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RESEARCH PAPER

Comparative effects of different modes of renin angiotensin system inhibition on hypercholesterolaemia-induced atherosclerosis

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BACKGROUND AND PURPOSE

Inhibition of the renin angiotensin system (RAS) has been consistently demonstrated to reduce atherosclerosis. However, there has been no direct comparison among the three available pharmacological modes of inhibiting the RAS, which are inhibitors of renin, ACE and angiotensin II type 1 receptor. The purpose of this study was to determine the relative effects of these three modes of pharmacological RAS inhibition in reducing atherosclerosis by determining the dose–response relationships.

EXPERIMENTAL APPROACH

Male LDL receptor -/- mice were administered either vehicle or any of three doses of aliskiren, enalapril or losartan through s.c. infusion for 12 weeks. All mice were fed a saturated fat-enriched diet during drug infusions. Systolic and diastolic BPs were measured during the study using a non-invasive tail-cuff system. Plasma cholesterol and renin concentrations, atherosclerotic lesions, and renal angiotensin II concentrations were determined at the termination of the study.

KEY RESULTS

Plasma renin concentrations were increased by all three drugs. None of the drugs changed plasma cholesterol concentrations. All drugs produced a dose-related decrease in BP. All three drugs also profoundly reduced atherosclerosis in a dose-dependent manner. The highest dose of each drug markedly attenuated lesion size, with no significant differences between the different drugs. The highest dose of each drug also similarly reduced renal angiotensin II concentrations.

CONCLUSION AND IMPLICATIONS

Drugs that inhibit the RAS, irrespective of their mode of inhibition, profoundly affect atherosclerotic lesion development in a dose-dependent manner.

Abbreviations

ACE, angiotensin-converting enzyme; Ang, angiotensin; apoE, apolipoprotein E; AT1, angiotensin II type 1 receptor; BP, blood pressure; LDL, low-density lipoprotein; PCR, polymerase chain reaction; RAS, renin angiotensin system

Introduction

The renin angiotensin system (RAS) contains a single precursor, angiotensinogen, that is cleaved by renin to form angiotensin (Ang)I. Ang I is subsequently cleaved by angiotensin-converting enzyme (ACE) to generate Ang II, the major bioactive peptide in the RAS. Renin and ACE are the critical enzymes for the synthesis of Ang II, while the Ang II type 1

(AT_1) receptor is the major receptor for the physiological and pathophysiological effects of Ang II. Over the last decade, the classic RAS has been expanded by the identification of alternative enzymes to synthesize or catabolize Ang II, a spectrum of other bioactive angiotensin peptides, and an increasing number of receptors that have been recognized to interact with these angiotensin peptides (Ferrario, 2002; Carey and Siragy, 2003; Santos *et al.*, 2003). Therefore, the RAS is a complex system exerting an array of responses, some being antagonistic to others.

Despite the complexity of the RAS, it has been consistently demonstrated that Ang II contributes to the development of atherosclerosis (Daugherty and Cassis, 1999; Daugherty *et al.*, 2000; Weiss *et al.*, 2001; Bruemmer *et al.*, 2003). Conversely, pharmacological inhibition of the classic RAS components decreases experimental atherosclerosis (Rader and Daugherty, 2008). Renin is the rate-limiting enzyme in the generation of angiotensin peptides. Previous studies have demonstrated that aliskiren, a renin inhibitor, blocks the generation of all angiotensin peptides (Lu *et al.*, 2008) and reduces atherosclerosis in animal models (Imanishi *et al.*, 2008; Lu *et al.*, 2008; Nussberger *et al.*, 2008; Weiss and Taylor, 2008). Studies have also consistently demonstrated that ACE or AT_1 receptor inhibition profoundly reduces atherosclerosis in a variety of animal models (Daugherty *et al.*, 2001; Candido *et al.*, 2002; 2004; Wassmann *et al.*, 2004; da Cunha *et al.*, 2005; Grothusen *et al.*, 2005). However, the mechanisms by which ACE inhibition or AT_1 receptor antagonism reduces atherosclerosis may be complex. For example, inhibition of ACE results in the accumulation of Ang I, which can thus be converted into Ang (1-7) through an ACE2-dependent pathway. In addition, ACE inhibitors also block the degradation of bradykinin, which has potent vasodilator properties. As to AT_1 receptor antagonists, although the direct inhibition of Ang II AT_1 receptor signalling could account for its anti-atherosclerotic effect, there may also be a contribution from the continuous presence of angiotensin peptides that interact with other receptors such as AT_2 or mas receptors. Therefore, inhibition of different sites within the RAS may reduce atherosclerosis through distinct mechanisms, indicating differences in anti-atherosclerotic effects depending on the mode of RAS inhibition.

