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Angiotensin-Converting Enzyme and AT_1-Receptor Inhibitors Restitute Hypertensive Internal Anal Sphincter in the Spontaneously Hypertensive Rats

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Abbreviations: ACE, angiotensin-converting enzyme; Ang II, angiotensin II; Angen, angiotensinogen; AoSM, aorta smooth muscle; ASM, anococcygeus smooth muscle; AT_1-R, Ang II receptor subtype 1; BSA, bovine serum albumin; CV, cardiovascular; HRP, horseradish peroxidase; IAS, internal anal sphincter; IASP, Internal anal sphincter pressure; IOD, integrated optical density; KPS, Krebs' physiological solution; MBP, muscle bath perfusates; NCM, nitrocellulose membrane; PBS, phosphate-buffered solution; RAS, renin-angiotensin system; RSM, rectum smooth muscle; RT, room temperature; RT-PCR, reverse transcriptase polymerase chain reaction; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; TBS-T, Tris-buffered solution-Tween 20®; WKY, Wistar-Kyoto rats

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ABSTRACT

The present study determined the effects of ACE inhibitor captopril and AT_1-R antagonist losartan on the internal anal sphincter pressures (IASP) in spontaneously hypertensive (SHR) vs. normotensive Wistar-Kyoto (WKY) rats. The SHR had significantly higher IASP (21.7 ± 0.8 mmHg) than the WKY (14.7 ± 0.9 mmHg), which was associated with the higher levels of Ang II in the plasma (50.3 ± 0.9 pg/ml) and in the muscle bath perfusates (MBP) (72.7 ± 11.8 pg/ml) as compared with the WKY rats ($p < 0.05$). Captopril and losartan decreased the IASP in the SHR and WKY rats, but they were more potent in the SHR. Captopril and losartan normalized the IASP in the SHR, while these agents may compromise rectoanal continence in the WKY rats. RT-PCR and Western Blots showed higher levels of angiotensinogen, renin, ACE, and AT_1-R in the IAS of SHR. Ang II caused concentration-dependent contraction of the IAS smooth muscle strips from WKY (pEC_{50} = 8.5 ± 0.1) and SHR (pEC_{50} = 8.6 ± 0.2). Losartan (100 nM) significantly ($p < 0.05$) inhibited this effect. From these data we conclude: 1). Hypertensive IAS in the SHR is primarily the result of RAS upregulation; 2). ACE inhibitors and AT_1-R antagonists simply relieve the hypertensive IAS; and 3). Differential effect of these inhibitors in the hypertensive vs. the normotensive IAS may explain the lack of incontinence as a side effect in hypertensive patients on ACE inhibitors and AT_1-R antagonists.

INTRODUCTION

Earlier studies from our laboratory have put forward evidence for the role of angiotensin II (Ang II) biosynthesis in the spontaneous myogenic tone of the internal anal sphincter (IAS) of rats (De Godoy et al., 2004). The studies show the expression of key components of the renin-angiotensin system (RAS): angiotensinogen (Angen), the Ang I-generating enzyme renin, and the Ang I-converting enzyme (ACE). Additional studies suggest an autocrine mechanism for Ang II generation in the regulation of the IAS basal tone (De Godoy et al., 2004; De Godoy and Rattan, 2005). These findings are in line with a broad range of reports in different tissues (Dzau et al., 1987; Dzau et al., 1988; Eggena et al., 1990; Griendling et al., 1993; Katwa et al., 1996; Bataller et al., 2003). These data expand the role of the RAS from endocrine to the local regulation.

The role of Ang II in different organ systems can be determined by the classical ACE inhibitors or by Ang II type 1 receptor (AT_1-R) antagonists, captopril and losartan, respectively. Captopril was first described in 1978 as a chronic antihypertensive in the hypertensive rats by inhibiting the conversion of Ang I into Ang II by ACE (Rubin et al., 1978). Losartan was introduced later as an alternative to captopril for the treatment of hypertension (Celik et al., 1995).

In previous "*in vitro*" studies we showed that inhibition of Ang II generation decreases the IAS basal tone (De Godoy et al., 2004; De Godoy and Rattan, 2005), suggesting the potential use of Ang II inhibitors in certain gastrointestinal disorders characterized with the hypertensive IAS. However, direct role of RAS and Ang II in the hypertensive IAS pressure (IASP) has not been examined.

The goal of the present studies was to evaluate the role of RAS, and the effects of Ang II inhibitors in the IASP of Wistar-Kyoto normotensive rats (WKY) and spontaneously hypertensive rats (SHR). We carried out "*in vivo*" and "*in vitro*", functional, biochemical, and molecular biology experiments in these animal models. We also examined the effects of captopril and losartan in the IASP. We systematically compared these parameters in the tonic smooth muscles of the IAS vs. those of the rectum (RSM), and the aorta (AoSM). The RSM and AoSM represent phenotypes of phasic smooth muscles.

Materials and Methods

Measurements of Intraluminal Pressures in the IAS Pressure (IASP). WKY and SHR were anaesthetized with isoflurane (initially with 5% isoflurane and then maintained with 1 % isoflurane throughout the length of the experiment). The IASP was measured via a solid-state transducer/catheter model 2-Fr Mikro-Tip (Millar Instruments, Houston, TX), following the previously described method of Terauchi et al (Terauchi et al., 2005). The catheter assembly was initially introduced into the rectum and then positioned precisely in the IAS via slow pull-through (about 7-8 mm from the anal verge). High pressures of the IAS consisted of rhythmic fluctuations superimposed on the steady tone. The IASP was recorded using a PowerLab/8SP recorder and analyzed via the software Chart 4 PowerLab (ADInstruments, Inc., Colorado Springs, CO).

Systolic Blood Pressure Measurements. The systolic blood pressure (SBP) was recorded via a catheter (inside diameter 0.38 mm, Clay Adams, Parsippany, NJ), attached to a Statham transducer (Medex, Inc., Carlsbad, CA). All recordings and analyses were carried out using Chart 4 PowerLab (ADInstruments, Inc.).

Administration of Agents. IASP and SBP were monitored for 30 min following the additive administration of captopril and losartan (1 to 50 mg/kg each, over 1 min of infusion). These doses have been shown to be selective in their actions (Celik et al., 1995; Tramontana et al., 2001).

Muscle Bath Studies. Animals were sacrificed by decapitation and the smooth muscle of IAS, RSM, and thoracic aorta were prepared for the recording of isometric tension as described before (Moummi and Rattan, 1988; De Godoy et al., 2004; Fukada et al., 2005). Briefly, circular smooth muscle strips of the IAS and RSM (~ 1 mm x 10 mm), and aorta rings (5 – 6 mm in length) were prepared in the oxygenated KPS.

Isometric tension was recorded via force transducer (model FT03; Grass Instruments, Quincy, MA) on a PowerLab/8SP data acquisition system (ADInstruments, Inc.). Following the equilibration period of 90 min, we measured the development of tone in the WKY and SHR groups in the basal state. The basal tone in the IAS was calculated with reference to the baseline determined at the end of each experiment with 50 mM EDTA (Biancani et al., 1985; Moummi and Rattan, 1988).

Cumulative concentration-response curves for Ang II (0.1 nM – 10 μM) in the IAS were obtained before and after 100 nM losartan, both in the background of 10 μM captopril. The changes in IAS basal tone were expressed as the percentage of maximal contraction by 10 μM bethanechol. The losartan concentration used in this study has been previously shown to be effective (Fan et al., 2002; De Godoy et al., 2004). The experimental protocols for *in vivo* and *in vitro* studies were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University and were in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Ang II Determinations. Arterial blood (2 ml) was rapidly collected into a chilled (4ºC) tube containing 1mg/ml of EDTA and 1 μM amastatin (inhibitor of the enzymes that degrade Ang II;

