and confocal microscopy. The specificity of the immunostaining was evaluated by omission of the primary antibody. Confocal laser microscopy was performed using an Olympus FV1000, USA with a 60× oil objective. Quantitative colocalization analysis was performed in dual-color confocal microscopy images of cells stained with fluorescence-labeled antibodies. Colocalization coefficients

Fig. 2 Immunofluorescence/cytochemical localization of caveolin-1 and angiotensin II AT_1R in VSMCs from SHR and WKY rats. VSMCs from SHR (**a**) and WKY (**b**) without and with losartan treatment were double-labeled with an antibody against caveolin-1 and with an antibody against AT_1R followed by antirabbit Cy3-conjugated and antimouse fluorescein isothiocyanate-conjugated secondary antibodies. Images are representative of two different experiments. In SHR cells stimulated with angiotensin II for 15 min, AT_1R immunoreaction (*green*) becomes more prominent in cell membranes (*e* in **a**), low caveolin-1 immunoreaction (*red*) was demonstrated (*f* in **a**), and the merged image shows colocalization (*g* in **a**). After losartan administration to VSMC SHR, low AT_1R staining (*i* in **a**) and increased immunoreactive caveolin-1 (*j* in **a**) were shown in the plasma membrane, with colocalization of both proteins (*k* in **a**). In WKY VSMCs after losartan treatment, slight AT_1R and caveolin-1 staining was identified with an absence of colocalization (k in a). Images are representative of two different experiments. *Scale bars* 20 μm. Magnification ×640. Colocalization of both proteins was determined using the Pearson's coefficient (*q*), ***$p<0.001$ versus SHR VSMCs at basal conditions. Data are expressed as mean±SEM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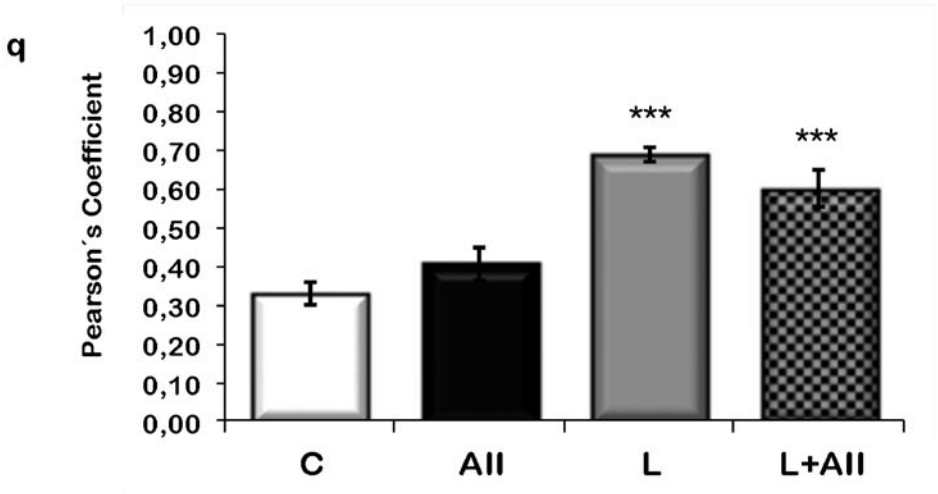
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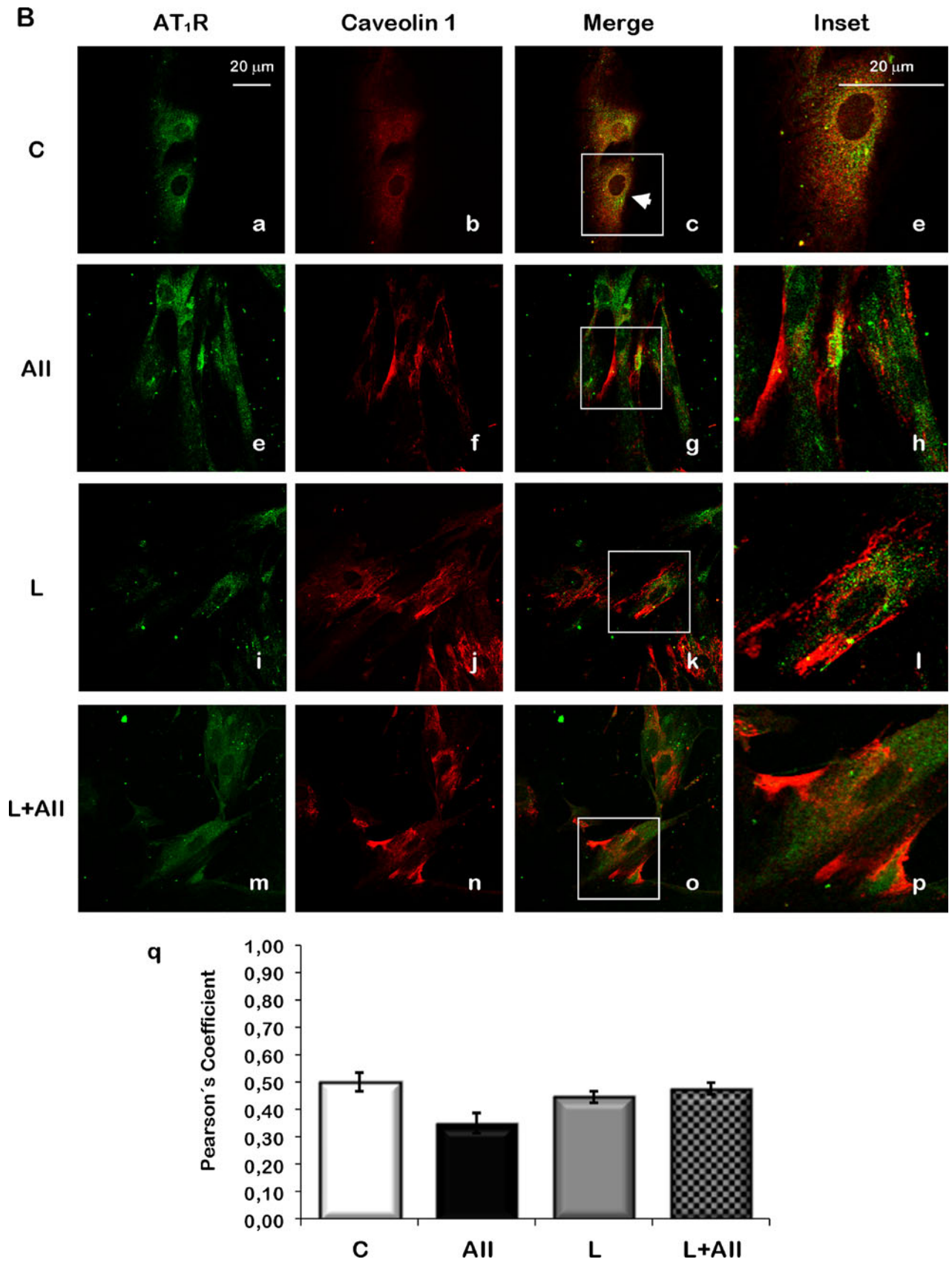

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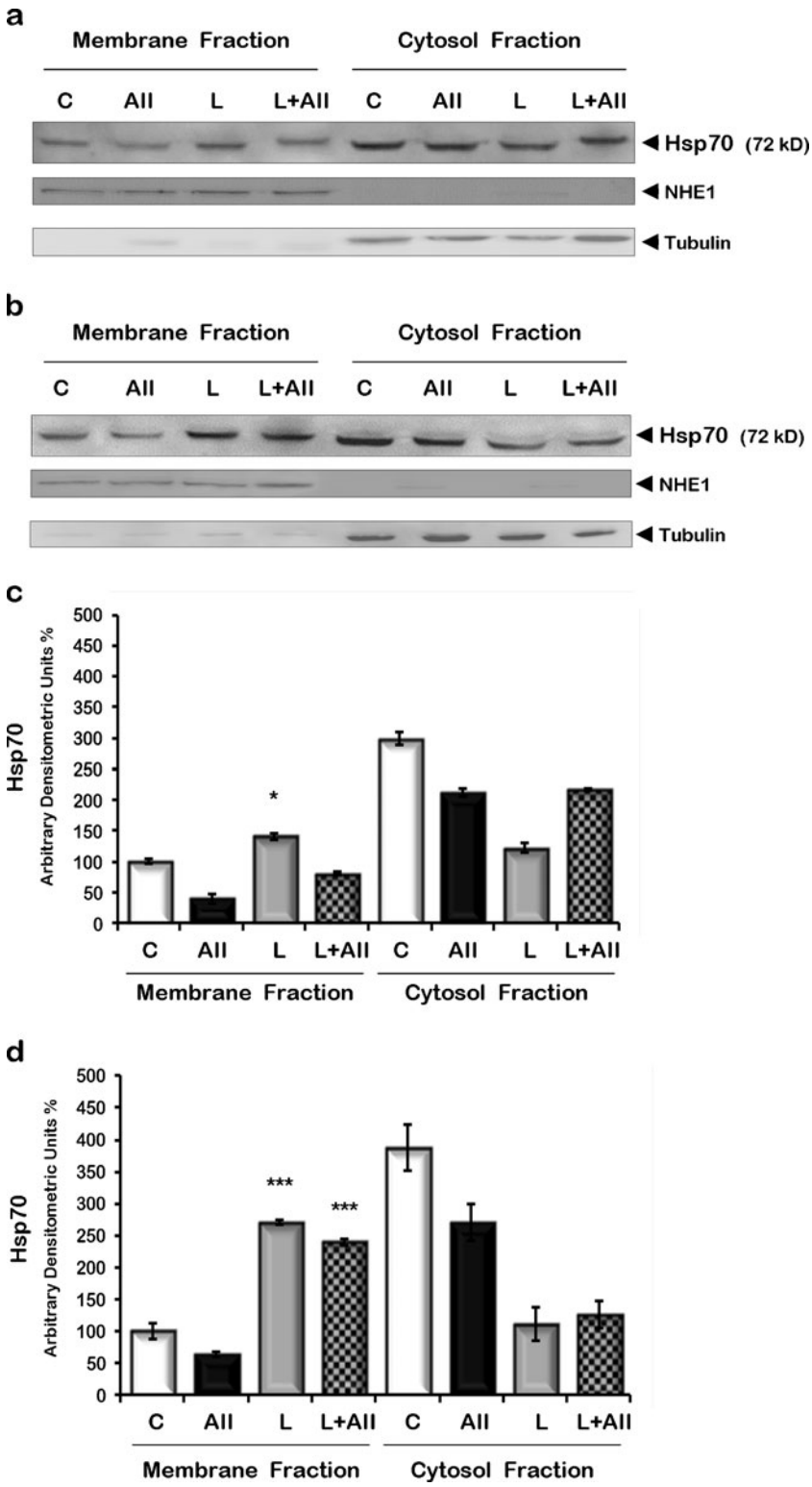


