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Effects of Chronic Receptor Blockade on Intracardiac Angiotensin II and Aldosterone Content in an Angiotensin II-infused Model of Hypertension

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

In

Quantitative and Systems Biology

By

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Abstract

Accumulation of angiotensin II (Ang II) in tissues is an Ang II receptor-mediated process. In pigs, acute angiotensin receptor blockade (ARB) reduced the heart-to-plasma ratio of Ang II following acute infusion. However in rats, chronic ARB treatment increased heart Ang II levels, suggesting that a differential response to ARB treatment may exist in the mammalian heart. Furthermore, the changes in heart aldosterone following chronic ARB treatment are not well described. To address the discrepancy in heart Ang II concentrations following ARB treatment, and to address the functional relevance of increased aldosterone in the heart two studies were undertaken. The first study consisted of three groups (n= 6) of rats, chronically studied: (1) control; (2) angiotensin II (Ang II; 80 ng/min for 28 d); and (3) angiotensin II + olmesartan (ARB; 10mg/kg/d for 21 d). Ang II-infusion increased intracardiac Ang II by 40% and intrarenal Ang II over 2-fold, and chronic ARB treatment decreased Ang II by 48% in the heart and over twofold in the kidney, suggesting that accumulation of Ang II in the heart is receptor-mediated as in the kidney. Ang II increased plasma aldosterone 2.5-fold and was exacerbated by ARB treatment. Intracardiac aldosterone was exacerbated by ARB treatment in the study. Suppression of intracardiac Ang II with ARB is consistent with the existing view of Ang II receptor-mediated uptake by tissues.

Ang II stimulates the release of aldosterone, and the chronic blockade of the AT1 in the previous study increased aldosterone, as did the chronic blockade

of mineralocorticoid receptor (MR). To evaluate the contribution of increased aldosterone to cardiovascular damage, an MR agonist was used in a second study, with and without an ARB to differentially assess the contributions of the respective receptors. This study included two additional experimental groups and a different ARB: 1) control (n=11); 2) angiotensin II (n=14), (Ang II; 80 ng/min for 28 d); 3) angiotensin II + losartan (n=17) (ARB; 10mg/kg/d for 21 d); 4) angiotensin II + MR blocker (n=14)(EPL; 100 mg eplerenone/kg/d in diet; Pfizer, St. Louis, MO, and 5) angiotensin II + ARB + MR blocker (Combo)(n=14).

8-isoprostane (8-iso-prostaglandin $PGF_{2\alpha}$) is a marker of oxidative damage and studies have shown it to be correlated to risk factors in coronary heart disease (40, 75). Nitrotyrosine is a marker of inflammation and nitrositive damage linked to heart disease (19, 56). Measuring these biomarkers indicates oxidative/nitrositive damage and inflammation. Blockade of the MR had no effect on blood pressure (158.7 ± 6.9 vs 165.1 ± 15.9 Ang II), but substantially increased cardioprotective actions demonstrated by a 57% decrease of urinary 8-isoprostane that was further abated when combined with the ARB achieving an additional decrease of 27%. Additionally, the nitrotyrosine concentrations were also ameliorated by receptor blockade; the combo blockade (54% of Control) have a 68% decrease from the Ang II (144% of Control). The data suggests that damage to the cardiovascular system by Ang II and aldosterone occurs via their respective receptors and in the case of aldosterone, independent from high blood pressure. There is evidence from the combined studies that in spite of increased aldosterone with ARB, there is a reduction in cardiac hypertrophy, inflammation

and nitrositive damage by blocking AT1 and MR. Ang II and aldosterone both contribute to cardiovascular damage via their respective receptors and blocking either one still resulted in decreased oxidative damage. Blocking both receptors did seem to add some additional improvement, but not significantly. These mechanisms may be better explained by studying the respective signaling pathways or looking closer at local hormone production that perhaps initiate receptor cross-talk. Additional studies are needed to further elucidate alternate pathways.

Acknowledgements

There are of course so many people that were instrumental in the completion of this project. Foremost has to be my advisor Dr. Rudy Ortiz, who patiently taught me the fine art of working with rats and how to analyze our findings. My greatest appreciation is to Rudy and Akira Nishiyama for the experience of conducting my research in Japan. This provided education and personal growth that I am sure I would never have achieved otherwise. I will treasure those days forever.

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Chapter 1

General Introduction

Hypertension

The Center for Disease Control and Prevention has estimated about 33 percent of adults in the United States have high blood pressure and another 28 percent are pre-hypertensive, meaning their blood pressure is higher than normal and are at a higher risk of developing hypertension (71). Hypertension is the underlying cause for heart disease and stroke, which are the first and third causes of death in the United States, respectively. Treatment of hypertension is difficult because there is no single cause. In fact, in the case of essential hypertension the cause is still unknown. Medically, the symptoms are treated without addressing the root cause. If more effective treatment strategies are to be developed, the underlying components involved in this complex disease needs to be better understood.

Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system (RAAS) plays a key role in the regulation of blood pressure through coordinated effects on the heart, blood vessels and kidneys. RAAS is an inter-related system of enzymes and hormones that function in the homeostatic control of arterial pressure and

extracellular volume (3). When functioning properly, RAAS is a precise negative feedback loop carefully regulating multiple cardiovascular and renal functions (3). However, when RAAS becomes dysregulated, it plays a pivotal role in the pathogenesis of cardiovascular and renal disorders (3, 41).

The kidneys sense a reduction in blood volume or pressure and release renin, the rate-limiting enzyme in the system (76). Angiotensinogen is produced constitutively by the liver and released into circulation providing a substrate for renin to produce angiotensin I by cleaving two peptides (11). Angiotensin I has little biological effect and becomes cleaved by angiotensin converting enzyme (ACE), and is transformed into the eight-peptide hormone, angiotensin II (Ang II) in circulation. Ang II can act as an endocrine, autocrine/paracrine and intracrine hormone (5, 68). Ang II directly constricts vascular smooth muscle cells, modulates myocardial contractility, and stimulates aldosterone production from the adrenal cortex via angiotensin receptors (AT1a and AT1b) (35, 82). Aldosterone acts on the kidneys to increase fluid and sodium retention to increase blood pressure and thus suppresses further secretion of renin (35).

Angiotensin II

The circulating levels of Ang II are chronically elevated during congestive heart failure and hypertension. The inappropriate elevation of Ang II is partially responsible for many consequences on the heart and kidneys (28, 45). RAAS is attributed to the remodeling process in myocardial hypertrophy and Ang II is the primary contributing factor (18). Ang II binds to plasma membrane receptors of

which there are two subtypes: Type 1 (AT1) and Type 2 (AT2). These receptors exhibit differential distribution in various tissues, with AT1 being more prevalent in cardiac and smooth muscle (42, 72, 77). The AT1 receptor mediates the actions of Ang II such as vasoconstriction, inflammation, cardiac contractility, oxidative stress and aldosterone release (32, 87). Ang II via the AT1 receptors also facilitates cardiac remodeling, growth and apoptosis (3, 48). The mounting evidence that various tissues, including the heart, have intracellular RAAS has provided new insights to the causes of cardiovascular injury and metabolic disorders (4, 12). Although Ang II mediation through the AT1 receptor has been well described in cardiovascular and renal diseases, there has been controversy as to whether the sequestration of Ang II in the heart is due to local production or AT1-mediated uptake.

Pharmaceutical companies have developed a class of drugs that are selective AT1 receptor antagonists or angiotensin receptor blockers (ARB) (30). These include losartan, olmesartan, candesartan, among others that have been prescribed in a clinical setting for the treatment of hypertension (7). Losartan, for example has a high binding affinity, and specificity to AT1 is 30,000 times more selective for AT1 than AT2 and has a slow disassociation rate (30). Thus, from an investigative perspective, the use of ARBs to block the AT1-mediated effects of Ang II is especially meaningful in studies that include models of inappropriately elevated Ang II such as Ang II-infusion as a tool to evaluate the mechanisms of angiotensin uptake by tissues.

Aldosterone

Aldosterone is a steroid hormone that is produced in the zona glomerulosa in the cortex of the adrenal gland and that is secreted in response to elevated levels of circulating Ang II or potassium (2, 3). Aldosterone regulates sodium and potassium, activates the sympathetic nervous system, stimulates myocardial and vascular fibrosis, and causes baroreceptor dysfunction (43, 94, 96). Aldosterone binds to a cytosolic mineralocorticoid receptor (MR), where it is translocated to the nucleus. MR agonists such as eplerenone or spironolactone have been developed to block this action and prevent nuclear transcription (46). It has been suggested that aldosterone mediates and exacerbates the effects of Ang II (83, 86). Studies have also shown that aldosterone contributes to vascular remodeling when Ang II is elevated (70). High affinity Type 1 aldosterone binding sites, that have a higher affinity for aldosterone versus other circulating steroids, have been reported in the heart; evenly distributed in the atrium and ventricle (63). There is evidence that aldosterone has an important role in cardiovascular disease that is independent of Ang II (73, 86). Some studies suggest that aldosterone can also be produced locally in the myocardium and in the blood vessels (25, 80). Aldosterone increases AT1 receptor density and potentiates Ang II induced hypertrophy in vascular smooth muscle cells (25, 89). Up-regulation of AT1 receptors and MR may result in increased tissue levels of Ang II and aldosterone, and if unblocked could lead to local tissue damage.

Experimental model

Rats are by far the most common model for hypertension research (64).

The most commonly used model of cardiovascular disease is the Spontaneously

Hypertensive rat (SHR) with over 4000 Medline references in the last 10 years,

often with the Wistar Kyoto rat (WKY) as the normotensive control (20). The SHR

are pre-hypertensive for the first 6–8 weeks of their lives with systolic blood

pressures around 100–120 mmHg (1, 20). To study mineralocorticoid

hypertension the deoxycorticosterone (DOCA)–salt model is useful as an

angiotensin-independent model that shows a markedly depressed renin–

angiotensin system (26). Another model is the transgenic rat line

[TGR(mREN2)27], a model of hypertension with a precisely defined monogenetic

defect, which may allow a clearer understanding of the role of local renin–

angiotensin systems in cardiovascular disease (38, 58, 59). The Ang II-induced

model of hypertension is useful because it is quick to become hypertensive and

demonstrates a chronic elevation in blood pressure and activation of RAAS. This

model has long been validated for systemic hypertension research (24, 59, 61,

100). In the Ang II-infused model of hypertension, Ang II is constantly

administered with an osmotic pump that is surgically implanted (24, 67, 78).