(De Godoy et al., 2004). The blood was centrifuged at 4,000g for 10 min and the plasma (1 ml) was immediately applied to an octadecasilyl(C_8)-silica cartridge (Waters Corp., Milford, MA). Samples were also collected as 2 ml of the muscle bath perfusates (MBP) following the incubation of the IAS, RSM, and AoSM tissues for 30 min in the presence of 1μM amastatin, and immediately applied to C_8 cartridges. All samples were washed with 10 ml of 0.1% trifluoroacetic acid. The peptides were eluted with 6 ml methanol: water: trifluoroacetic acid (80:19:1, v/v/v). The eluate was dried in a vacuum centrifuge and stored at 4 ºC until the further procedures (Bagby et al., 1979; Aihara et al., 1999). The eluate was dissolved in 300 μl of PBS, transferred onto a 96-well React-Bind™ NeutrAvidin™-coated ELISA plate (Pierce, Rockford, IL), and incubated for 1 h at RT. Non-specific binding was blocked with blocking buffer (PBS, BSA 0.1 %, and 0.05 % Tween®-20) for 30 min at RT. Then, each well was washed 3 times with washing buffer (PBS and 0.05% Tween®-20) and incubated for 60 min at RT with 100 μl of rabbit anti-Ang II antibody diluted in blocking buffer. Wells were washed and incubated for 60 min at RT with the donkey adsorbed anti-rabbit secondary antibody in blocking buffer. Wells were washed and exposed to the 1-Step TMB Substrate (Pierce, Rockford, IL) for 30 min at RT. Finally, the reaction was stopped by 2 M H_2SO_4, and the plate was read at 450 nm in an ELISA plate reader. Our preliminary experiments determined optimal conditions to be 1:1,000 and 1:2,000 for primary and secondary antibodies (De Godoy and Rattan, 2005). The levels of Ang II in plasma and MBP were determined in the basal state, and in the presence of captopril or losartan (50 mg/kg and 10 μM each in the case of plasma and MBP, respectively).

RT-PCR. Total RNA was isolated and purified from different tissues by the acid guanidine–phenol–chloroform method (Chomczynski and Sacchi, 1987) and quantified by measurement of absorbance at 260 nm in a spectrophotometer. Total RNA (2 μg) was subjected to first-strand

cDNA synthesis using oligo dT primers (Promega, Madison, WI) and Omniscript RT Kit (Qiagen, Germantown, MD) in a final volume of 20 µl at 42°C for 60 min. PCR primers specific for Angen, renin, ACE, AT_1-R, and α-actin cDNA were designed as shown on Table 1. PCR was performed in a Promega 2x Master Mix (Promega, Madison, WI) in a final volume of 25 µl, using a Perkin-Elmer Thermal Cycler (PerkinElmer Life and Analytical Sciences, Inc., MA). The PCR conditions (in the case of Angen, renin, and α-actin) consisted of 94ºC for 5 min (for the initial denaturation phase) followed by 35 cycles of 94ºC for 30s (denaturation), 57ºC for 30s (annealing), and 72ºC for 1 min (extension). In the end, it was allowed a final extension at 72ºC for 7 min. In the case of ACE and AT_1-R, the annealing temperature was set at 60ºC. The PCR products were separated on 1.5% (w/v) agarose gel containing ethidium bromide and were visualized with UV light. The relative densities of Angen, renin, ACE, and AT_1-R were calculated by normalizing the integrated optical density (IOD) of each blot with that of α-actin.

Western Blot Analysis. Western Blot studies were performed to determine the relative distribution of Angen, renin, ACE, and AT_1-R following the previous method (Fan et al., 2002). Briefly, the tissues isolated and dissected as described above were subjected to homogenization, protein extraction, and determination by the method of Lowry et al. (Lowry et al., 1951). The proteins (20 µg) were then separated by gel electrophoresis and transferred onto a nitrocellulose membrane (NCM) at 4°C.

The NCM was then incubated with the specific primary antibodies (mouse IgG, 1:1,000 for Angen and renin; goat IgG, 1:1,000 for ACE; rabbit IgG 1:1,000 for AT_1-R) for 2 h at RT. After washing with TBS-T, the NCMs were incubated with HRP-labeled secondary antibody (1:10,000) for 1 h at RT. The corresponding bands were visualized with enhanced

chemiluminescence substrate using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and Hyperfilm MP (Amersham Bioscience, Corp., NJ).

NCMs were then stripped of antibodies using the Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL) for 5 min at RT, and then reprobed for α-actin using the specific primary (mouse IgG 1:10,000 for α-actin) and secondary (1:10,000) antibodies. Bands corresponding to different proteins were scanned (SnapSacn.310; Agfa, Ridgefield Park, NJ) and the IODs determined using Image-Pro Plus 4.0. The relative densities were calculated by normalizing the IOD of each blot with that of α-actin.

Drugs and Antibodies. Ang II, amastatin, bethanechol, captopril, and HOE-140 were from Sigma-Aldrich (St. Louis, MO). Losartan was a generous gift from Merck (Rahway, NJ). Ang II antibody was from Peninsula Laboratories, Inc. (San Carlos, CA). Angen antibody was from Research Diagnosis, Inc. (Flanders, NJ). All other antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All PCR primers were from MWG (MWG-Biotech Inc., High Point, NC).

Data Analysis. Results were expressed as means ± S.E.M. Concentration-response/dose-response curves were analyzed using a non-linear interactive fitting program (GraphPad Prism 3.0, Graph Pad Software Incorporated, CA). Agonist potencies and maximum responses were expressed as pEC_{50} (negative logarithm of the molar concentration of agonist producing 50% of the maximum response) and E_{max} (maximum effect elicited by the agonist), respectively. Inhibitor potencies and maximum inhibition were expressed as log ID_{50} (logarithm of the mg/kg dose of inhibitor producing 50% of the maximum inhibition) and I_{max} (maximum inhibition elicited by the inhibitor), respectively. Statistical significance was tested by the one-way analysis of variance (ANOVA) followed by the Dunnett post-hoc test when three or more

different groups were compared. To compare only two different groups, the unpaired Student t-test was used. A 'p' value less than 0.05 was considered to be statistically significant.

Results

Comparisons of the Intraluminal Pressures (IASP), and Systemic Blood Pressure (SBP) in WKY vs. SHR. The IASP in the SHR group was significantly higher than in the WKY group with the values of 21.7 ± 0.8 mmHg and 14.7 ± 0.9 mmHg, respectively (*; $p < 0.05$; n = 6-7; Fig. 1A). SBP was also significantly ($p < 0.05$) higher in the SHR than in the WKY group with the values of 172.2 ± 1.9 mmHg and 115.5 ± 2.8 mmHg, respectively (*; $p < 0.05$; n = 6; Fig. 1B).

Effects of Captopril and Losartan on the IASP. Captopril and losartan significantly ($p < 0.05$) reduced the IASP in both strains of rats (Figs. 2 and 3). However, these agents were more efficacious and potent in the SHR (Table 2). For example, 5 mg/kg captopril produced significantly greater fall in the IAS in the SHR vs. WKY rats, 6.7 ± 0.8 and 1.5 ± 0.3 mmHg, respectively (*; $p < 0.05$; n = 4; Fig. 2). Interestingly, the final IASP in the case of WKY rats following 50 mg/kg captopril was significantly lower (7.9 ± 0.4 mmHg, Fig. 4A) than in the SHR (9.4 ± 0.4 mmHg, Fig. 4B). An actual defecation reflex was observed following captopril (50 mg/kg) in 1 out of 4 WKY rats, where the pressures from the IAS fell down to the levels not significantly different from those of the rectum (3.5 ± 2.5 mmHg). This suggests that higher doses of captopril may compromise IAS tone, one of the components of the rectoanal continence.

Likewise, 5 mg/kg of losartan produced significantly greater fall in the IASP in the SHR vs. WKY rats, 7.0 ± 0.6 and 3.3 ± 0.3 mmHg (*; $p < 0.05$; n = 4; Fig. 3). It is noteworthy that in the SHR although captopril and losartan were more potent in causing the fall in the IASP in absolute

terms, these agents simply normalized the IASP to the levels not significantly different from the WKY group (Table 2).

Effects of Captopril and Losartan on the Systolic Blood Pressure (SBP). Captopril and losartan caused significant fall in the SBP of SHR but not in the WKY rats (Fig. 5). However, the final values in the case of SHR even after the higher doses of these agents were not significantly different from those of WKY rats. The final SBP after 50 mg/kg of captopril and losartan in the case of SHR was 122.2 ± 2.3, and 123.0 ± 6.3 mmHg, respectively, and these values in the case of WKY rats before the administration of any of these agents were 114.3 ± 2.3, and 117.1 ± 5.7 mmHg, respectively ($p > 0.05$; $n = 4$). Therefore, both ACE inhibitor and AT_1-R antagonist caused similar normalization of elevated SBP and IASP in the SHR.