Fig. 3 Losartan-mediated Hsp70 translocation to the membrane in VSMCs from SHR. Cells were stimulated with angiotensin II (100 nmol/L) for 15 min, with losartan (100 nmol/L) for 90 min, and with losartan (100 nmol/L) for 90 min plus angiotensin II stimulation for the last 15 min. Semiquantitative immunoblotting of VSMC membrane and cytosol fractions from WKY (**a**) and SHR (**b**) was performed. NHE1 and tubulin were used as membrane and cytosol fraction controls, respectively. Densitometric analysis: the ratio of Hsp70/NHE1 or Hsp70/Tubulin was normalized to membrane WKY (**c**) and SHR (**d**), respectively. *$p<0.05$; ***$p<0.001$ significantly different from controls. *Bars* means±SEM; $n=4$

were determined with JACoP Plugin Mac Biophothonics Image J 1.43 m (Wayne Rasband NIH, USA) using the overlap coefficient according to Pearson. The resulting coefficient value was the average of select image areas containing predominantly colocalization from at least 15 cells that were examined in each group.

Statistical analysis

Data are presented as means±SEM. Groups were compared using one-way analysis of variance and the Bonferroni post hoc test was used to compensate for multiple testing procedures. A value of $p<0.05$ was significant.

Results

Angiotensin II AT_1R and caveolin-1 in vascular smooth muscle Cells

As well as its structural role, caveolin-1 is directly implicated in interactions with signaling proteins. In VSMCs, angiotensin II induces rapid (<2 min) translocation of a portion of the AT_1R from heavy membrane fractions to caveolin-1-enriched lipid rafts, and AT_1R and caveolin-1 become associated (Ushio-Fukai and Alexander 2006). If the agonist-occupied AT_1 receptor is internalized via caveolae, angiotensin II stimulation might result in decreasing caveolin protein levels due to internalization (Ishizaka et al. 1998). Figure 1 shows higher AT_1R levels in SHR VSMCs than in WKY VSMCs and focused on the SHR cells, the higher levels of AT_1R induced by angiotensin II are associated with a slight reduction in caveolin-1 protein levels. Likewise, losartan treatment decreased AT_1R and increased caveolin-1 protein levels in VSMCs from SHR.

Next, we explored the direct relationship between caveolin-1 and AT_1R by immunofluorescence confocal microscopy. Minimal caveolin-1 staining and high AT_1R immunoreaction were identified in membrane surfaces from angiotensin II-stimulated SHR cells (f in Fig. 2a). After L treatment, increased caveolin-1 protein expression and reduced AT_1R labeling were shown in SHR VMSC membranes when compared to angiotensin II-stimulated VSMCs. An increase overlap between these proteins (caveolin-1 and AT_1 R) is shown in losartan and losartan plus angiotensin II treatment in the merged images (k and o in Fig. 2a) vs control SHR VSMCs. In WKY VSMCs, minimal AT1 receptor expression was identified as expected, moderate membrane caveolin-1 staining occured, and colocalization between these proteins was negligible after losartan treatment (k in Fig. 2b).

Angiotensin II AT_1R inhibition treatment induced the expression of Hsp70 protein levels at the plasma membrane in SHR

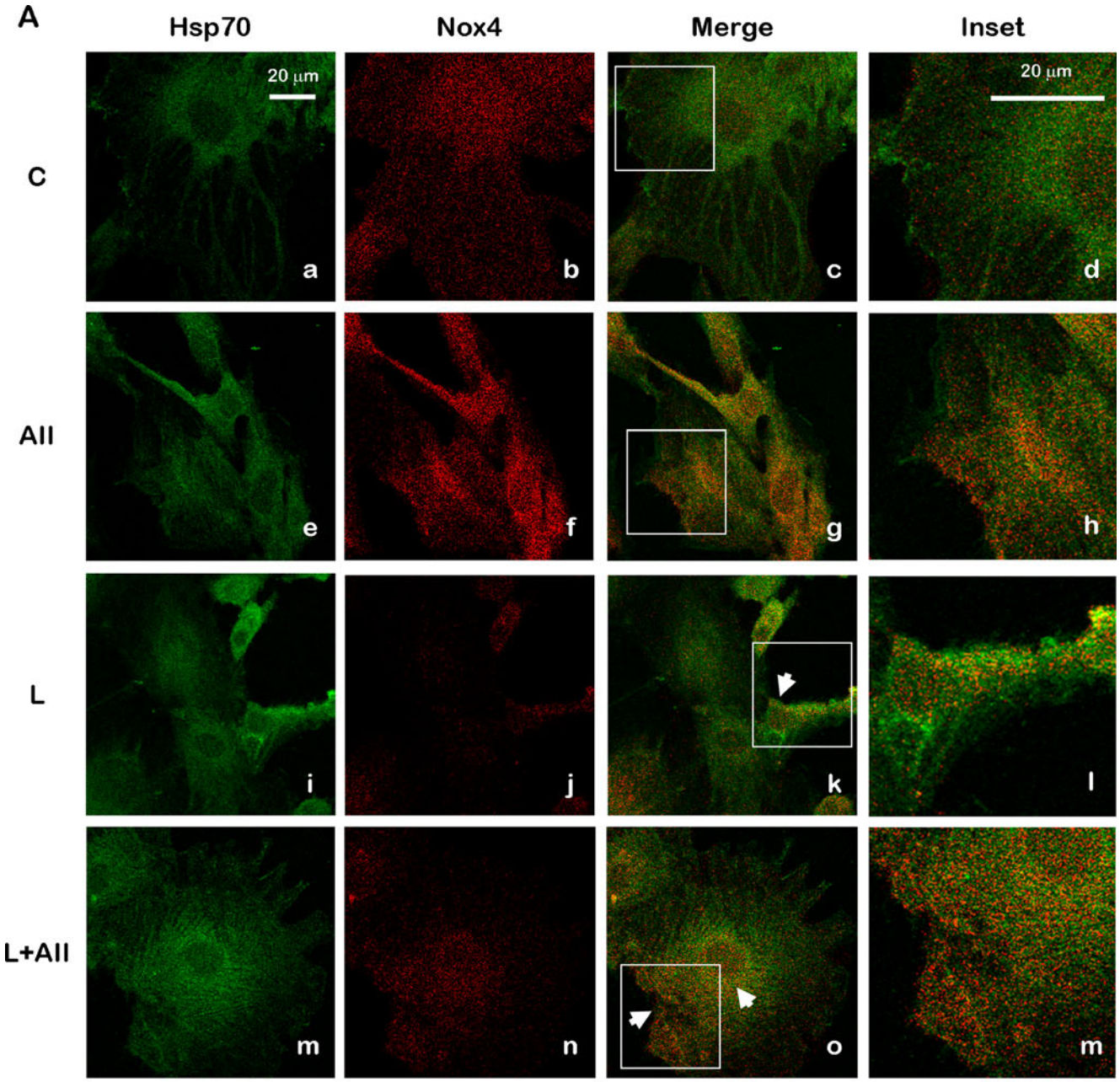
To get insight into the mechanisms involved in AT_1R inhibition in SHR, we looked for the expression of chaperone Hsp70 protein levels. We hypothesized that angiotensin II AT_1R antagonist treatment would mediate Hsp70 translocation to the plasma membrane. Cells were treated with angiotensin II (100 nmol/L) for 15 min, with losartan (100 nmol/L) for 90 min, and with losartan (100 nmol/L) added to the cells for 90 min plus angiotensin II stimulation for the last 15 min. After 90 min of L treatment and L+AII, an increased Hsp70 expression was confirmed in SHR VSMC membranes when compared with SHR VSMCs at basal conditions and with angiotensin II-stimulated SHR VSMC membranes (Fig. 3).