Despite the consistent demonstration that inhibition of the RAS profoundly reduces atherosclerosis (Daugherty *et al.*, 2001; Candido *et al.*, 2002; 2004; Wassmann *et al.*, 2004; da Cunha *et al.*, 2005; Imanishi *et al.*, 2008; Lu *et al.*, 2008; Nussberger *et al.*, 2008; Rader and Daugherty, 2008; Weiss and Taylor, 2008), no studies have determined the maximal effect or directly compared the relative anti-atherosclerotic effects of the three different modes for pharmacological inhibition of the RAS. Hypercholesterolaemia is critical for the development of atherosclerosis (Rader and Daugherty, 2008). RAS activation is a critical contributor to hypercholesterolaemia-induced atherosclerosis in mice as demonstrated in our previous studies (Daugherty *et al.*, 2004; Lu *et al.*, 2008). In this study, we used LDL receptor -/- male mice fed a saturated fat-enriched diet to determine the dose-related response of the three modes of pharmacological inhibition of the RAS on the reduction of atherosclerosis.

Methods

Mice and diet

LDL receptor -/- (B6.129S7-Ldlr^{tm1Her}; Stock #002207) male mice that have been backcrossed ten times into the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained in a barrier facility and fed with a normal mouse laboratory diet. To induce hypercholesterolaemia, mice were fed with a diet supplemented with saturated fat (milk fat 21% wt/wt) and cholesterol (0.2% wt/wt; Diet# TD.88137; Harlan Teklad, WI, USA). At the end of the experiment, the mice were anaesthetized by an i.p. injection of ketamine (100 $mg \cdot kg^{-1}$) and xylazine (10 $mg \cdot kg^{-1}$). Blood was collected with EDTA (1.8 $mg \cdot mL^{-1}$) via right ventricular puncture, kept on ice, centrifuged (376 $g \times 20$ min) at 4°C and stored at –80°C. After collection of blood, the right atrium was incised, and saline was infused through a left ventricular puncture to remove blood. Aortas were removed and kept in 10% formalin, while other organs such as livers and kidneys were snap- frozen in liquid nitrogen and stored at –80°C. All animal care and experimental procedures were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Drug administration

Model 2004 Alzet mini-osmotic pumps (Durect Corporation, Cupertino, CA, USA) were implanted into LDL receptor -/- male mice at the age of 8 weeks, and replaced every 4 weeks to continuously deliver drugs for a total of 12 weeks (Daugherty and Cassis, 1999; Daugherty *et al.*, 2000). Ten groups of mice ($n = 15$ per group) were studied as follows: vehicle (PBS); aliskiren 2.5, 12.5 or 25 $mg \cdot kg^{-1} \cdot day^{-1}$; enalapril 0.25, 1.25 or 2.5 $mg \cdot kg^{-1} \cdot day^{-1}$; and losartan 2.5, 12.5 or 25 $mg \cdot kg^{-1} \cdot day^{-1}$. Doses of each drug were chosen based on estimates that would encompass a range of partial to complete inhibition of their respective targets (Daugherty *et al.*, 2001; Lu *et al.*, 2008). Aliskiren was provided by Novartis. Enalapril (Cat# E6888) and losartan (Cat# 61188) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All study mice were fed the saturated fat-enriched diet, which was started 1 day after the pump implantation throughout the drug infusions.

BP measurements

Systolic and diastolic BPs were measured using a non-invasive tail-cuff system (Coda 8; Kent Scientific Corporation, Torrington, CT, USA) (Daugherty *et al.*, 2009). The measurements were performed for four sequential days prior to and at every 4 weeks during drug infusions.

Measurement of plasma components

Plasma cholesterol concentrations and lipoprotein–cholesterol distributions were determined as described previously (Daugherty *et al.*, 2000). Plasma renin concentrations were measured by incubation of plasma samples (8 μL) with an excess of rat angiotensinogen in the presence of EDTA (0.02 M) for 30 min at 37°C. Ang I generated in the samples was quantified by radioimmunoassay using a commercially available kit (Cat# 1553; DiaSorin, MN, USA) (Lu *et al.*, 2008).

Quantification of atherosclerosis

Atherosclerosis was quantified on aortic intima of arches, thoracic aortas and abdominal aortas by an *en face* method as described previously (Daugherty and Whitman, 2003; Daugherty and Rateri, 2005). Lesion size was measured in the aortic arch region that included ascending aorta, arch and part of descending aorta (from the aortic orifice of left subclavian artery to 3 mm below), the thoracic aortic region that was defined as from the end of the aortic arch region to the last intercostal arteries, and the abdominal aortic region from the last intercostal arteries to the aortic bifurcation.

Characterization of atherosclerotic tissues

Serial cross sections in aortic roots were cut on a cryostat as described previously (Daugherty and Whitman, 2003). Oil Red O staining was performed to visualize lipid-laden macrophages and collagen was stained using Gomori Trichrome. Immunostaining of smooth muscle alpha actin was performed using a rabbit polyclonal antibody (Cat# ab5694; Abcam, Cambridge, MA, USA) as described previously (Lu *et al.*, 2007b).