Comparison of Ang II Levels II in plasma of WKY and SHR. Ang II levels in the plasma samples from the SHR were significantly higher than in the WKY rats (50.3 ± 0.9 pg/ml vs. 13.2 ± 3.0 pg/ml, $n = 3$) (Fig. 6). Pretreatment with captopril (50 mg/kg) significantly reduced the circulating levels of Ang II in both groups of rats, confirming the efficacy of this agent as an ACE inhibitor.

Losartan (50 mg/kg) caused no significant change in the Ang II levels in the plasma samples from WKY rats, but caused significant increase in SHR (*; $p < 0.05$; Fig. 6). The higher levels of Ang II in the plasma may be explained on the bases of the displacement of Ang II from the binding sites from different tissues.

Comparison of Basal IAS tone, and Ang II Levels in the Muscle Bath Perfusates (MBP) from the IAS, RSM, and AoSM, in WKY and SHR. In agreement with the *in vivo* data, we observed a significant increase in the basal tone in the IAS from SHR vs. WKY. The IAS tone

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in these groups of animals was 0.065 ± 0.013 g/mg and 0.025 ± 0.009 g/mg of the tissue weight, respectively (*; $p < 0.05$; n = 6; Fig. 7A).

Ang II levels in the MBP from the IAS (from both WKY and SHR) were higher than those from the RSM and AoSM (Fig. 7B). These differences were more dramatic between IAS and RSM from SHR, 72.7 ± 11.8 pg/ml and 16.0 ± 3.8 pg/ml, respectively (*; $p < 0.05$; n =3; Fig. 7B).

Interestingly, in agreement with the data from the plasma samples, losartan pretreatment (10 μM) caused an increase in the levels of Ang II in the MBP from the IAS of SHR but not WKY rats, from 72.7 ± 11.8 to 115.7 ± 6.0 pg/ml (*; $p < 0.05$; Fig. 7C). However, pretreatment of the IAS tissues with captopril (10 μM), as expected, caused significant reduction in the levels of Ang II both in the WKY and SHR.

Western Blots to Determine the Relative Expression of Angiotensinogen (Angen), Renin, ACE, and AT_1-R in the WKY and SHR. Angen was observed as a doublet: 65 kDa and 55 kDa bands (Fig. 8A). As previously demonstrated in rat pancreas (Leung et al., 1999) and in rat IAS (De Godoy and Rattan, 2005), the higher molecular weight band represents the proAngen, and the lower molecular weight band represents the renin substrate Angen. SHR samples expressed significantly ($p < 0.05$) higher levels of Angen (but not proAngen) than in the WKY (Fig. 8A). Expression of renin was also significantly higher in the IAS of SHR vs. WKY rats (*; $p < 0.05$; Fig. 8B), but not in the RSM and AoSM ($p > 0.05$).

ACE and AT_1-R were found to be expressed in significantly higher levels in the SHR vs. WKY rats in the IAS and AoSM (*; $p < 0.05$; Fig. 9A,B). In the RSM of SHR however, the levels of AT_1-R were not significantly different from WKY rats.

RT-PCR of Angen, Renin, ACE, and AT_1-R in the WKY and SHR. To determine the role of RAS in the elevated IASP and the IAS tone in SHR vs. WKY rats, we further evaluated the transcriptional patterns of the genes coding Angen, renin, ACE, and AT_1-R. AoSM lacked RT-PCR products for renin (Fig. 10B). The expression patterns of AT_1-R were significantly higher (Fig. 10D), lower in the case of Angen (Fig. 10A), and no significant differences in the case of renin (Fig. 10B) and ACE (Fig. 10C). These differences in the RT-PCR data (in contrast to the higher levels of Ang II, and translational expression of different components of the RAS in the SHR vs. WKY rats) suggest the regulation of RAS in Ang II generation to be at the post-transcriptional level.

Contractile Effect of Ang II in the IAS. Ang II in the presence of 10 μM captopril produced concentration-dependent contraction of the IAS smooth muscle strips from WKY and SHR, with similar E_{max} or pEC_{50} values (Table 3). Losartan (in the presence of captopril) produced a significant ($p < 0.05$) inhibition of the Ang II response. However, the smooth muscle strips from the SHR were more resistant to losartan as compared with the WKY group ($p < 0.05$; Table 3; Figure 11).

Discussion

Present studies demonstrate hypertensive IAS in SHR, and its restitution to the normotensive levels following the administration of ACE and AT_1-R inhibitors. Using a multipronged approach of functional, molecular biology, and Ang II bioassay measurements the studies show that the hypertensive IAS in these animals is associated with the upregulation of the RAS.

There is abundance of literature to show the relationship between the upregulation of the RAS and the development of cardiovascular (CV) hypertension (Bagby et al., 1979; Hubner et

al., 1995; Bolterman et al., 2005) in the SHR. In all of these studies normotensive WKY rats have been used as control. Considering recent concept on the role of RAS in the IAS tone (Rattan, 2005; De Godoy and Rattan, 2005), the present studies provide important information on the role of RAS in the pathophysiology of the IAS tone in the SHR. SHR develop significantly higher IASP (on anorectal manometry for the intraluminal pressures in *in vivo* studies), and IAS tone (in *in vitro* studies), suggesting a relationship between CV and IAS hypertensions. Similar relationship has been reported in certain patients (Celik et al., 1995) with CV hypertension associated with hypertensive IAS and constipation. These symptoms disappeared after the treatment with antihypertensive agents. Above changes in the IAS tone in the SHR appear to be selective since no such hyperactivity was observed in the RSM in these animals. Such data provide important future directions to explore the effects of ACE and AT_1-R inhibitors in the motility disorders characterized with IAS hypertension.

Since their advent, captopril and losartan have been extensively used as antihypertensive agents because of their ability to inhibit Ang II generation and interaction with the AT_1-R, respectively (Rubin et al., 1978; Celik et al., 1995). The present data also demonstrate the normalization of the hypertensive IAS with both captopril (Fig. 2) and losartan (Fig. 3) in the SHR in par with the basal IASP in the WKY rats. Similar results were also observed in parallel showing normalization of SBP of the SHR following these inhibitors (Fig. 5). Beyond normalization, these agents do not produce any hypotensive effect neither in the CV or IAS in the SHR. This is in agreement with previous reports showing the absence of adverse effects of captopril and losartan in normotensive rats and humans (Lee et al., 1991; Horita et al., 2004; Cosentino et al., 2005).

Present data support the previous concept that the basal tone in the IAS of normal rats is partially mediated by the interaction of Ang II with the AT_1-R (De Godoy et al., 2004; De Godoy and Rattan, 2005). We also observed in one out of four normotensive animals that the higher dose of captopril induces defecation reflex resulting in the expulsion of fecal pellets (Fig. 4A). This effect was not encountered in any of the SHR (Fig. 4B) where the higher doses of captopril or losartan do not reduce the basal IASP below the normotensive levels. Such observations may provide an explanation for the lack of symptomatic anal incontinence following captopril specifically in the hypertensive patients.

Above symptomatic changes in the IAS of normotensive rats were not observed with losartan. A possible mechanism for the distinct effects of captopril in the normotensive animals may be potentiation effect by captopril on bradykinin. Accordingly, in addition to the ACE inhibition, captopril is known to inhibit the degradation of bradykinin, a circulating peptide that produces smooth muscle relaxation (Engel et al., 1972). In support of this notion, we have observed that bradykinin is selectively and significantly more potent in producing IAS relaxation in the case of WKY in comparison with the SHR (data not include).

In agreement with the earlier concept of the autocrine control of RAS in the IAS (De Godoy and Rattan, 2005), the levels of Ang II in the IAS MBP were significantly higher as compared with the RSM and AoSM in normotensive WKY rats (Fig. 7B,C). These levels in the IAS increase further several folds in the SHR. The authenticity of Ang II measurements in the MBP and in the plasma (Fig. 6) was ascertained by the precipitous fall in these levels in the presence of ACE inhibitor.

In contrast to the effect of captopril on Ang II levels in the plasma and MBP, losartan increases Ang II levels in the SHR. These findings may be explained on the bases of Ang II

displacement from the receptor binding sites by competition with the AT_1-R antagonist (Zhu et al., 2004).

Higher levels of Ang II in the SHR were accompanied with the upregulation of the RAS in all tissues examined. Interestingly, however, AoSM does not express RT-PCR (Fig. 10B) and Western blot products (Fig. 8B) for renin. In contrast to the clear demonstration of the RAS upregulation at the translational level in SHR, at the transcriptional level, all tissues show lower levels of Angen gene transcripts, and no significant differences in the renin and ACE (shown by RT-PCR data). These observations suggest that the upregulation of RAS occurs at the post-transcriptional level (Figs. 8-10). Upregulation of the AT_1-R in the SHR however, may occur both at the transcriptional and translational levels (Figs. 9B and 10D).

