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Aldosterone does not mediate angiotensin II-induced atherosclerosis and abdominal aortic aneurysms

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1 We have demonstrated previously that infusion of angiotensin II (AngII) into hyperlipidemic mice augments atherosclerosis and results in the formation of abdominal aortic aneurysms (AAA). The purpose of this study was to determine the role of aldosterone in these AngII-induced vascular pathologies.

2 Male apolipoprotein E−/− (apoE) mice were infused with either vehicle or aldosterone (50 or 200 ng kg^{-1} min^{-1}). Arterial blood pressure was determined throughout the study and serum lipid concentrations and vascular pathology were quantified after 28 days of infusion.

3 Infusion of aldosterone did not influence body weight or serum cholesterol concentrations. Kidney weight was increased dose-dependently by aldosterone infusion. Systolic blood pressure was not significantly altered by aldosterone. Plasma aldosterone concentrations were increased dose-dependently by infusion of aldosterone. However, there was no effect of aldosterone on the extent of atherosclerosis and AAAs were not formed.

4 Implantation of pellets containing spironolactone (16 mg kg^{-1} day^{-1}) in AngII-infused apoE−/− mice (1000 ng kg^{-1} min^{-1}) had no effect on AngII-induced elevations in blood pressure. Plasma aldosterone concentration was not influenced by coadministration of spironolactone with AngII. Spironolactone administration did not influence the extent of atherosclerosis. Moreover, spironolactone had no significant effect on AngII-induced AAA (incidence of AAA formation: 80 *versus* 70% for vehicle *versus* spironolactone, respectively; not significant).

5 These studies demonstrate that the AngII-induced vascular pathologies of atherosclerosis and AAA formation are not mediated through aldosterone.
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Abbreviations: AAA, abdominal aortic aneurysms; AngII, angiotensin II; apoE, apolipoprotein E

Introduction

We have demonstrated previously that infusion of angiotensin II (AngII) into hyperlipidemic mice augments the development of atherosclerotic lesions and promotes the formation of abdominal aortic aneurysms (AAA) (Daugherty & Cassis, 1999; Daugherty *et al*., 2000; Manning *et al*., 2002). The AngII augmentation of atherosclerosis is associated with infiltration of macrophages, while formation of AAA is due to medial dissection followed by inflammation and remodeling (Daugherty *et al*., 2000; Saraff *et al*., 2003). The formation of AngII-induced AAAs was attenuated by the coinfusion of losartan, inferring AT1 receptor mediated effects (Daugherty *et al*., 2001).

Elaboration of aldosterone from the adrenal cortex is a well-characterized response that is mediated *via* AT1 receptors. Several of the effects of AngII infusion in rats and mice have been attributed to aldosterone, including vascular inflammation in the rat heart (Rocha *et al*., 2002), cardiac injury (Dechend *et al*., 2001), and vascular changes associated with oxidative stress (Diep *et al*., 2002). Infusion of aldosterone into uninephrectomized rats receiving 1% sodium chloride resulted in vascular inflammation in the heart (Rocha *et al*., 2002). Thus, it is conceivable that aldosterone contributes to the marked vascular inflammation that is promoted by AngII infusion into hyperlipidemic mice (Daugherty *et al*., 2000; 2001).

In this study, we hypothesized that elaboration of aldosterone during AngII infusion contributes to the development of atherosclerosis and AAA formation. To test this hypothesis, we determined if aldosterone infusion mimicked the effects of AngII on these vascular pathologies. In addition, the role of aldosterone in AngII-infused hyperlipidemic mice was determined by coadministration of spironolactone, an aldosterone receptor antagonist.