Kidney Ang II measurements

Kidney samples (n = 5 per group) from the study mice were weighed and homogenized in 10 volumes of ice-cold buffer containing HCl (0.1N), ethanol (80%), o-phenanthroline (0.5 mM), pepstatin (0.1 mM) and captopril (10 μM). Homogenates were centrifuged at 20 000× g for 20 min at 4°C. The supernatant was stored at –20°C for 12 h, centrifuged and diluted (1:1) with orthophosphoric acid (0.1%). Samples were stored at 4°C for 6 h, centrifuged, and the supernatant diluted (1:1) with orthophosphoric acid (0.02%). Angiotensin peptides were partially purified using C18 mini-columns equilibrated with methanol (4 mL) and water (8 mL). Samples were applied to columns using gentle pressure, columns were washed twice with water (4 mL), and peptides were eluted with methanol (3 mL). Eluate was vacuum evaporated and reconstituted in the buffer for radioimmunoassay using a rabbit anti-AngII antibody (Cat# T-4005; Bachem/Peninsula Laboratories, San Carlos, CA, USA).

Statistical analyses

Version 9.2 of SAS (SAS Institute Inc., Cary, NC, USA) and version 11 of SigmaPlot (Systat Software Inc., San Jose, CA, USA) were used for statistical analyses. A $P < 0.05$ was considered significant except as noted below. To compare study groups on continuous responses assessed once on each specimen, we employed one-way ANOVA followed by pairwise comparisons with P-values adjusted by the Tukey–Kramer method; observations were weighted, and when necessary, square-root transformed to justify application of one-way ANOVA. To determine the association between two continuous responses assessed once, we computed a Pearson correlation. We also estimated a standardized coefficient for a linear regression model relating the two variables while controlling for group membership. To compare groups on continuous responses assessed repeatedly, we fit a linear mixed model expressing the mean response as a function of group membership and time; because the software did not adjust the P-values in pairwise comparisons, we required a P-value < 0.01 to declare statistical significance. Data are presented as mean ± SEM.

Results

Characteristics of study mice

All doses of the three drugs were well-tolerated as determined by daily visual inspection and steadily body weight gain (Table 1). Plasma cholesterol concentrations (Table 1) and lipoprotein–cholesterol distributions (data not shown) were not influenced by any dose or mode of the RAS inhibition compared with the vehicle. While all doses of enalapril and losartan increased plasma renin concentrations, only the highest dose of aliskiren increased plasma renin concentra-

Table 1

Characteristics of mice

Infusion	Dose ($mg \cdot kg^{-1} \cdot day^{-1}$)	Body weight (g) Baseline	Final	Plasma cholesterol concentrations ($mg \cdot mL^{-1}$)
Vehicle		24.2 ± 0.5	34.1 ± 1.1	15.8 ± 1.1
Aliskiren	2.5	24.6 ± 0.5	37.0 ± 1.0	15.9 ± 0.7
	12.5	24.3 ± 0.4	33.9 ± 0.5	16.9 ± 0.8
	25	24.1 ± 0.5	33.4 ± 0.9	15.4 ± 0.9
Enalapril	0.25	23.7 ± 0.3	36.1 ± 0.9	17.2 ± 0.8
	1.25	23.6 ± 0.4	36.3 ± 0.9	16.1 ± 0.5
	2.5	24.2 ± 0.5	30.8 ± 1.5	15.8 ± 0.6
Losartan	2.5	24.3 ± 0.5	38.4 ± 1.0	16.4 ± 0.7
	12.5	24.7 ± 0.5	36.2 ± 1.1	15.8 ± 0.8
	25	24.7 ± 0.4	34.3 ± 1.0	15.9 ± 1.3

Values are presented as mean ± SEM. Comparisons of body weight and plasma cholesterol concentrations among the 10 study groups (n = 8–15 per group) were performed by one-way ANOVA.

tions (Figure 1). However, there was no significant difference in plasma renin concentrations among the three doses of aliskiren (Figure 1A). Both enalapril (Figure 1B) and losartan (Figure 1C) dose-dependently increased plasma renin concentrations. The magnitude of change in plasma renin concentrations was equivalent in mice infused with the highest doses of enalapril and losartan.

Comparison of three modes of pharmacological RAS inhibition on systolic and diastolic BP reduction

Changes in systolic and diastolic BP (at week 12 during drug infusion vs. baseline) were compared. Each dose of all three drugs produced dose-related decreases in both systolic and diastolic BPs (Figures 2 and 3). The highest doses of each drug reduced both systolic and diastolic BPs to a similar level.