Previous studies from our laboratory have shown that Ang II causes direct contraction of the rat IAS via AT_1-Rs (Fan et al., 2002; De Godoy and Rattan, 2005). In the present studies we compared the effects of different concentrations of Ang II in the basal tone of WKY and SHR IAS after treatment with captopril. Captopril was used to eliminate interference of endogenous Ang II production. The data reveal no significant difference in the potencies of Ang II between WKY and SHR (Fig. 11) in spite of the increase in the number of AT_1-R in the SHR. The maximal biological response may be achieved at agonist concentrations lower than those required to occupy all the available receptors (Zhu, 1993). Because of this, above findings in the IAS do not negate the physiological relevance of increase in the number of AT_1-R in the SHR. Based on the theory of spare receptors (Zhu, 1993) we hypothesized that if increase in the AT_1-R are of functional importance, less Ang II would be necessary to compete with losartan to produce 50% of the maximal response in the SHR than in the WKY. Our observations are in agreement with this concept (Fig. 11, Table 3).

In summary, the present data demonstrate hypertensive IAS in the SHR associated with the upregulation of RAS in the IAS. This data, along with the medical report associating CV hypertension with the hypertensive IAS suggest a link between the two. The ability of captopril and losartan to normalize the elevated IASP in the hypertensive SHR suggests the potential use of such agents in certain anorectal disorders associated with hypertensive IAS (Sun et al., 1992; Cook et al., 2001; Azpiroz and Whitehead, 2002). Based on the data in the SHR, normalization of the IAS tone to the levels of normotensives (without lowering it further) may provide plausible explanation for the lack of anorectal incontinence in the hypertensive patients on ACE and AT_1-R inhibitors.

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Footnotes

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Legends for Figures

Fig. 1: Comparison of the basal levels of (**A**) internal anal sphincter pressure (IASP), and (**B**) systolic blood pressure (SBP) between the WKY and the SHR reveals higher levels in the SHR group (*; $p < 0.05$; unpaired Student t-test; n = 6 to 7). Data in this and the subsequent figures represent the mean ± SEM.

Fig. 2: Effects of different doses of the ACE inhibitor captopril on the IASP of WKY rats and SHR (**A**), and the respective (**B, C**) representative tracings. Captopril is more potent in causing fall in the IASP of SHR vs. WKY rats. (*; $p < 0.05$; one-way ANOVA; n = 4).

Fig. 3: Effects of different doses of the Ang II AT_1-receptor antagonist losartan on the IASP of WKY rats and SHR (**A**), and their respective tracings (**B, C**). Losartan is more potent in causing fall in the IASP in the SHR as compared with the WKY rats (*; $p < 0.05$; one-way ANOVA; n = 4).

Fig. 4: Representative tracings showing the effects of 50 mg/kg of captopril on the IASP of (**A**) WKY and (**B**) SHR. Note that in this example, captopril decreases the IASP of the WKY group to levels close to the rectal pressures and elicits rectoanal inhibitory reflex (RAIR) as shown by a sudden fall in the IASP accompanied with the expulsion of fecal pellets. Such reflex was not observed in the SHR group.

Fig. 5: Systolic blood pressure (SBP) data. Effects of different doses of captopril and losartan on the SBP of WKY rats (panels **A** and **B**), and of SHR (panels **C** and **D**). Note that captopril and losartan significantly and dose-dependently restitute the SBP in the SHR, while there was no significant effect in the WKY rats. (*; $p < 0.05$; one-way ANOVA; n = 3).

Fig. 6: Ang II levels in the plasma of the arterial blood of WKY and SHR. Ang II levels in the basal state (control, C) in the SHR are significantly higher than in the WKY rats. Captopril

(Cap) at 50 mg/kg reduces the circulating levels of Ang II in both strains of rats. Losartan (Los; 50 mg/kg) does not change the circulating levels of Ang II in the WKY rats, but significantly increases in the SHR (*; $p < 0.05$; one-way ANOVA; n =3).

Fig. 7: A. Basal tone in the internal anal sphincter (IAS) in *in vitro* studies, showing increase in the basal tone in the SHR group as compared with the WKY (*; $p < 0.05$; n = 4). **B.** Note several fold higher levels of Ang II in the muscle bath perfusates (MBP) of the IAS, rectum smooth muscle (RSM), and aorta smooth muscle (AoSM) in the SHR vs. WKY rats. Ang II control (C) levels in the IAS are several folds higher than in the RSM and the AoSM in the WKY rats, and they increase still higher in the SHR. The panel **C** shows 10 μM captopril (Cap) significantly reduces the levels of Ang II in both strains of rats, while losartan (Los) has no significant effect in the WKY group. In the SHR group, however, losartan significantly increases Ang II levels in the MBP of the IAS. (*; $p < 0.05$; one-way ANOVA; n = 3)

Fig. 8: Western blots for angiotensinogen (Angen) (**A**) and renin (**B**) in internal anal sphincter (IAS), rectum smooth muscle (RSM), and aorta smooth muscle (AoSM) from the WKY and the SHR. Note the presence of Angen bands as a doublet: at 65 kDa (precursor of Angen) and at 55 kDa (actual Angen as previously shown in the rat pancreas (Leung et al., 1999)). The bar graph shows higher relative density of the bands at 55 kDa Angen and renin in the SHR tissues, especially in the IAS. Data are shown as the percent relative density of 3 independent determinations calculated as the IOD of the band of interest by the IOD of the band of α-actin (43 kDa).

Fig. 9: Western blots showing higher expression of ACE (**A**) and AT_1-R (**B**) in the internal anal sphincter (IAS) than in rectum smooth muscle (RSM), and aorta smooth muscle (AoSM) and their upregulation in the SHR vs. WKY group. Data are shown as the percent relative density of

3 independent determinations calculated as the IOD of the band of interest normalized by the IOD of the band of α-actin (43 kDa).

Fig. 10: Gene transcripts of (**A**) angiotensinogen, (**B**) renin, (**C**) angiotensin-converting enzyme (ACE), and the (**D**) Ang II type 1 receptor (AT_1-R) of internal anal sphincter (IAS), rectum smooth muscle (RSM), and aorta smooth muscle (AoSM) samples from the WKY and the SHR. Data are shown as the percent relative density calculated as the IOD of the band of interest normalized by the IOD of the band of α-actin (mean ± SEM; n = 3).

Fig. 11: Concentration-response curves for Ang II in the internal anal sphincters isolated from WKY and SHR. These experiments were carried out in the presence of ACE inhibitor captopril (10 μM) to eliminate the effect of endogenous Ang II production. Note that the smooth muscle strips from the SHR group are more resistant to AT_1-R inhibition by losartan than the WKY (*; $p < 0.05$; n = 4).

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Table 1. Primers used in the PCR for amplification of cDNA encoding angiotensinogen, ACE, renin, and α-actin in the internal anal sphincter (IAS), rectal smooth muscle (RSM) and aortic smooth muscle (AoSM) of WKY and SHR

Primer	Strand	Sequence (5' – 3')	Accession No.
Angiotensinogen	Forward	CCTCGCTCTCTGGACTTATC	NM_134432
	Reverse	CAGACACTGAGGTGCTGTTGT	
ACE	Forward	CGCAGAGCTGGGAGAACATTTACGA	NM_012544
	Reverse	GATAGTTGTCTGGTAACGGTGGACG	
Renin	Forward	ACACTGCCTGTGAGATTCACAACC	M16984
	Reverse	CCTGGCTACAGTTCACAACGTA	
AT_1-R	Forward	CTACAGCATCATCTTTGTGGTGGGA	NM_031009
	Reverse	CGTAGACAGGCTTGAGTGGGACTT	
α-actin	Forward	GATCACCATCGGGAATGAACG	NM_007392
	Reverse	CTTAGAAGCATTTGCGGTGGAC	

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Table 2. Values of $logID_{50}$ and I_{max} in the IAS of WKY and SHR

Group	**$logID_{50}$[a]**	**I_{max}[b] (mmHg)**
WKY (Captopril)	1.0 ± 0.1	6.0 ± 0.8
WKY (Losartan)	0.5 ± 0.13	6.3 ± 2.1
SHR (Captopril)	0.42 ± 0.1[c]	12.3 ± 2.3[c]
SHR (Losartan)	0.42 ± 0.2	10.0 ± 2.7[c]

[a]$logID_{50}$ is defined as the logarithm of mg/kg dose of the inhibitor that produces 50 % inhibition. Data represent the mean ± S.E.M. of 6 - 7 independent determinations.
[b]I_{max} is defined as the maximal inhibition elicited by the inhibitor.
[c]$p < 0.05$ compared with WKY.