Methods

Mice and drug administration

Male apolipoprotein E−/− (apoE) mice (8 weeks old) (backcrossed 10× into a C57BL/6J background) were generated

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from an in-house breeding stock that was originally obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). All mice were maintained in a barrier facility and fed a normal laboratory diet *ad libitum*. For infusion of aldosterone, vehicle (9% ethanol, 87% propylene glycol, 4% water; $n=7$) or aldosterone (50 ($n=7$) or 200 ($n=13$) ng kg^{-1} min^{-1}, Sigma, St Louis, MO, U.S.A.) were administered subcutaneously *via* Alzet osmotic minipumps (Model 2004) for 28 days. To determine the role of aldosterone in AngII-infused vascular pathologies (Daugherty & Cassis, 1999; Daugherty *et al*., 2000), male apoE−/− mice (2 months of age) were implanted in the subscapular region with pellets containing either vehicle ($n=10$) or spironolactone (25 mg pellet, subcutaneous delivery of 16 mg kg^{-1} day^{-1}; Innovative Research of America, Sarasota, FL, U.S.A.; $n=10$). This dose of spironolactone has been previously demonstrated to exhibit significant blockade of the mineralocorticoid receptor in rats and mice (de Gasparo *et al*., 1987; Beggah *et al*., 2002; Virdis *et al*., 2002; Michel *et al*., 2004). After 1 week, mice were infused with AngII (1000 ng kg^{-1} min^{-1}) using an Alzet minipump for 28 days.

Measurement of serum components

On the final day of each study, blood was removed from anesthetized mice by ventricular puncture for measurement of cholesterol in sera (10 μl) and aldosterone (50 μl) in plasma. Serum cholesterol concentrations were determined by a commercially available enzymatic assay kit (Wako Chemicals, Richmond, VA, U.S.A.). Aldosterone concentration in plasma was determined with a commercial kit (Diagnostic Systems Laboratories Inc., Webster, TX, U.S.A.).

Blood pressure measurements

Systolic blood pressure was measured on conscious, restrained mice using the Visitech tail cuff system (BP-2000 Visitech Systems). Blood pressure was measured at the same time of day, 5 days a week on a 37°C heated stage. Mice were subjected to 10 preliminary and 10 recorded measurements, and a minimum of five measurements on each mouse were required for inclusion of data.

Quantification and characterization of atherosclerosis and AAA

Atherosclerosis was quantified on the aortic intima as described previously (Daugherty & Whitman, 2003). Aneurysms in the abdominal aorta were quantified by the percent incidence and the weight of the abdominal aorta.

Statistics

Data were analyzed with two-way ANOVA using SigmaStat. Data were tested for use of parametric or nonparametric *post hoc* analysis, as appropriate, and multiple comparisons were performed using Tukey's tests. $P<0.05$ values were considered to be statistically significant. All data are represented as means ± s.e.m.

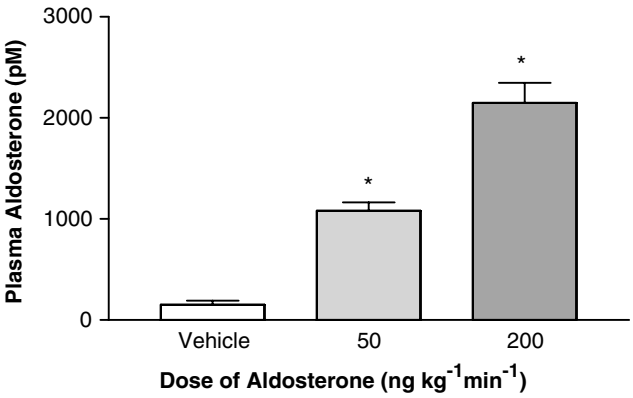

Figure 1 Effect of aldosterone infusion on plasma aldosterone concentrations in apoE−/− mice. Infusion of aldosterone (50 or 200 ng kg^{-1} min^{-1}) elevated plasma aldosterone concentrations 7- and 14-fold, respectively (vehicle, $n=7$; 50 ng kg^{-1} min^{-1}, $n=7$; 200 ng kg^{-1} min^{-1}, $n=13$; pumps implanted on day 0). Data are mean ± s.e.m. *Significantly different from vehicle ($P<0.001$).

Results

Infusion of aldosterone into apoE−/− mice dose-dependently increased plasma aldosterone concentration (Figure 1; $P<0.001$). There were no significant effects of aldosterone on body weight or serum cholesterol concentrations (Table 1). However, kidney weight increased dose-dependently in aldosterone-infused mice (Table 1; $P<0.001$). Systolic blood pressure was not significantly altered by infusion of aldosterone (Figure 2). The total area of the aortic arch was not influenced by infusion of aldosterone (Table 1). Atherosclerotic lesions were very small in 3-month-old apoE−/− mice (<1% lesion surface area) and were not altered by infusion of aldosterone (data not shown). Moreover, there were no observable aneurysms formed in the abdominal aorta of aldosterone-infused mice.

