Angiotensin II promotes KV7.4 channels degradation through reduced interaction with HSP90 (heat shock protein 90)

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ANGIOTENSIN II PROMOTES K_V7.4 CHANNELS DEGRADATION THROUGH REDUCED INTERACTION WITH HSP90

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ABSTRACT

Voltage gated Kv7.4 channels have been implicated in vascular smooth muscle cells (VSMCs) activity as they modulate basal arterial contractility, mediate responses to endogenous vasorelaxants, and are down-regulated in several arterial beds in different models of hypertension. Angiotensin II (Ang II) is a key player in hypertension that affects the expression of several classes of ion channels. In this study we evaluated the effects of Ang II on the expression and function of vascular $K_v7.4$. Western blot and quantitative PCR revealed that in whole rat mesenteric artery Ang II incubation for 1-7h decreased $K_v7.4$ protein expression without reducing transcript levels. Moreover, Ang II decreased XE991 (K_v7) –sensitive currents, and attenuated membrane potential hyperpolarization and relaxation induced by the K_v7 activator ML213. Ang II also reduced $K_v7.4$ staining at the plasma membrane of VSMCs. Proteasome inhibition with MG132 prevented Ang II-induced decrease of $K_v7.4$ levels, and counteracted the functional impairment of ML213-induced relaxation in myography experiments. Proximity Ligation Assays showed that Ang II impaired the interaction of $K_v7.4$ with the molecular chaperone HSP90, enhanced the interaction of $K_v7.4$ with the E3 ubiquitin ligase CHIP, and increased $K_v7.4$ ubiquitination. Similar alterations were found in mesenteric VSMCs isolated from Ang II-infused mice. The effect of Ang II was emulated by 17-AAG that inhibits HSP90 interactions with client proteins. These results show that Ang II downregulates $K_v7.4$ by altering protein stability through a decrease of its interaction with HSP90. This leads to the recruitment of CHIP and $K_v7.4$ ubiquitination and degradation via the proteasome.

Keywords: Angiotensin II/Heat shock protein/KCNQ/$K_v7.4$/Proteasome

INTRODUCTION

Hypertension is associated with increased arterial contractility and resistance to receptor-mediated vasodilators. K_v7 channels ($K_v7.1$ – $K_v7.5$) are voltage-gated potassium (K^+) channels encoded by KCNQ genes that regulate the contractile state of vascular smooth muscle at rest and contribute markedly to receptor-mediated vasorelaxations[1, 2]. Arterial smooth muscle cells express $K_v7.1$, $K_v7.4$ and $K_v7.5$, with negligible $K_v7.2$ and $K_v7.3$ levels[3], and blockers of these channels such as XE991 or linopirdine can contract most arteries or enhance vasoconstrictor responses[4-6]. Conversely, agents that enhance K_v7 activity are effective vasorelaxants[4-7]. In addition, K_v7 blockers, as well as knockdown of $K_v7.4$ impairs vasorelaxations produced by receptor agonists in various arteries[2, 8, 9]. Notably, many receptor-mediated vasorelaxations are impaired in arteries from hypertensive animals, and any remaining relaxation is no longer sensitive to K_v7 blockade[8, 10]. The functional impairment is linked to a reduction of $K_v7.4$ protein abundance in renal, mesenteric and coronary arteries from hypertensive animals, which is not correlated with a reduction in gene transcript (*KCNQ4*), suggesting the involvement of post transcriptional mechanisms. Whilst some molecular determinants of the pathological changes in K_v7 channel expression occurring in the vasculature during hypertension have started to be unveiled[11], the mechanisms that dictate the membrane abundance of $K_v7.4$ channels in smooth muscle are yet to be fully elucidated. K^+ channel trafficking can be regulated by several processes, such as the protein internalization/endosomal recycling pathway[12], and altered protein folding via changes in the interaction with molecular chaperones such as Heat Shock Proteins (HSPs)[13]. Here, we aim to uncover the processes governing the handling of $K_v7.4$ in vascular smooth muscle following treatment with Angiotensin II (Ang II), a molecule strongly associated with cardiovascular

dysfunction, to gain insight into the processes that control $K_v7.4$ membrane abundance during hypertension.

METHODS

Detailed methods are available in the Online Supplement (http://hyper.ahajournals.org). The authors declare that all supporting data are available within the article (and its online supplementary files).

Animals

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and were approved by the local ethics committees (St George's Animal Welfare Committee, Barts and The London School of Medicine, University of Reading Animal Welfare and Ethical Review Board). 10-12 weeks-old male Wistar rats (Charles River, U.K.) and C57BL/6J mice (12-18 weeks of age) were killed by cervical dislocation. Animals were housed in a climatically controlled environment, on a 12h light/dark cycle, with free access to water and standard food ad libitum.

Statistical analysis

All data are expressed as mean ± s.e.m. One- or two-way ANOVA test followed by a Dunnett's or Tukey's multiple comparisons test, and Student's t-test (paired or unpaired) were used to determine statistical significance between groups, according to the different experiments. Differences were considered statistically significant when $p<0.05$.

RESULTS

Effects of Ang II on $K_v7.4$ levels and function

We first investigated the effects of Ang II on gross $K_v7.4$ levels in protein lysates from whole mesenteric arteries. Western blot experiments showed that 100nmol/L Ang II reduced $K_v7.4$ protein levels by ~50% after 1h compared to control vessels, by ~70% after 3h, and by ~80% after 7h treatment (Fig 1A). Incubation with Ang II did not reduce *KCNQ4* mRNA levels even after 7h (Fig 1B), suggesting a post-transcriptional mechanism. Similar results were also observed in rat aorta (Fig S1A-B, http://hyper.ahajournals.org). To circumvent any possible non-specific binding of the $K_v7.4$ antibody we investigated the effects of Ang II treatment on a human smooth muscle cell line (SGVSM-9) transfected with EGFP-tagged $K_v7.4$, using an anti-GFP antibody. In SGVSM-9 cells transfected with EGFP-$K_v7.4$, in-cell western blot experiments showed that treatment for 1h with 100nmol/L Ang II induced a ~20% decrease in EGFP-$K_v7.4$ with respect to control cells (Fig 1C). Moreover, in CHO cells expressing the angiotensin II receptor type 1 (AT1R) and EGFP-$K_v7.4$, incubation of 100nmol/L Ang II for 1h decreased EGFP-$K_v7.4$ protein expression by ~30%, as measured by Western blot (Fig S1C, http://hyper.ahajournals.org).