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Table 3. Values of pEC_{50} and E_{max} for Ang II in the IAS of WKY and SHR (in the presence of 10 μM captopril)

Group	**pEC_{50}[a]**	**E_{max}[b] (%)**
WKY	8.5 ± 0.1	36.6 ± 5.1
WKY (Losartan 100 nM)	6.4 ± 0.1[c]	28.4 ± 5.4
SHR	8.6 ± 0.2	44.3 ± 8.0
SHR (Losartan 100 nM)	7.2 ± 0.2[de]	43.1 ± 4.7[e]

[a]pEC_{50} is defined as the –log EC_{50} (concentration of the agonist that produces 50 % contraction).

Data represent the mean ± S.E.M. of 4 independent determinations.

[b]E_{max} is defined as the maximal contraction elicited by the agonist.

[c]$p < 0.05$ compared with WKY.

[d]$p < 0.05$ compared with SHR.

[e]$p < 0.05$ compared with WKY (100 nM Losartan)

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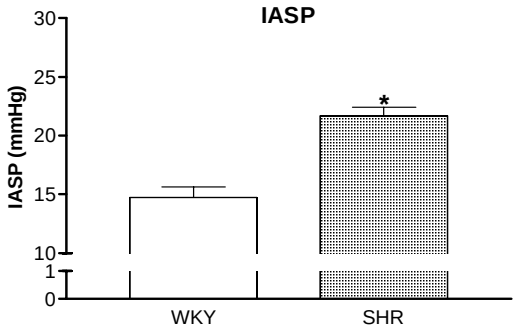
Figure 1

A



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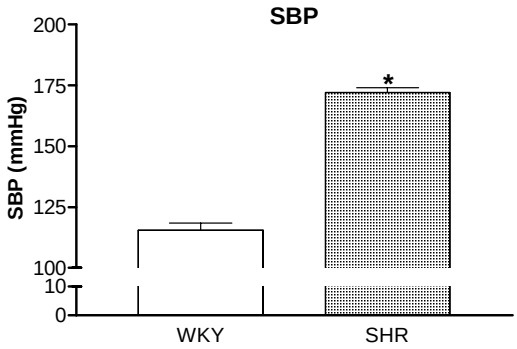