Systolic blood pressure increased in AngII-infused mice, and was not influenced by spironolactone (Figure 3). Plasma aldosterone concentrations were increased in AngII-infused mice (Figure 4) compared to vehicle-infused mice (Figure 1), and were similar to those induced by the low dose of aldosterone (50 ng kg^{-1} min^{-1}; Figure 1). Coadministration of spironolactone had no significant effect on plasma aldosterone concentrations (Figure 4). Administration of spironolactone to AngII-infused mice did not influence AngII-induced atherosclerosis (Figure 5). Infusion of AngII resulted in AAA formation in 80% of mice, which was not significantly influenced by spironolactone (Figure 6a). The weight of the abdominal aorta (an index of AAA severity) was also not significantly influenced by spironolactone (Figure 6b).

Discussion

A well-known physiological effect of AngII is the stimulation of synthesis and secretion of aldosterone from the adrenal cortex (Catt *et al*., 1987). In agreement with previous studies, infusion of AngII to hyperlipidemic mice resulted in the elaboration of aldosterone. However, blockade of the effects of aldosterone from coadministration of a mineralocorticoid receptor antagonist had no effect on AngII-induced vascular

Table 1 Characteristics of saline- and aldosterone-infused mice

Infusion	n	*Body weight* (g)	*Kidney weight* (g)	*Serum cholesterol* (mmol l^{-1})	*Aortic arch area* (μm)
Vehicle	7	26.6±1.1	0.37±0.03	8.0±0.5	15.28±0.32
Aldosterone 50 ng kg^{-1} min^{-1}	7	26.1±0.9	0.44±0.01*	7.3±0.3	15.08±0.50
Aldosterone 200 ng kg^{-1} min^{-1}	13	27.8±0.7	0.51±0.02*	7.2±0.4	14.27±0.88

*Significantly different from saline, $P<0.001$.

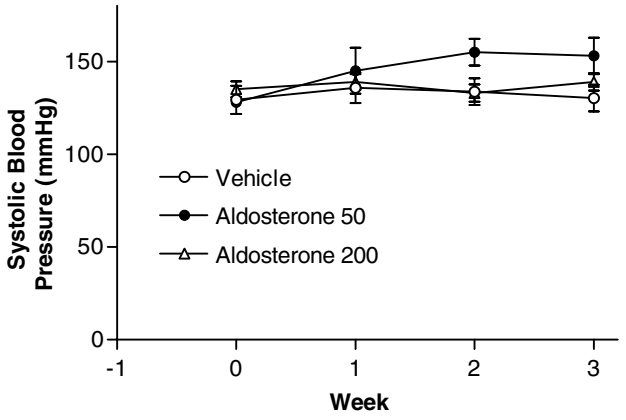

Figure 2 Effect of aldosterone infusion on systolic blood pressure in apoE−/− mice. Infusion of aldosterone (50 ($n=7$) or 200 ($n=13$) ng kg^{-1} min^{-1}, begun on day 0; vehicle, $n=7$) did not alter systolic blood pressure. Data are mean±s.e.m. of weekly measurements.

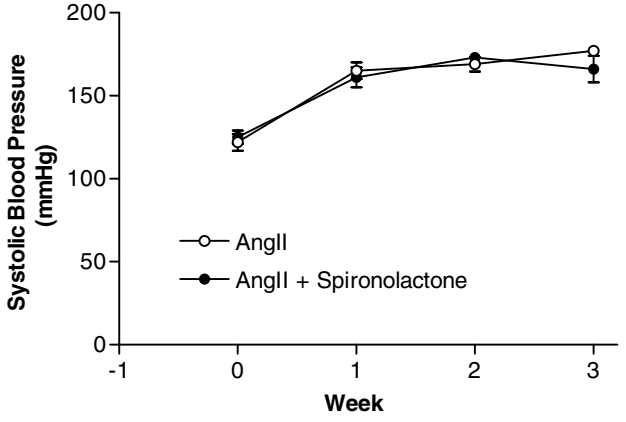

Figure 3 Effect of spironolactone on systolic blood pressure in AngII-infused apoE−/− mice. Mice were implanted with pellets containing spironolactone 1 week prior to minipump implantation (saline or AngII, 1000 ng kg^{-1} min^{-1}; day 0). Infusion of AngII increased blood pressure to the same extent in mice administered spironolactone (AngII, $n=8$; AngII/spironolactone, $n=10$). Data are mean±s.e.m. of weekly measurements.

