The HMG-CoA reductase inhibitor rosuvastatin and the angiotensin receptor antagonist candesartan attenuate atherosclerosis in an apolipoprotein E-deficient mouse model of diabetes via effects on advanced glycation, oxidative stress and inflammation

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Abstract

Aims/hypothesis We evaluated the anti-atherosclerotic effect of the 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, rosuvastatin, and the angiotensin II receptor blocker (ARB), candesartan, alone and in combination, in the streptozotocin-induced diabetic apolipoprotein E-deficient (*Apoe*^{−/−}) mouse.

Methods Control and streptozotocin-induced diabetic *Apoe*^{−/−} mice received rosuvastatin (5 mg kg^{−1} day^{−1}), candesartan (2.5 mg kg^{−1} day^{−1}), dual therapy or no treatment for 20 weeks. Aortic plaque deposition was assessed by Sudan IV staining and subsequent visual quantification. The abundance of proteins was measured using immunohistochemistry.

Results Diabetes was associated with a fourfold increase in total plaque area. Rosuvastatin attenuated plaque area in diabetic mice in the absence of lipid-lowering effects. The anti-atherosclerotic effect of rosuvastatin was comparable to that observed with candesartan. A similar beneficial effect was seen with dual therapy, although it was not superior to

monotherapy. Rosuvastatin treatment was associated with attenuated accumulation of AGE and AGE receptor (RAGE) in plaques. Similar beneficial effects on markers of oxidative stress were seen with the ARB and statin. Candesartan was more effective at reducing macrophage accumulation and collagen I abundance in plaques compared with rosuvastatin. The combined effect of candesartan and rosuvastatin was superior in reducing macrophage infiltration, monocyte chemoattractant protein-1 level, vascular AGE accumulation and RAGE abundance in the vascular wall. Furthermore, the combination tended to be more effective in reducing smooth muscle cell infiltration and connective tissue growth factor abundance in plaques.

Conclusions/interpretation Rosuvastatin has direct anti-atherosclerotic effects in diabetic macrovascular disease. These effects are independent of effects on lipids and comparable to the effects observed with candesartan.

Keywords Angiotensin II · Atherosclerosis · Diabetes · Diabetic–hypercholesterolaemic mouse · Statin

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Abbreviations

ARB	angiotensin II receptor blocker
AT1	angiotensin subtype 1 receptor
CML	carboxymethyllysine
CTGF	connective tissue growth factor
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
MCP-1	monocyte chemoattractant protein-1
RAC-1	ras-related C3 botulinum toxin substrate 1
RAGE	AGE receptor

Introduction

It is well established that people with diabetes have a greater risk of heart disease than those without diabetes. Indeed, macrovascular complications account for more than 50% of deaths in people with diabetes. While it is understood that both metabolic and haemodynamic factors such as hyperglycaemia, hyperlipidaemia and dysregulation of the renin–angiotensin system contribute to diabetes-associated atherosclerosis [1–3], other less traditional pathways, such as oxidative stress and advanced glycation, are now considered to play an important role in the development of diabetic macrovascular disease [4, 5].

Type 2 diabetes is often associated with a range of concomitant disorders including hypertension and dyslipidaemia. 3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors, also known as statins, are commonly used for the treatment of dyslipidaemia. A number of large studies have demonstrated that statin therapy is associated with a reduction in cardiovascular events in people both with and without type 2 diabetes [6–12]. While a reduction in LDL-cholesterol was observed in many of these studies, it is now considered that statins also mediate pleiotropic anti-atherogenic effects that are independent of their effects on lipoproteins, and that this action may contribute to their efficacy in reducing cardiovascular events. Indeed, HMG-CoA reductase inhibitors have been shown to attenuate, although not in the context of diabetes, many of the stages critical to atherosclerotic plaque development including monocyte chemotaxis [13], neutrophil–endothelial cell interaction [13], smooth muscle cell apoptosis [14], migration [15] and proliferation [16], as well as plaque stability [17].