We then evaluated the effects of Ang II on K_v7 function. Whole cell K^+ currents were recorded from isolated mesenteric artery myocytes and the K_v7 current was identified as the component sensitive to the selective blocker XE991 (1µmol/L) (Fig S2A, http://hyper.ahajournals.org). Incubation of 100nmol/L Ang II for 3h decreased the amplitude of XE991-sensitive current by about 60% (Fig 1D). ML213 is an activator of $K_v7.2$, $K_v7.4$ and $K_v7.5$ channels that has been characterized in several smooth muscles, including VSMCs from mesenteric arteries[14]. In sharp microelectrode studies on whole mesenteric arteries ML213 hyperpolarized the membrane potential by approximately 17mV; a subsequent application of the K_{ATP} channel activator levcromakalin (10µmol/L) further increased membrane resting potential (Fig 1E and Fig S2B, http://hyper.ahajournals.org, control). Treatment with Ang II for 2h blunted ML213-induced hyperpolarization (increase of membrane potential by ~2mV) but did not affect the membrane

hyperpolarization caused by levcromakalin (Fig 1E and Fig S2B, http://hyper.ahajournals.org, Ang II). ML213-induced relaxation of pre-contracted mesenteric arteries is abolished by the K_v7 blockers XE991 (Fig S3, http://hyper.ahajournals.org) and linopirdine[14]. Incubation of mesenteric arteries with Ang II impaired the relaxation of pre-contracted mesenteric arteries produced by ML213 (Fig 1F and S2D, Fig S3, http://hyper.ahajournals.org), consistent with the loss of $K_v7.4$ function.

Effects on Ang II on $K_v7.4$ subcellular distribution

$K_v7.4$ subunits consistently show a clear and abundant peripheral distribution, following the shape of the cell membrane of the VSMC, whilst $K_v7.1$ was mainly intracellular and $K_v7.5$ signal was altogether less apparent and only occasionally localized at the plasma membrane (Fig S3, http://hyper.ahajournals.org). The $K_v7.4$ staining overlapped with the signal of the plasma membrane marker wheat germ agglutinin in control conditions (WGA, Fig. 2A). Treatment with 100nmol/L Ang II (3h) reduced the membrane localization of $K_v7.4$ concomitant with a decrease in the global level of $K_v7.4$ (Fig 2A). Fluorescence-intensity profiles along cross-sections of VSMCs showed that in control cells the $K_v7.4$ signal was higher in the membrane than cytosol (membrane:cytoplasm ratio 1.57±0.12), while in Ang II treated cells $K_v7.4$ fluorescence was similar between the two regions (membrane:cytoplasm ratio 0.88±0.10) (Fig 2B).

Role of endosomal and proteasomal pathways in Ang II-mediated $K_v7.4$ degradation

To gain insight about the cellular mechanisms that mediated the shift in $K_v7.4$ localization and reduction in total protein levels, mesenteric arteries were incubated with the endosome inhibitor dynasore or the proteasome inhibitor MG132 in presence or absence of Ang II. In Western blot experiments co-incubation of dynasore (DYN), used at concentrations (100µmol/L) shown to

be effective in previous studies[15], did not fully prevent the reduction of K_v7.4 produced by 3h incubation with Ang II (Fig. 2C). Conversely, MG132 (20 µmol/L) fully prevented the Ang II-induced reduction of K_v7.4 (Fig. 2C). MG132 also counteracted the impairment of ML213-induced relaxation of mesenteric arteries by Ang II in myography experiments (Fig 2D).

Role of oxidative stress in Ang II-induced K_v7.4 degradation

Ang II is known to increase oxidative stress[16] which induces proteasome-mediated protein degradation[17]. We therefore evaluated whether the observed increase of K_v7.4 protein degradation induced by Ang II was mediated by oxidative stress. In VSMCs, treatment with Ang II for 30min increased the production of reactive oxygen species (ROS) measured by the fluorescence dye ROS Deep Red (by ~50% compared to time-matched control), similarly to that caused by 1mmol/L H_2O_2 (increase by ~70%, Fig 3A). In contrast, longer incubation (3h) with Ang II did not significantly enhance ROS levels with respect to time-matched control, whereas H_2O_2 was still able to increase ROS levels (by ~40%, (Fig 3A). The ROS scavenger N-Acetyl-L-Cysteine (NAC, 1mmol/L) counteracted Ang II-induced ROS production (Fig S5, http://hyper.ahajournals.org), but did not prevent either K_v7.4 protein down-regulation (Fig 3B) or the impairment of ML213-induced relaxation in mesenteric arteries when co-incubated with Ang II (Fig 3C).

Role of HSPs in Ang II-mediated K_v7.4 degradation

HSP70/90 machinery is abundantly expressed in eukaryotic cells, and is a key regulator of protein homeostasis in both physiologic and pathologic conditions[18]. Moreover, HSP70/90 interact with K_v7.4 in heterologous-expression systems[19]. Neither HSP70 nor HSP90 transcript/protein levels changed in Ang II-treated mesenteric arteries compared to control vessels (Fig S6, http://hyper.ahajournals.org). Therefore, we investigated whether Ang II-

induced downregulation of K_v7.4 was mediated by an altered interaction with HSPs using Proximity Ligation Assay (PLA). No significant differences in the number of interactions between K_v7.4 and HSP70 were observed in isolated VSMCs treated with Ang II when compared to controls (Fig 4A). In contrast, a significant reduction (by ~40%) in K_v7.4:HSP90 interactions was detected in VSMCs upon incubation with Ang II (Fig 4B). HSP70/90 machinery is linked to the proteasome system via the co-chaperone and E3 ubiquitin-ligase CHIP (**C**-terminus of **H**sp70-**I**nteracting **P**rotein). Incubation of VSMCs with Ang II enhanced the interaction of K_v7.4 with CHIP by ~30% (Fig 4C), and increased the number of ubiquitin molecules interacting with K_v7.4 by ~65% when compared to control (Fig 4D). These data suggested that Ang II reduced K_v7.4:HSP90 interactions thus enhancing protein ubiquitination via an increased interaction of Kv7.4:HSP70 complex with CHIP. To corroborate this, we studied the effect of 17-AAG (17-Demethoxy-17-(2-propenylamino) geldanamycin), a geldanamycin-analog which inhibits HSP90 function, on total K_v7.4 protein levels. Incubation of mesenteric arteries with 1µmol/L 17-AAG caused a reduction of K_v7.4 protein by ~60% (Fig 5A), increased the number of interactions between K_v7.4 and CHIP (Fig 5B), and the number of ubiquitin molecules interacting with K_v7.4 (Fig 5C).

Role of HSPs-CHIP-ubiquitin system in K_v7.4 degradation in vivo

Since K_v7.4 protein is down-regulated in Ang II-infused mice[7], we investigated whether the observed alterations of HSPs-CHIP-ubiquitin system could be detected in the same *in vivo* model of prolonged exposure to Ang II. PLAs showed that the number of interactions of K_v7.4 with HSP70 in VSMCs isolated from mesenteric arteries of Ang II-infused mice was not significantly different from those isolated from saline-infused mice (Fig 6A), whereas a significant decrease in the number of K_v7.4:HSP90 interactions was observed in VSMCs from Ang II- with respect to saline-infused mice (Fig 6B). Moreover, an increased interaction of

K_v7.4 with CHIP (Fig 6C) and ubiquitin (Fig 6D) was detected in VSMCs from Ang II-mice when compared to control groups.