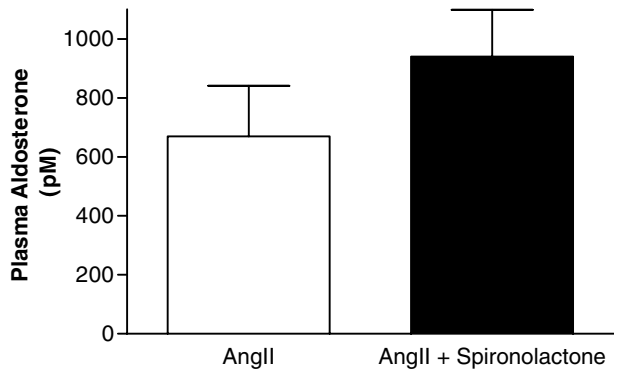

Figure 4 Effects of spironolactone on plasma aldosterone concentrations in AngII-infused apoE−/− mice. Mice were implanted with pellets containing spironolactone 1 week prior to minipump implantation (saline or AngII, 1000 ng kg^{-1} min^{-1}; day 0). Plasma aldosterone concentrations were not influenced by the administration of spironolactone (AngII, $n=8$; AngII/spironolactone, $n=10$). Data are mean±s.e.m.

Figure 5 Effect of spironolactone on the development of atherosclerosis in apoE−/− mice infused with AngII. Mice were implanted with pellets containing spironolactone 1 week prior to minipump implantation (saline or AngII, 1000 ng kg^{-1} min^{-1}; day 0). The extent of atherosclerosis was not influenced by the administration of spironolactone (AngII, $n=8$; AngII/spironolactone, $n=10$). Data are mean±s.e.m.

disease. Moreover, infusion of aldosterone to hyperlipidemic mice at a dose that elevated the systemic aldosterone concentration to a level induced by AngII did not influence atherosclerosis, or result in AAA pathology. Thus, infusion of aldosterone did not mimic the effects of AngII infusion. These results demonstrate that despite elaboration of aldosterone in AngII-infused hyperlipidemic mice, aldosterone does not contribute to the vascular pathologies from AngII infusion in this model.

For more than 50 years it has been known that the adrenal hormone aldosterone is stimulated by AngII, and that aldosterone regulates the transport of sodium and potassium in kidney epithelial cells by binding to the cytoplasmic mineralocorticoid receptor (Brown, 2003). More recently, several lines of evidence suggest that aldosterone interacts directly with the vasculature, including localization of the mineralocorticoid receptor on cells of the vascular wall (Hatakeyama *et al*., 1994; Takeda *et al*., 1996; 1997). In addition, aldosterone can be synthesized by vascular cells,