Comparison of three modes of pharmacological RAS inhibition on atherosclerosis

Atherosclerotic lesions were quantified as % lesion area in aortic arches, thoracic aortas and abdominal aortas by *en face* measurement. LDL receptor -/- mice fed the saturated fat-enriched diet for 12 weeks developed readily discernable lesions in aortic arches, modestly sized lesions in thoracic aortas, and minimal lesions in abdominal aortas (data not shown). All three modes of pharmacological RAS inhibition profoundly reduced hypercholesterolaemia-induced atherosclerosis in a dose-dependent manner in both aortic arches (Figure 4 and Supporting Information Figure S1) and thoracic aortas (Supporting Information Figure S2). There were no differences in the maximal reductions in lesion size between the groups.

Oil Red O staining demonstrated the predominance of lipid-laden macrophages in atherosclerotic lesions. The presence of collagen was not readily distinguishable, whereas only sparse positive staining of smooth muscle actin was detected in atherosclerotic lesions (data not shown).

Correlation analysis between BP changes and lesion size

We performed Pearson correlation analyses to determine whether % lesion area in aortic arches was correlated with systolic or diastolic BP changes. The Pearson correlation of % lesion area with systolic BP was –0.421 ($P < 0.0001$), and with diastolic BP was –0.360 ($P = 0.0002$). However, if group membership was controlled by fitting a linear regression model with BP changes as the dependent variable, and both % lesion area and group membership as the independent variables, the estimated standardized regression coefficient for % lesion area with either systolic or diastolic BP was –0.093 ($P = 0.43$ and 0.45, respectively).

Comparisons of renal Ang II concentrations in study mice

We were unable to directly measure Ang II concentrations in aortas. Instead, we measured Ang II concentrations in kidney tissues from mice infused with either PBS or the highest dose of each drug. There was a significant but equivalent reduction

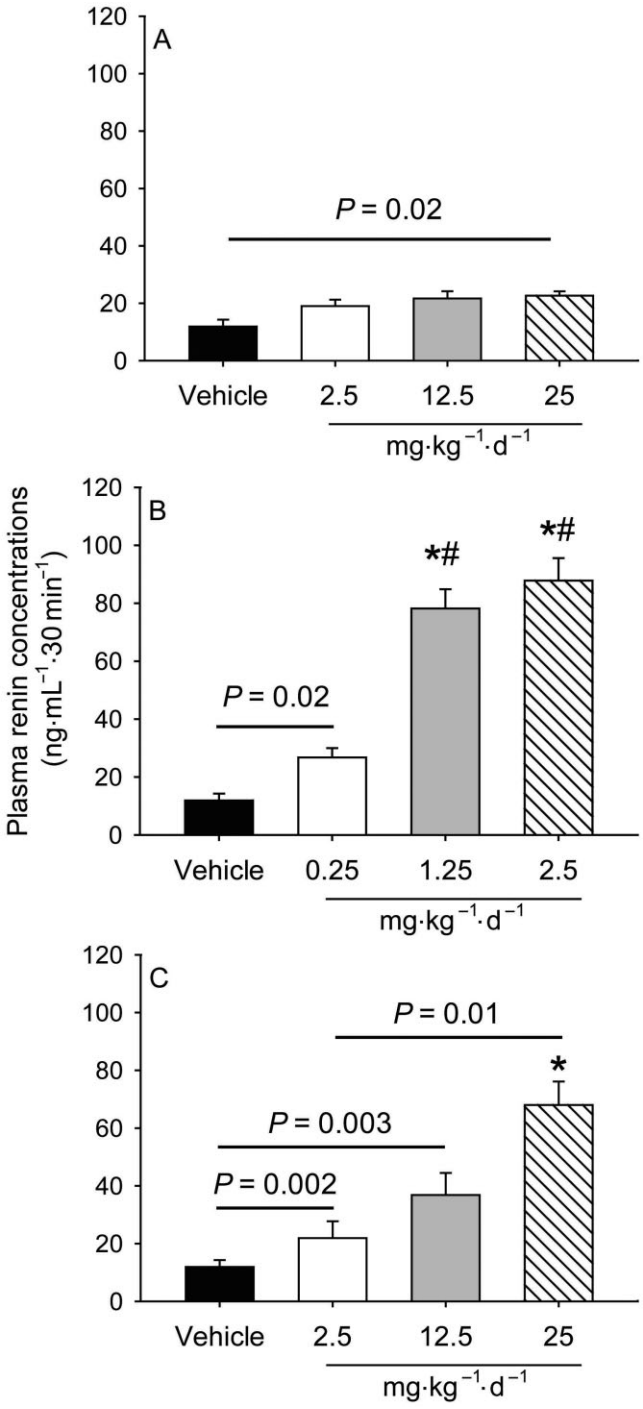

Figure 1

Comparison of different modes of RAS inhibition on changes in plasma renin concentrations. Plasma renin concentrations were measured using a radioimmunoassay kit ($n = 7$ per group). Histograms represent means and bars represent SEM. $^{*}P < 0.0001$ versus the vehicle, and $^{\#}P < 0.0001$ versus enalapril 0.25 mg·kg^{-1}·day^{-1}. (A) Effects of aliskiren; (B) enalapril and (C) losartan.