Fig. 4 Colocalization of Hsp70 with Nox4/p22phox in losartan treated VSMCs from SHR and WKY. Immunofluorescence confocal microscopy. Confocal images of VSMCs from SHR (**a**) and WKY (**b**) were double-labeled with Hsp70 (*green*) and Nox4 (*red*) antibodies. Nuclei are labeled with Hoechst (*blue*). *Arrows* indicate areas of colocalization (*yellow*) in the merge. As shown, in losartan-treated VSMCs from SHR increased Hsp70 immunoreaction (*i* in **a**) colocalizes with downregulated Nox4, with a perinuclear distribution and at the cell periphery (*k* in **a**). In addition, Hsp70 labeling with a fibrillar pattern extending along actin fibers was shown in L- and L+AII-treated VSMCs. In losartan plus angiotensin II, perinuclear colocalization of both proteins with punctate distribution is shown (*o* in **a**). Losartan treatment in VSMCs from WKY shows areas of colocalization of both proteins in the merge (*k* in **b**). *Scale bars* 20 μm. Magnification ×640. VSMCs from SHR (**c**) and WKY (**d**) were double labeled with anti-Hsp70 (*green*) and anti-p22phox (*red*) antibodies. *Arrows* indicate areas of colocalization (*yellow*) in the merge. Confocal microscopy shows colocalization of increased Hsp70 with p22phox labeling in a punctuate pattern, principally around the nucleus and at the cell periphery, similar to Nox4 in losartan (*k* in **c**) and losartan plus angiotensin II-treated VSMCs from SHR (*o* in **c**). Perinuclear colocalization of both proteins after losartan and losartan plus angiotensin II treatment was observed in VSMCs from WKY (*k* and *o* in **d**). *Scale bars* 20 μm. Magnification ×640. Colocalization of Hsp70 with Nox4 or with p22 phox was determined using the Pearson's coefficient (*q*), ***$p<0.001$ versus SHR VSMCs at basal conditions. *$p<0.05$ versus WKY VSMCs at basal conditions. Data are expressed as mean±SEM

Involvement of Hsp70 on decreased Nox4/p22phox NADPH oxidase subunit expression after angiotensin II AT_1R inhibition

Next, we studied the after-effects of angiotensin II AT_1R inhibition to see whether the membrane translocated Hsp70 interacts with NADPH oxidase subunits p22phox and Nox4 or not. Colocalization and coimmunoprecipitation strategies were used.

As shown in Fig. 4, by immunofluorecence, SHR losartan-treated VSMCs show higher Hsp70 expression with decreased Nox4 expression when compared with SHR VSMCs at basal conditions (C). Colocalization of both proteins was found with a perinuclear distribution and at the cell periphery where the cell contacts the underlying matrix in a pattern reminiscent of focal adhesions, which is the pattern of Nox4 distribution previously reported (Hilenski et al. 2004). Hsp70 labeling appeared fibrillar, extending along actin fibers after losartan and losartan plus angiotensin II treatment. A clear overlap between increased Hsp70 and downregulated p22phox was demonstrated exhibiting a punctate staining pattern in perinuclear distribution and also at the plasma membrane in a pattern similar to Nox4 in losartan-treated VSMCs and in losartan plus angiotensin II-treated VSMCs from SHR when compared with SHR VSMCs at basal conditions (C; Fig. 4). In SHR VSMCs (AII), increased expression of p22phox was observed. Lower staining of both proteins Nox4 and p22phox was shown in losartan-treated VSMCs from WKY (j in Fig. 4b and j in Fig. 4d, respectively). Perinuclear colocalization of Hsp70/p22phox was observed in losartan-treated VSMCs from WKY (k in Fig. 4b).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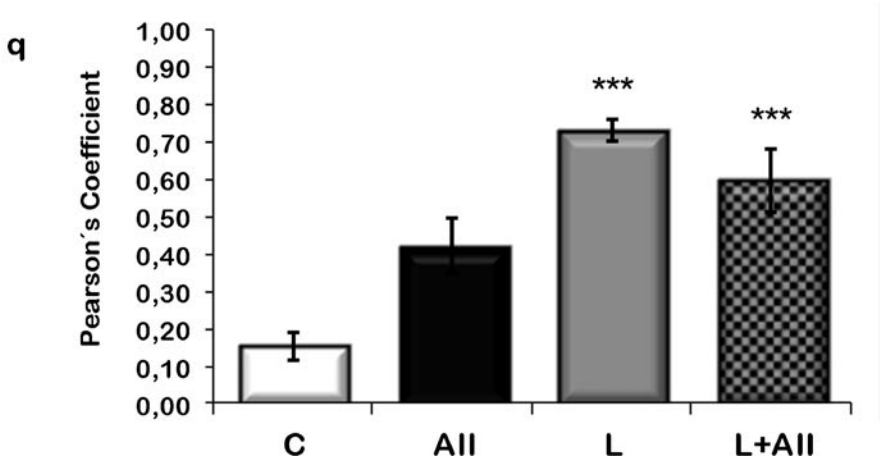
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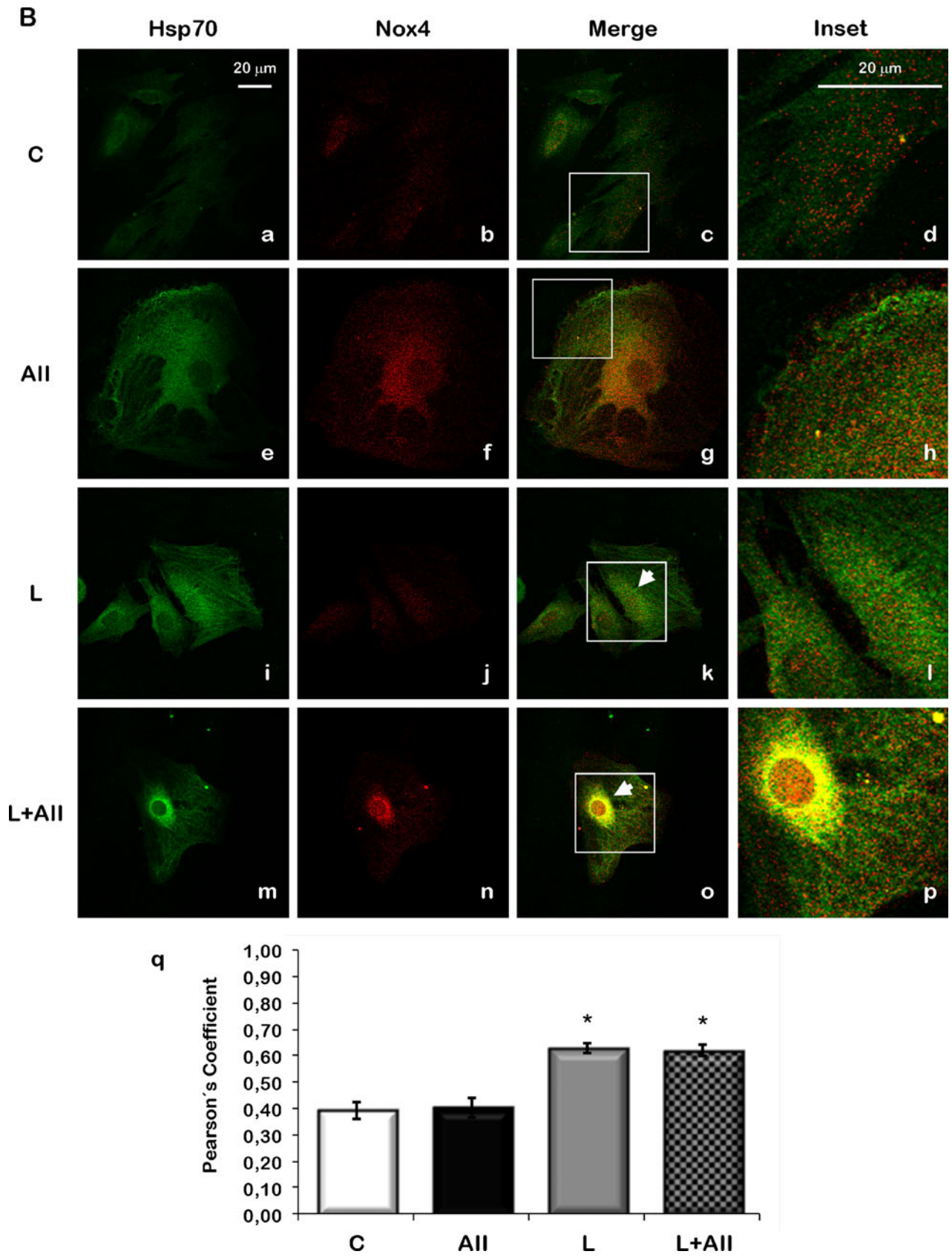