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Figure 2

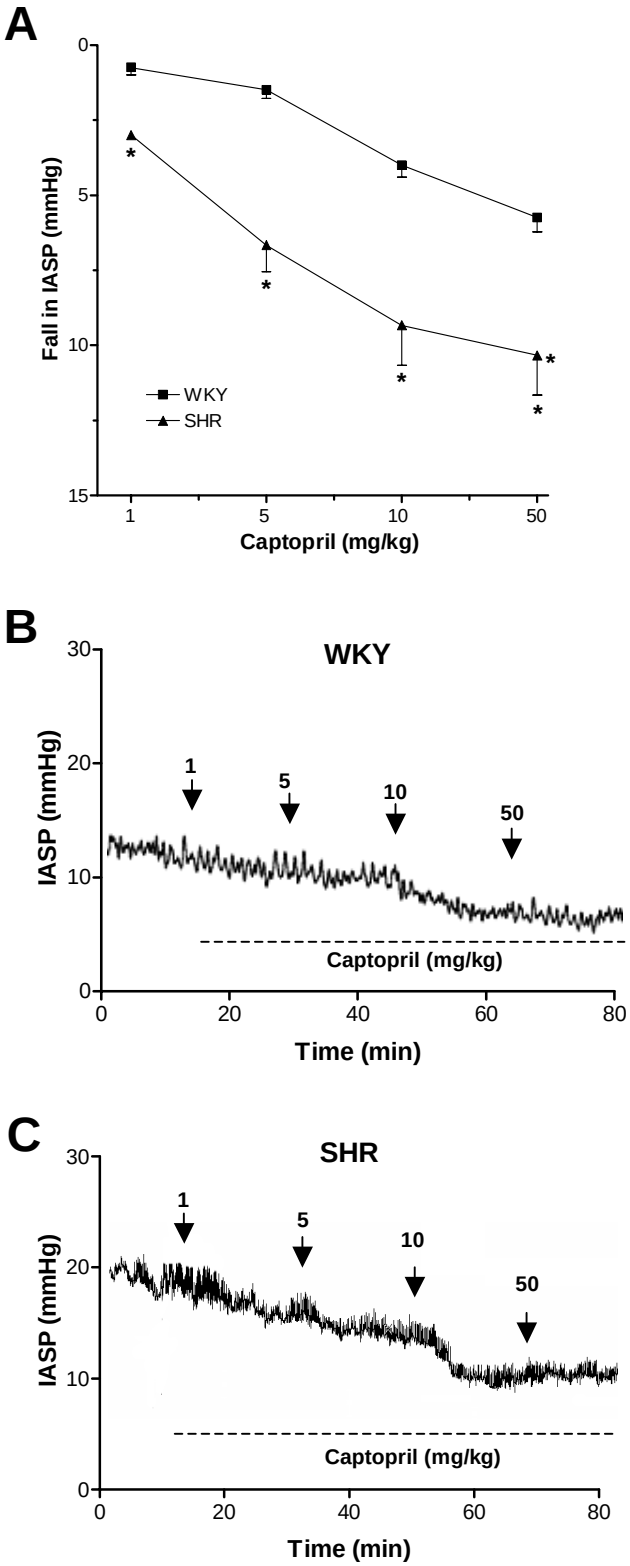

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Figure 3

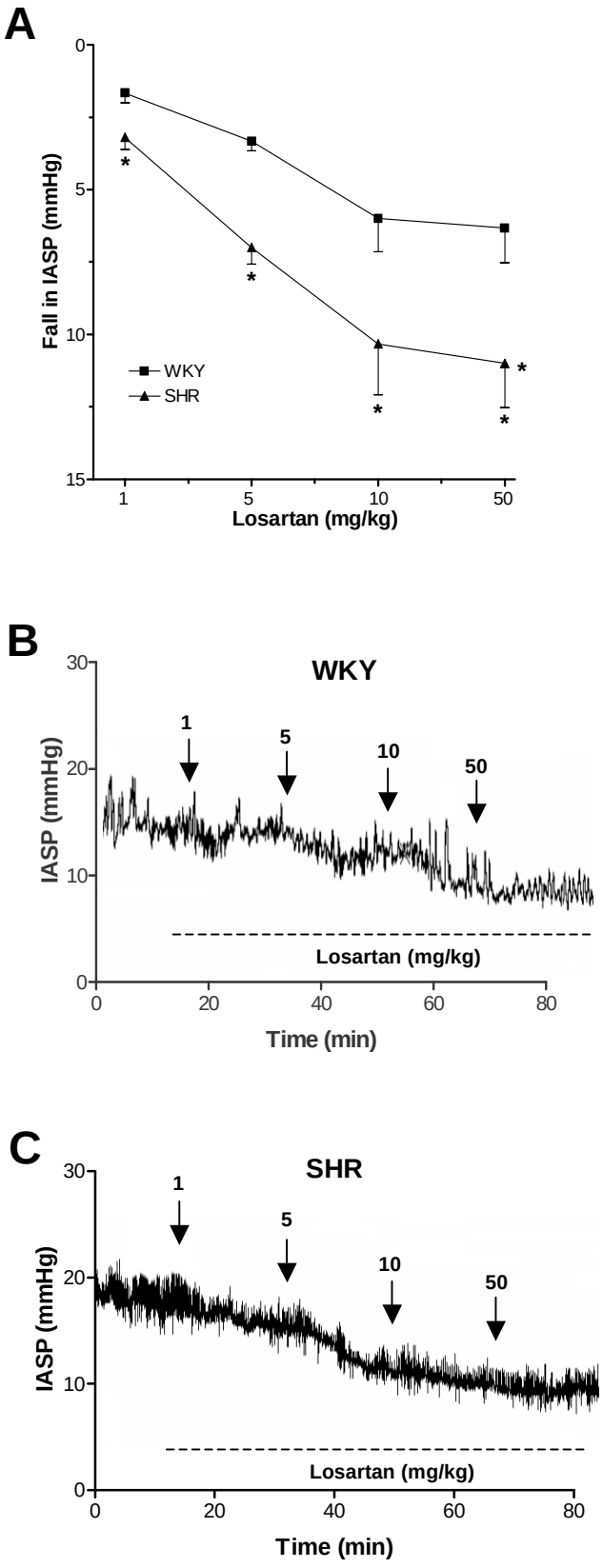

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Figure 4

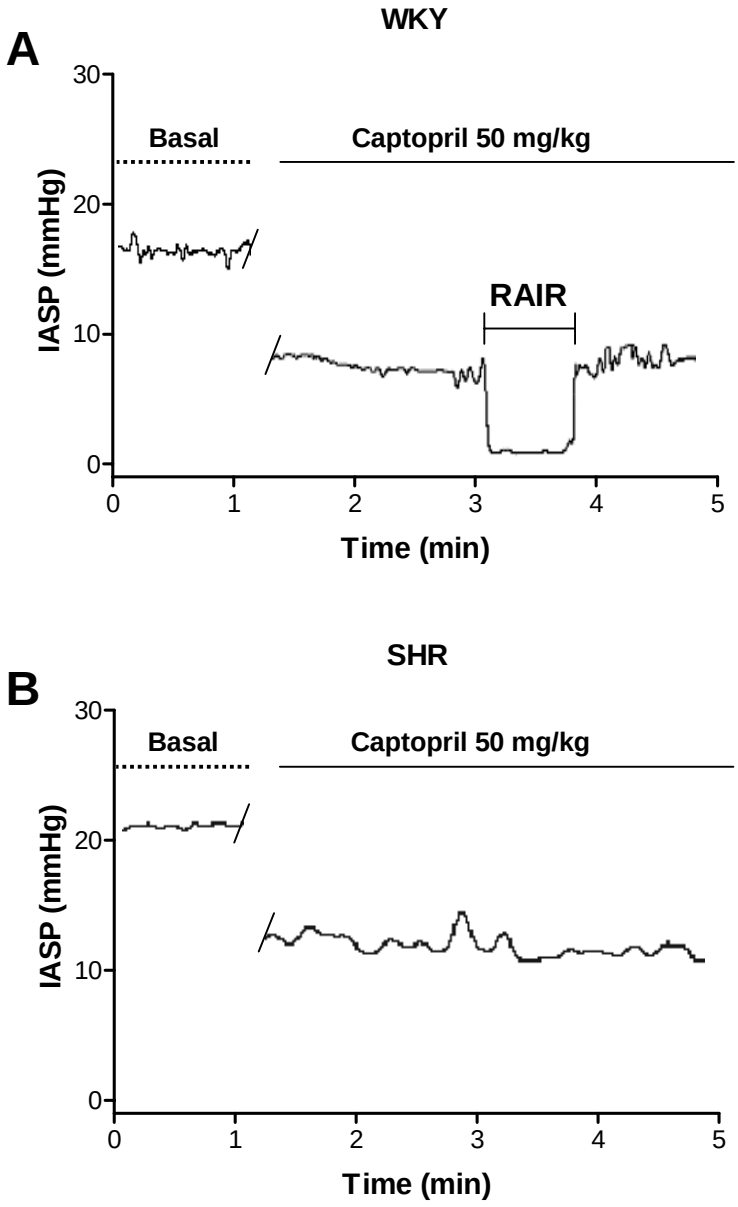