Angiotensin II receptor blockers (ARBs) antagonise the angiotensin subtype 1 receptor (AT1) and are widely used as blood pressure-lowering agents in the absence and presence of diabetes. The prevalence of hypertension is greater in patients with diabetes [18], and hypertension itself is an independent cardiovascular risk factor [19]. A reduction in cardiovascular events has been seen in a number of large studies on agents that interrupt the renin–angiotensin system in patients without and with diabetes [20–22], including ARBs [23]. The cardioprotective effects seen with these agents may be due not only to blood pressure reduction, but also to additional mechanisms resulting from a reduction of angiotensin II's direct actions on the vasculature. It is now well established that angiotensin II mediates various direct, pro-atherogenic effects on the vasculature, including the upregulation of monocyte migration [24], endothelial cell adhesion [25], and smooth muscle cell proliferation and migration [26, 27], as well as increased reactive oxygen species [28], pro-inflammatory cytokines [29] and growth factors [30]. Moreover, various components of the renin–angiotensin

system have been shown to be present at the level of the vessel wall [2, 3, 30].

The aim of the current study was initially to assess such anti-atherosclerotic effects of the HMG-CoA reductase inhibitor rosuvastatin that are independent of its lipid-lowering effects, in a model of atherosclerosis accelerated by diabetes, specifically in the streptozotocin-induced diabetic, hyperlipidaemic apolipoprotein E-deficient (*Apoe*^{−/−}) mouse. These effects were then compared with those seen in this model after treatment with the ARB candesartan. Finally, the anti-atherosclerotic effects of combined rosuvastatin and candesartan treatment were assessed and compared with rosuvastatin and candesartan alone, with concomitant evaluation of potentially relevant pathways.

The diabetic *Apoe*^{−/−} mouse model is associated with accelerated atherosclerosis exhibiting lesions similar to those seen in humans, ranging from fatty streaks to complex plaques with necrotic cores and cholesterol clefts [2, 3]. Indeed, our laboratory has shown that the diabetic *Apoe*^{−/−} mouse is an excellent model for studying the effects of various agents on diabetes-associated atherosclerosis, particularly agents that influence the haemodynamic and metabolic pathways that are activated in the diabetic state [31, 32].

Methods

Animals

We used 6-week-old male homozygous *Apoe*^{−/−} mice (back-crossed twenty times from the C57BL/6 strain; Animal Resource Centre, Canning Vale, WA, Australia), which were housed at the Precinct Animal Centre at the Alfred Monash Research Education Precinct (Melbourne, Australia) and studied according to the principles devised by the Animal Welfare Committee of the Baker Heart Research Institute and Alfred Hospital. A subset of mice was rendered diabetic by five daily intraperitoneal injections of streptozotocin (Boehringer-Mannheim, Mannheim, Germany), at a dose of 55 mg/kg in citrate buffer [2]. Control (buffer alone) and diabetic mice were further randomised to receive: (1) the HMG-CoA reductase inhibitor rosuvastatin (5 mg kg^{−1} day^{−1}; AstraZeneca, Macclesfield, UK); (2) the AT1 receptor antagonist candesartan (2.5 mg kg^{−1} day^{−1}; AstraZeneca); (3) dual therapy by gavage at the above-mentioned doses for 20 weeks; or (4) no treatment (*n*=6–8 per group).

Systolic blood pressure was assessed by a non-invasive tail cuff system in conscious mice at the end of the study [33]. At the conclusion of the study, animals were anaesthetised by an intraperitoneal injection of pentobarbitone

sodium/phenytoin sodium (100 mg/kg body weight) (Euthatal; Sigma-Aldrich, Castle Hill, NSW, Australia). Blood was collected from the left ventricle and centrifuged (6,000×g), and plasma and erythrocytes were stored at −20°C and 4°C respectively for subsequent analysis. Animals were killed and the aortas were rapidly dissected and snap frozen in liquid nitrogen and stored at −80°C or stored in buffered formalin (10%, vol./vol.) for subsequent measurement of plaque area and immunohistochemical studies.

Metabolic parameters

Glycohaemoglobin was determined in lysates of erythrocytes by high-pressure liquid chromatography (BioRad, Richmond, CA, USA) [34]. Plasma cholesterol, triacylglycerol and glucose levels were measured by an autoanalyser technique (Hitachi 917; Hitachi, Tokyo, Japan); LDL-cholesterol was calculated according to the Friedewald formula [35].