Reasearch Question and Hypotheses

The heart possesses all the molecular components necessary to support a

fully functional intracardiac RAAS (4, 12). Local aldosterone synthesis is possible

and may be upregulated in the presence of increased Ang II induced by chronic ARB treatment as previously reported (100). Thus, if the accumulation of Ang II in the rat heart is exacerbated with chronic ARB treatment, then the content of aldosterone in the heart should be similarly increased if local production is viable. Additionally, if the levels of intracardiac Ang II are reduced in the heart following chronic ARB treatment, then in theory, local aldosterone synthesis should be reduced as well. The intracardiac synthesis of aldosterone remains a controversial topic that has not been fully evaluated in the Ang II-infused rat following chronic treatment with an ARB (23, 80, 86). Thus, the regulation of the heart RAAS including AT1 and MR content and binding following the infusion of Ang II remains poorly examined and warrants further investigation. Elevated plasma aldosterone following eplerenone treatment is a well established occurrence, and because increased plasma aldosterone has been shown to increase Ang II receptor binding and number (89, 99), the mechanism exists for treatment with MR antagonist to exacerbate the intracardiac Ang II content. Ortiz et al. also showed an increase in intrarenal and intra-adrenal Ang II when treated with eplerenone (61), leaving the possibility open for a similar effect in the heart. However, the effects of MR blocker on cardiac Ang Ii have not been fully studied and are poorly understood. Data on the regulation of AT1 And MR in the heart in response to chronic ARB and EPL treatment during Ang II infusion is lacking. A better understanding of mechanisms regulating cardiac RAAS during Ang II-dependent hypertension will enhance our appreciation for the contributions of receptor mediated response to cardiovascular complications associated with

inappropriately elevated systolic blood pressure and RAAS.

We hypothesized that chronic ARB treatment decreases intracardiac Ang II, similar to that observed in the kidney, associated with a decrease in heart aldosterone. We hypothesize that in an Ang II-infused model of hypertension, oxidative/nitrositive damage is mediated through the MR and AT1 independent of increased blood pressure.

Chapter 2

Chronic Angiotensin Receptor Blockade Suppresses Intracardiac Angiotensin II in Ang II-Infused Rats.

Introduction

The accumulation of renal angiotensin II (Ang II) is mediated primarily by an angiotensin receptor type 1(AT1)-mediated process (16, 27, 67, 73, 74). However, Zou et al. (100) reports that chronic (2 weeks) infusion of Ang II did not increase the tissue levels of Ang II in the heart or adrenals. Furthermore, chronic treatment with the angiotensin receptor blocker (ARB) losartan in the Ang II-infused rat increased intracardiac Ang II four-fold and intra-adrenal Ang II by 80%, suggesting that the process of receptor-mediated sequestration of Ang II may be tissue-specific. Conversely, in pigs, acute (10 min) treatment with an ARB (L-158, 809) resulted in about a 10-fold decrease in heart-to-plasma ratio of acutely (15 min) infused radiolabelled Ang II, suggesting that, at least acutely, the accumulation of Ang II in the heart is receptor-mediated (90). Thus, the data on the accumulation of Ang II in the heart following either acute or chronic Ang II infusion are incongruous and warrant further evaluation to clarify the inconsistency of the response to ARB treatment.

As a component of the renin–angiotensin system, elevated Ang II stimulates aldosterone secretion, resulting in elevated plasma aldosterone. As

aldosterone has been reported to increase Ang II receptor (i.e. AT1) number and binding *in vitro* (69, 83, 89), a potential mechanism by which Ang II infusion results in accumulation of tissue Ang II can be through a plasma aldosterone-dependent increase in Ang II receptor number and binding. Therefore, in theory, ARB treatment can contribute to decreasing tissue Ang II levels (in addition to displacing Ang II) by reducing plasma aldosterone, which decreases AT1 number and binding, and thus, tissue Ang II levels. However, the relationships between aldosterone and Ang II levels in the heart in Ang II-dependent hypertension are not well described.

The present study elucidated the effects of chronic ARB treatment on intracardiac Ang II and aldosterone content in Ang II-infused rats. We hypothesized that chronic ARB treatment decreases intracardiac Ang II, similar to that observed in the kidney, associated with a decrease in heart aldosterone.

Methods

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of both Kagawa Medical University and the University of California in accordance to the guidelines for the care and use of animals established by these institutions.

Animals and Procedures.

Male Sprague-Dawley rats (Clea Japan Inc., Tokyo, Japan) approximately 200-225g were randomly assigned to three experimental groups (n=6/group): 1)

control, 2) angiotensin II (Ang II; 80 ng/min) and 3) angiotensin II + olmesartan (ARB; 10 mg/kg/d in diet; Daiichi-Sankyo Co. Ltd., Tokyo*).* Control and Ang II rats were maintained on a normal rat chow diet (Daiichi-Sankyo Co. Ltd., Tokyo). The dosage of ARB is consistent with that used previously to successfully block Ang II (51, 53). Food consumption rates in this study were measured weekly to verify treatment goals were met. Systolic blood pressure (SBP) was monitored daily by radiotelemetry implanted in the animals and used to evaluate the effectiveness of the Ang II infusion and ARB (59, 60). Animals were anesthetized with sevoflurane prior to implanting a biotelemetric transducer (PA-C40, DSI, St. Paul, MN) subcutaneously in the dorsal flank with the catheter lead extending inguinally and into the femoral artery and into the abdominal aorta. The animals were allowed to recover for 7 days from the effects of the surgery. The animals were again anesthetized with sevoflurane and implanted with an osmotic mini-pump (Durect Corp, Cupertino, CA) subcutaneously to infuse Ang II (80 ng/min; Phoenix Pharmaceuticals, Belmont, CA) for 28 days. Each animal was maintained individually in metabolic cages in a temperature and light controlled room. Throughout the experiment, animals had unrestricted access to water and their specific diet. Ang II infusion began on day 1 of the study and the animals were allowed 7 days to become hypertensive prior to treatment to mimic the conditions in which ARB is prescribed. The ARB diet began on day 7 for a period of 21 days. On the last day of the study, 24-hr urine voids were recorded and an aliquot was collected for analysis of total protein. Urine samples were diluted 1:100 prior to measurement of total protein by photospectrometry (BioRad,

Hercules, CA). Urinary total protein excretion ($U_{TP}V$) was calculated as the

product of urine volume and urinary total protein concentration.

Dissections

On day 28, animals were weighed, decapitated, and trunk blood collected

into chilled vials containing 5 mM EDTA plus protease inhibitor cocktail (PIC;

Sigma, St. Louis, MO) for the measurement of plasma aldosterone, Ang II and

plasma renin activity (PRA). The heart was removed patted dry and weighed.

Sections were placed in 10 ml of cold methanol and homogenized for Ang II

extractions or 10 ml of PBS and homogenized for aldosterone extraction. The

left kidney was removed, cleaned, weighed, and placed in a glass vial containing

10 ml of methanol, and homogenized for extraction of Ang II. The right kidney

was removed, cleaned, weighed, flash-frozen in liquid nitrogen and stored at

−80°C for future analysis. The right adrenal was removed, cleaned, weighed,

placed in a glass vial containing 10 ml of cold PBS, and homogenized for

extraction of aldosterone.

Hormone analyses

PRA was measured using a commercially available RIA kit (DiaSorin,

Stillwater, MN, USA). Adrenal and heart aldosterone were extracted from their

homogenates with ethyl acetate. Heart, adrenal and plasma aldosterone

concentration was measured by a commercially available RIA kit (Siemens

Healthcare Diagnostics, Los Angeles, CA). Heart and kidney homogenates and

plasma (1.0 mL) were extracted for Ang II measurements with details of the Ang II extraction and assay (Phoenix Pharmaceuticals, Burlingame, CA) procedures described previously (10).

Western Blot analyses

All chemicals and reagents used in Western blot analyses were obtained from Bio-Rad (Hercules, CA), Pierce (Rockford, IL), and Santa Cruz Biotechnology Corp (Santa Cruz, CA), unless otherwise noted. Heart segments were harvested as described above followed by homogenization in 1 ml hypotonic saline containing PIC. The homogenate was spun down and the pellet was re-suspended in 1 ml lysis buffer containing PIC and 1% Triton X-100. The soluble fraction was collected and protein concentration was determined using the BCA assay. Tissue homogenates containing 50 µg of total protein was analyzed by standard Western blot technique as previously described using CYP11B2 (Chemicon, Billerica, MA) and AT1 antibodies (61). In addition to normalizing the gels by loading 50 µg of total protein, densitometry values were also normalized for β-actin expression of the blots. Western blots were analyzed on a Bio-Rad Chemidoc imager and band intensity was quantified using Analysis One software (Bio-Rad).

Statistics

Data are presented as means±SE. Plasma and organ measurements were compared by one-way analysis of variance and linear regression model.

For all cases, if significance (P< 0.05) was detected, a Fisher's protected least significant difference test was applied *post hoc*. Statistics were performed using Statview software (SAS, Cary, NC, USA).

Results

Blood pressure, and body and heart mass

The infusion of Ang II increased mean SBP 46% (174±10 mmHg) above control (119±7 mmHg) and ARB treatment was effective in reducing the pressure to control levels (125±6 mmHg) by day 28, demonstrating the effectiveness of both the Ang II infusion and ARB treatment (Figure 1). By day 28, mean body mass was 11% lower than control in the Ang II group, and ARB treatment had recovered mean body mass to 8% of control, which was not significantly different. Absolute heart mass was greater in both Ang II and ARB groups above control (Table 1). Relative heart mass in the Ang II group was 46% greater than control and 14% greater than ARB treated rats, suggesting that blockade of Ang II receptor had partially alleviated the hypertrophy (Table 1).

Intraorgan angiotensin II

Ang II infusion increased intracardiac Ang II by 40% (53.1±2.1 versus 74.0±6.3 fmol/g) from control (Figure 2). Treatment with ARB decreased mean intracardiac Ang II by 32% (50±7 fmol/g), which is similar to control levels. Infusion of Ang II increased mean intrarenal Ang II over two-fold (96±6 vs 207±13 fmol/g) and ARB treatment (92±7 fmol/g) returned levels to control (Figure 2).