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Figure 5

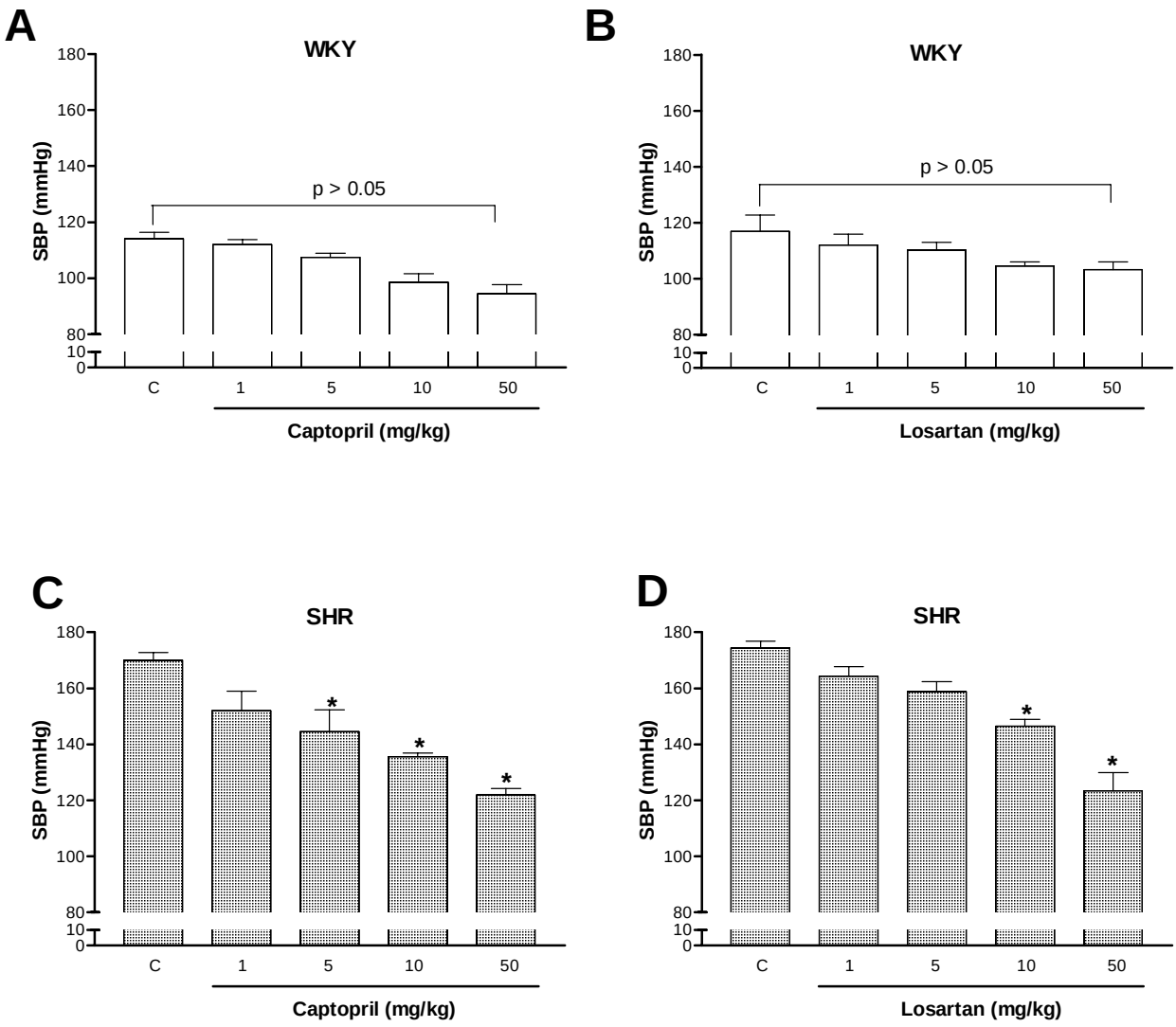

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Figure 6

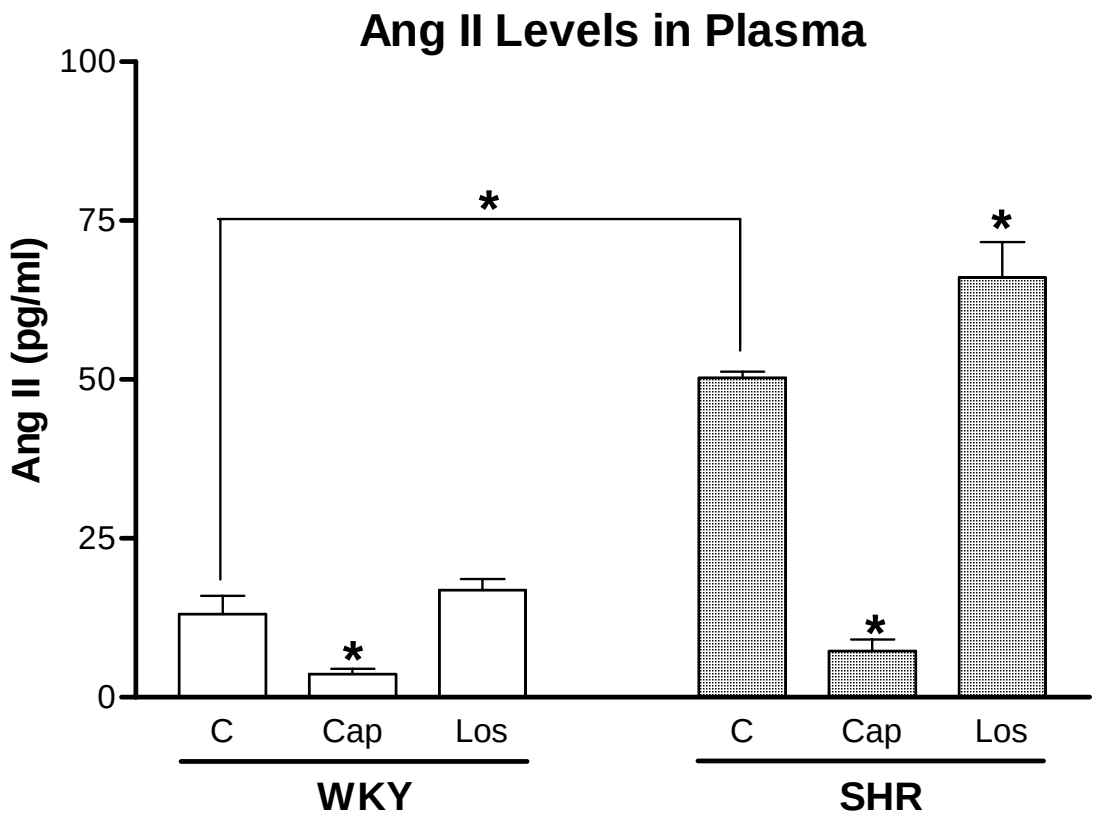

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Figure 7

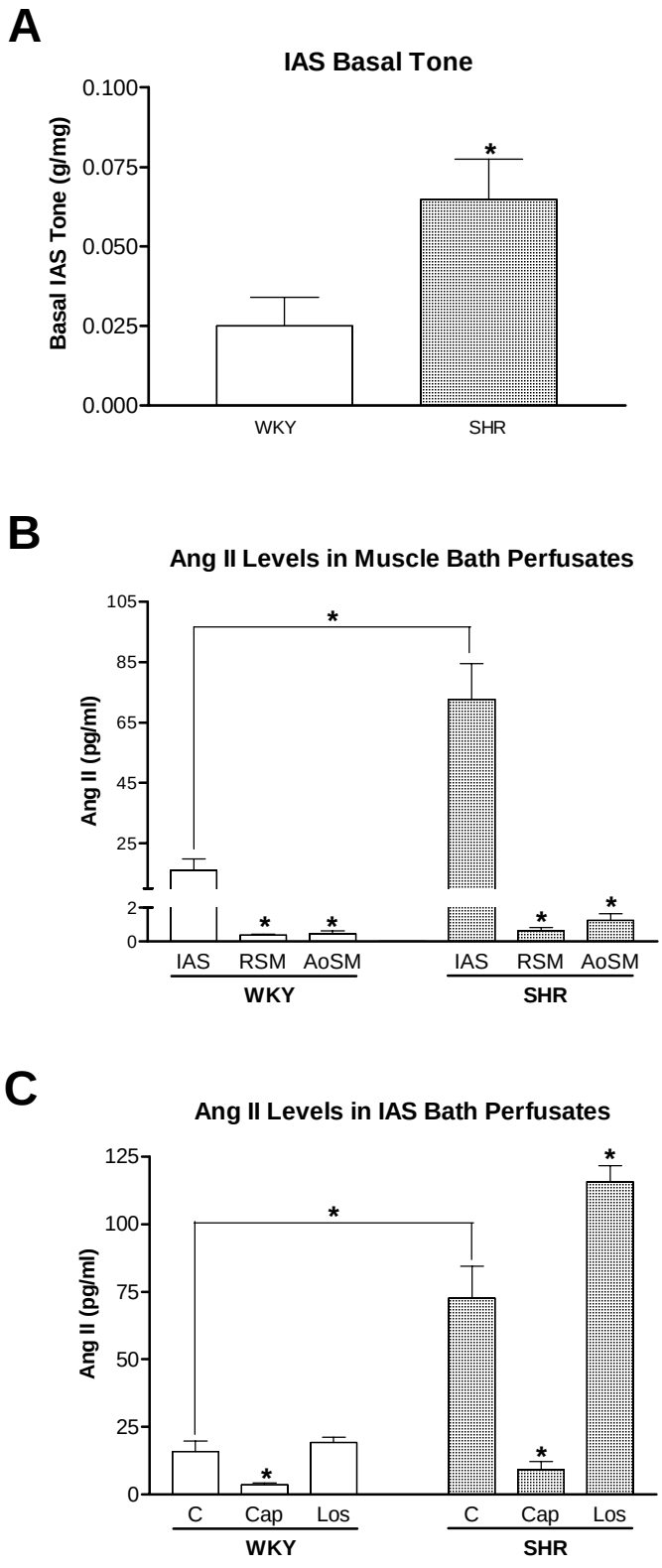

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Figure 8