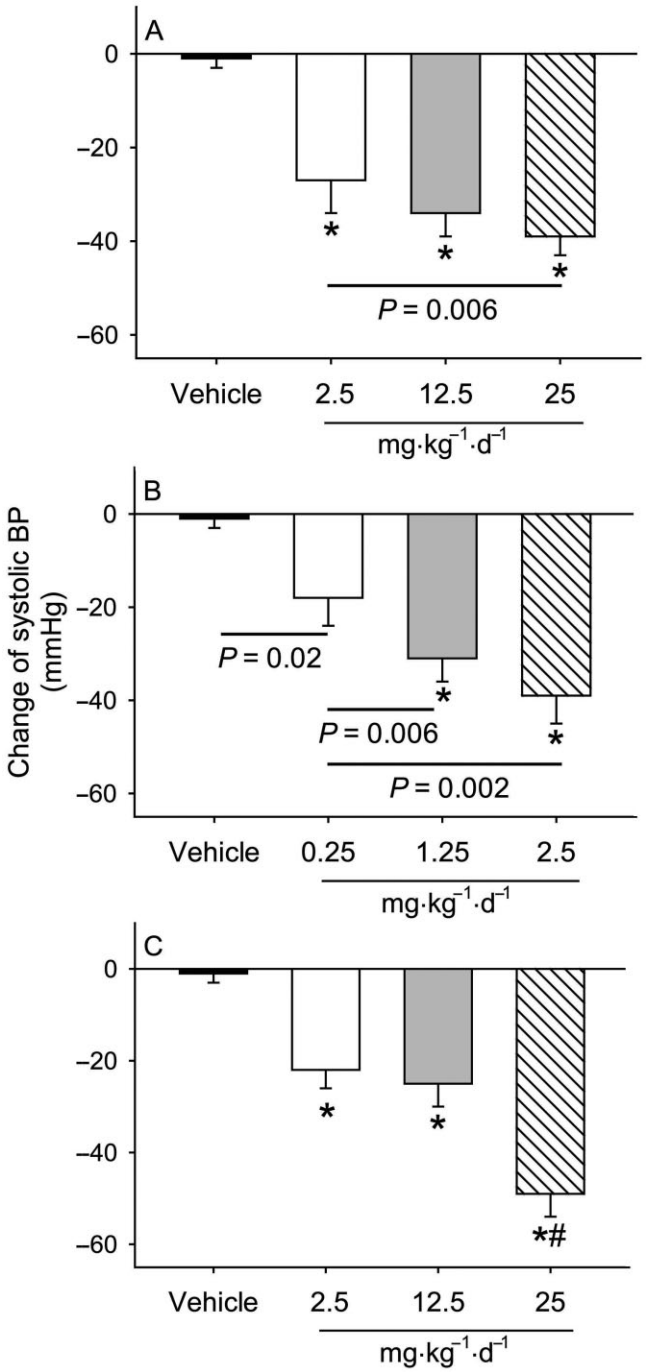

Figure 2

Comparison of different modes of RAS inhibition on changes in systolic BP. Changes in systolic BP were compared in LDL receptor -/- mice between 1 week before pump implantation (baseline) and at 12 weeks after drug administration at the indicated doses (n = 8–14 per group). Histograms represent means and bars represent SEM. *P < 0.0001 versus the vehicle, and $^{\#}P$ < 0.0001 versus the two lower doses of losartan (2.5 and 12.5 mg·kg^{-1}·day^{-1}). (A) Effects of aliskiren; (B) enalapril and (C) losartan.