Intraorgan aldosterone and CYP11B2

While Ang II infusion did not significantly increase intracardiac CYP11B2 expression, treatment with ARB increased expression 230% of control levels (Figure 3). The changes in intracardiac aldosterone paralleled those observed for intracardiac CYP11B2. Intracardiac aldosterone was not significantly increased after Ang II infusion (2.2 ± 0.3 versus 2.7 ± 1.0 pmol/g); however, treatment with ARB increased mean intracardiac aldosterone nearly 4-fold (7.8 ± 1.7 pmol/g) compared to control (Figure 4). The 54% increase in mean adrenal aldosterone following Ang II infusion did not reach statistical significance compared to control; however, treatment with ARB induced over a 3-fold increase in mean adrenal aldosterone compared to control (Table 2). Ang II infusion had no significant effect on intrarenal aldosterone (583 ± 166 versus 663 ± 197 pmol/g); however, ARB treatment decreased levels by 35% (380 ± 71 pmol/g) of control (Table 2).
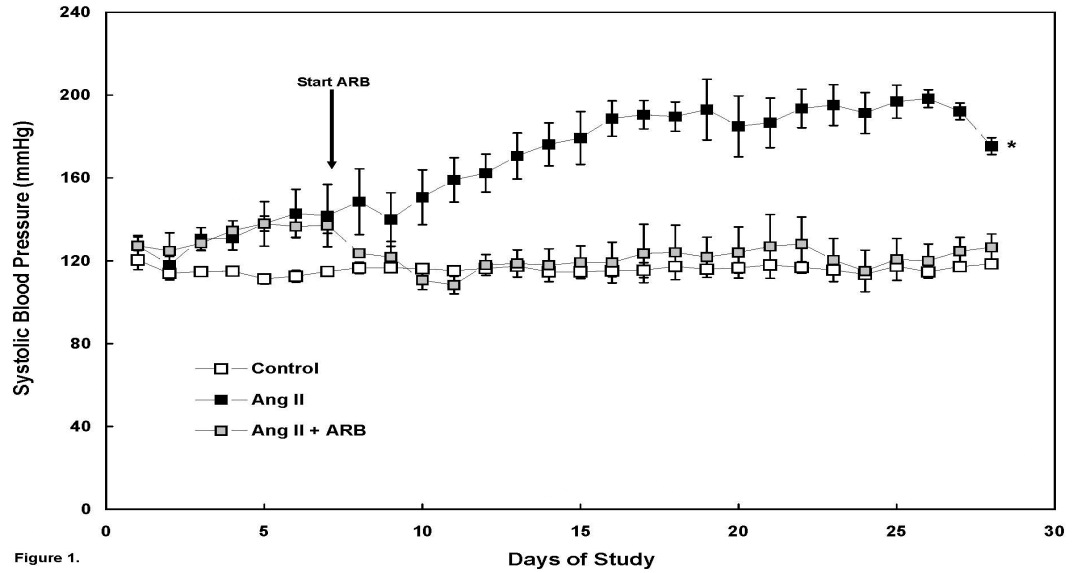
Figure 1.

Figure 1. Mean (±SE) systolic blood pressure (n=6) in Control, Angiotensin II (Ang II) and Ang II + angiotensin receptor blocker (ARB)-treated animals. The arrow indicates when the ARB treatment was induced (day 7) with a diet containing olmesartan (10mg/kg/d). *Denotes significant (p<0.05) difference from Control.

Table 1. Mean (± SE) body mass (BM), heart mass, and relative heart mass from different from Control, Angiotensin II (Ang II), and Ang II + angiotensin receptor blocker (ARB)-treated rats after 28 days.

	Control	Ang II	Ang II + ARB
BM (g)	400 ± 14	355 ± 10*	370 ± 12
Heart Mass (g)	1.12 ± 0.06	1.46 ± 0.06*	1.33 ± 0.06*
Relative Heart Mass (g/ 100 g BM)	0.28 ± 0.01	0.41 ± 0.02*	0.36 ± 0.01$^{*, \#}$

* Significantly (p<0.05) different than control, # significantly (p<0.05) different than Ang II group.
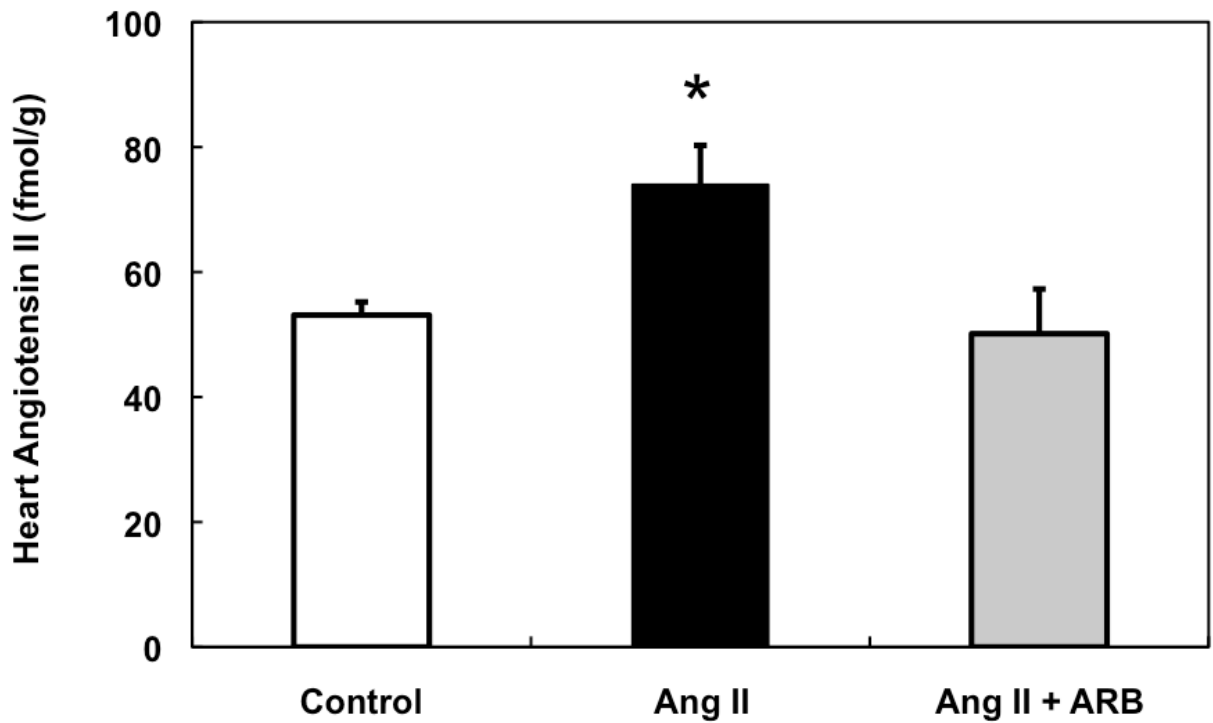
Figure 2. Mean (±SE) heart angiotensin II (Ang II) content from control, angiotensin II (Ang II) and Ang II + angiotensin receptor blocker (ARB)-treated animals. Ang II infusion (80 ng/min x 4 weeks) was initiated on day 1 and treatment with ARB (10 mg/kg/d x 3 weeks) was initiated on day 7. * Denotes significant (P< 0.05) difference from control. # Denotes significant (P< 0.05) difference from Ang II.

Table 2. Mean (± SE) plasma and tissue Ang II, aldosterone and PRA after 28 days of Ang II-induced hypertension and 21 days of ARB treatment.

	Control	Ang II	Ang II + ARB
Plasma Ang II (fmol/mL)	32 ± 4	38 ± 3	70 ± 7[*,#]
Plasma Aldosterone (nM/ml)	1.4 ± 0.1	3.5 ± 1.2[*]	5.6 ± 1.0[*,#]
PRA (ng Ang I/L/h)	4.4 ± 1.0	0.6 ± 0.2[*]	0.2 ± 0.1[*]
Adrenal Aldosterone (pmol/mg of tissue)	235± 50	363 ± 94[*]	755 ± 169[*,#]
Kidney Ang II (fmol/g of tissue)	96 ± 6	207 ± 14[*]	92 ± 7[#]

* Significantly (p<0.05) different than control, # significantly (p<0.05) different than Ang II.
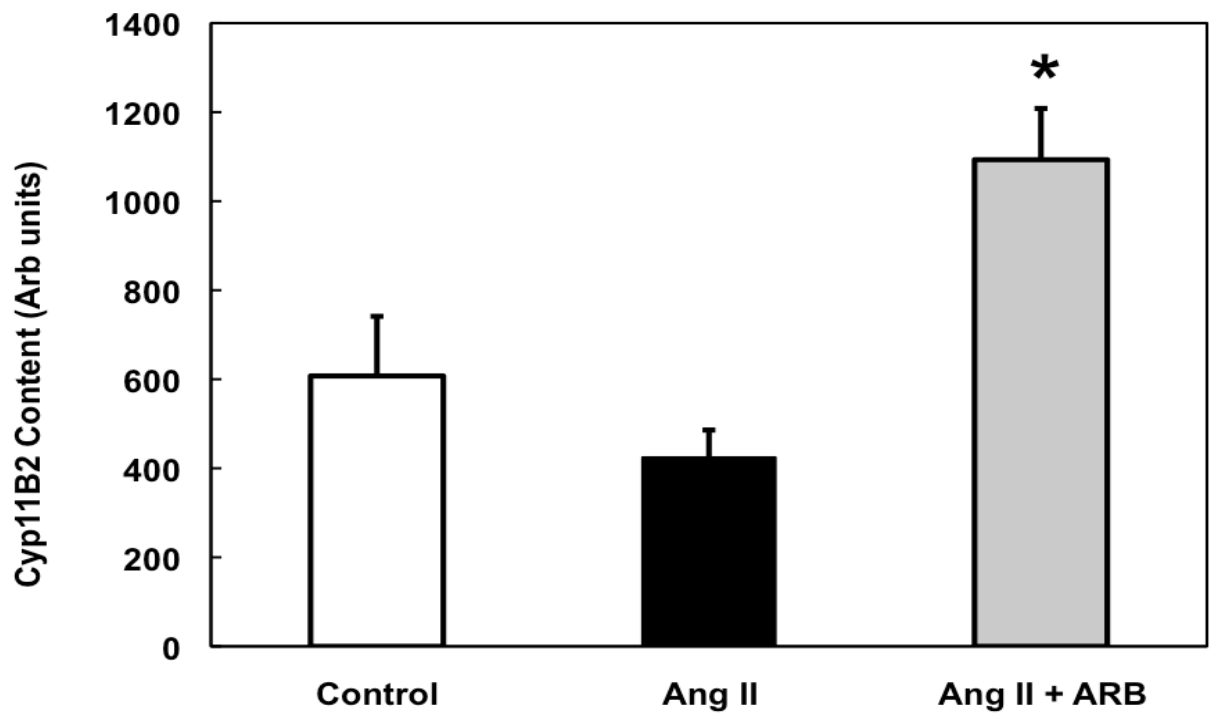
Figure 3. Mean (±SE) Cyp11B2 densitometry in arbitrary units difference from control, angiotensin II (Ang II) and Ang II + angiotensin receptor blocker (ARB)-treated animals. *Denotes significant (P< 0.05) difference from control.
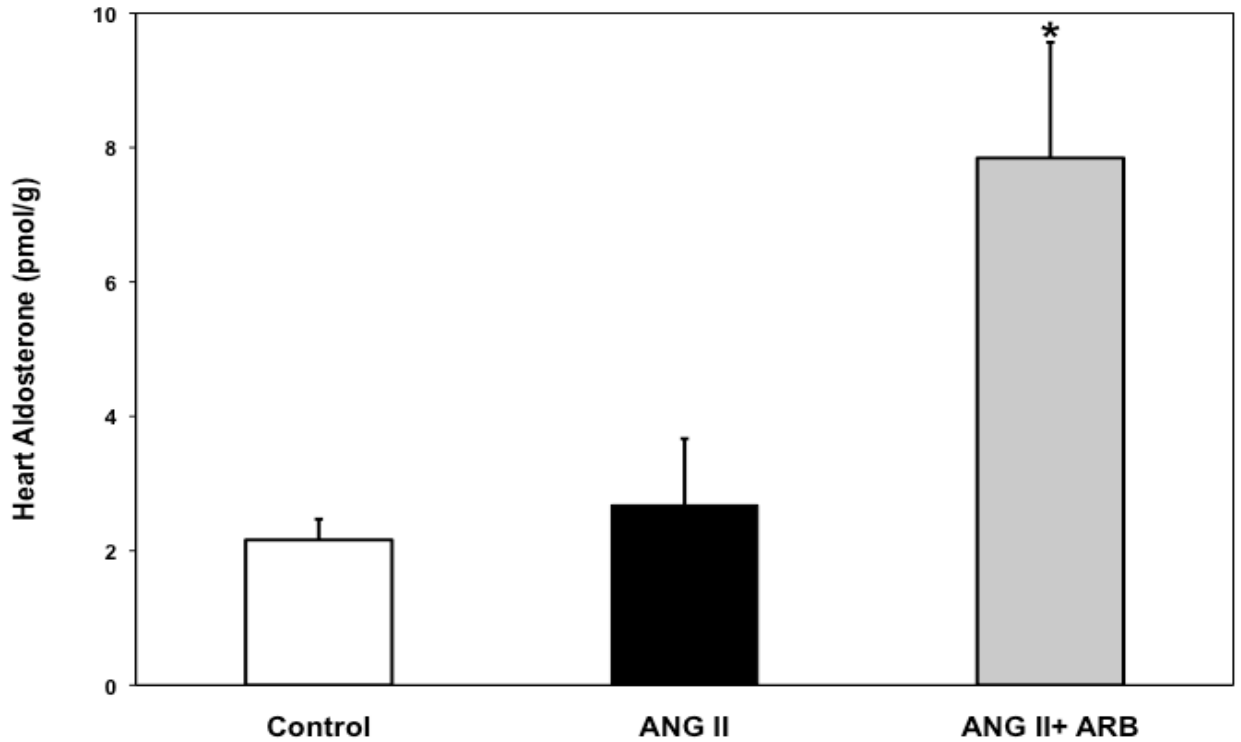
Figure 4. Mean (±SE) heart aldosterone content from control, angiotensin II (Ang II) and Ang II + angiotensin receptor blocker (ARB)-treated animals. Ang II infusion (80 ng/min x 4 weeks) was initiated on day 1 and treatment with ARB (10 mg/kg/d x 3 weeks) was initiated on day 7. *Denotes significant (P< 0.05) difference from control.