DISCUSSION

Ang II reduces K_v7 expression and function via increased proteasome-mediated degradation

In this study, we present novel and detailed mechanistic information showing how Ang II, a key mediator of the pathological changes occurring in vascular disease[16], modulates K_v7.4 channel expression, which is down-regulated in hypertension[7]. Ang II has been demonstrated to acutely (up to 25 min) suppress the XE991-sensitive K_v current in mesenteric VSMCs[20], but no insight regarding the molecular mechanisms involved was provided, and the effects of longer exposure of Ang II on K_v7 expression and function were not studied. Our data show that treatment with Ang II (1-7h) reduced the expression of K_v7.4 protein, decreased the amplitude of voltage-dependent currents sensitive to the pan-K_v7 channels blocker XE991 in VSMCs, blunted the hyperpolarization of the VSMCs membrane induced by the K_v7 activator ML213, and impaired the vasorelaxant effects induced by the same drug in whole mesenteric arteries. In contrast, Ang II did not affect the ability of the K_{ATP} channel activator levcromakalin to hyperpolarize VSMCs. ML213 effects in smooth muscle are abolished by K_v7 blockers like XE991 (Fig S3, http://hyper.ahajournals.org) or linopirdine[14], strongly suggesting the involvement of K_v7 channels in ML213-induced arterial relaxation. ML213 has been reported to be more specific for channels formed by K_v7.2 (whose expression in VSMCs in negligible[3]) or K_v7.4 subunits[21], but one study suggested that it also enhances the current mediated by K_v7.5[22], which contributes with K_v7.4 to the formation of the mature channel in the vasculature[1, 23]. Due to the lack of K_v7 subunit-specific blockers, we could not rule out the involvement of K_v7.5 in ML213-induced relaxation. However, our immunofluorescence data (Fig S4, http://hyper.ahajournals.org) together with previous evidence[2, 8, 9, 23] infer that the

reduction of vasorelaxant effects of ML213 prompted by Ang II was mediated by decreased K_v7.4 levels in VSMCs. Interestingly, ML213 still produced some relaxation in Ang II-treated arteries even though its ability to hyperpolarize was largely (but not entirely) lost by incubation with Ang II. A previous study revealed that 1µM ML213 produced considerable relaxation of pre-contracted arteries without marked membrane hyperpolarization[14]. This apparent discrepancy in electro-mechanical coupling probably reflects the differences in experimental conditions, as isometric tension studies involved a pre-contracted and therefore depolarized artery compared to the microelectrode recordings performed on unstimulated arteries. Like other K_v7.2-7.5 activators ML213 works through stabilization of the open configuration that will be more apparent under depolarized conditions. However, further investigation regarding the role of K_v7 channels in regulating the electro-mechanical coupling in VSMCs is required.

K_v7.4 down-regulation was also confirmed in clonal cells expressing heterologous K_v7.4 channels, and was not accompanied by a reduction in mRNA levels, indicating that Ang II reduced K_v7.4 by post-transcriptional mechanisms. Modulation of protein trafficking, recycling and degradation has a considerable impact on K^+ channels expression and function[24] and dysregulation of recycling/degradation pathways has been linked to channelopathies such as hereditary arrhythmias or cystic fibrosis[24]. Our findings reveal that degradation of K_v7.4 protein by Ang II treatment and the subsequent functional impairment of K_v7-dependant vasorelaxation was prevented by proteasome inhibition. Ang II has been shown to induce proteasome degradation of large conductance calcium activated BK channels via increased endosome-mediated internalization in VSMCs, and Kv11.1 (ERG) channels in over-expression systems[15, 25]. Interestingly, in contrast to the above studies where Ang II effects were noticeable after prolonged treatment (7-24h), we observed downregulation and altered subcellular distribution of K_v7.4 after as little as 1h. Moreover, the lack of effect of dynasore in fully restoring K_v7.4 protein levels suggest that the endosomal pathway does not play a primary role

in Ang II-induced $K_v7.4$ down-regulation in contrast to BK_{Ca} channels[15]. Previous studies have established that Ang II causes oxidative stress in target cells[16], and that the increased ROS levels activate the ubiquitin-proteasome system, as a protective mechanism to prevent the accumulation/aggregation of oxidized proteins[17]. In our experiments, Ang II caused a rapid increase in ROS levels (30min) which did not persist with longer exposure (3h), and the ROS scavenger N-Acetyl-L-Cysteine did not prevent the down-regulation of $K_v7.4$ levels and function prompted by Ang II. Although we cannot exclude the possibility that sustained oxidative stress reduces $K_v7.4$ levels, our data suggest that the early burst of ROS production induced by Ang II was not sufficient to alter $K_v7.4$ abundance, and that the activation of the proteasome system triggered by oxidative stress was not the main mechanisms involved in Ang II-induced downregulation of $K_v7.4$.

Ang II-induced decrease of $K_v7.4$:HSP90 interaction promotes the ubiquitination of $K_v7.4$

Ang II affects protein folding in hypertensive mice[26], regulates the activity of HSPs, which mediate some maladaptive responses occurring in hypertension[27], and also regulate ion channel folding. HSP90 in particular, plays a pivotal role in the folding of the cystic fibrosis transmembrane conductance regulator (CFTR), the ClC-2 chloride channel[28], HERG channel[13] and the K_{ATP} potassium channel[29]. Moreover, enhancement of the HERG:HSP90 interaction is responsible for the increased membrane trafficking induced by estradiol[30]. Reduced binding of HSP90 decreases the interaction of client proteins with the HSP40-HSP70-HOP chaperone pathway that promotes forward trafficking, and increases interaction with HSP70-ubiquitin ligase CHIP complexes, which lead to proteasome-mediated degradation[31]. Our data reveal that the molecular chaperones HSP70/90 interact with $K_v7.4$ in native VSMCs, and that Ang II treatment selectively reduced the interaction of $K_v7.4$ with HSP90 in this physiological environment. In heterologous expression systems, modulating HSP90 isoforms levels either by overexpression or silencing strategies revealed a differential role in $K_v7.4$ maturation of the

two major HSP90 isoforms, namely α and β, with β-isoform promoting protein folding, and α-isoform enhancing CHIP-mediated proteasomal degradation[32]. Although some distinctive features exist, HSP90 isoforms exert similar actions, with many functions being characteristic of both isoforms and only associated more frequently to one of them[33]. Moreover, their expression is differentially regulated, with a constitutive form (HSP90β) and an inducible one (HSP90α). Although we did not investigate the contribution of α- or β-isoform, in our experiments we did not observe any change in HSP90 levels, suggesting that HSP90 expression was not modified by Ang II incubation, and that a different mechanism was responsible for the alteration in K_v7.4:HSP90 binding. Covalent modifications, including phosphorylation, acetylation and nitrosylation, influence the activity of HSP90 and consequently the maturation of selected clients. Phosphorylation and acetylation slow down HSP90 activity, resulting in a reduced maturation of client proteins[34, 35]. Therefore, Ang II might activate different pathways ultimately altering HSP90 and/or K_v7.4, modifying their ability to interact. Irrespective of the molecular mechanism, the ultimate effect was an increased interaction of K_v7.4 with CHIP and an enhanced ubiquitination of K_v7.4 as determined by PLA. This hypothesis was corroborated by studies with 17-AAG, a pharmacological inhibitor of HSP90, that in our experiments reproduced the effects of Ang II in terms of K_v7.4 down-regulation, interaction with CHIP and ubiquitination. Our results are in line with previous evidence showing that pharmacological inhibition of HSP90 induces rapid degradation of client proteins through the ubiquitin-proteasome pathway[13, 36].