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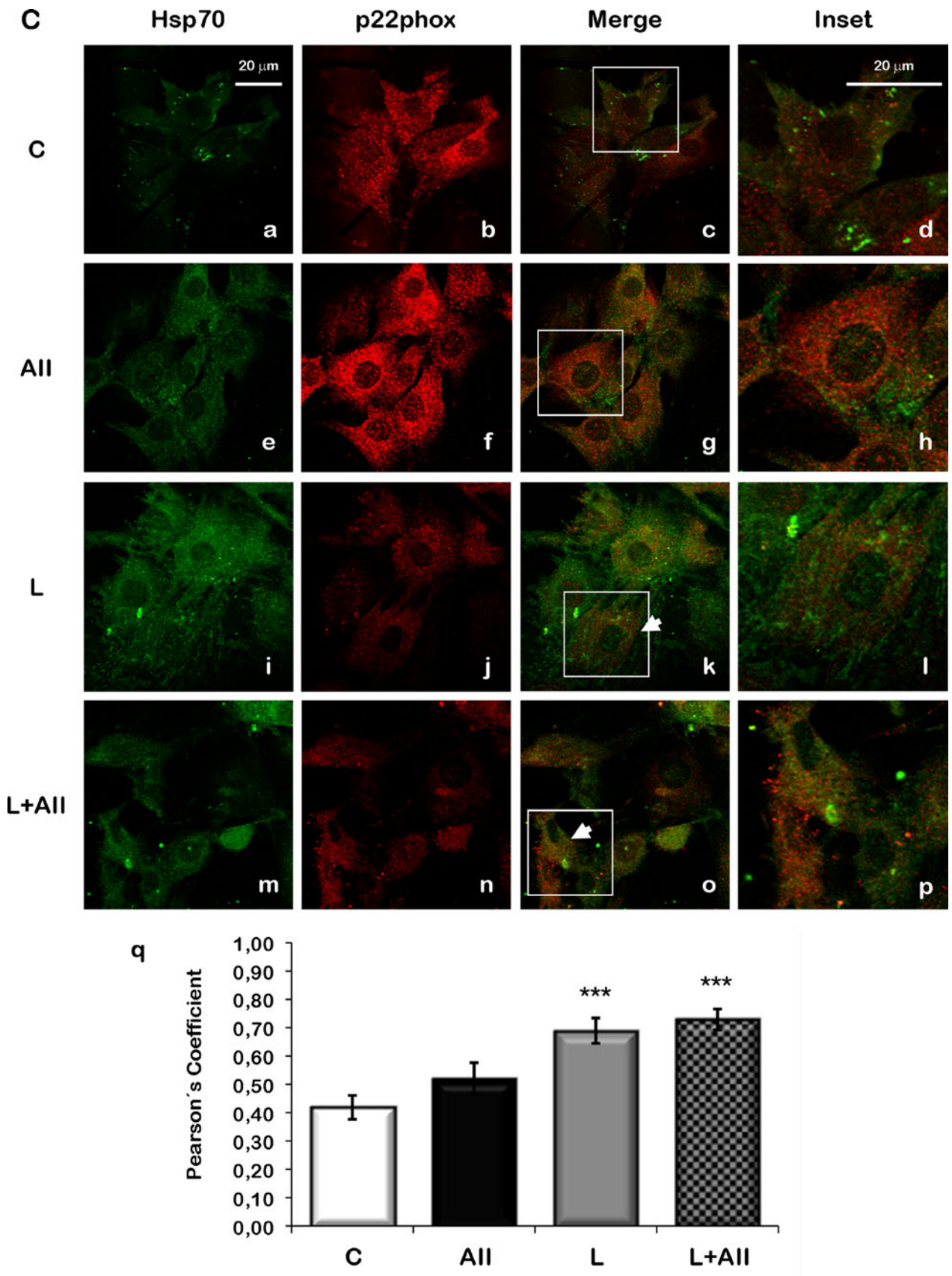

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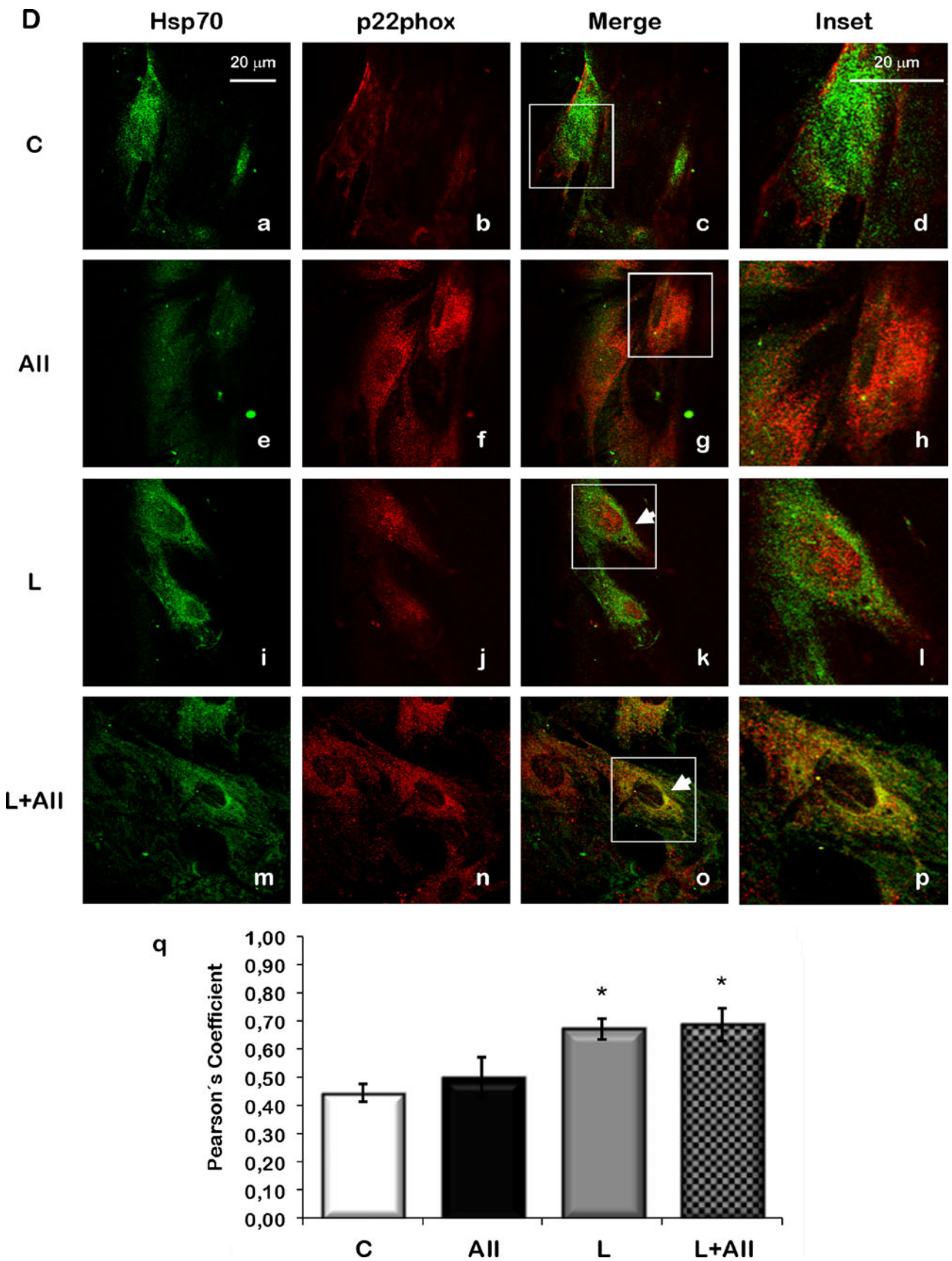

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Together, these data suggest that increased Hsp70 is associated with decreased p22phox and Nox4 staining in specific subcellular compartments and at the plasma membrane in losartan SHR VSMCs. To assess the possibility that Hp70 and Nox4/p22phox may become physically associated on AT_1R inhibition, membrane fractions from SHR and WKY VSMCs stimulated with angiotensin II with or without previous losartan treatment were immunoprecipitated with an anti-Nox4 antibody. Immunoprecipitates were then subjected to western blot analysis with mAbs against Hsp70, Nox4, p22phox, and RhoA. As shown in Fig. 5, in losartan-treated SHR VSMCs (L) and in SHR VSMCs after L+AII, the increased level of Hsp70 contrasts with the decreased immunoprecipitation of Nox4 and p22phox occurring in membrane fractions. Increased membrane Nox4 and p22phox and lower Hsp70 protein expressions were shown in SHR VSMCs (AII) when compared with losartan-treated VSMCs from SHR.

As both RhoA GTPase and Nox proteins are membrane targeted, one might expect rapid and coordinated activation of these proteins shortly after receptor engagement. We looked for RhoA signaling pathway in the interaction between Hsp70 and NADPH oxidase subunits Nox4/p22phox. In this way, immunoprecipitated Nox4 that was western blotted for RhoA showed decreased Nox4 interaction with RhoA after losartan incubation in SHR VSMC membranes. RhoA activation was demonstrated through the enhanced RhoA expression in membrane fraction from angiotensin II stimulated SHR VSMCs. This effect was not observed in L- and L+AII-treated VSMCs. Coprecipitation was not observed in VSMC membranes incubated without Nox4 (data not shown).

Hsp72 knockdown effect on Nox4/p22phox NADPH subunit proteins in VSMCs from SHR after angiotensin AT_1R inhibition

To further validate the association of Hsp70 and Nox4/p22phox, we transfected VSMCs from SHR and WKY with shHsp72-p-SIREN-RetroQ vector to transiently silence Hsp72 expression and either with control EV. SHR VSMCs transfected with the plasmid to knockdown Hsp72 expression and stimulated with angiotensin II for 15 min, increased p22phox protein levels when compared to SHR cells without transfection and to cells transfected with control empty vector. Western blot analysis revealed that Hsp72 depletion was associated with higher Nox4 and p22phox in L+AII-treated VSMCs from SHR, related to losartan SHR VSMCs without transfection and control empty vector. Higher Nox4 and p22phox expression was revealed by western blot analysis in Hsp72 depleted SHR VSMCs when compared with non transfected cells, although treated with losartan (b and d in Fig. 6).