Plasma hormones

Infusion of Ang II in both the untreated and ARB-treated groups suppressed PRA compared with control (Table 2). Plasma Ang II levels was not different between control and Ang II-infused groups; however, following treatment with ARB there was a two-fold increase (Table 2). Infusion of Ang II induced a 2.5-fold increase in mean plasma aldosterone and ARB treatment increased levels an additional 60% from Ang II (Table 2**)**.

Intracardiac AT1 Receptors.

Ang II infusion decreased intracardiac AT1 receptor expression 10% of control levels, and ARB treatment decreased AT1 expression an additional 12% from Ang II (Figure 5).

Urinary total protein excretion

After 28 d, Ang II infusion increased (P<0.01) $U_{TP}V$ nearly 2.5-fold (27±3.5 mg/d) from control (11±1.8 mg/d) and ARB had no further effect (28±4.7).
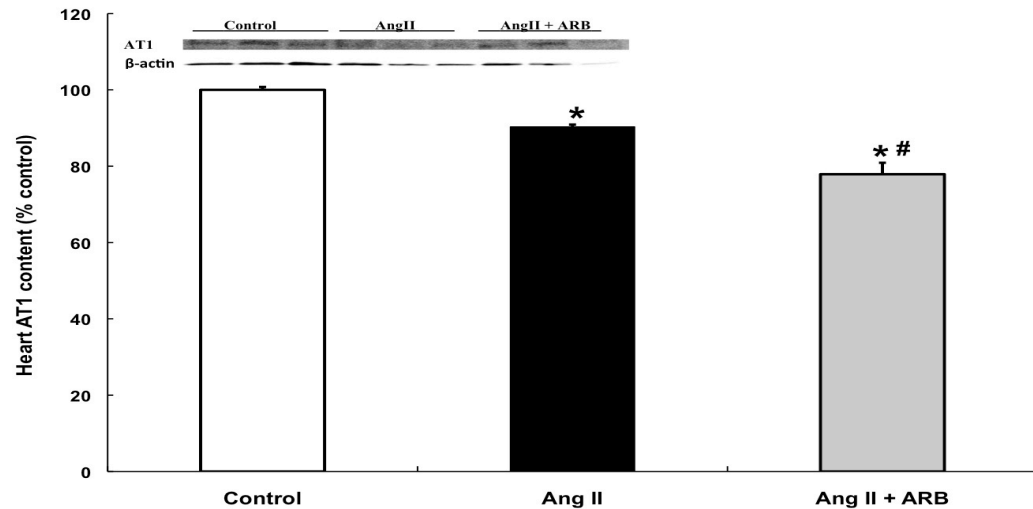
Figure 5. Mean (±SE) heart angiotensin receptor type 1 (AT1) protein content from Control, angiotensin II (Ang II) and Ang II + angiotensin receptor blocker (ARB)-treated animals. Ang II infusion (80 ng/min x 4 wk) was initiated on day 1 and treatment with ARB (10 mg/kg/d x 3 wk) was initiated on day 7. * Denotes significant (p<0.05) difference from Control; # denotes significant (p<0.05) difference from Ang II.

Discussion

Accumulation of angiotensin II in kidneys has been shown to be a receptor-mediated process (10, 24, 39, 90, 99, 100); however, there is some discrepancy in the response of the heart to elevated Ang II during acute and chronic Ang II infusion and between acute and chronic ARB treatment. While chronic infusion of Ang II was previously shown to increase intrarenal Ang II levels two-fold, simultaneous measures of Ang II levels in the heart or adrenals were not affected with infusion suggesting that the accumulation of Ang II is tissue-specific (100). In the same study, ARB treatment in the Ang II-infused rat completely ameliorated the intrarenal Ang II levels; however, the heart and adrenal Ang II levels were increased four-fold and 80%, respectively, further suggesting that the accumulation of Ang II in extrarenal tissues is not a receptor-mediated process (100). Conversely, acute ARB treatment reduced the heart-to-plasma ratio of radio labeled Ang II nearly 10-fold in the acutely infused pig heart, suggesting that the accumulation of Ang II in the mammalian heart is angiotensin receptor mediated as in the kidney (90). In accordance with our hypothesis, Ang II levels in the heart in the present study increased during chronic infusion and were completely ameliorated with chronic ARB treatment, suggesting that the accumulation of Ang II in the heart is an AT1 receptor-mediated process similar to that observed in the kidney.

The increase of plasma Ang II levels following ARB treatment and in the presence of reduced PRA suggests that increased plasma Ang II levels are the

consequence of reduced tissue sequestration and increased displacement from their receptors, and substantiates the idea that regulation of intracardiac Ang II levels is AT1-mediated during Ang II infusion. Furthermore, the complete amelioration of intrarenal Ang II (and SBP) following ARB treatment in the present study provides evidence of the effectiveness of the blockade of the angiotensin receptor (61, 81, 100), and suggests that the blockade of angiotensin receptors was systemic and not just localized in the heart. Interestingly, despite the robust blockade of the angiotensin receptors, ARB treatment failed to recover the Ang II-induced suppression of PRA. While ARB treatment in Ang II-infused Sprague-Dawley rats has been shown to recover the Ang II-induced suppression of PRA (98, 100) an important and significant difference between this and those previous studies is the timing of the initiation of ARB. In the present study, ARB (olmesartan) was not provided until after the first week of Ang II infusion when the animals were hypertensive (approximately 74% of maximal SBP), whereas in the previous studies, (98, 100) ARB (losartan or candesartan) was provided simultaneously at the onset of the study when animals are still normotensive and PRA is not suppressed. In normotensive subjects with suppressed PRA (<0.65 ng Ang I/mL/h), various ARBs (including olmesartan and valsartan) failed to recover PRA levels within 24 h following an acute dosage (31) suggesting that ARB treatment is not effective at recovering PRA levels once they are maximally suppressed (as may be the case with Ang II infusion), and alludes to a potential change in sensitivity of the juxtaglomerular apparatus to Ang II. This phenomenon clearly warrants further investigation.

Another important and novel discovery of the present study is the increase in heart aldosterone levels following chronic Ang II receptor blockade. In the present study, chronic ARB treatment was associated with a nearly fourfold increase in intracardiac aldosterone. Aldosterone is synthesized from deoxycorticosterone by a mitochondrial cytochrome P450 enzyme, aldosterone synthase (CYP11B2) (17, 37). The CYP11B2 enzyme in the heart was elevated following ARB treatment, in agreement with the heart aldosterone data. In addition, chronic ARB treatment in the present study did not prevent adrenal aldosterone production, and thus the exacerbated levels of plasma and adrenal aldosterone are consistent with the phenomenon of aldosterone 'breakthrough' or 'escape' (21, 55, 89). Treatment with ARB failed to reduce aldosterone production and secretion, and instead, was associated with a 'breakthrough' or 'escape' event in the present study suggesting that aldosterone escape is independent of AT1 mediation and likely the result of compensatory changes in response to chronic blockade of adrenal angiotensin receptors. While aldosterone breakthrough has been reported (21, 55), to the best of our knowledge, this is the first report of local aldosterone breakthrough in the heart following RAS inhibition. Thus, both local and systemic aldosterone breakthrough events occurred simultaneously in the present study, suggesting that diagnosis of systemic aldosterone breakthrough in patients may be associated with local breakthrough in the heart necessitating co-therapy with a mineralocorticoid receptor antagonist. The similarity in the levels of aldosterone between plasma and heart following ARB treatment provides evidence for effective sequestration

of circulating aldosterone by the heart in the presence of inappropriately elevated plasma aldosterone, and would argue against the notion of any significant aldosterone production by the heart (23). A limitation of the present study is the lack of data that address the mechanisms by which ARB induced a breakthrough event. Elucidation of the cellular mechanisms of aldosterone escape warrant further investigation, especially if it can be determined that elevated tissue levels associated with escape are detrimental. Aldosterone has been reported to increase Ang II receptor (i.e. AT1) number and binding *in vitro* (69, 83, 89), however in this study we found that in spite of the increased in aldosterone, the ARB was able to constrain it's upregulation.

It is well established that the progression of renal disease is associated with increased proteinuria. A review of the clinical data suggests that ARB treatment reduces proteinuria but the results are variable (36). Data regarding the effectiveness of ARB treatment on alleviating the proteinuria in Ang II-dependent hypertensive models are scarce. Olmesartan (ARB) has been shown to effectively alleviate the proteinuria after 14 d in Ang II-infused rats (78) (78). However, in the present study, a similar dose of olmesartan failed to alleviate proteinuria in Ang II-infused rats after three weeks of treatment. As inappropriately elevated aldosterone contributes to the progression of renal injury as indicated by increased proteinuria,(27, 57, 62) the discrepancies in these results may be attributed to differences in aldosterone levels, which unfortunately were not reported in the previous study (36). Thus, the exacerbation of aldosterone by ARB treatment in the present study likely impaired the ability of

the ARB to alleviate the proteinuria.