PERSPECTIVES

We show for the first time in vascular smooth muscle that Ang II enhances K_v7.4 degradation via the ubiquitin-proteasome system, through a reduced interaction with HSP90. The specific pathway(s) activated by Ang II, as well as the possible molecular modifications occurring in all the interactors (including additional co-chaperones) will need to be further investigated.

Indeed, Ang II can activate diverse intracellular pathways, including non G-protein signaling, and modulate several processes, such as inflammation[16], which might in turn regulate HSPs activity and therefore protein stability and degradation. However, the present study has identified a novel and dynamic regulation of K_v7.4 that impacts considerably on vascular physiology. Noticeably, we showed that these alterations also occur *in vivo* after chronic exposure to Ang II, a well-known model of hypertension where K_v7.4 expression and function is reduced[7], suggesting that the observed reduction of K_v7.4 protein stability and enhanced degradation play a role also in the pathological changes occurring in hypertension. Since modulators of HSP90 function are being developed therapeutically[37-39], these data provide a possible new therapeutic strategy for the treatment of hypertension.

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DISCLOSURE

None

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NOVELTY AND SIGNIFICANCE

What is new. We showed that Ang II decreases the expression and function of Kv7.4 potassium channels in vascular smooth muscle cells by reducing its interaction with chaperone protein HSP90 and altering protein stability.

What is relevant. Kv7.4 is an important mediator of arterial relaxation and is down-regulated in hypertension, a condition where Ang II is dysregulated. Increasing HSP90 activity might represent a new strategy to treat hypertension.

Summary. Ang II down-regulates Kv7.4 by disrupting its interaction with HSP90, which lead to the recruitment of the ubiquitin-ligase CHIP and the subsequent protein degradation via the proteasome.

FIGURE LEGENDS

Fig. 1. Effects of Ang II on K_v7.4 expression and function. (A) Top: representative western blot showing K_v7.4 (detected with an anti-K_v7.4 antibody) and β-actin in mesenteric arteries after incubation with 100nmol/L Ang II for 1, 3 or 7 hours. Bottom: Quantification of western blot experiments. n=4-6. *=p<0.05: **=p<0.01 (Student's t-test). (B) Quantitative PCR showing kcnq4 mRNA levels after incubation with 100nmol/L Ang II for 7h. Data are expressed using the $2^{-\Delta Ct}$ formula. (C) In cell western blot showing the levels of EGFP-K_v7.4 (detected with an anti-GFP antibody) in transfected SGVSM-9 after incubation with 100nmol/L Ang II for 1h. Data are expressed as percentage of the average of the controls for each experimental session. n=12-14 wells per experimental point from 3-4 sessions. ***=p<0.001 (Student's t-test). The inset shows a SGVSM9 cell transfected with EGFP-K_v7.4, with GFP fluorescence (green pseudocolor) and DAPI staining (blue pseudocolor), proving the effective expression of EGFP-Kv7.4 protein. Scale bar: 5µm. (D) Mean current-voltage relationship (I-V) of the XE991-sensitive current in mesenteric VSMCs after 3 hours incubation in absence (control, black) or in presence (Ang II, grey) of 100nmol/L Ang II. n=5 cells per experimental point from 5 rats. **=p<0.01: ****=p<0.0001 (two-way ANOVA). (E) Changes in resting membrane potential (ΔE_m) of VSMCs in whole mesenteric artery induced by 10µmol/L ML213 (black columns) and 10µmol/L levcromakalim (grey columns) after 2 hours incubation in absence (control) or in presence (Ang II) of 100nmol/L. (F) Isometric tension recordings in mesenteric arteries incubated for 3 hours in absence (CTL, black) or presence (Ang II, grey) of 100nmol/L Ang II. The graph shows the relaxation induced by increasing concentration of ML213 in arteries pre-contracted with 1µmol/L U46619. Data are expressed as percentage of the maximum contraction to U46619. n=4-5. **=p<0.01; ***=p<0.001 (two-way ANOVA).

Fig. 2. Effects of Ang II on K_v7.4 subcellular distribution in mesenteric artery myocytes and role of endocytosis and proteasome in Ang II-induced down-regulation of K_v7.4. (A) Immunofluorescence experiments showing K_v7.4 (red pseudocolor) in absence (CTL) or in presence (Ang II) of 100nmol/L Ang II. Staining of the plasma membrane marker wheat germ agglutinin (WGA, green pseudocolor) and nuclear marker DAPI (blue presudocolor) are also shown. Scale bar: 5μm. (B) Top: fluorescence intensity profiles for K_v7.4 (red) and WGA (green) along the yellow line drawn in the pictures, expressed as Arbitrary Units (AU), in control (CTL) or Ang II-treated VSMCs. Regions where WGA intensity was <5AU were considered as cytosol (C), while portions where intensity was above the threshold were identified as plasma membrane (M). Bottom: bar graph showing the ratio between the average fluorescence intensities in the plasma-membrane and cytosol in VSMCs. For each cell, the mean of the ratios obtained along three random lines was calculated. n=15 cells per experimental point, obtained from 3-4 rats in 3-4 sessions. ***=p<0.001 (Student's t-test). (C) Top: representative western blot showing K_v7.4 and β-actin proteins in mesenteric arteries after incubation for 3h with different combinations of 100nmol/L Ang II, 100μmol/L dynasore (DYN), and 20μmol/L MG132, as indicated. Bottom: quantification of western blot experiments. Normalized K_v7.4 intensities are expressed as percentage of control. n=6: **=p<0.01, ns=non significant (one-way ANOVA). (D) Isometric tension recordings in mesenteric arteries incubated for 3 hours in control conditions or with 100nmol/L Ang II in absence of presence of 20μmol/L MG132. The graph shows the relaxation to ML213 in arteries pre-contracted with 300nmol/L U46619. Data are expressed as percentage of the maximum contraction to U46619. n=4-6. *=p<0.05: **=p<0.01: ****=p<0.0001 (two-way ANOVA).