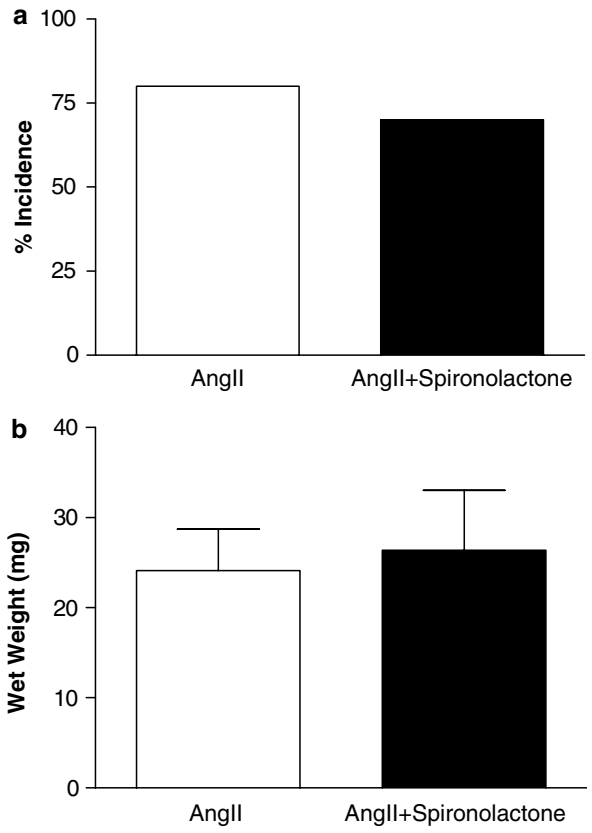

Figure 6 Effect of spironolactone on AAA incidence (a) and abdominal aorta tissue weight (b) in AngII-infused apoE−/− mice. Mice were implanted with pellets containing spironolactone 1 week prior to minipump implantation (saline or AngII, 1000 ng kg^{-1} min^{-1}; day 0). (a) AngII resulted in AAA formation in 80% of the mice. Spironolactone had no effect on AAA incidence (AngII, $n=8$; AngII/spironolactone, $n=10$). (b) Abdominal aorta tissue weight as an index of AAA severity. Data are mean ± s.e.m.

including endothelial and vascular smooth muscle cells (Bunnemann *et al*., 1993; Takeda *et al*., 1995; Rudolph *et al*., 2000). Cardiac and vascular inflammatory effects of aldosterone were demonstrated when uninephrectomized rats were infused with aldosterone and given 1% sodium chloride in their drinking water (Rocha *et al*., 2002; Sun *et al*., 2002; Takeda *et al*., 2002). In the coronary vasculature, inflammatory lesions from aldosterone infusion were preceded by elevations in cyclooxygenase-2, macrophage chemoattractant protein-1, and osteopontin (Rocha *et al*., 2002). Importantly, these vascular inflammatory effects of aldosterone were linked to AngII-induced hypertension, with inhibition of vascular inflammation from AngII infusion in rats treated with eplerenone (Rocha *et al*., 2002). For these reasons, we hypothesized that aldosterone contributes to the inflammatory vascular pathologies of atherosclerosis and AAA formation elicited by the infusion of AngII to hyperlipidemic mice (Daugherty *et al*., 2000; 2001).

In this study, we found that AngII infusion increases the plasma aldosterone concentrations in hyperlipidemic mice, demonstrating the well-known effect of AngII to stimulate aldosterone. However, our results demonstrate that elevations in aldosterone from AngII infusion do not contribute to atherosclerosis and AAA formation in hyperlipidemic mice. First, infusion of aldosterone in this study at a dose that elevated plasma aldosterone to levels induced by AngII did not mimic the pathologies elicited by AngII. Further, a higher dose of aldosterone that resulted in a marked increase (14-fold) in circulating aldosterone concentrations did not appreciably alter atherosclerosis or cause aneurysm-like pathology. In contrast, using the same experimental design, a 1 month infusion of AngII resulted in a striking effect to augment atherosclerosis and cause aneurysm formation in 90% of AngII-infused mice. Thus, elevating circulating aldosterone to levels induced by AngII did not reproduce the vascular pathologies of AngII infusion, suggesting that aldosterone is not the primary mediator of these AngII-induced pathologies.

While a variety of investigators have infused aldosterone into rats (Rocha *et al*., 1999; 2002; Virdis *et al*., 2002), relatively few studies have examined infusion of aldosterone to mice (Grubb & Boucher, 1997; Keidar *et al*., 2004; Wang *et al*., 2004). At aldosterone infusion doses (1 μg g^{-1} day^{-1}) in normolipidemic mice far in excess of those used in this study (200 ng kg^{-1} min^{-1}), the plasma aldosterone concentration increased 55-fold; however, no vascular pathologies were noted and blood pressure was not determined (Grubb & Boucher, 1997). Aldosterone has been recently reported to constrict the preglomerular afferent arteriole in the rabbit kidney, an effect that was attenuated by endothelial-derived nitric oxide (Arima *et al*., 2004). The lack of effect of aldosterone on blood pressure in the present study may have resulted from buffering of aldosterone-induced vasoconstriction by endothelial-derived nitric oxide.