Assessment of plaque area

To evaluate the atherosclerotic lesions, two approaches were used: visual analysis and histological section analysis. The visual approach was used to obtain information about the distribution and extent of atherosclerosis throughout the aorta, whereas microscopic analysis was used to evaluate lesion composition. In brief, as previously described, the aorta was cleaned of peripheral fat under a dissecting microscope and stained with Sudan IV–Herxheimer's solution (0.5% wt/vol.; Sigma Chemical, St Louis, MO, USA) [2]. Aortic images were digitised using a dissecting microscope (Olympus SZX9; Olympus Optical, Tokyo, Japan) equipped with a high-resolution camera (Zeiss, Heidelberg, Germany). Lesion area measurements were performed by calculating the proportion of aortic intima surface area occupied by the red stain in each of the segments.

Immunohistochemistry

Serial sections were dewaxed and rehydrated as described previously [2]. For detailed information, please see Electronic supplementary material (ESM; Methods and ESM Table 1).

Statistical analysis

Data were analysed by ANOVA using Statview V (Brainpower, Calabasas, CA, USA). Post hoc comparisons of group means were performed by Fisher's least significant difference method. Data are shown as mean±SEM unless otherwise specified. A *p* value of less than 0.05 was regarded as statistically significant.

Results

Metabolic parameters and blood pressure

Diabetes was associated with reduced body weight gain in streptozotocin-treated *Apoe*^{−/−} mice compared with non-diabetic control *Apoe*^{−/−} mice (Table 1). Body weights were not influenced by any of the treatments, either as monotherapies or in combination (Table 1). Food intake was increased in diabetic animals, but was not affected by any of the treatments (Table 1).

Diabetes was associated with a marked elevation in glycated haemoglobin and plasma glucose levels (Table 1). Neither candesartan nor rosuvastatin nor dual therapy had any effect on these parameters.

Diabetes was associated with a marked increase in plasma total cholesterol levels and LDL-cholesterol levels (*p*<0.05). Neither candesartan nor rosuvastatin nor dual therapy had an effect on any of these parameters (Table 1). Triacylglycerol and HDL concentrations were increased in

Table 1 Characteristics of mice at the conclusion of the 20 week study

Parameters	Control	Diabetic	D + Ca	D + RSV	D + Ca/RSV
Body weight (g)	30±0	24±0*	24±1	23±1	25±1
Food intake (g)	2.1±0.2	7.0±0.4*	6.4±0.3	6.5±0.2	6.4±0.3
Glycated haemoglobin (%)	5.3±0.2	14.8±0.5*	14.8±0.5	14.8±0.8	14.8±0.8
Glucose (mmol/l)	9.7±0.4	29.0±1.4*	29.4±1.2	27.2±1.2	28.2±1.0
Total cholesterol (mmol/l)	11.6±0.5	24.9±2.4*	23.3±1.6	26.8±1.9	27.4±2.6
HDL-cholesterol (mmol/l)	2.7±0.2	3.8±0.4*	4.3±0.4	4.1±0.5	4.0±0.6
LDL-cholesterol (mmol/l)	8.3±0.4	19.5±2.1*	18.0±1.1	21.2±1.4	22.0±2.1
Triacylglycerol (mmol/l)	1.3±0.2	3.9±0.8*	2.2±0.4	3.4±0.6	3.0±0.4
Systolic BP (mmHg)	100±4	108±2*	96±4 [†]	107±3	100±4

Data are expressed as mean ± SEM

D + Ca, diabetic + candesartan; D + RSV, diabetic + rosuvastatin; D + Ca/RSV, diabetic + candesartan + rosuvastatin

**p*<0.05 vs control group; [†]*p*<0.05 vs diabetic group; *n*=6–8 per group

Fig. 1 Quantification of visual plaque area for **a** total aorta, **b** arch, **c** thoracic and **d** abdominal aorta. Data are expressed as mean $\pm$ SEM. * p <0.05, ** p <0.01 vs control; † p <0.05, †† p <0.01 vs diabetic. C, control; D, diabetic; D + C, diabetic + candesartan; D + R, diabetic + rosuvastatin; D + C/R, diabetic + candesartan + rosuvastatin

diabetic *Apoe*^{-/-} mice compared with non-diabetic controls (Table 1), but neither treatment significantly reduced these parameters.

The induction of diabetes was associated with a small but significant increase in systolic blood pressure in *Apoe*^{-/-} mice (Table 1). Candesartan had a modest blood pressure-reducing effect, whereas rosuvastatin and the combination had no effect on blood pressure.