Fig. 3. Role of oxidative stress in the downregulation of K_v7.4 induced by Ang II. (A) Left: representative images showing the fluorescence of the ROS-sensitive dye ROS Deep Red in mesenteric VSMCs after incubation for 30 min (upper panels) or 3 hours (lower panels) in

control medium (CTL) or in presence of 100nmol/L Ang II or 1mmol/L H_2O_2. Right: quantification of ROS Deep Red fluorescence intensity in VSMCs, expressed as Arbitrary Units (AU). n=35-48 cells obtained from 4-5 rats per experimental point in 4-5 sessions. *=p<0.05, ns=not significant (one-way ANOVA). (B) The inset shows a representative western blot of $K_v7.4$ and β-actin proteins in mesenteric arteries after incubation for 3 hours with different combination of 100nmol/L Ang II and 1mmol/L of N-Acetyl-L-Cysteine (NAC), as indicated. The bar graph shows the quantification of western blot experiments. n=6. *=p<0.05 (one-way ANOVA). (C) Isometric tension recordings in mesenteric arteries incubated for 3 hours in control condition or with 100nmol/L Ang II in absence of presence of 1mmol/L NAC. The graph shows the relaxation to ML213 in arteries pre-contracted with 1μmol/L U46619. Data are expressed as percentage of the maximum contraction to U46619. n=6. **=p<0.01, ***=p<0.001 ****=p<0.0001 (two-way ANOVA).

Fig. 4. Effects of Ang II on $K_v7.4$ interaction with Heat Shock Proteins and ubiquitin. Proximity Ligation Assay showing the interaction of $K_v7.4$ with HSP70 (A), HSP90 (B), CHIP (C), and ubiquitin (D) in mesenteric artery myocytes. Representative fluorescence and bright field (insets) confocal mid-cell xy sections of mesenteric VSMCs in absence (CTL) or presence (Ang II) of 100nmol/L Ang II are shown for each panel. Red puncta indicate target proteins are in close proximity (<40 nm). Bar graphs show the quantification of the mean number of PLA signals per mid-cell xy section. n=17-40 cells from 3-4 rats per experimental point in 3-4 sessions. *=p<0.05, ***=p<0.001, ****=p<0.0001 (Student's t-test). Nuclei (DAPI staining, blue) are also shown. Scale bar: 5μm.

Fig. 5. Effects of HSP90 inhibition on $K_v7.4$ levels, interaction with CHIP, and ubiquitination. (A) Left: representative western blot showing $K_v7.4$ and β-actin in mesenteric arteries incubated with DMSO (CTL) or 1μmol/L 17-AAG. Right: quantification of western blot experiments. n=5. *=p<0.05 (Student's t-test). (B-C) Proximity Ligation Assay showing

the interaction of K_v7.4 with CHIP (B) and ubiquitin (C) in mesenteric artery myocytes. Representative fluorescence and bright field (insets) confocal mid-cell xy sections of mesenteric artery myocytes incubated with DMSO (CTL) or 1μmol/L 17-AAG are shown for both panels. Red puncta indicate target proteins are in close proximity (<40nm). Nuclei (DAPI staining) are shown in blue pseudocolor. Scale bar: 5μm. Bar graphs show the quantification of the mean number of PLA signals per mid-cell xy section. n=24-44 cells from 3-4 rats per experimental point in 3-4 sessions. *=p<0.05, ****=p<0.0001 (Student's t-test).

Fig. 6. K_v7.4 interaction with Heat Shock Proteins, CHIP and ubiquitin in Ang II-infused mice. Proximity Ligation Assay showing the interaction of K_v7.4 with HSP70 (A), HSP90 (B), CHIP (C), and ubiquitin (D) in mesenteric artery myocytes obtained from mice infused for 28 days with saline solution or Ang II. Representative fluorescence and bright field (insets) confocal mid-cell xy sections of mesenteric VSMCs from saline- or Ang II- infused mice are shown for each panel. Red puncta indicate target proteins are in close proximity (<40 nm). Bar graphs show the quantification of the mean number of PLA signals per mid-cell xy section. n=35-40 cells from 4 mice per experimental group. *=p<0.05, **=p<0.01, ****=p<0.0001 (Student's t-test). Nuclei (DAPI staining, blue) are also shown. Scale bar: 5μm.

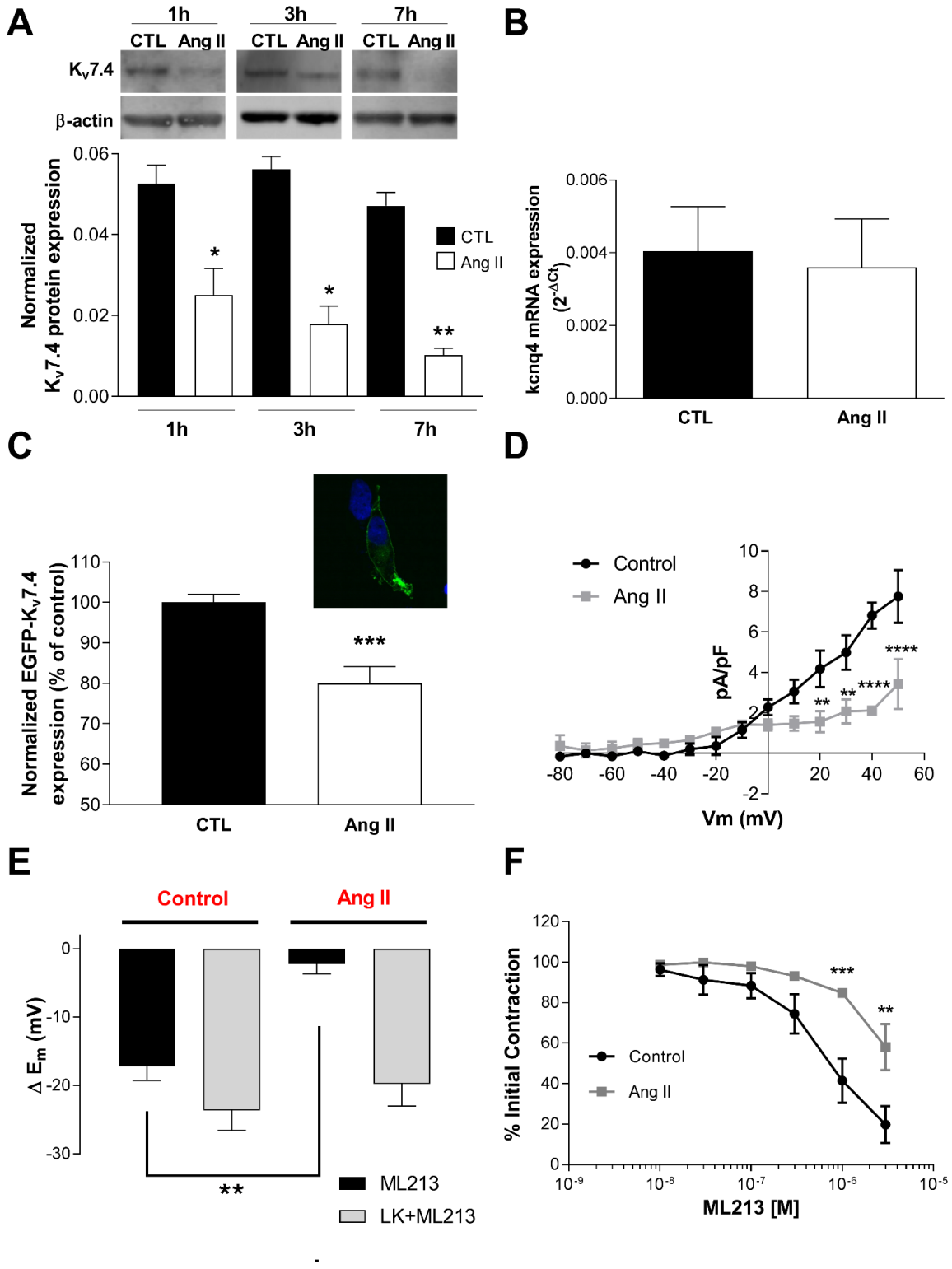
A
1h
3h
7h
CTL Ang II
CTL Ang II
CTL Ang II
Kv7.4
β-actin
Normalized Kv7.4 protein expression
0.06
0.04
0.02
0.00
*
*
**
CTL
Ang II
1h
3h
7h
B
kcnq4 mRNA expression (2-ΔCt)
0.006
0.004
0.002
0.000
CTL
Ang II
C
Normalized EGFP-Kv7.4 expression (% of control)
110
100
90
80
70
60
50
***
CTL
Ang II
D
Control
Ang II
pA/pF
10
8
6
4
2
-2
-80 -60 -40 -20 20 40 60
**
**
****
****
Vm (mV)
E
Control
Ang II
Δ Em (mV)
0
-10
-20
-30
**
ML213
LK+ML213
F
% Initial Contraction
120
100
80
60
40
20
0
***
**
Control
Ang II
10-9 10-8 10-7 10-6 10-5
ML213 [M]