Perspectives

The present results suggest that chronic treatment with an ARB promotes aldosterone escape locally at the level of the heart despite the benefit of reducing arterial blood pressure. Therefore, patients on chronic ARB treatment may be more susceptible to aldosterone-induced cardiovascular injury if the aldosterone is unabated. Further studies are needed to confirm that the elevated levels of intracardiac aldosterone are physiologically relevant and functional. Future studies that focus on mineralocorticoid receptor regulation in the heart during disruption of RAS are needed to confirm whether or not local aldosterone breakthrough has the potential to be physiologically relevant. Alternatively, increased intracardiac aldosterone may induce intracellular events that are independent of mineralocorticoid receptor activation that would require interventions to inhibit the intracellular signaling of those mechanisms.

Chapter 3

Combined angiotensin and mineralocorticoid receptor antagonism improves biomarkers for inflammation and oxidative stress in the heart of Ang II-Infused Rats

Introduction

Blood pressure and volume homeostasis is primarily regulated by angiotensin II (Ang II) (87, 97). Chronically elevated Ang II can have severe consequences such as congestive heart failure and hypertension (97). Important vascular effects of Ang II include its pro-inflammatory properties and the induction of oxidative stress (87, 97). Inflammation and the generation of free radicals contributes to the activation of the fibrotic process and hypertrophy (8, 92). Ang II stimulates the release of aldosterone from the adrenal cortex, which in turn can have additional inflammatory and oxidative effects (29, 95). Additionally, studies indicate that angiotensin II exerts its cardiovascular effects mainly through an AT_1-mediated stimulation of reactive oxygen species (ROS; $\bullet O_2^-$ and H_2O_2) through NADPH oxidase activation (66, 87, 88). Aldosterone has also been implicated as a stimulus for ROS production *via* up-regulation of NADPH oxidase (57, 84, 91). Infusion of aldosterone in rats increased NADPH subunit expression, markers of oxidative stress, and MAPK activity (57).

Mineralocorticoid receptor (MR) antagonism offers cardioprotection against oxidative damage despite increasing intra-cardiac aldosterone during Ang II-dependent hypertension (14, 15). Recent data from the Randomized

Aldactone Evaluation Study (RALES) revealed an additional effect of mineralocorticoid receptor antagonism with spironolactone on overall mortality in patients with advanced heart failure treated with angiotensin-converting enzyme (ACE) inhibitors and loop diuretics (65). 8-iso-protaglandnin-F_2 (8-isoprostane) and nitrotyrosine have been described as stable ROS-mediated tissue injury markers (6, 13, 47). 8- isoprostane is the stable end product of arachidonic acid oxidation generated by ROS on membrane phospholipids (93). Nitrotyrosine is the stable end product of cell membrane protein-bound tyrosine nitration by peroxynitrite caused by increased nitric oxide (47, 50). Furthermore, data exists to support that a combination of Ang II and aldosterone receptor blockade may be beneficial in reducing vascular injury in cardiovascular disease (52). However, the combined benefits of AT1 and MR blockade on cardiac aldosterone and oxidative/nitrositive stress in an Ang II-infused model of hypertension has not been described. We hypothesize that in an Ang II-infused model of hypertension, oxidative/nitrositive damage is mediated through the MR and AT1 independent of increased blood pressure.

Methods

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of both Kagawa Medical University and the University of California in accordance to the guidelines for the care and use of animals established by these institutions.

Animals and Procedures

Male Sprague-Dawley rats (Clea Japan Inc., Tokyo, Japan) or (Charles River, Wilmington, MA) approximately 200-225g were randomly assigned to five experimental groups: 1) control (n=11), 2) angiotensin II (n=14)(Ang II; 80 ng/min; Sigma-Aldrich), 3) Ang II + ARB (n=17)(ARB; 10 mg losartan/kg/d in diet; Merck & Co, Inc*),* 4) Ang II + MR blockade (n=14) (EPL; 100 mg eplerenone /kg/d in diet; Pfizer, St. Louis, MO), and 5) Ang II + ARB + MR blockade (Combo)(n=14). Control and Ang II rats were maintained on a normal rat chow diet (Research Diets, Inc., New Brunswick, NJ). The dosage of ARB is consistent with that used previously to successfully block Ang II (16, 51, 53). This dosage of eplerenone (0.1% in diet) has been shown to result in optimal pharmacokinetic characteristics for effective *in vivo* inhibition of MR in the rat (9). Food consumption rates in this study were measured weekly to verify that treatment goals were met. Systolic blood pressure (SBP) was monitored daily by radiotelemetry implanted in the animals and used to evaluate the effectiveness of the Ang II infusion, ARB and EPL (59, 60). Animals were anesthetized with 5% isoflurane prior to implanting a biotelemetric transducer (PA-C40, DSI, St. Paul, MN) in the peritoneal cavity with the catheter lead extending into the abdominal aorta. The animals were allowed to recover for 7 days from the effects of the surgery. The animals were again anesthetized with 5% isoflurane and implanted with an osmotic mini-pump (Durect Corp, Cupertino, CA) subcutaneously behind the neck to infuse Ang II (80 ng/min; Sigma-Aldrich, St. Louis, MO) for 28 days.

Each animal was maintained individually in metabolic cages in a temperature and light controlled room. Throughout the experiment, animals had unrestricted access to water and their specific diet. Ang II infusion began on day 1 of the study and allowed 7 days to become hypertensive prior to treatment to mimic the conditions in which ARB and/or EPL are prescribed. The ARB, EPL and Combo diets began on day 7 for a period of 21 days. On the last day of the study, 24-hr urine voids were recorded and aliquots were collected for later analysis.

Dissections.

On day 28, animals were weighed, decapitated, and trunk blood collected into chilled vials containing 5 mM EDTA plus protease inhibitor cocktail (PIC; Sigma, St. Louis, MO) for the measurement of plasma aldosterone, Ang II and plasma renin activity (PRA). The heart was removed patted dry and weighed. Sections were placed in 10 ml of cold methanol and homogenized for Ang II extractions or 10 ml of PBS and homogenized for aldosterone extraction. Additional pieces were flash-frozen in liquid nitrogen and stored at –80°C for future analysis. The right adrenal was removed, cleaned, weighed, placed in a glass vial containing 10 ml of cold PBS, and homogenized for extraction of aldosterone. The left adrenal gland was removed, cleaned weighed and flash-frozen in liquid nitrogen and stored at –80°C for future analysis.

Hormone analyses

PRA was measured using a commercially available RIA kit (DiaSorin,

Stillwater, MN, USA). Adrenal and heart aldosterone were extracted from their homogenates with ethyl acetate. Heart, adrenal, and plasma aldosterone concentrations were measured by commercially available RIA kit (Siemens Healthcare Diagnostics, Los Angeles, CA). Heart homogenates and plasma (0.8 mL) were extracted for Ang II measurements with details of the Ang II extraction and assay (Phoenix Pharmaceuticals, Burlingame, CA) procedures described previously (10).

Western Blot analyses

All chemicals and reagents used in Western blot analyses were obtained from Bio-Rad (Hercules, CA), Pierce (Rockford, IL), and Santa Cruz Biotechnology Corp (Santa Cruz, CA), unless otherwise noted. Heart segments were harvested as described above followed by homogenization in 1 ml hypotonic saline containing PIC. The homogenate was spun down and the pellet was re-suspended in 1 ml lysis buffer containing PIC and 1% Triton X-100. The soluble fraction was collected and protein concentration was determined using the BCA assay. Tissue homogenates containing 30 µg of total protein was analyzed by standard Western blot technique as previously described using AT1 antibodies (61). In addition to normalizing the gels by loading 30 µg of total protein, densitometry values were also normalized for β-actin expression of the blots. Western blots were analyzed on a Bio-Rad Chemidoc imager and band intensity was quantified using Analysis One software (Bio-Rad).

Urinalysis

Aldosterone was extracted from urine using ethyl acetate and assayed by commercially available RIA (Siemens Healthcare Diagnostics, Los Angeles, CA). Urine samples were diluted 1:8 prior to measurement of total angiotensinogen (IBL America, Minneapolis, MN) by ELISA (34). Urine samples were diluted 1:10 prior to measurement of 8-isoprostane (Cayman Chemical, Ann Arbor, MI) by ELISA (62). Urine samples were extracted by dichloromethane for measurement of corticosterone by commercially available EIA (Arbor Assays, Ann Arbor, MI).

Statistics

Mean (±SE) plasma and organ measurements were compared by one-way analysis of variance and linear regression model. For all cases, if significance ($P < 0.05$) was detected, a Fisher's protected least significant difference test was applied *post hoc*. Statistics were performed using Statview software (SAS, Cary, NC, USA).

Results

Blood pressure, and body and heart mass

The infusion of Ang II increased mean SBP 141% (117 ± 7 mmHg vs 165 ± 16 mmHg), and ARB treatment was effective in reducing the pressure to control levels (117 ± 4 mmHg) by day 28, demonstrating the effectiveness of both the Ang II infusion and ARB treatment. Treatment with EPL (159 ± 7 mmHg) had

no significant effect on blood pressure, but when combined with the ARB, co-

therapy was able to completely ameliorate SBP (117 ± 4 mmHg) (Figure 6).