The chaperone Hsp70 is included within losartan's effect on NADPH oxidase activity

It has been previously shown that basal ROS production in VSMCs is attributable to Nox4 activity (Zuo et al. 2004). To determine whether Hsp70 could be involved in the regulation of oxidase functioning in VSMCs, we measured NADPH oxidase activity in membrane fractions from WYK and SHR VSMCs transfected with the vector to knockdown Hsp72 expression, and with the empty vector. In SHR, depletion of Hsp72 on NADPH oxidase activity caused a tenfold increase in VSMCs (L+AII) and a twofold

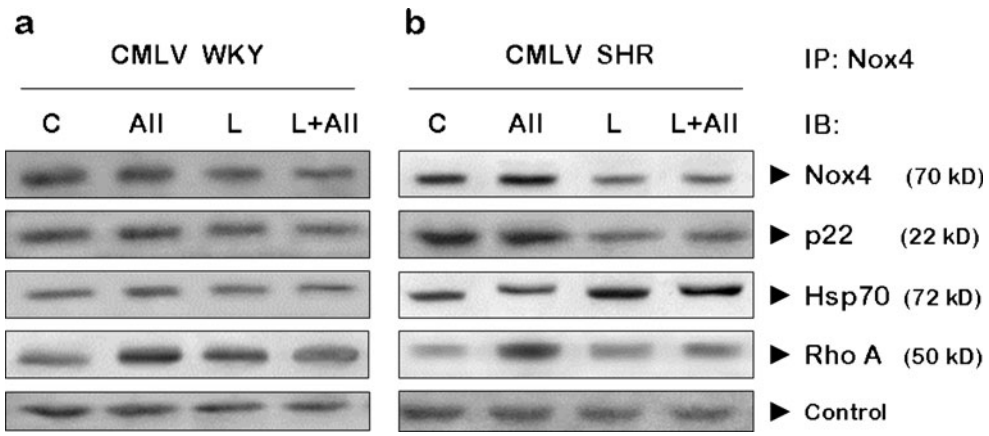

Fig. 5 Hsp70 association with Nox4/p22phox NADPH oxidase subunits and RhoA in SHR and WKY VSMCs after angiotensin AT1R inhibition. Representative immunoprecipitation. WKY and SHR VSMC membrane fractions stimulated with angiotensin II, treated with losartan and with losartan plus angiotensin II were immunoprecipitated with anti-Nox4 antibody and immunoblotted with p22phox, Hsp70, and RhoA antibodies. Increased Hsp70 coprecipitation with lower Nox4 and p22phox expression was shown in SHR VSMCs (L) membrane fractions and in SHR VSMCs (L+AII) membrane fractions related to SHR VSMCs at basal condition (C). Both, decreased Nox4 and a lower expression of RhoA while interacting were shown in SHR VSMCs (L) related to SHR VSMCs (AII) membrane fractions

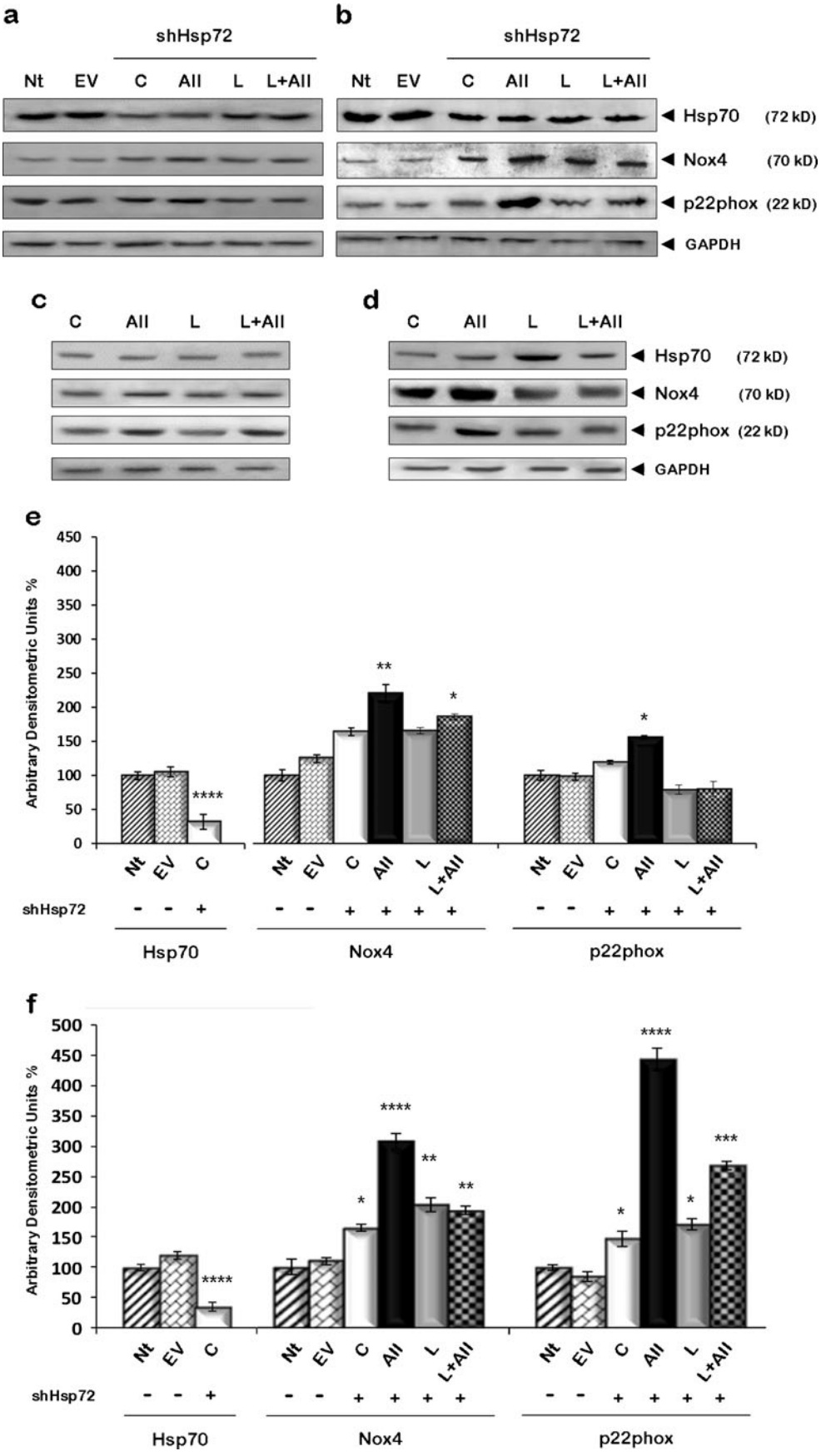

Fig. 6 Hsp72 knockdown effect on Nox4/p22phox NADPH subunit proteins in VSMCs from SHR after angiotensin AT_1R inhibition. VSMCs from WKY (**a**) and SHR (**b**) with and without losartan treatment were transfected with shHsp72-p-SIREN-RetroQ plasmid vector to knockdown Hsp72 or the control empty vector (EV). VSMCs lysates were immunoblotted with Hsp70, Nox4, and p22phox antibodies GAPDH as a loading control. No significant differences on Hsp70 protein expression were shown between VSMCs Nt and EV. Hsp72 depletion is shown in Fig. 6a and b. Hsp72 depletion was associated with higherNox4 expression in SHR VSMCs (L) and (L+AII) compared with SHR VSMC Nt control $^{**}p<0.01$, for both. Hsp72 depletion was associated with higher p22phox expression in SHR VSMCs (L) and (L+AII) vs SHR VSMC Nt control. $^{*}p<0.01$; $^{**}p<0.001$, respectively. Hsp72 knockdown was associated with higher Nox4 and p22phox protein in SHR VSMCs (AII) vs VSMCs Nt control; $^{****}p<0.0001$ and $^{****}p<0.0001$, respectively (**f**). Bars are means±SEM of three experiments. Nontransfected VSMC membrane fractions from WKY (**c**) and SHR (**d**) stimulated with angiotensin II (100 μmol/L) for 15 min (AII), treated with losartan (100 μmol/L) for 90 min (L) and treated with losartan for 75 min plus angiotensin for 15 min (L+AII) were subjected to western blot analysis with anti-Hsp70, anti-Nox4, and antip22phox antibodies, GAPDH as control

increase in VSMCs (L) when compared to VSMCs transfected with the empty vector and with nontransfected VSMCs. In addition, as shown in Fig. 7, after Hsp72 knockdown, a more than tenfold increase on NADPH oxidase activity was demonstrated in transfected VSMCs stimulated with angiotensin II from SHR (AII) when compared to nontransfected SHR VSMCs and with VSMCs from WKY. For these results, after Hsp72 silencing in VSMCs from SHR, losartan could not prevent angiotensin II-enhanced Nox4 and p22phox expression and NADPH oxidase activity.

Hsp70 in the regulation of Nox4 and p22phox localization, stress fiber formation, and focal adhesion integrity

The colocalization of Hsp70, with decreased Nox4 and p22phox and the lower protein levels of RhoA after immunoprecipitation strategy, raise the possibility that Nox4 and p22phox localization is impaired when Hsp70 is increased, and that Hsp70 is involved in cytoskeletal modulation. To determine whether Hsp70 may contribute to the regulation of stress fiber formation and focal adhesion integrity after losartan treatment, immunofluorescence confocal microscopy was performed.

Two-color confocal microscopy was performed to visualize α-actin and Hsp70 in the same cells using fluorophore-conjugated anti-α-actin and specie-specific anti-Hsp70, respectively. The distribution of α-actin between cortical filaments and stress fibers differed considerably between losartan- and non-losartan-treated SHR VSMCs (see Fig. 8). In SHR VSMCs stimulated with AII, α-actin was found mostly in stress fibers extending through cytoplasm and little staining was observed at the cell cortex. There was a reorganization of the actin cytoskeleton with fibers dispersed throughout the interior of the VSMCs, running parallel to the long axis of the cells. In losartan-treated SHR VSMCs, α-actin staining was localized at the cell periphery and colocalization with Hsp70 was shown. Intense perinuclear Hsp70 staining colocalized with soluble α-actin was observed in losartan-treated SHR VSMCs. Moreover, losartan treatment in SHR VSMCs resulted in wavy, disorganized stress fibers in the interior of the cell, as detected by soluble actin staining that colocalized with Hsp70. In contrast, after Hsp72 depletion in VSMCs from SHR, we observed actin polimerazation that resulted in the thickening of actin stress fibers although these cells were treated with losartan for 90 min (Fig. 8).

We next demonstrated colocalization of Nox4 with increased vinculin staining; this last protein is a focal adhesion marker in angiotensin II-stimulated VSMCs from SHR. The localization of increased Nox4 and decreased Hsp70 labeled in focal adhesions from SHR VSMCs and the apparent loss of these structures in losartan-treated SHR VSMCs (Fig. 3) raise the possibility that Hsp70 may be involved in the regulation of focal adhesion integrity after AT_1R inhibition in these cells.