Fig 1

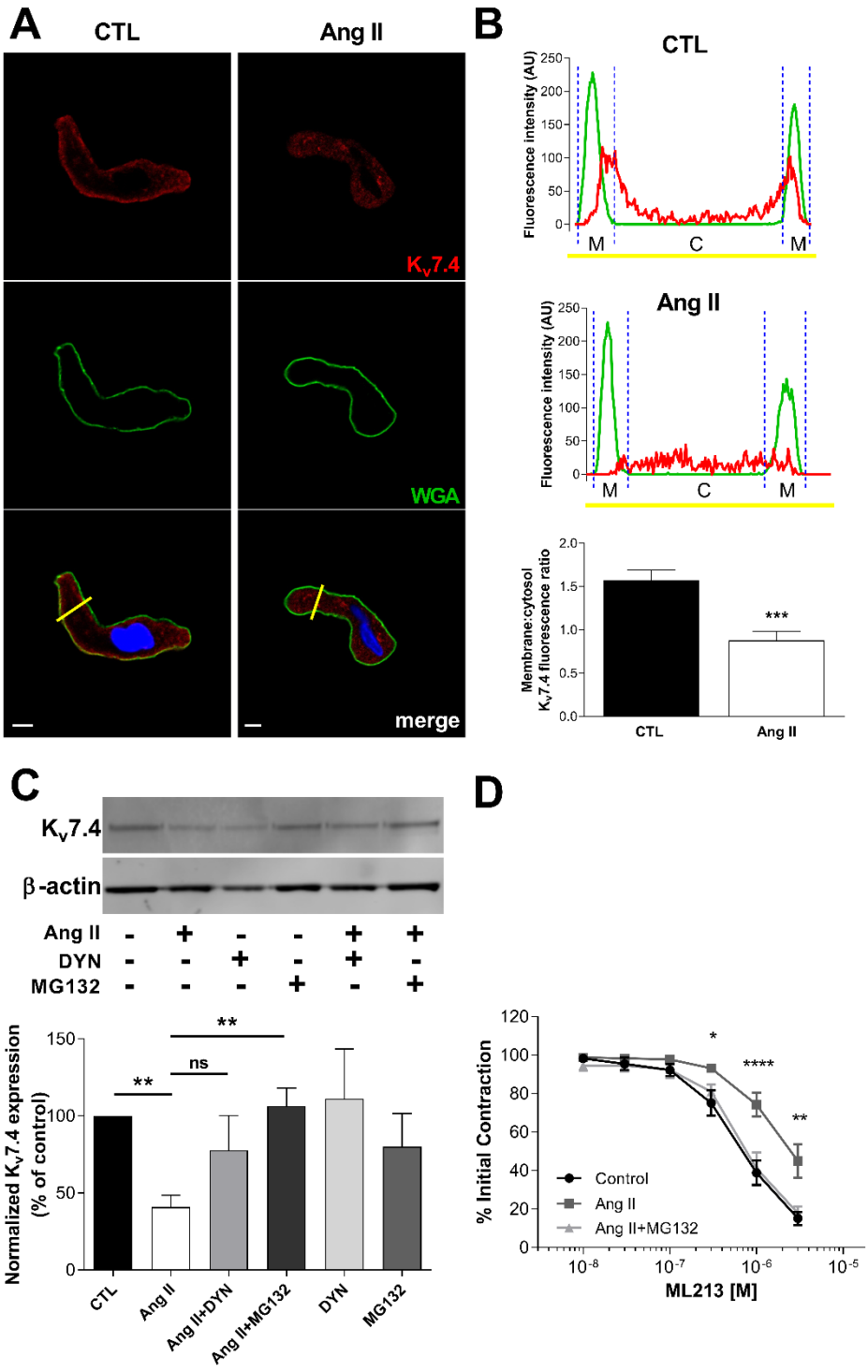
A
CTL
Ang II
K_v7.4
WGA
merge
B
CTL
Fluorescence intensity (AU)
M
C
M
Ang II
Membrane:cytosol K_v7.4 fluorescence ratio
***
CTL
Ang II
C
K_v7.4
β-actin
Ang II
DYN
MG132
Normalized K_v7.4 expression (% of control)
**
ns
**
CTL
Ang II
Ang II+DYN
Ang II+MG132
DYN
MG132
D
% Initial Contraction
Control
Ang II
Ang II+MG132
*
****
**
ML213 [M]