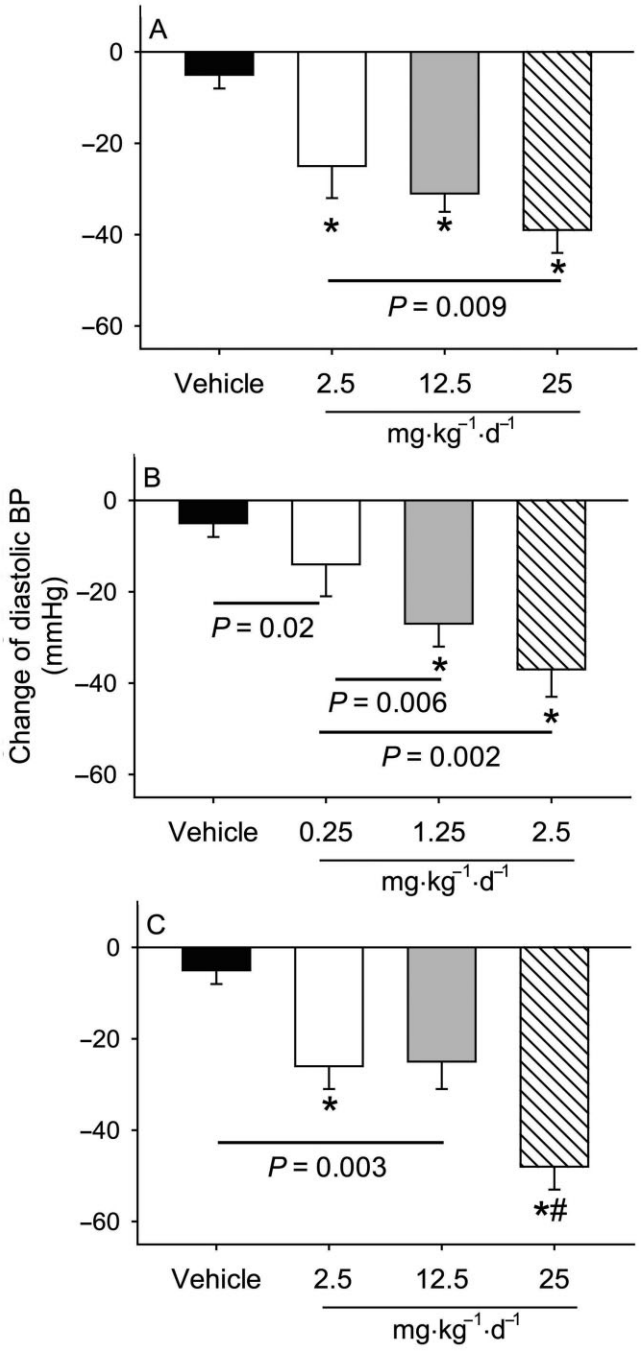

Figure 3

Comparison of different modes of RAS inhibition on changes of diastolic BP. Changes in diastolic BP were compared in LDL receptor -/- mice between 1 week before pump implantation (baseline) and at 12 weeks after drug administration at the indicated doses (n = 8–14 per group). Histograms represent means and bars represent SEM. *P < 0.0001 versus the vehicle, and $^{\#}P$ < 0.0001 versus the two lower doses of losartan (2.5 and 12.5 mg·kg^{-1}·day^{-1}). (A) Effects of aliskiren; (B) enalapril and (C) losartan.

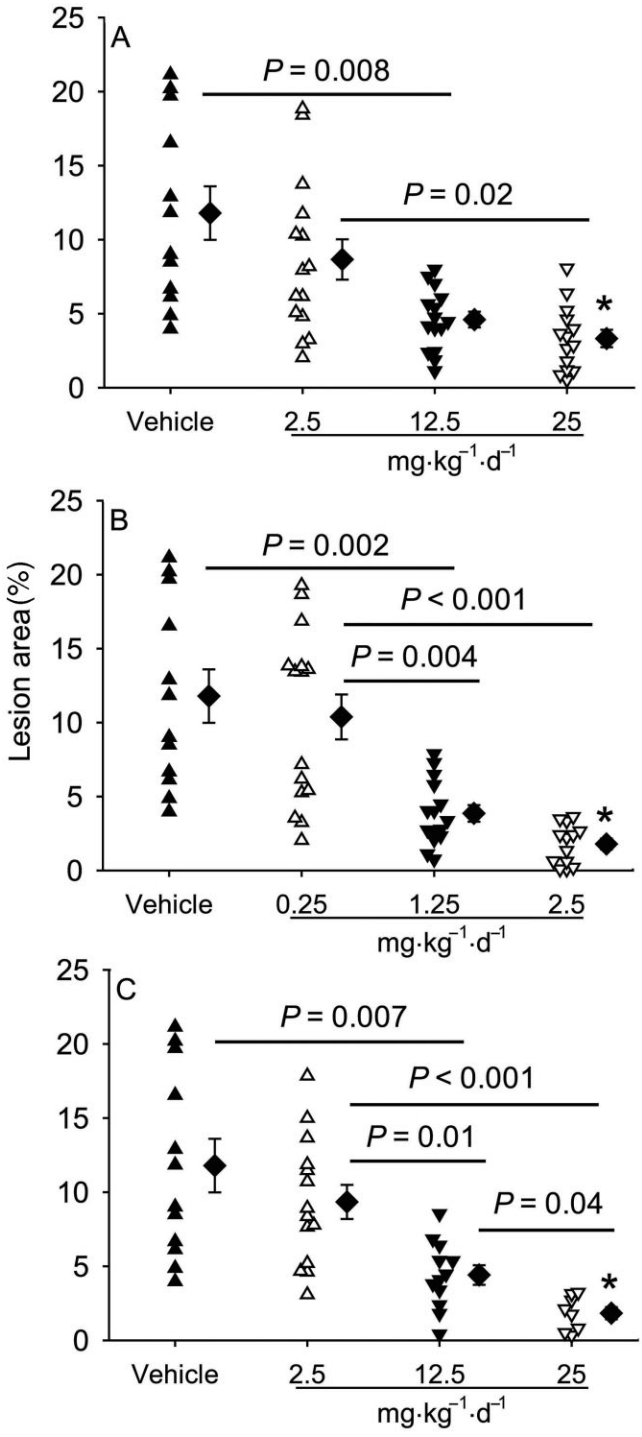

Figure 4

Comparison of different modes of RAS inhibition on atherosclerotic lesion size in aortic arches. The lesion area (as a % of whole area) was measured on the intimal surface of aortic arches (n = 8–14 per group). Triangles represent the values for individual mice, diamonds represent means, and bars are SEM. *P < 0.001 versus the vehicle. (A) Effects of aliskiren; (B) enalapril and (C) losartan.