To validate the role of Hsp70 in mediating the effects on Nox4/vinculin, we transfected VSMCs with plasmid pSIREN-RetroQ to silence Hsp72and/or the control empty vector. Immunofluorescence confocal microscopy revealed that Hsp72 depletion was associated with higher Nox4 and presence of vinculin staining in losartan plus angiotensin II-treated VSMCs from SHR when related to losartan-treated SHR VSMCs without transfection and to control empty vector. Indeed, as shown in Fig. 9, Nox4 labeling appeared fibrillar, extending along actin fibers beyond the vinculin-positive focal adhesions in some cells. The depletion of Hsp72 in SHR VSMCs without losartan treatment led to increased vinculin staining when compared with transfected losartan-treated cells. Thus, Hsp70 appears to be a negative regulator of these important cytoskeletal structures in the presence of losartan.

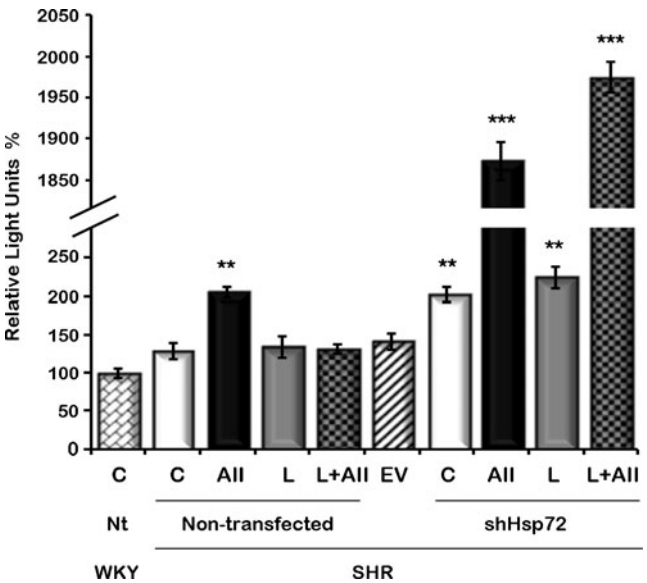

Fig. 7 Hsp72 knockdown effect on NADPH oxidase activity in VSMCs from SHR after angiotensin AT_1R inhibition. Lucigenin-enhanced chemiluminescence was used to measure NADPH oxidase activity in membrane fractions from non transfected VSMCs (Nt) (*left*) from WKY and SHR and from SHR VSMCs transfected with shHsp72-p-SIREN-RetroQ plasmid vector to knockdown Hsp72 or the control empty vector (EV) (*right*). No significant differences on NADPH activity were shown between SHR VSMC Nt and EV. Increased activity was shown in nontransfected VSMCs (AII) from SHR vs nontransfected VSMCs from WKY, $^{**}p<0.01$ SHR VSMCs transfected with the vector to knockdown Hsp72 without treatment (**c**) and treated with angiotensin II for 15 min (AII) showed increased NADPH activity when compared to non-transfected SHR VSMCs (control, C), $^{**}p<0.01$and $^{***}p<0.001$, respectively. SHR VSMCs transfected with shHsp72 and treated with losartan (L) or losartan plus angiotensin II (L+AII) showed increased NADPH activity when compared with SHR VSMC Nt control; $^{**}p<0.01$and $^{***}p<0.001$, respectively. Bars are means±SEM of four independent experiments

Discussion

In this study, through Hsp72 protein knockdown, we demonstrate that the chaperone Hsp70 is required for the antioxidative and cytoskeleton modulation effects of AT_1R inhibition. The role of Hsp70 as a negative regulator of Nox4/p22phox after losartan treatment in VSMCs from SHR is shown. Moreover, Hsp70 contributes to actin disorganization, decreased cytoskeleton polymerization, and a loss of focal adhesion-like structures in the presence of losartan (Fig. 10).

Direct interaction with caveolin is required to traffic the AT_1R through the exocytic pathway. This interaction represents an important focus for the dynamic control of receptor signaling in VSMCs (Wyse et al. 2003). Caveolin-1 plays an essential role in AT_1R targeting into caveolin-1-enriched lipid rafts and Rac1 activation, which are required for the proper organization of ROS-dependent angiotensin II signaling (Zuo et al. 2004). After AT_1R inhibition, we showed upregulation of caveolin-1 protein expression and a slight decrease in caveolin-1 expression after angiotensin II stimulation in SHR VSMCs. It is possible that angiotensin II stimulation results in caveolae internalization and translocation to intracellular compartments (Ushio-Fukai and Alexander 2006). Previously, caveolin-1 protein degradation has been demonstrated in VSMCs stimulated with angiotensin II for prolonged intervals. As expected from an interaction restricted to the exocytic pathway, only 2 % of caveolin can be recovered from the VSMC lysates bound to AT_1R (Ishizaka et al. 1998).

To assess the apparent association between AT_1R and caveolin protein expression, we performed colocalization experiments demonstrating that upregulated AT_1R colocalized with decreased caveolin-1 expression in SHR VSMC membranes related to losartan-pretreated SHR VSMCs. Here, we identify the role of Hsp70 in the mechanism responsible for the effect of losartan on ROS through the regulation of Nox4/p22phox.

The chaperone plays a critical role in the recovery of cells from stress and in cytoprotection (Riordan et al. 2004). In doing so, Hsp70 holds, translocates, or refolds stress-denatured proteins preventing their irreversible aggregation with other proteins in the cell (Nollen and Morimoto 2002). Albeit, on the other hand, expression levels of Nox4 have been reported to be as much as 100-fold greater than other Nox isoforms in VSMCs. Localized at focal adhesions, within the nucleus, in the endoplasmic reticulum, and stress fibers (Burridge and Chrzanowska-Wodnicka 1996), Nox4 is functionally linked to oxygen sensing, proinflammatory responses, migration, proliferation, and differentiation (Clempus et al. 2007) suggesting that it regulates fundamental cellular processes (Bedard and Krause 2007). Notwithstanding, the mechanism by which Nox4 activity is regulated remain unclear. Previously, we provided evidence of the interaction between the translocated Hsp70 and Nox4 downregulation in SHR microdissected

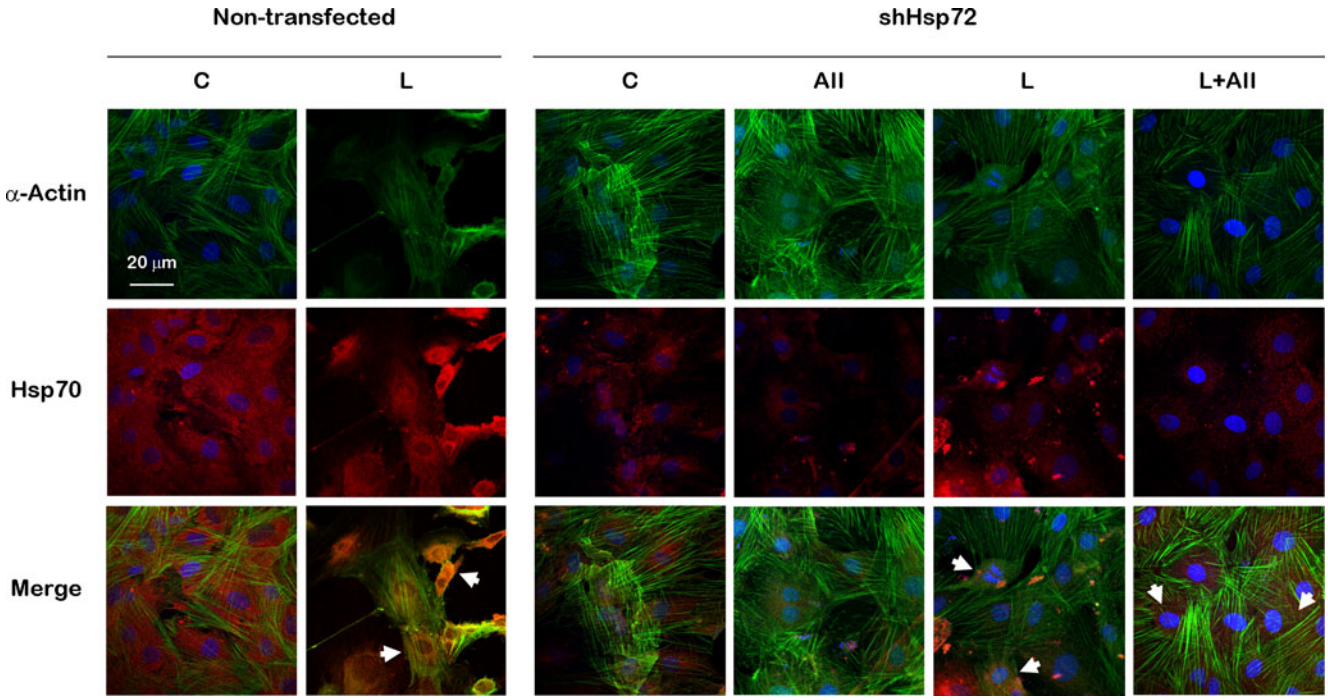

Fig. 8 Hsp72 knockdown on *α-actin* distribution and polimerization in VSMCs from SHR. Nontransfected VSMCs without and with losartan treatment (*left*), VSMCs transfected with shHsp72-p-SIREN-RetroQ plasmid vector to knockdown Hsp72 and stimulated with angiotensin II for 15 min (AII), treated with losartan 90 min (L) and losartan plus angiotensin II (L+AII), and VSMCs transfected with control empty vector (EV) (images not shown), were double labeled with anti-Hsp70 (*red*) and anti-α-actin (*green*) antibodies. *Arrows* indicate areas of colocalization (*yellow*) in the merge. *Scale bars* 20 μm. Magnification 640×