Fig 2

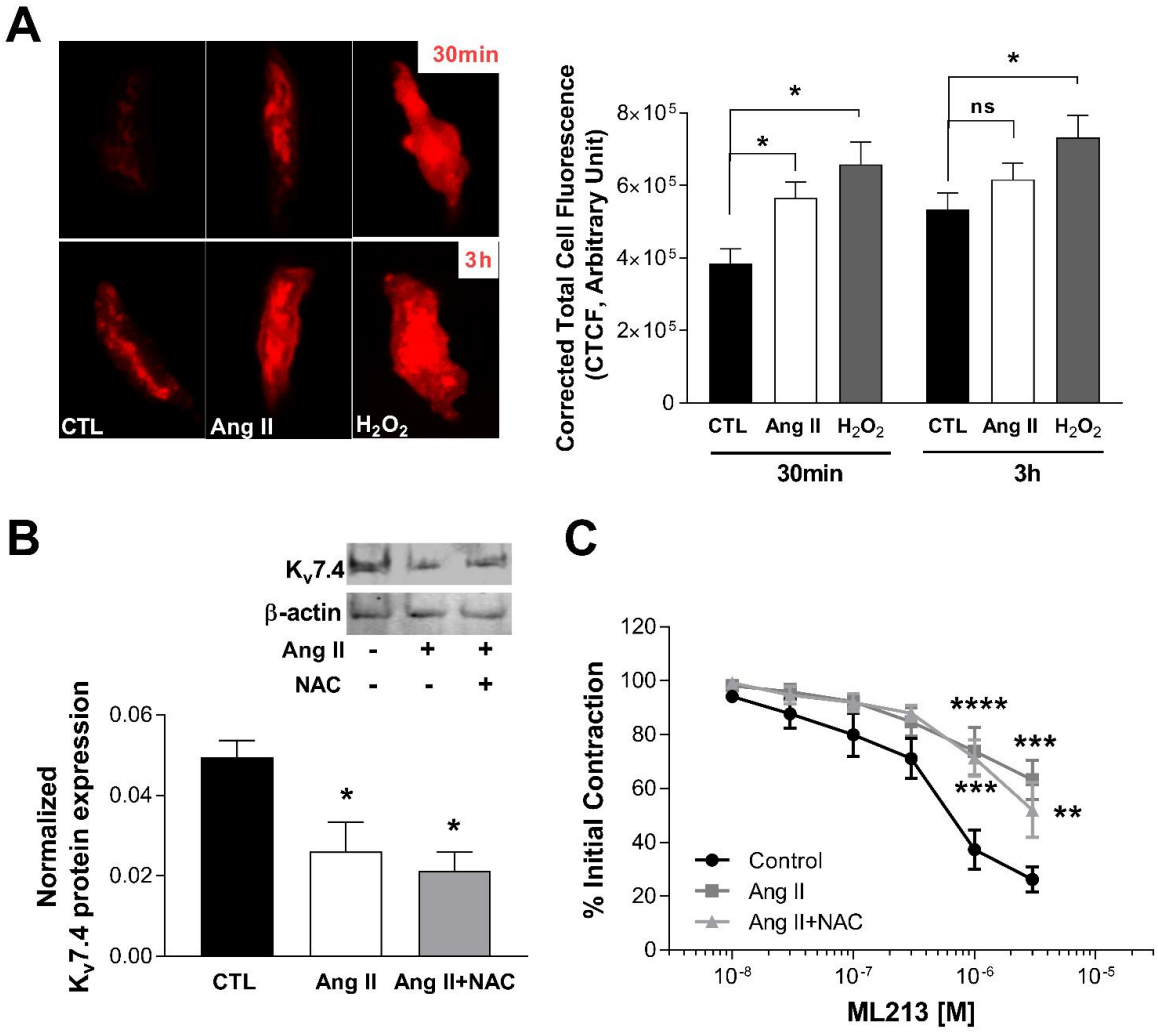
A
30min
3h
CTL
Ang II
H2O2
Corrected Total Cell Fluorescence (CTCF, Arbitrary Unit)
8×10^5
6×10^5
4×10^5
2×10^5
0
*
*
*
ns
CTL
Ang II
H2O2
CTL
Ang II
H2O2
30min
3h
B
Kv7.4
β-actin
Ang II - + +
NAC - - +
Normalized Kv7.4 protein expression
0.06
0.04
0.02
0.00
*
*
CTL
Ang II
Ang II+NAC
C
% Initial Contraction
120
100
80
60
40
20
0
****
***
***
**
Control
Ang II
Ang II+NAC
10^-8
10^-7
10^-6
10^-5
ML213 [M]

Fig 3

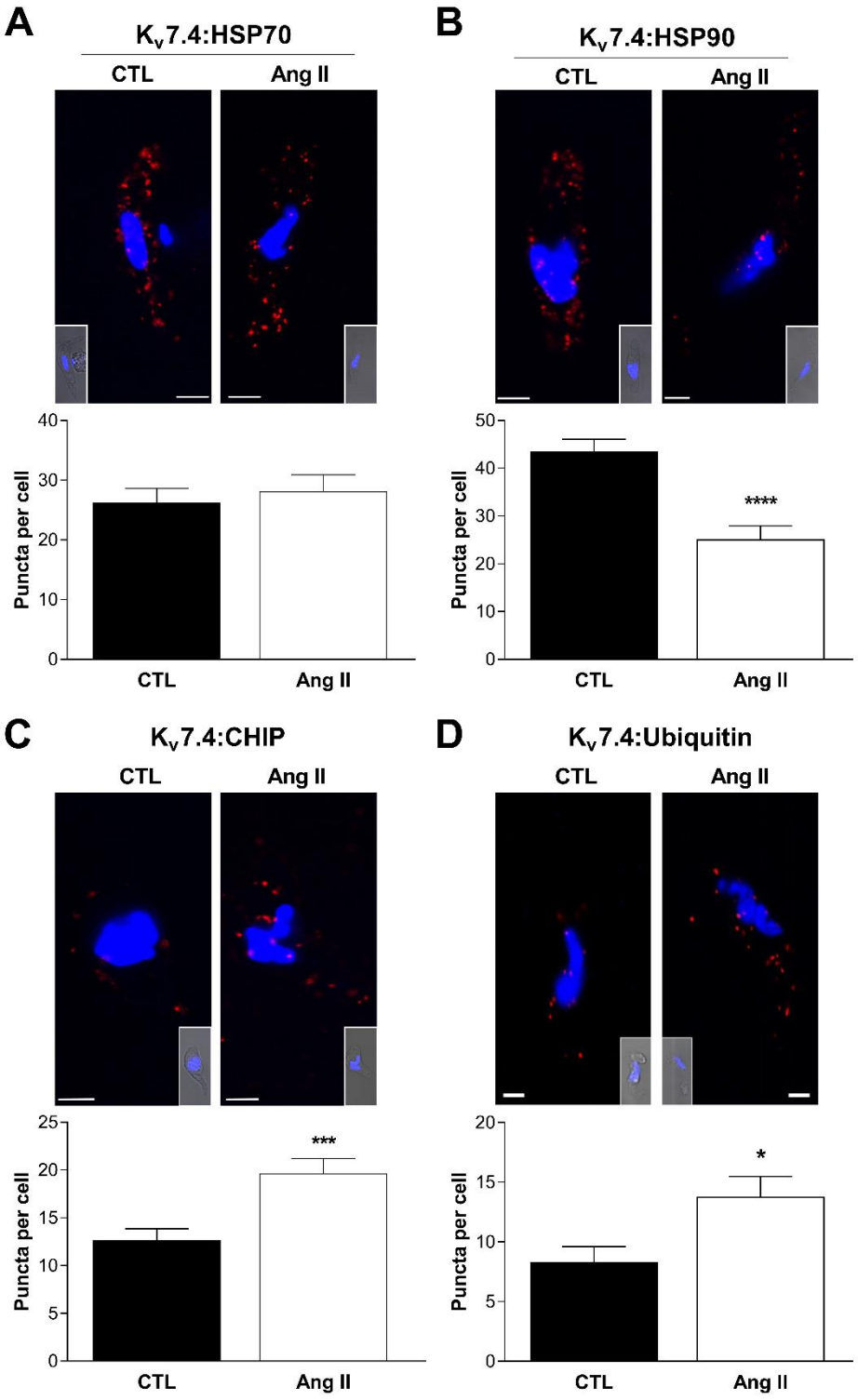
A
K_v7.4:HSP70
CTL
Ang II
Puncta per cell
40
30
20
10
0
CTL
Ang II
B
K_v7.4:HSP90
CTL
Ang II
Puncta per cell
50
40
30
20
10
0
****
CTL
Ang II
C
K_v7.4:CHIP
CTL
Ang II
Puncta per cell
25
20
15
10
5
0
***
CTL
Ang II
D
K_v7.4:Ubiquitin
CTL
Ang II
Puncta per cell
20
15
10
5
0
*
CTL
Ang II