By day 28, Ang II decreased mean body mass (413± 9 vs 352 ± 14 g) by 14%,

and ARB treatment had recovered mean body mass to 8% of control, which was

not significantly different (Table 3). Both EPL and Combo were similar to control
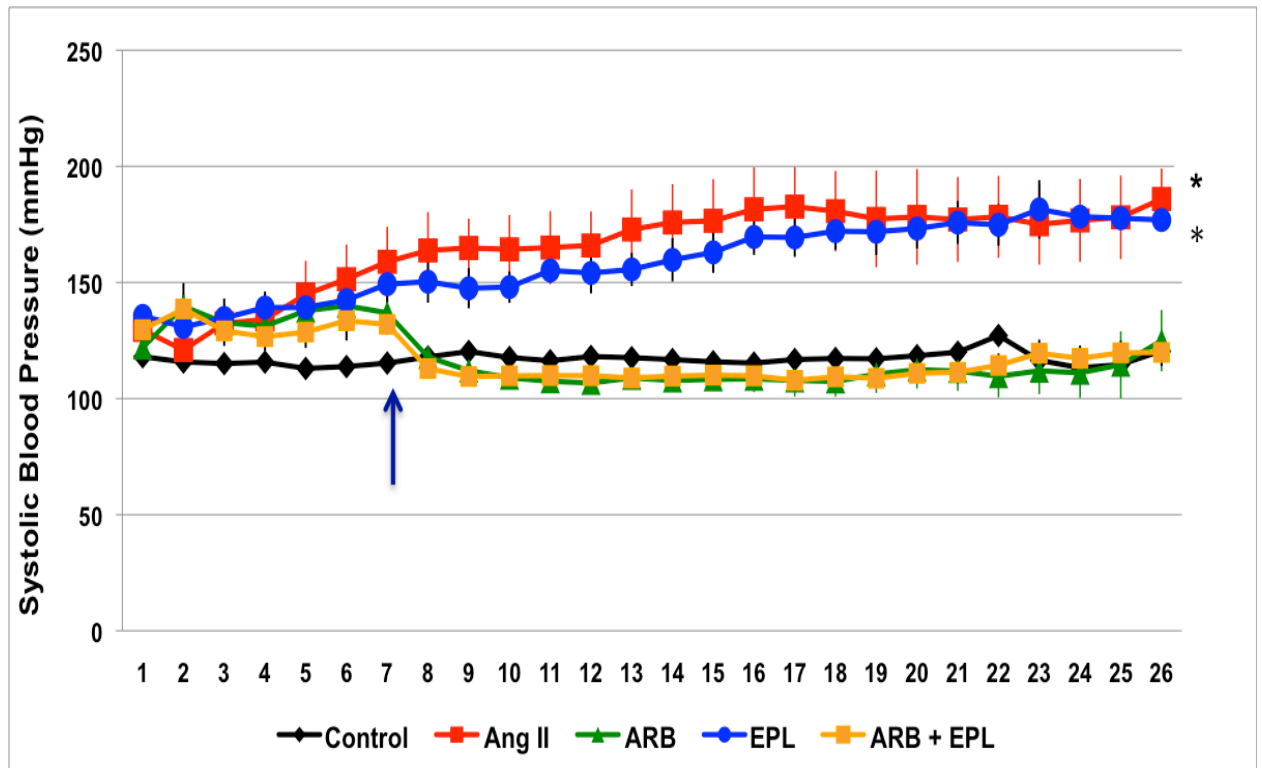
Figure 6. Mean (±SE) systolic blood pressure (n=14/group) from Control, angiotensin II (Ang II; 80 ng/min x 4 wk), Ang II + ARB (ARB; 10 mg losartan /kg/d in diet x 3 wk), Ang II + MR blocker (EPL; 100 mg eplerenone /kg/d in diet x 3 wk), and Ang II + ARB + MR blocker (Combo x 3 wk). The arrow indicates when treatment was initiated (day 7) with a diet containing the receptor blockers. * Denotes significant (p<0.05) difference from Control.

Table 3. Mean (± SE) body mass, heart mass, and relative heart mass different from Control, Ang II, and Ang II + angiotensin receptor blocker (ARB), Ang II + eplerenone (EPL), Ang II + ARB and EPL (Combo) treated rats after 28 days

	Control	ANG II	ARB	EPL	COMBO
Body Mass (g)	413 ± 9.3	352 ± 13.5*	410 ± 7.3$^{\#}$	356 ± 8.8*$^{\%}$	421 ± 8.8$^{\#\$}$
Heart Mass (g)	1.23±0.47	1.29±0.04*	1.14±0.04*$^{\#}$	1.10±0.03*$^{\#}$	1.25±0.03$^{\#}$
Relative Heart Mass (g/ 100 g BM)	0.31±0.01	0.37±0.01*	0.28±0.01*$^{\#}$	0.32±0.01$^{\#\%}$	0.28±0.01$^{\#}$

* Denotes significantly ($p<0.05$) different from Control. # Denotes significantly different ($p<0.05$) from Ang II. % Denotes significantly ($p<0.05$) different from ARB. $ Denotes significantly ($p<0.05$) different from EPL.

(Table 3). Ang II increased mean absolute heart mass (1.23±0.47 vs 1.29±0.04 g), and ARB and EPL treatments reduced mean absolute heart mass (Table 3). Combo had no significant effect on mean absolute heart mass compared to control (1.25±0.03 vs 1.23±0.47) (Table 3). Ang II-infusion increased mean relative heart mass (0.37±0.01 vs. 0.31±0.01 g/100g BM) by 19%; ARB and Combo ameliorated the increase in mean relative heart mass (0.28±0.01 g/100g BM), suggesting that blockade of Ang II receptor alleviated the hypertrophy, EPL (0.32±0.01 g/100g BM) was not as effective in reducing hypertrophy as ARB. (Table 3)

Angiotensinogen

Urinary angiotensinogen was significantly elevated in the Ang II group (1738 ± 376 vs. 22 ± 7 ng/ml/day Control), and ARB, EPL and Combo were not significantly different from Control (Figure 7) suggesting increased Ang II production.

Hormone Measurements

Plasma renin activity was significantly suppressed in all Ang II-infused groups compared to control, but activity was not different among them (Table 4). Plasma Ang II concentration increased nearly 3-fold with infusion and nearly 6-fold with ARB treatment. The Combo group was significantly lower than the ARB group (41.7 ± 6.5 versus 156.7 ± 29.3 fmol/mL) but not significantly different from the other groups (Table 4).

Plasma aldosterone in the EPL group is nearly 36-fold higher than the control group (1682 ± 142 versus 45 ± 11 nmol/ml) and significantly higher than all other groups. The Ang II, ARB and Combo groups are all similar to control with the Combo group being significantly higher than the ARB group (Table 4) Urinary aldosterone in the EPL group showed a 3-fold increase over the control group (48.1 ± 10 versus 11.8 ± 4.9 pmol/ml) and was significantly higher than all other groups. None of the other groups were significantly different than control (Table 4).

All groups had significantly higher concentrations of heart aldosterone than the control. The EPL group again was about 3.5-fold higher (15.8 ± 1.4 versus 3.59 ± .3 pmol/g tissue) concentration than control (Figure 8). The EPL group had the significantly highest concentration of aldosterone in the adrenal glands as well (Table 4).

Similar to the aldosterone data, urinary corticosterone concentration in the EPL group was 4 fold higher than Control. There was no significant difference between Control, Ang II and ARB groups. The Combo group had significantly higher concentration of excreted Corticosterone than the Control, Ang II and ARB groups, but was significantly 2-fold less than the EPL group (Figure 9).

Figure 7. Mean (±SE) urinary angiotensinogen (n= 6/group) from Control, angiotensin II (Ang II; 80 ng/min x 4 wk), Ang II + ARB (ARB; 10 mg losartan /kg/d in diet x 3 wk), Ang II + MR blocker (EPL; 100 mg eplerenone /kg/d in diet x 3 wk), and Ang II + ARB + MR blocker (Combo x 3 wk). # Denotes significant ($p<0.05$) difference from Ang II.

Table 4.Mean (± SE) plasma and tissue Ang II, aldosterone and PRA after 28 days of Ang II-induced hypertension and 21 days of ARB treatment.

	Control	ANG II	ARB	EPL	COMBO
PRA (ng Ang I/L/h)	2.9±0.4	0.8±0.3*	0.6±0.2	0.3±0.1	0.2±0.1
Plasma Ang II (fmol/ml)	26.3±0.8	77.7± 20.1*	156.7± 29.3*#	81.6± 9.4*%	41.7± 6.5%
Plasma Aldosterone (nM/ml)	45±11	810± 30*	85 ± 13#	1682±142*#%§	154 ± 25#$
Urine Aldosterone (pmol/ml)	11.8±4.9	18.8±2.5	27.5±2.8	48.1±10.0*,#,$,%§	19.2 ± 3.9
Adrenal Aldosterone (pmol/mg of tissue)	1195 ± 233	2164	1336 ± 244	5703 ± 2150*%§	486 ± 210

* Denotes significantly ($p<0.05$) different from Control. # Denotes significantly different ($p<0.05$) from Ang II. % Denotes significantly ($p<0.05$) different from ARB. $ Denotes significantly ($p<0.05$) different from EPL. § Denotes different from Combo.

Oxidative Stress Biomarkers

Urinary 8-isoprostane was significantly elevated 15% in the Ang II group (32 ± 4.5 vs 27.2 ± 3.3 μg/d) compared to Control, suggesting that elevated Ang II increased oxidative stress. Treatment with ARB reduced 8-isoprostane by 57% (13.8 ± 3.1 μg/d) from Ang II and 49% less than control suggesting that ARB not only prevented oxidative damage from Ang II uptake but offered additional protection from injury. EPL decreased it by 65% (11.3 ±1.7 μg/d) from Ang II, and co-therapy offered the greatest reduction of 8-isoprostane at 84% (5.2 ± 0.8 μg/d)(Figure10). This data suggests that aldosterone is involved in oxidative damage, but to a lesser degree than the Ang II, and by blocking both you get minimal improvement. Nitrotyrosine (NT) levels in the heart showed Ang II increased NT levels to 144% of Control (Figure 11). ARB treatment reduced NT by 75% of Ang II, and MR antagonism (EPL) reduced heart NT by 66% of Ang II, and co-therapy reduced NT further to 68% of Ang II (Figure 11).