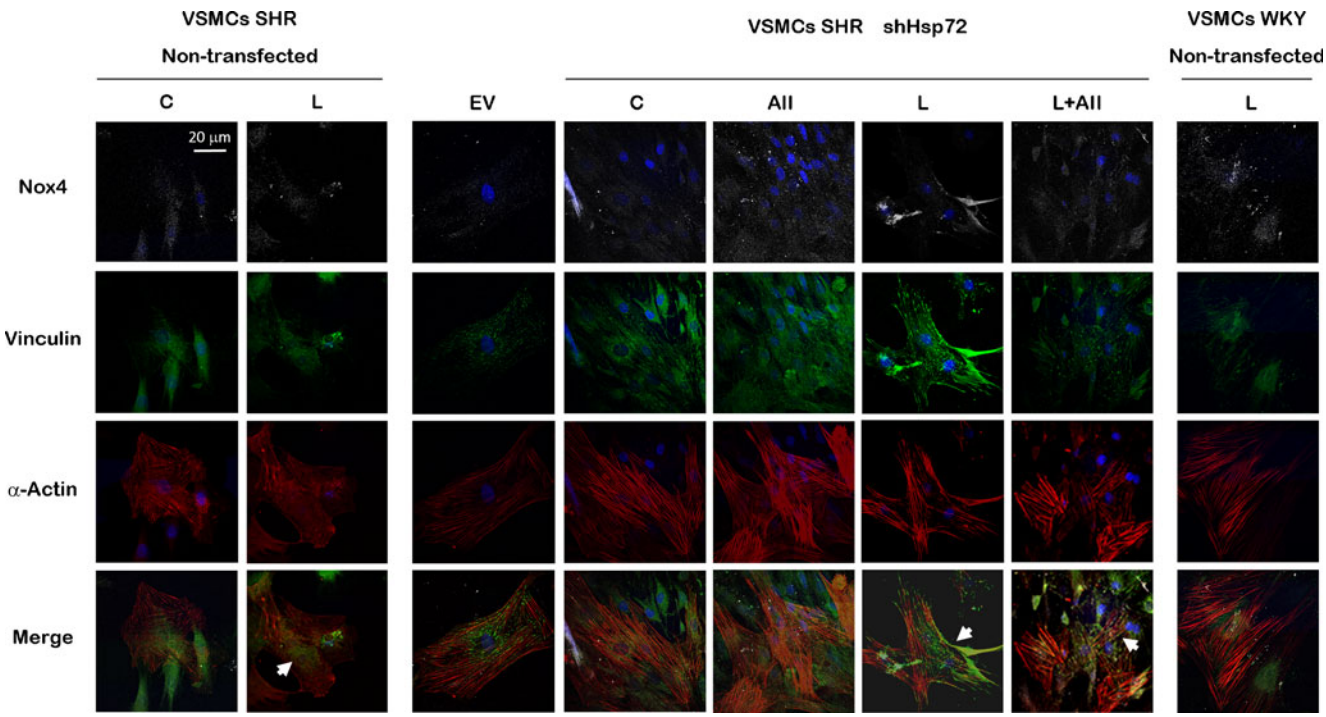

Fig. 9 Hsp72 knockdown on Nox4 expression, stress fiber formation and focal adhesion integrity in VSMCs from SHR. Nontransfected SHR (*left*) and WKY (*right*) VSMCs without and with losartan treatment. SHR VSMCs transfected with control empty vector and VSMC SHR (central) transfected with shHsp72-p-SIREN-RetroQ plasmid vector (shHsp72) were labeled with anti-Nox4 (*gray*), antivinculin (*green*), and α-actin (*red*) antibodies. Nuclei were labeled with Hoechst (*blue*) images acquired at the focal adhesion (*upper*) and stress fiber (*lower*) planes are depicted. *Arrows* indicate areas of colocalization in the merge at the focal adhesion (*upper*) and stress fiber (*lower*). *Scale bars* 20 μm. Magnification ×640

proximal tubule membranes after angiotensin II AT_1R inhibition (Bocanegra et al. 2010). Here, we demonstrated association of Hsp70 with Nox4/p22phox after the recruitment of Hsp70 to the membrane fraction in L- and L+AII-treated VSMCs from SHR. Nox4 labeling was confined to the plasma membrane at the cell periphery in a pattern reminiscent of focal adhesions. Nox4 labeling was also showed around the nucleus. p22phox revealed labeling in a punctate perinuclear distribution and at the cell periphery in a pattern similar to Nox4. These results indicate that Nox4 is preferentially located in different subcellular compartments and that p22phox is found in these locations as well. Colocalization of Hsp70 with Nox4 and p22phox was shown in VSMCs from SHR after losartan administration. The increased level of Hsp70 contrasted with the lower Nox4 and p22phox labeling occurring in focal adhesions, along stretch fibers, and at the VSMCs plasma membranes. In addition, we observed the physical interaction of increased expression of Hsp70 and decreased protein levels of Nox4 /p22phox in losartan-treated SHR VSMC membranes. Interestingly, Hsp72 knockdown was associated with the upregulation of Nox4 and p22phox protein expressions in losartan plus angiotensin II-treated VSMCs from SHR when related to losartan-treated VSMCs without transfection and control empty vector. Furthermore, Hsp72 silencing in losartan plus angiotensin II-treated SHR VSMCs led to similar values of NADPH oxidase generation, as it did with wild-type SHR VSMCs, suggesting the chaperone involvement in the antioxidant effect of losartan by its physical and functional interaction with the NADPH oxidase subunits Nox4/p22phox. It is possible that other Nox isoforms may be critically contributing to NADPH oxidase activity (Bedard and Krause 2007), although basal ROS production in VSMCs has been assigned to Nox4 activity (Montezano et al. 2011). p22phox requirement for Nox4-dependent ROS generation has been demonstrated in functional studies (Kawahara et al. 2005; Martyn et al. 2006). p22phox mutants lacking the proline-rich COOH terminus are still fully active in supporting Nox4 activity, even though such mutants are not sufficient for Nox1, 2, and 3 activation. Nox4 does not require cytosolic subunits for its activity (Martyn et al. 2006; Geiszt et al. 2000). However, although it is known that Nox4 requires p22phox, there has been no systematic search for the proteins that bind to Nox4/p22phox subunits. Recent work by Lyle et al. has identified polymerase [DNA-directed] delta-interacting protein 2 as a putative regulator of Nox4 activity in VSMCs (Lyle et al. 2009).

Our present results suggest Hsp70 interacts with Nox4/p22phox as a negative regulating chaperone of Nox4/p22phox protein expression and activity in the losartan mechanism on the VSMC complex. However, there appears to be more than one level of regulation of Nox4 by Hsp70. Nox4 and p22phox have been detected in stress fibers and focal adhesions (Lyle et al. 2009). In this regard,

we observed colocalization of upregulated Hsp70 with lower Nox4 and p22phox immunoreaction while the Nox4 expression was no longer localized in focal adhesions. In contrast, Hsp72 knockdown may insure proper Nox4 and p22phox localization to focal adhesions by vinculin-positive staining raising the possibility of chaperone involvement in the negative modulation of cytoskeleton integrity.

Both focal adhesion turnover and stress fiber formation are mediated through RhoA activation. Rho activation leads to the assembly of contractile actin–myosin filaments (stress fibers) and of associated focal adhesion complexes (Ridley and Hall 1992). In our study, decreased membrane Nox4 protein levels in losartan-treated VSMCs from SHR were shown to interact with lower levels of RhoA in the presence of enhanced Hsp70 protein levels in coinmunoprecipitation studies. The association of Nox4 with the cytoskeleton may have functional consequences in addition to providing compartmentalization of ROS production (Hilenski et al. 2004). Of note, previous work shows an association of oxidase subunits and O_2^- producing activity with the submembranous actin network (Benna et al. 1994) and with actin associated proteins, including coronin and cofilin (Benna et al. 1999). Nox4 association with stress fibers may influence cytoskeletal rearrangement. Since that event, Cytochalasin D abolished Nox4 and p22phox labeling in focal adhesions at the termini of actin filaments suggesting that these subunits are associated with or regulated by the actin cytoskeleton in VSMCs. Nox4 is required for the maintenance of α-actin-based stress fibers in VSMCs. Therefore, siNox4 induced a dramatic decrease in intensity and distribution of VSMCs α-actin along fibers (Clempus et al. 2007). Thus, there are functional and physical links among the actin cytoskeleton, actin-associated proteins, focal adhesion proteins, and the NADPH oxidase Nox4.

Two situations are described in the literature where the properties of actin are modulated by sHsps; the actin polymerization is inhibited in vitro by some sHsps acting as capping proteins and the actin cytoskeleton is protected by some sHsps against the disruption induced by various stressful conditions (Mounier and Arrigo 2002). In our study, Hsp70 appears to be involved in targeting Nox4 and α-actin in stress fibers and interaction among α-actin protein, Hsp70, and the Nox 4 subunit of NADPH oxidase after losartan treatment was demonstrated. This interaction resulted in wavy, disorganized stress fibers, which was detected by a decrease of filamentous actin staining and an increase in soluble actin staining leading to actin polymerization inhibition. In a reverse manner, Hsp72 depletion leads to an enhanced intensity of filamentous actin staining, increasing the thickness of stress fibers, which indicates actin polymerization. Moreover, after Hsp72 depletion, Nox4 labeling appeared fibrillar, extending along actin fibers beyond the vinculin-positive focal adhesions. Understood in conjunction with the observations reported here, Hsp70 appears to be a negative regulator of these important cytoskeletal structures in the presence of losartan.