Fig 4

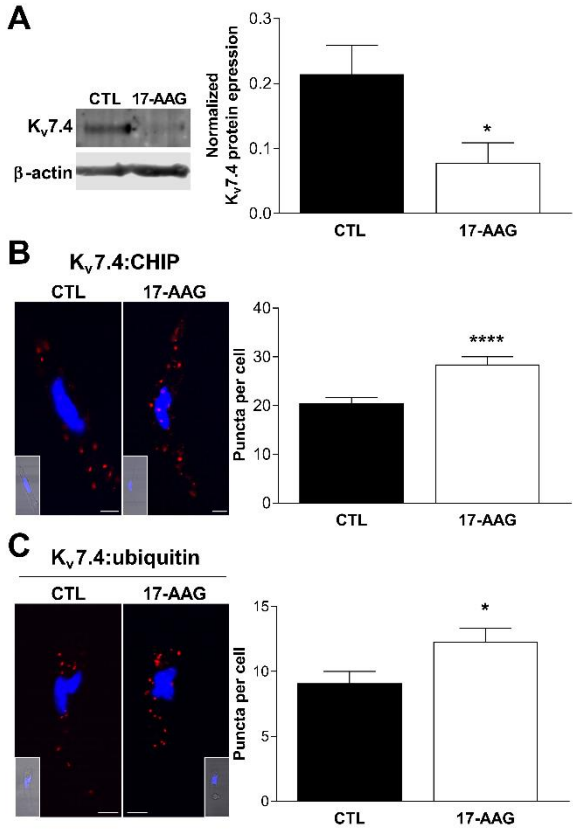
A
CTL
17-AAG
$K_v7.4$
β-actin
Normalized $K_v7.4$ protein epression
0.3
0.2
0.1
0.0
*
CTL
17-AAG
B
$K_v7.4$:CHIP
CTL
17-AAG
Puncta per cell
40
30
20
10
0
****
CTL
17-AAG
C
$K_v7.4$:ubiquitin
CTL
17-AAG
Puncta per cell
15
10
5
0
*
CTL
17-AAG

Fig 5

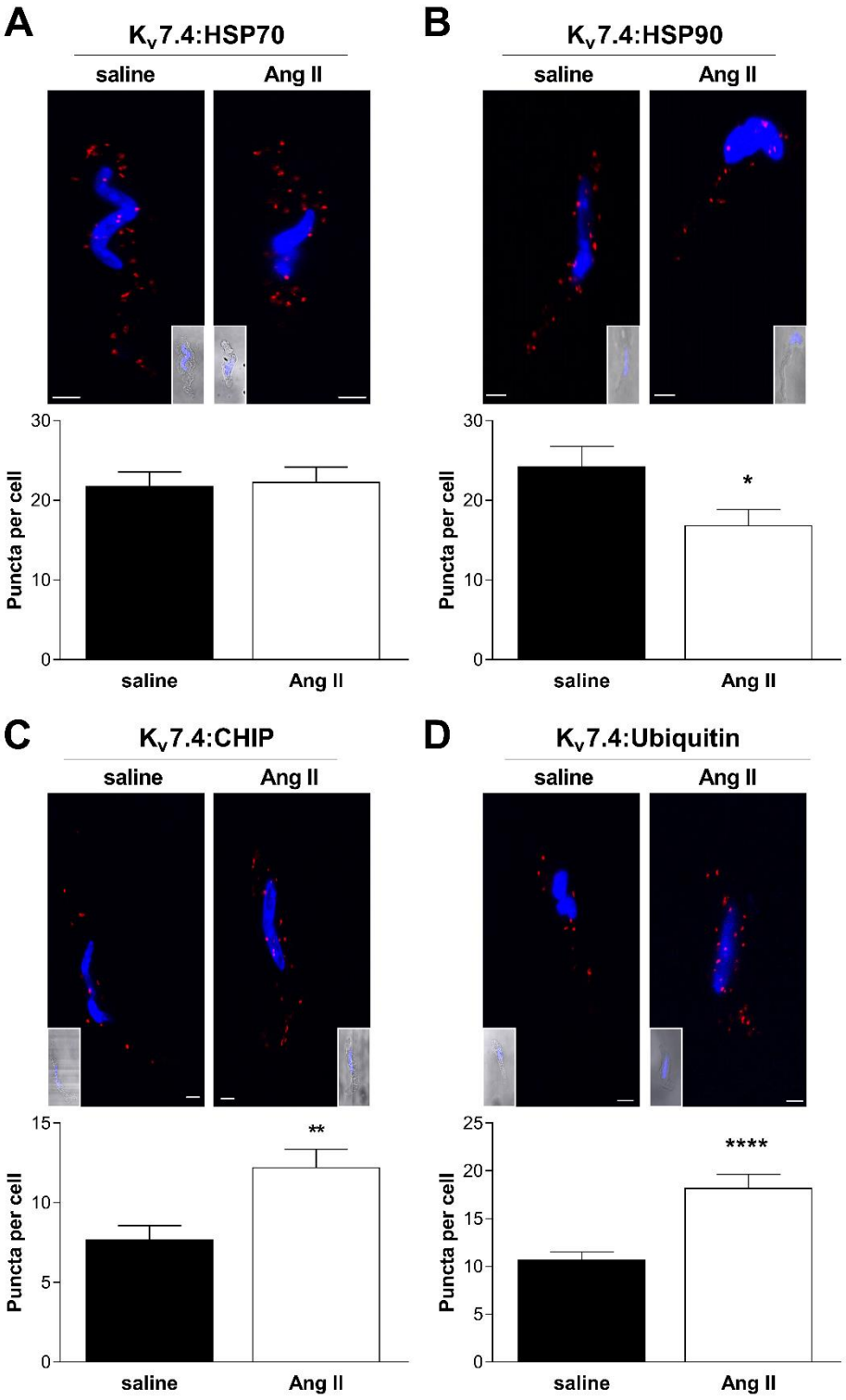
A
K_v7.4:HSP70
saline
Ang II
Puncta per cell
30
20
10
0
saline
Ang II
B
K_v7.4:HSP90
saline
Ang II
*
Puncta per cell
30
20
10
0
saline
Ang II
C
K_v7.4:CHIP
saline
Ang II
**
Puncta per cell
15
10
5
0
saline
Ang II
D
K_v7.4:Ubiquitin
saline
Ang II
****
Puncta per cell
25
20
15
10
5
0
saline
Ang II

Fig 6