in kidney Ang II concentrations in mice infused with the highest dose of each drug, compared with the PBS-infused control mice (Supporting Information Figure S3).

Discussion and conclusions

The present study demonstrated that inhibition of the RAS by drugs that operate through three distinct pharmacological modes reduces atherosclerotic lesion size in a dose-dependent manner in hypercholesterolaemic mice. The dose–response curves showed no marked differences in the relative anti-atherosclerotic effects for these three different modes of RAS inhibition. The RAS inhibition was achieved by s.c. infusion of the drugs through osmotic minipumps to ensure continuous and constant delivery during a 12 week period. To our knowledge, this is the first study to directly compare the three currently available pharmacological modes of inhibition of the RAS on atherosclerosis using multiple doses of each drug within a single study.

The effects of renin inhibition on atherosclerosis have been determined recently in a number of studies (Imanishi *et al.*, 2008; Lu *et al.*, 2008; Nussberger *et al.*, 2008; Weiss and Taylor, 2008; Poss *et al.*, 2010). Conversely, many studies have used experimental models to demonstrate the consistent anti-atherosclerotic effects of ACE inhibitors and AT_1 receptor antagonists (Aberg and Ferrer, 1990; Charpiot *et al.*, 1993; Schuh *et al.*, 1993; Strawn *et al.*, 2000; Johnstone *et al.*, 2004; Wassmann *et al.*, 2004; Weiss and Taylor, 2008). Although all three modes of inhibiting the RAS reduced lesion size, it is unclear whether there are differences in efficacy. Renin is the rate-limiting enzyme that acts on its unique substrate of angiotensinogen in the formation of angiotensin peptides. Unlike renin, ACE has both RAS-related and non-RAS-related substrates (Nishimoto *et al.*, 2001). In addition, ACE inhibition influences other substrates of ACE such as bradykinin, which also affects experimental atherosclerosis (Merino *et al.*, 2009). AT_1 receptor antagonism prevents the actions of Ang II by inhibiting its binding to the AT_1 receptor. However, it simultaneously increases plasma concentrations of Ang II (Muller *et al.*, 1997; Strawn *et al.*, 2000) and possibly other angiotensin peptides. Increased plasma Ang II concentrations during AT_1 receptor antagonism may stimulate AT_2 receptors; however, the role of AT_2 receptors in atherosclerosis is unclear (Daugherty *et al.*, 2001; 2004; Iwai *et al.*, 2005; Johansson *et al.*, 2005; Sales *et al.*, 2005; Hu *et al.*, 2008; Koitka *et al.*, 2010).

Given the different modes of inhibiting the RAS, it is possible that ACE and AT_1 receptor inhibition reduces atherosclerosis through different pathways from renin inhibition. There have been reports that ACE inhibition and AT_1 receptor antagonism have differential effects on atherosclerosis (Schuh *et al.*, 1993), while several other studies have demonstrated that single doses of drugs targeting different sites within the RAS produce comparable reductions in atherosclerotic lesion size (Ortlepp *et al.*, 2002; Imanishi *et al.*, 2008; Nussberger *et al.*, 2008; Weiss and Taylor, 2008). To directly compare the anti-atherosclerotic effects and thus assist in clarifying the conflicting findings in the literature, the present study, via applying multiple doses of each drug concurrently, provided

strong evidence that the three modes of pharmacological RAS inhibition similarly reduce atherosclerosis in mice.

While the reduction in intrarenal Ang II concentration, as measured in the present study, may represent the effectiveness of the three modes of RAS inhibition, it does not imitate the changes in Ang II production in aortas that may directly contribute to the development of atherosclerosis. Unfortunately, measurement of Ang II levels in mouse aortas is not technically feasible (Weiss and Taylor, 2008). We have demonstrated previously that angiotensinogen, renin and ACE are present in atherosclerotic lesions of LDL receptor -/- mice (Daugherty *et al.*, 2004). Angiotensinogen and renin are predominantly co-localized with lipid-laden macrophages, whereas ACE is present in all the major cell types in lesions. Although all these components of the RAS are present in atherosclerotic lesions, the contribution of the local expression of angiotensinogen or ACE to lesion formation has not been defined. Conversely, deletion of renin from bone marrow-derived cells decreases atherosclerosis (Lu *et al.*, 2008). Ang II exerts its effects on atherosclerosis by binding to AT_{1A} receptors (Daugherty *et al.*, 2004; Wassmann *et al.*, 2004). However, we did not find that AT_{1A} receptors on bone marrow-derived cells played a critical role in the development of hypercholesterolaemia-induced atherosclerosis (Lu *et al.*, 2008), although contradictory findings have been reported in different mouse models (Cassis *et al.*, 2007; Fukuda and Sata, 2008; Kato *et al.*, 2008; Koga *et al.*, 2008; Tsubakimoto *et al.*, 2009; Endtmann *et al.*, 2011).