Recent studies demonstrate that chronic hyperaldosteronism fails to induce cardiac remodeling and fibrosis, suggesting a requirement for high salt in the effects from excess aldosterone (Wang *et al*., 2004). It is conceivable that infusion of aldosterone to mice on a high salt diet may have resulted in vascular pathologies similar to those elicited by AngII. However, results from this study and previous findings in our laboratory demonstrate that the vascular pathologies of augmented atherosclerosis and AAA formation from AngII infusion occur without the need for salt administration.

Relatively few studies have examined the effect of aldosterone in experimental models of atherosclerosis. In New Zealand rabbits placed on a 1% cholesterol diet, the mineralocorticoid receptor antagonist eplerenone improved endothelial function, suggesting that aldosterone has potential proatherosclerotic effects (Rajagopalan *et al*., 2002). Using the same approach of antagonism of the mineralocorticoid receptor with chronic eplerenone treatment (12 weeks) in apoE−/− mice, results demonstrated a 35% reduction in atherosclerosis (Keidar *et al*., 2004). Recent studies demonstrated a modest (33%) but significant increase in atherosclerotic lesion area in the aortic root with chronic infusion of aldosterone to apoE−/− mice (Keidar *et al*., 2004). While the focus of our studies was to determine the role of aldosterone in the AngII model of augmented atherosclerosis and AAA formation, it is recognized that aldosterone may play a more extended role in the development of atherosclerosis.

To determine directly whether aldosterone contributes to AngII-induced atherosclerosis or AAA formation, we administered the non selective mineralocorticoid receptor antagonist, spironolactone, to AngII-infused apoE−/− mice. The dose and route for spironolactone administration used in this study have been demonstrated previously to exert effective mineralocorticoid blockade (de Gasparo *et al*., 1987; Rocha

et al., 1998; Abdallah *et al*., 2001; Dorrance *et al*., 2001; Beggah *et al*., 2002; Virdis *et al*., 2002; Griffin *et al*., 2003; Michel *et al*., 2004). The lack of effect of spironolactone on blood pressure in AngII-infused mice in this study is consistent with previous results obtained administering similar spironolactone doses to stroke-prone SHR (Rocha *et al*., 1998). Moreover, a similar dose and route of spironolactone administration has been demonstrated to effectively block AngII-induced vascular changes and oxidative stress in rats (Diep *et al*., 2002). Finally, recent studies in mice administered $20\,mg\,kg^{-1}\,day^{-1}$ of spironolactone demonstrate effective mineralocorticoid receptor antagonism (Beggah *et al*., 2002; Michel *et al*., 2004).

While several studies demonstrate effective mineralocorticoid blockade using a similar dose and route of spironolactone administration, spironolactone does exhibit nonspecific effects. For example, spironolactone exhibits some degree of antagonist effects at the androgen receptor. Recent studies from our laboratory demonstrate that castration of male hyperlipidemic mice markedly reduces AngII-induced AAA (Henriques *et al*., 2004). Thus, if spironolactone was exhibiting antagonist properties at the androgen receptor, we would have anticipated reductions in the incidence of AngII-induced AAA. In summary, based on prior literature for spironolactone use in rodents and our knowledge of androgen receptor effects in AngII-induced AAA, the dose of spironolactone administered in this study should have provided effective and relatively specific mineralocorticoid receptor blockade, but did not influence AngII-induced atherosclerosis or AAA formation. Future studies should determine the effect of eplerenone, a specific aldosterone receptor antagonist, on AngII-induced atherosclerosis and AAA formation.

In conclusion, despite elevations in circulating aldosterone from AngII infusion, blockade of the mineralocorticoid receptor did not influence the AngII-induced vascular pathologies of atherosclerosis or AAA formation. Moreover, infusion of aldosterone at doses that mimic AngII-induced increases in systemic aldosterone concentrations did not reproduce the pathologies elicited by AngII. These results demonstrate that aldosterone does not contribute significantly to AngII-induced atherosclerosis or AAA formation in hyperlipidemic mice.

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