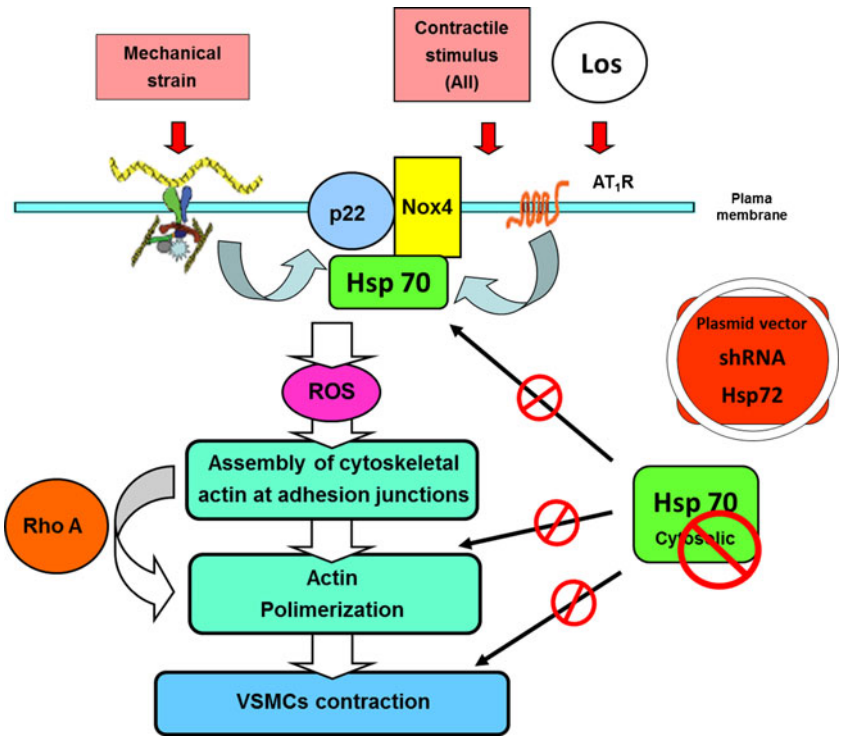

Fig. 10 Potential involvement of Hsp70 as a negative regulator of Nox4/p22 phox in losartan-induced actin depolymerization and protection against oxidative stress in VSMCs from SHR

Conclusion

Our data shows a functional association of Hsp70 with Nox4/p22phox providing a possible mechanism in explaining an antioxidative function of losartan along with subsequent modulation of cytoskeletal integrity in VSMCs from SHR. Our research points to a new understanding of the chaperone Hsp70 included within the mechanisms of losartan for therapeutic intervention in ROS-mediated vascular injury.

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References

Anwar A, Siegel D, Kepa JK, Ross D (2002) Interaction of the molecular chaperone Hsp70 with human NAD(P)H:quinone oxidoreductase 1. J Biol Chem 277:14060–14067

Bedard K, Krause KH (2007) The NOX family of ROS-generating NADPHoxidases: physiology and pathophysiology. Physiol Rev 87:245–313

Benna JE, Ruedi J, Babior B (1994) Cytosolic guanine nucleotide-binding protein rac2 operates in vivo as a component of the neutrophil respiratory burst oxidase: transfer of rac2 and the cytosolic oxidase components p47 phox and p67 phox to the submembranous actin cytoskeleton during oxidase activation. J Biol Chem 269:6729–6734

Benna JE, Dang PM, Andrieu V, Vergnaud S, Dewas C, Cachia O, Fay M, Morel F, Chollet-Martin S, Hakim J, Gougerot-Pocidalo MA (1999) p40phox associates with the neutrophil Triton X-100-insoluble cytoskeletal fraction and PMA-activated membrane skeleton: a comparative study with p67phox and p47phox. J Leukoc Biol 66(6):1014–1020

Bocanegra V, Manucha W, Peña MR, Cacciamani V, Vallés PG (2010) Caveolin-1 and Hsp70 interaction in microdissected proximal tubules from spontaneously hypertensive rats as an effect of losartan. J Hypertens 28(1):143–155

Briones AM, Tabet F, Callera GE, Montezano AC, Yogi A, He Y, Quinn MT, Salaices M, Touyz RM (2011) Differential regulation of Nox1, Nox2 and Nox4 in vascular smooth muscle cells from WKY and SHR. J Am Soc Hypertens 5(3):137–153

Burridge K, Chrzanowska-Wodnicka M (1996) Focal adhesions, contractilityand signaling. Annu Rev Cell Dev Biol 12:463–519

Callera GE, Touyz RM, Tostes RC, Yogi A, He Y, Malkinson S, Schiffrin EL (2005) Aldosterone activates vascular p38MAP kinase and NADPH oxidase via c-Src. Hypertension 45:773–779

Clempus RE, Sorescu D, Dikalova AE, Pounkova L, Jo P, Sorescu GP, Schmidt HH, Lassegue B, Griendling KK (2007) Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. Arterioscler Thromb Vasc Biol 27:42–48

Cruzado MC, Risler NR, Miatello RM, Yao G, Schiffrin EL, Touyz RM (2005) Vascular smooth muscle cell NAD(P)H oxidase activity during the development of hypertension: Effect of angiotensin II and role of insulin like growth factor-1 receptor transactivation. Am J Hypertens 18(1):81–87

Geiszt M, Kopp JB, Varnai P, Leto TL (2000) Identification of renox, an NAD(P)H oxidase in kidney. Proc Natl Acad Sci USA 97:8010–8014

Gunst SJ, Zhang W (2008) Actin cytoskeletal dynamics in smooth muscle: a new paradigm for the regulation of smooth muscle contraction. Am J Physiol Cell Physiol 295:C576–C587

Hartl FU (1996) Molecular chaperones in cellular protein folding. Nature 381:571–579

Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK (2004) Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 24:677–683

Hitomi H, Kiyomoto H, Nishiyama A (2007) Angiotensin II and oxidative stress. Curr Opin Cardiol 22:311–315

Ishizaka N, Griendling KK, Lassegue B, Alexander RW (1998) Angiotensin II type1 receptor: relationship with caveolae and caveolin after initial agonist stimulation. Hypertension 32:459–466

Kawahara T, Ritsick D, Cheng G, Lambeth JD (2005) Point mutations in the proline-rich region of p22phox are dominant inhibitors of Nox1- and Nox2-dependent reactive oxygen generation. J Biol Chem 280:31859–31869

Lyle AN, Deshpande NN, Taniyama Y, Seidel-Rogol B, Pounkova L, Du P, Papaharalambus C, Lassegue B, Griendling KK (2009) Poldip2, a novel regulator of Nox4 and cytoskeletal integrity in vascular smooth muscle cells. Circ Res 105:249–259

Martyn KD, Frederick LM, von Loehneysen K, Dinauer MC, Knaus UG (2006) Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. Cell Signal 18:69–82

Montezano AC, Touyz RM (2012) Oxidative stress, Noxs, and hypertension: experimental evidence and clinical controversies. Ann Med 44(Suppl 1):S2–S16

Montezano AC, Burger D, Ceravolo GS, Yusuf H, Montero M, Touyz RM (2011) Novel Nox homologues in the vasculature: focusing on Nox4 and Nox5. Clin Sci (Lond) 120(4):131–141

Mounier N, Arrigo AP (2002) Actin cytoskeleton and small heat shock proteins: how do they interact? Cell Stress Chaperones 7(2):167–176

Nollen EA, Morimoto RI (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins. J Cell Sci 115:2809–2816

Ramirez-Sanchez I, Ceballos-Reyes G, Rosas-Vargas H, Cerecedo-Mercado D, Zentella-Dehesa A, Salamanca F, Coral-Vazquez RM (2007) Expression and function of utrophin associated protein complex in stretched endothelial cells: dissociation and activation of eNOS. Front Biosci 12:1956–1962

Ridley AJ (2001) Rho family proteins: coordinating cell responses. Trends Cell Biol 11(12):471–477

Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389–399

Riordan M, Garg V, Thulin G, Kashgarian M, Siegel NJ (2004) Differential inhibition of Hsp72 and Hsp25 produces profound impairment of cellular integrity. J Am Soc Nephrol 15:1557–1566

Stephanou A, Latchman DS (1999) Transcriptional regulation of the heat shock protein genes by STAT family transcription factors. Gene Expr 7:311–399

Touyz RM (2005) Reactive oxygen species as mediators of calcium signaling by angiotensin II: implications in vascular physiology and pathophysiology. Antioxid Redox Signal 7:1302–1314

Touyz RM, Tolloczko B, Schiffrin EL (1994) Mesenteric vascular smooth muscle cells from SHR display increased Ca^{-2} responses to angiotensin II but not to endothelin-1. J Hypertens 12:663–673

Ushio-Fukai M, Alexander RW (2006) Caveolin-dependent angiotensin II type1 receptor signaling in vascular smooth muscle. Hypertension 48:797–803

Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, Griendling KK (1999) Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. J Biol Chem 274:22699–22704

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84

Wyse BD, Prior IA, Qian H, Morrow IC, Nixon S, Muncke C, Kurzchalia TV, Thomas WG, Parton RG, Hancock JF (2003) Caveolin interacts with the angiotensin II type 1 receptor during exocytic transport but not at the plasma membrane. J Biol Chem 278:23738–23746

Yoneda M, Sanada H, Yatabe J, Midorikawa S, Hashimoto S, Sasaki M, Katoh T, Watanabe T, Andrews PM, José PA, Felder RA (2005) Differential effects of angiotensin II type-1 receptor antisense oligonucleotides on renal function in spontaneously hypertensive rats. Hypertension 46(1):58–65

Zuo L, Ushio-Fukai M, Hilenski LL, Alexander RW (2004) Microtubules regulate angiotensin II type 1 receptor and rac1 localization in caveolae/lipid rafts. Arterioscler Thromb Vasc Biol 24:1–7