It has been demonstrated consistently, in both human and animal studies, that plasma renin concentrations are increased by the three pharmacological approaches we used to inhibit the RAS (Wiggins and Kelly, 2009). While some human studies have shown that plasma renin concentrations were increased more profoundly in patients treated with aliskiren, compared with ACE inhibitors or AT_1 receptor antagonists (Nussberger *et al.*, 2002; 2007; Uresin *et al.*, 2007; Wiggins and Kelly, 2009), others did not find differences in plasma renin concentrations between patients administered aliskiren and ACE inhibitors or AT_1 receptor antagonists (Azizi *et al.*, 2004; Menard *et al.*, 2006). In this study, different magnitudes of plasma renin increases may reflect factors inherent within the mode of the assay and the method of blood collection in anaesthetized mice. For example, it has been reported that monoclonal antibodies used in some commercial renin assays may directly interact with renin inhibitors, and factors such as incubation duration may also affect the values from such renin assays (Menard *et al.*, 2006). In the present study, plasma renin concentrations were determined by measuring generation of Ang I from the addition of an excess of rodent angiotensinogen (Cassis *et al.*, 2004). The lower concentrations of plasma renin in mice infused with aliskiren might have resulted from residual aliskiren in the assay that inhibited the production of Ang I. In addition, it is well recognized that anaesthesia can increase plasma renin concentrations (Oates and Stokes, 1974). Therefore, the stated measurements of plasma renin may be overestimated.

While the end point of this study was atherosclerotic lesions, we also delineated the relative role of the different modes of pharmacological RAS inhibition on systolic and diastolic BPs. Consistent with their effects on atherosclerosis, all three modes of inhibiting the RAS also dose-dependently reduced BP. Pearson correlation analyses of lesion size with BP changes indicates that these two parameters represent two distinct manifestations, and both are similarly affected by the three modes of RAS inhibition. Furthermore, while these analyses infer an associative link between BP changes and lesion size, this does not provide a conclusive demonstration that changes in BP *per se* directly contribute to the mechanisms of atherosclerosis (Lu *et al.*, 2007a). The role of BP has been approached in several studies that have compared the anti-atherosclerotic effects of RAS inhibition to other classes of drugs that lower BP. These include comparisons between irbesartan and hydralazine and between aliskiren and hydralazine, which all produced comparable BP reductions in apoE -/- mice, but only aliskiren and irbesartan reduced atherosclerotic lesions (Wassmann *et al.*, 2004; Poss *et al.*, 2010). Similarly, AT_1 receptor antagonism by irbesartan or candesartan and calcium antagonism by amlodipine equivalently reduced BP, but only AT_1 receptor blockade markedly reduced atherosclerosis (Candido *et al.*, 2004; Doran *et al.*, 2007). The findings from the literature and this study infer that the link between BP lowering effects of pharmacological RAS inhibition and their anti-atherosclerotic effects may involve a complex mechanism.

This study confirms the important effect of the RAS in the development of experimental atherosclerosis. Using a mouse model in which drug administration can be constantly maintained for 3 months, this study demonstrated that inhibitors of renin, ACE and AT_1 receptors markedly reduce atherosclerotic lesion formation in a similar dose-dependent manner.

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Conflict of interest

Aliskiren was provided by Novartis. Gene Liau was an employee of Novartis.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative en face images of aortic arches that were used for quantification of atherosclerotic lesions. Images are provided for examples of atherosclerosis in mice infused with PBS, aliskiren 25 $mg \cdot kg^{-1} \cdot d^{-1}$, enalapril 2.5 $mg \cdot kg^{-1} \cdot d^{-1}$ or losartan 25 $mg \cdot kg^{-1} \cdot d^{-1}$.
Figure S2 Comparison of RAS inhibition on atherosclerotic lesions in thoracic aorta. Percent lesion area was measured on the intimal surface of thoracic aortas (n = 8–14 per group). Triangles represent the values for individual mice, diamonds represent means, and bars are SEM. * denotes $P < 0.001$ versus the vehicle.
Figure S3 Comparison of RAS inhibition on renal AngII concentrations. Renal AngII concentrations (n = 5 per group) were measured using radioimmunoassay method. Histobars represent means and bars represent SEM. * denotes $P < 0.05$ versus the vehicle.

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