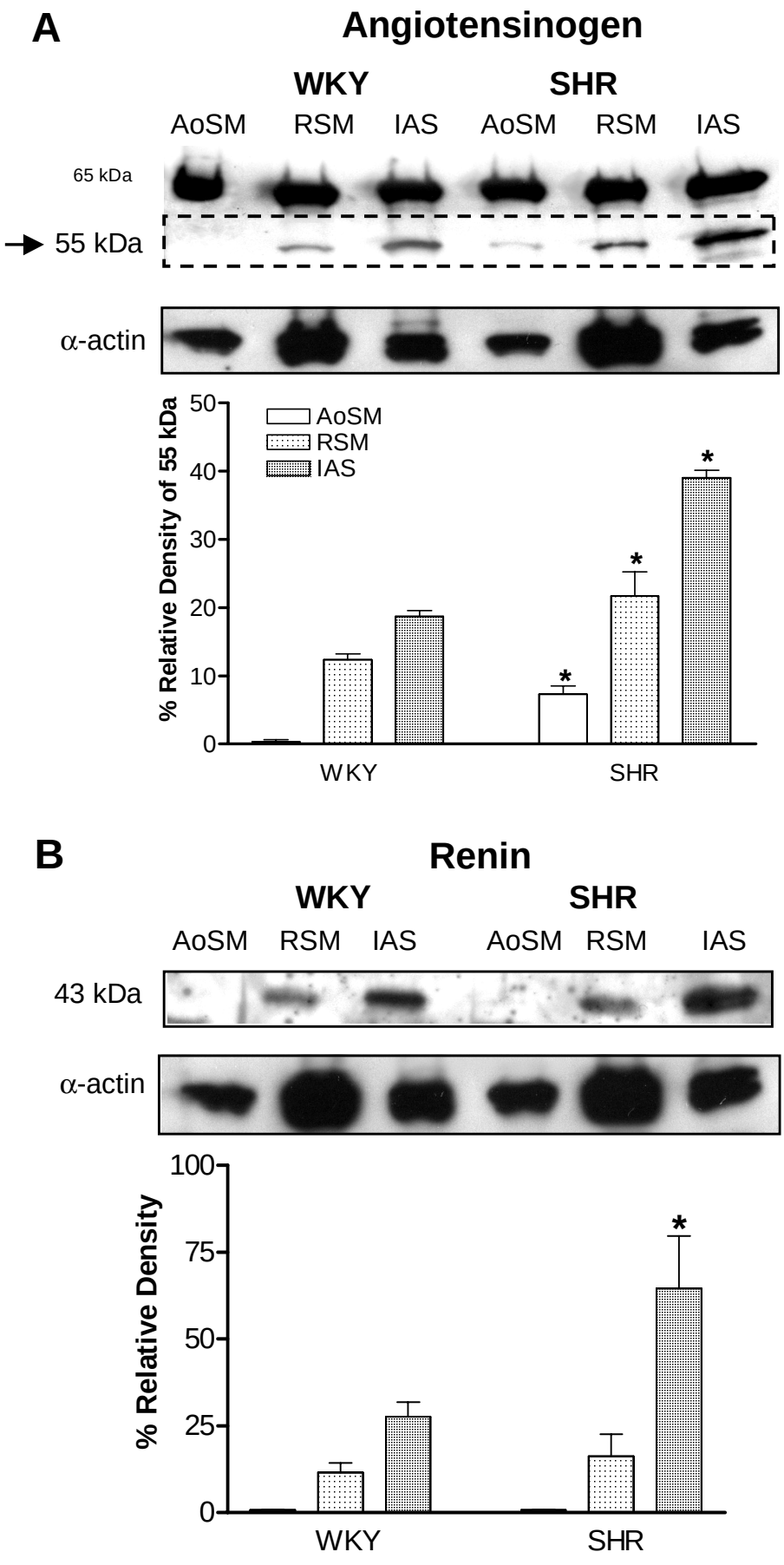

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Figure 9

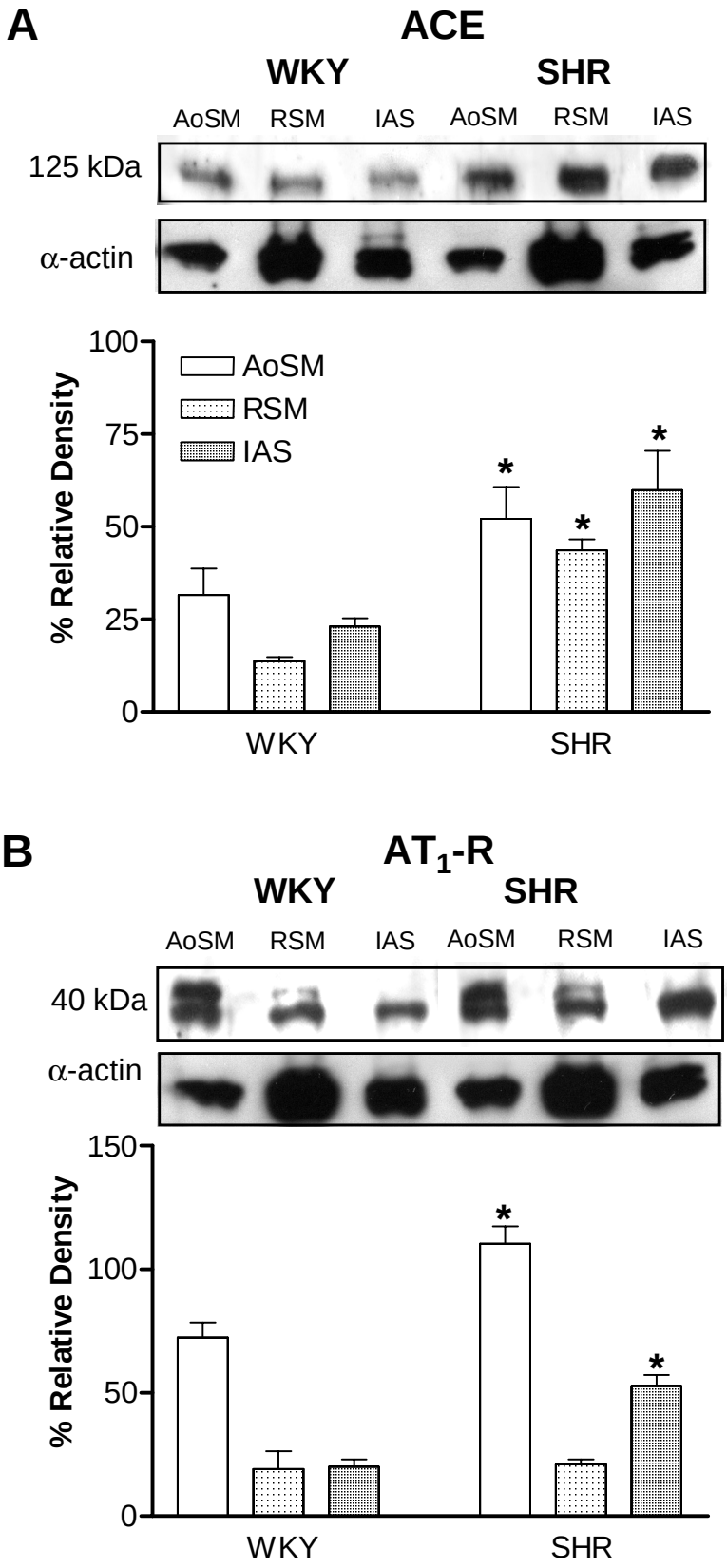

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Figure 10

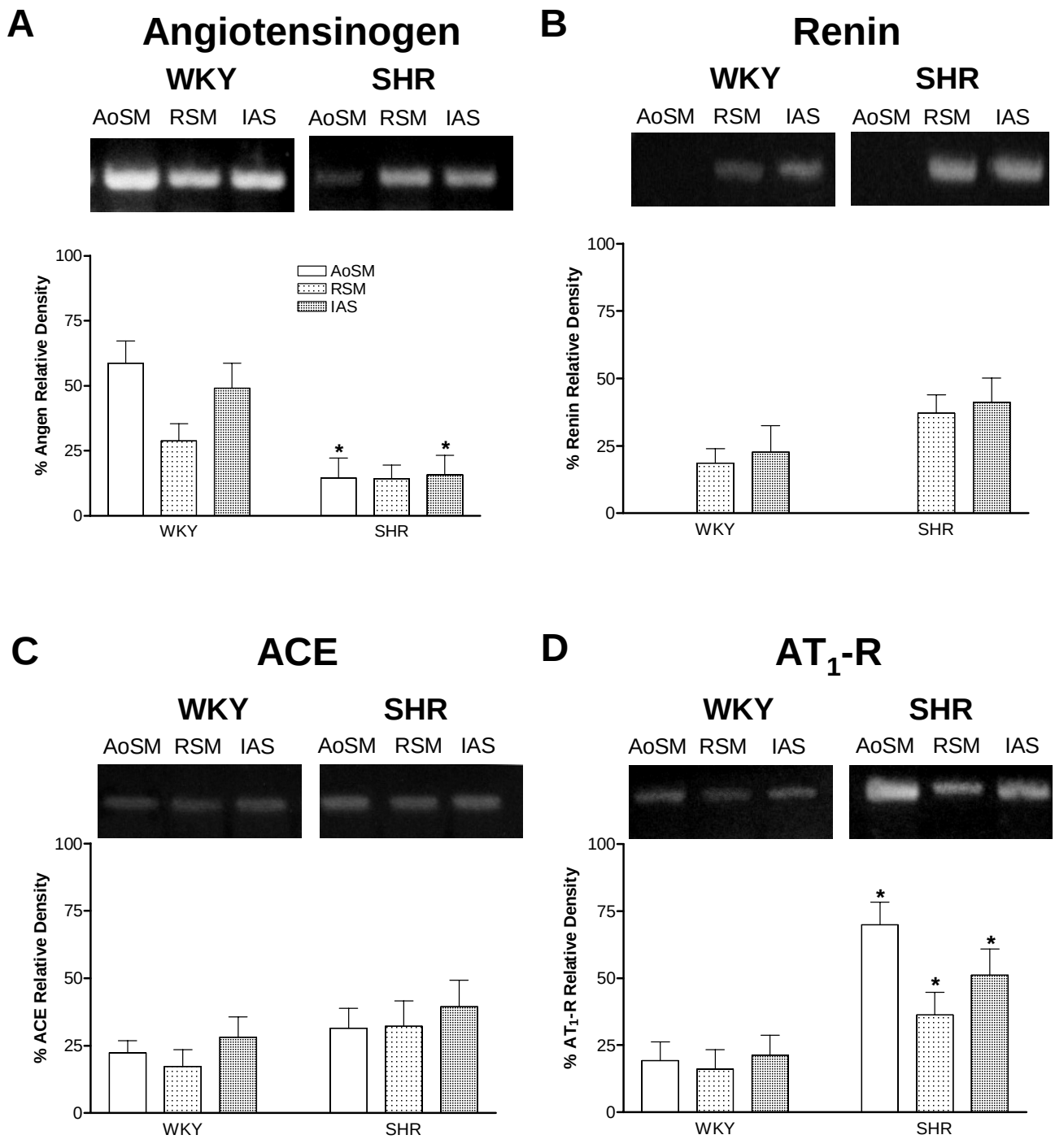

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Figure 11