Figure 8. Mean (±SE) Heart aldosterone (n=14/group) from Control, angiotensin II (Ang II; 80 ng/min x 4 wk), Ang II + ARB (ARB; 10 mg losartan /kg/d in diet x 3 wk), Ang II + MR blocker (EPL; 100 mg eplerenone /kg/d in diet x 3 wk), and Ang II + ARB + MR blocker (Combo x 3 wk). * Denotes significant (p<0.05) difference from Control. # Denotes significantly different (p<0.05) from Ang II. % Denotes significantly (p<0.05) different from ARB.
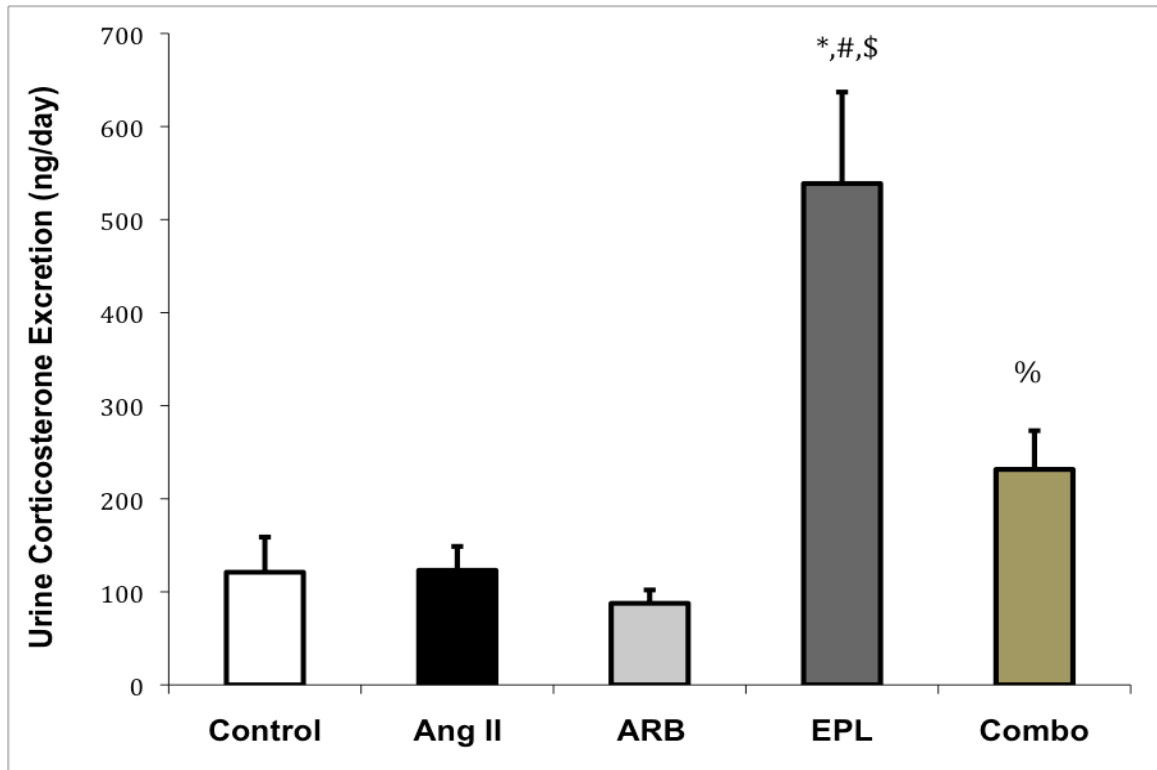
Figure 9. Mean (±SE) Urine corticosterone (n=14/group) from Control, angiotensin II (Ang II; 80 ng/min x 4 wk), Ang II + ARB (ARB; 10 mg losartan /kg/d in diet x 3 wk), Ang II + MR blocker (EPL; 100 mg eplerenone /kg/d in diet x 3 wk), and Ang II + ARB + MR blocker (Combo x 3 wk). * Denotes significantly (p<0.05) different from Control. # Denotes significantly different (p<0.05) from Ang II. % Denotes significantly (p<0.05) different from ARB. $ Denotes significantly (p<0.05) different from EPL.
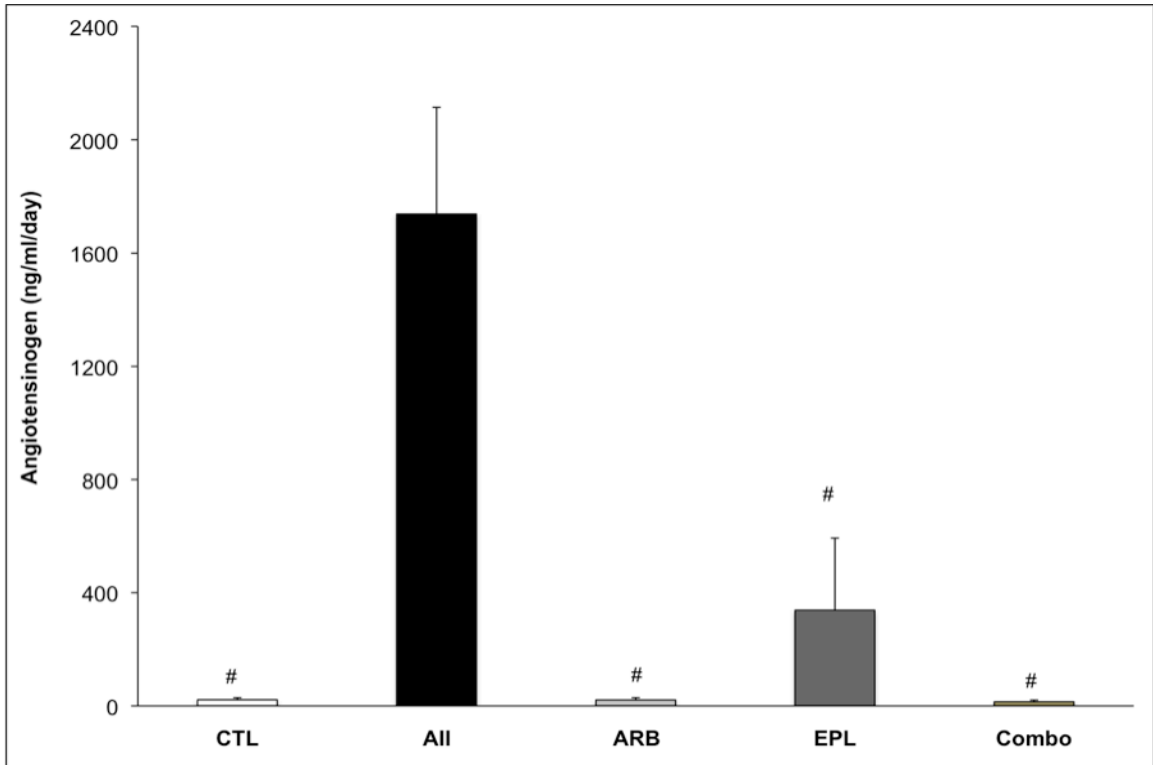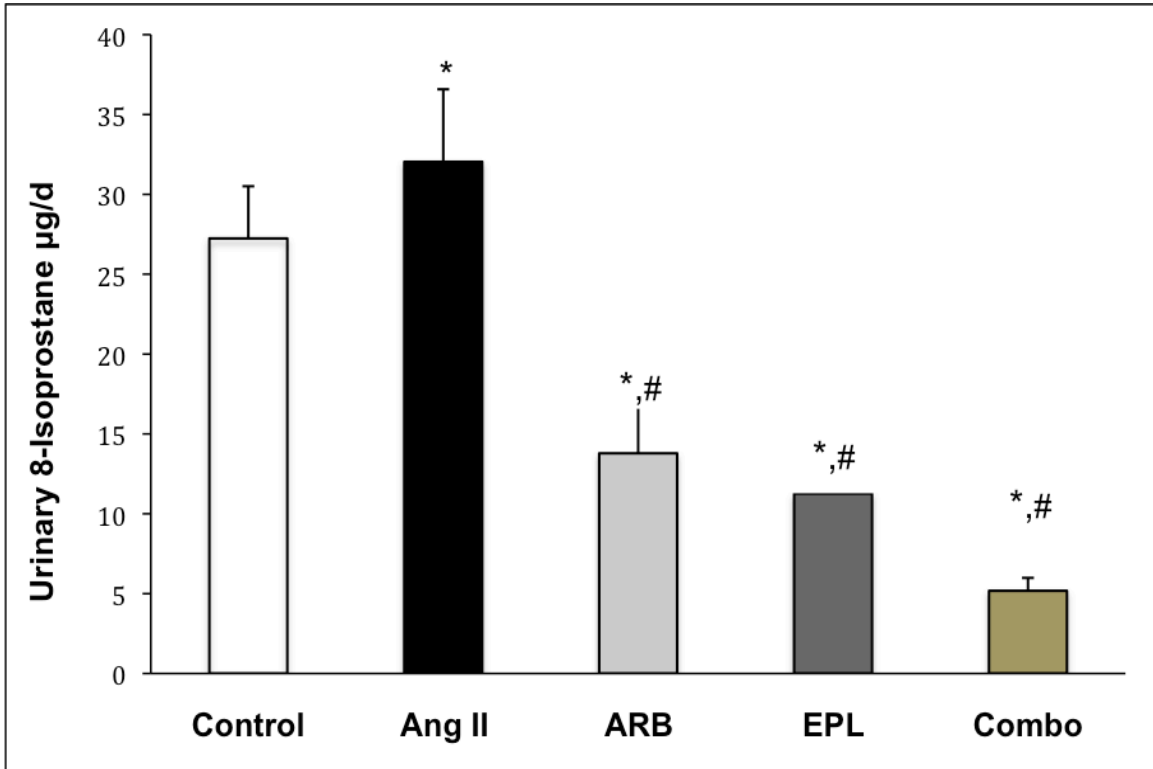
Figure 10. Mean (±SE) urinary 8-Isoprostane (n=14/group) from Control, angiotensin II (Ang II; 80 ng/min x 4 wk), Ang II + ARB (ARB; 10 mg losartan /kg/d in diet x 3 wk), Ang II + MR blocker (EPL; 100 mg eplerenone /kg/d in diet x 3 wk), and Ang II + ARB + MR blocker (Combo x 3 wk). * Denotes significantly (p<0.05) different from Control. # Denotes significantly different (p<0.05) from Ang II.
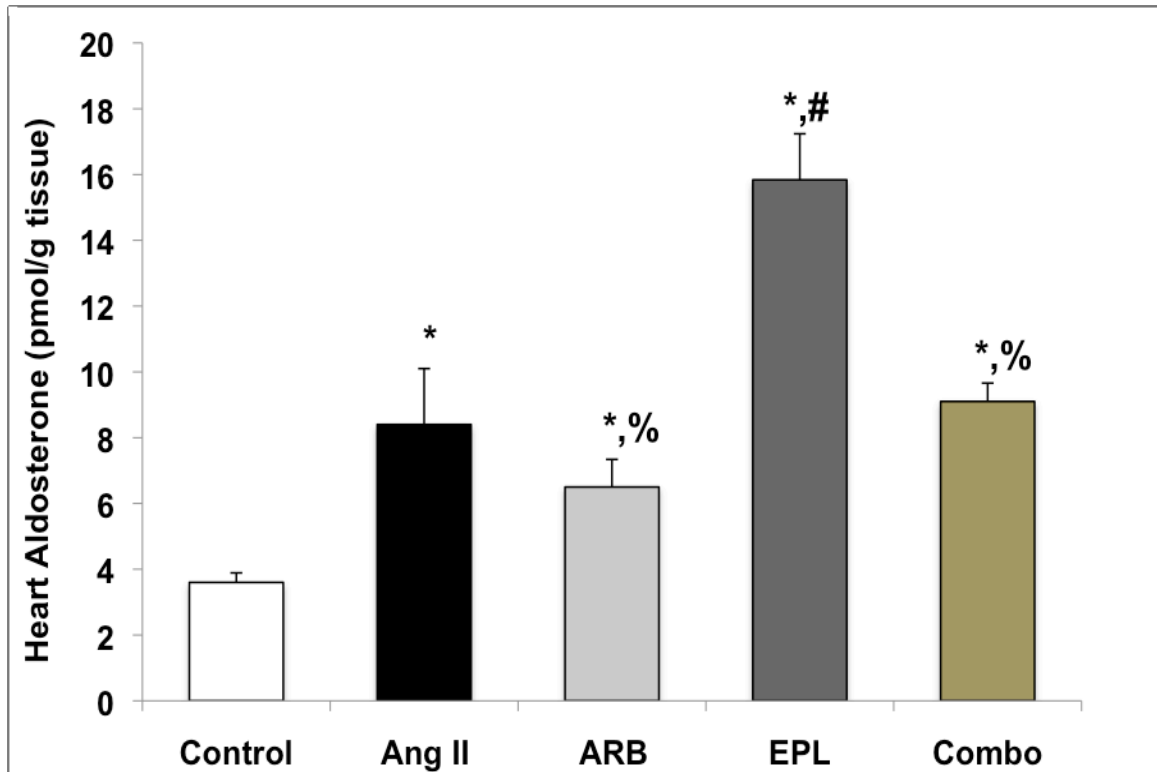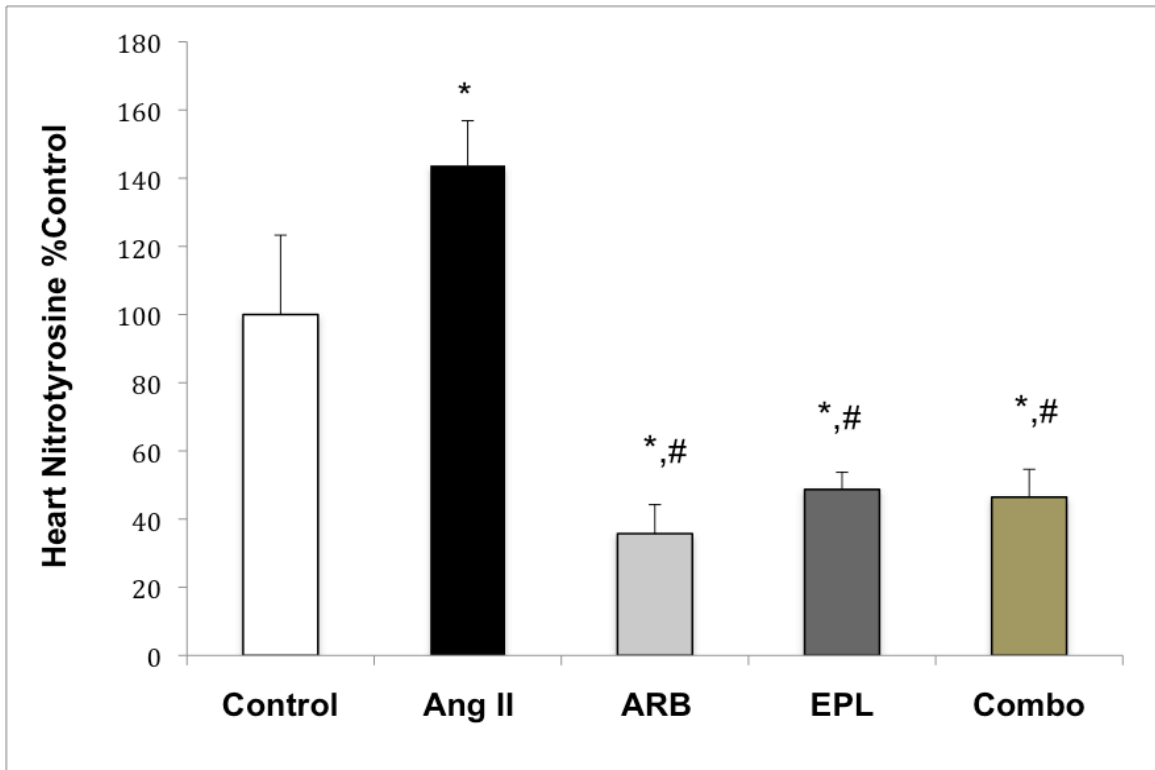
Figure 11. Mean (±SE) heart nitrotyrosine (n=14/group) from Control, angiotensin II (Ang II; 80 ng/min x 4 wk), Ang II + ARB (ARB; 10 mg losartan /kg/d in diet x 3 wk), Ang II + MR blocker (EPL; 100 mg eplerenone /kg/d in diet x 3 wk), and Ang II + ARB + MR blocker (Combo x 3 wk). * Denotes significantly (p<0.05) different from Control. # Denotes significantly different (p<0.05) from Ang II.

Discussion

Chronically elevated Ang II can have severe consequences on cardiovascular health such as congestive heart failure and hypertension (3, 4, 28). The blood pressure data in this study clearly shows that the Ang II-infusion was successful at increasing systolic blood pressure (SBP). The infusion of Ang II increased mean SBP and ARB treatment was effective in reducing the pressure to Control levels almost immediately after beginning treatment, demonstrating the effectiveness of both the Ang II infusion and ARB treatment. EPL had no significant effect on blood pressure, but when combined with the ARB was able to recover to control levels suggesting Ang II is primarily responsible for the increased blood pressure through AT1, which is in agreement with our prior findings (16).

Our data shows that Ang II-infusion increased aldosterone concentration in our model. Aldosterone up-regulates the expression of AT1 receptors in cardiac tissue and vascular smooth muscle cells (69, 74), increasing the availability for AT1-mediated stimulation of reactive oxygen species (ROS). Urinary 8-isoprostane results suggest that blocking AT1 receptor reduced 8-isoprostane in agreement with earlier studies (66, 87, 88). Furthermore, blocking MR not only prevented aldosterone induced tissue damage, but also offers protection despite the increased concentrations of heart and plasma aldosterone providing evidence that the MR blockade was sufficient to ameliorate the aldosterone-mediated consequences and suggests that the exacerbated plasma

aldosterone concentrations associated with MR blockade [cite all the epl/spiro papers showing increased aldo here] are not physiologically functional during the measurement period implemented in the present study. Combining treatment with ARB and MR blockade did not provide an additive effect on ameliorating the decrease in urinary 8-isoprostane suggesting that the levels reported here may represent some minimal threshold of effectiveness of the receptor blockade of both AT1 and MR. The changes in heart nitrotyrosine content were similar to those for urinary 8-isoprostane suggesting that the mechanisms inducing both oxidative and nitrositive stress in Ang II-infused hypertension are similar.

Left ventricular hypertrophy is commonly considered a marker for hypertension and heart disease (49). Inflammation and the generation of free radicals contributes to the activation of the fibrotic process and hypertrophy (8, 92). Relative heart mass in the Ang II group was increased and blockade of AT1 was able to prevent the Ang II-induced hypertrophy suggesting that Ang II induces hypertrophy via an AT1-mediated process. Blocking AT1 not only ameliorated the hypertrophy in the heart but also significantly improved it. The EPL group demonstrated increased hypertrophy when compared to ARB, but was not hypertrophic compared to Control and significantly less than Ang II suggesting that elevated aldosterone contributes to cardiac hypertrophy; however, minimally. This is further supported in the finding that the Combo group had similar mean relative heart mass as ARB suggesting that Ang II, largely independent of aldosterone, is primarily responsible for cardiac hypertrophy in

this model. EPL was able to reduce hypertrophy in salt sensitive models of hypertension (70), but did not have an effect in a low salt model (44). Spironolactone had no effect on cardiac hypertrophy in the Ren2 model (59). Collectively, these data suggest that aldosterone may play a measurable role in hypertrophy in the absence of elevated Ang II, but in the presence of inappropriately elevated Ang II, Ang II is primarily responsible for cardiac remodeling and possibly cardiac remodeling.

Angiotensinogen (AGT) is the precursor to Ang II, and it's production is stimulated by Ang II (33). Our earlier study showed that when treated with an ARB the tissue levels of Ang II are suppressed (16). The data suggest that ARB is successful in preventing augmentation of urinary AGT as previously demonstrated (29). It is reasonable to suggest that the Ang II measurements in the heart would mimic the AGT data, which is exacerbated by Ang II-infusion but is prevented from increased production by both ARB and EPL. Combining both blockers did not have any increased effects.

Corticosterone is an intermediate in the steroidogenic pathway from pregnenolone to aldosterone.

Ang II activates NADPH oxidase through AT1 (66, 87) and MR (57, 84) stimulating oxidative /nitrositive damage. Our data suggests that NADPH oxidase may be stimulated similarly by both Ang II and aldosterone or may reach a maximum stimulation when both hormones are elevated, and thus, blocking both receptors does not provide an additive effect. This study supports prior

studies that Ang II and aldosterone contributions to oxidative damage are mediated through their respective receptors. Blocking these receptors independently can reduce oxidative/nitrositive damage independent of blood pressure; however, combining ARB and EPL didn't additionally reduce oxidative damage, but does add the benefit of reduced blood pressure. Nonetheless, a significant contribution of the present study is the fact that a similar protective effect of MR blockade with that observed with ARB, despite the sustained increase in SBP, suggests that the protective benefits of MR blockade are independent of elevated SBP.

Perspective

The present results suggest that chronic treatment with an ARB and an MR agonist promotes a significant reduction of oxidative stress possibly providing cardioprotection. Although, chronic MR blockade exacerbated aldosterone, elevated Ang II was prevented from causing increased oxidative damage. Increased aldosterone did not increase oxidative stress; however, increased intracardiac aldosterone may induce intracellular events that are independent of mineralocorticoid receptor activation that would require interventions to inhibit the intracellular signaling of those mechanisms. Future studies that focus on mineralocorticoid receptor regulation in the heart during disruption of RAAS are needed to confirm whether or not local aldosterone is an alternate pathway for NAPDH activation when Ang II is blocked.

Conclusion

Because hypertension and the resulting pathologies continue to be a growing problem globally, the information we uncover can potentially have broad impact. The Ang II-induced model of hypertension is an effective tool for studying the effects of an inappropriately elevated RAAS and the contributions of the system's hormones and associated receptors. By manipulating receptor activation with the use of pharmaceuticals we are able to mimic human conditions. In the preceding two studies we were able to take a closer look at receptor activation under manipulated conditions to add further knowledge regarding the consequences of hypertension when RAAS is chronically elevated.

There was a question to whether Ang II was AT1 receptor mediated in heart in the same manner that has been shown in the kidney and adrenal glands or if Ang II sequestration is tissue specific. In our first study, In accordance with our hypothesis, Ang II levels in the heart increased during chronic infusion and were completely ameliorated with chronic ARB treatment, suggesting that the accumulation of Ang II in the heart is an AT1 receptor-mediated process similar to that observed in the kidney. Treatment with ARB failed to reduce aldosterone production and secretion, and instead, was associated with a 'breakthrough' or 'escape' event suggesting that aldosterone escape is independent of AT1 mediation and likely the result of compensatory changes in response to chronic blockade of adrenal angiotensin receptors. While aldosterone breakthrough has been reported (21, 55), to the best of our knowledge, this is the first report of

local aldosterone breakthrough in the heart following RAS inhibition.

In the second study ARB did not activate an aldosterone 'break through" event. In the first study, Olmesarten was the ARB used for blockade of AT1, but Losartan was used in the second study and this may be the cause of the different outcomes. Aldosterone 'break through' is a reported phenomenon in other studies as well as clinically so we are not certain as to the cause. Further study would be needed to determine this. To evaluate the contribution of increased aldosterone to cardiovascular damage, an MR agonist was used in a second study, with and without an ARB to differentially assess the contributions of the respective receptors. They not only ameliorated the hypertrophy in the heart but also significantly improved it. The data also suggests that Ang II, largely independent of aldosterone, is primarily responsible for cardiac hypertrophy in this model. The data suggests that damage to the cardiovascular system by Ang II and aldosterone occurs via their respective receptors and in the case of aldosterone, independent from high blood pressure. This study supports prior studies that Ang II involvement in oxidative damage is through an AT1-mediated process as well as aldosterone induced damage is also mediated through its receptor. Blocking these receptors independently can reduce oxidative/nitrositive damage independent of blood pressure, however combining ARB and EPL didn't additionally reduce oxidative damage, but does add the benefit of reduced blood pressure. Further studies looking at oxidant and anti-oxidant enzymes will perhaps provide clarification.

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