Plaque area

Diabetes was associated with a fourfold increase in total plaque area (p <0.01; Fig. 1a). This increase in plaque area was observed across the whole aorta (arch, thoracic and abdominal segments; p <0.05; Fig. 1b–d), but was most

apparent in the aortic arch. Both rosuvastatin (p <0.01) and candesartan (p <0.01) were associated with a reduction in total plaque area to levels near those seen in control mice. While dual therapy was also effective at normalising total plaque area (p <0.01), it was not superior to either agent alone. A similar profile of plaque deposition was seen in the aortic arch (Fig. 1b), with all three treatment regimens effective at attenuating atherosclerosis (p <0.01). While, in the thoracic aorta, there was a tendency for all three treatments to attenuate the increase in plaque deposition seen in diabetic mice, none of these reached significance. In the abdominal aorta, candesartan (p <0.01) as well as dual therapy (p <0.05) attenuated atherosclerosis.

Plaque and vessel wall composition

Inflammation Macrophage accumulation, as assessed by F4/80 staining, tended to be increased by 25% in the plaques of diabetic mice, but this did not reach statistical significance (Table 2). Both candesartan and the combination of candesartan and rosuvastatin attenuated macrophage infiltration (p <0.05) to levels below that seen in control *Apoe*^{-/-} mice. In the adjacent vascular wall there was no difference in F4/80 abundance in diabetic mice (Table 2). Dual therapy was associated with a greater than 50% reduction in macrophage infiltration (p <0.05), an effect not seen with either candesartan or rosuvastatin alone (p <0.01 for candesartan vs combination treatment). Monocyte chemoattractant protein-1 (MCP-1) production was significantly increased in the vascular wall and plaques of diabetic *Apoe*^{-/-} mice (Table 2). Candesartan and rosuvastatin alone had only moderate effects on MCP-1 abundance, but the combination of both treatments significantly reduced these in the vascular wall (p <0.05 versus diabetes,

Table 2 Quantification of plaque and vessel wall composition

	Control	Diabetic	D + Ca	D + RSV	D + Ca/RSV
F4/80					
Plaque	11.6 $\pm$ 6.2	16.1 $\pm$ 5.5	3.3 $\pm$ 1.4 [†]	7.6 $\pm$ 3.1	2.7 $\pm$ 2.3 [†]
Vessel wall	9.0 $\pm$ 0.9	6.8 $\pm$ 1.9	7.8 $\pm$ 1.5	4.5 $\pm$ 1.0	2.5 $\pm$ 1.0 [†]
MCP-1					
Plaque	31.2 $\pm$ 0.4	52.2 $\pm$ 4.6**	33.6 $\pm$ 0.5 ^{††}	47.9 $\pm$ 1.6	36.6 $\pm$ 0.5 ^{††}
Vessel wall	20.0 $\pm$ 3.3	33.1 $\pm$ 3.1**	26.7 $\pm$ 2.2	33.0 $\pm$ 2.7	25.1 $\pm$ 1.0 [†]
Collagen I					
Plaque	6.3 $\pm$ 4.1	12.9 $\pm$ 5.9	3.4 $\pm$ 1.9 [†]	3.6 $\pm$ 2.2	5.8 $\pm$ 3.3
Vessel wall	4.9 $\pm$ 0.7	16.3 $\pm$ 0.8*	12.0 $\pm$ 3.4	8.0 $\pm$ 2.6 [†]	11.0 $\pm$ 1.2
Collagen IV					
Plaque	1.5 $\pm$ 1.0	4.9 $\pm$ 2.7	3.8 $\pm$ 1.9	3.8 $\pm$ 1.2	1.8 $\pm$ 1.2
Vessel wall	9.7 $\pm$ 2.1	17.1 $\pm$ 1.7**	8.5 $\pm$ 1.7 ^{††}	7.7 $\pm$ 0.8 ^{††}	8.8 $\pm$ 1.0 ^{††}

Data are expressed as mean $\pm$ SEM

D + Ca, diabetic + candesartan; D + RSV, diabetic + rosuvastatin; D + Ca/RSV, diabetic + candesartan + rosuvastatin

* p <0.05, ** p <0.01 vs control group; † p <0.05, †† p <0.01 vs diabetic group; n =6–8 per group

$p < 0.05$ rosuvastatin versus combination treatment). In the plaque, candesartan and the combination therapy significantly reduced MCP-1 abundance ($p < 0.01$; Table 2).

AGE/AGE receptor axis Diabetes was associated with an approximately fivefold increase in the presence of the AGE, carboxymethyllysine (CML), in plaques of *Apoe*^{-/-} mice (Fig. 2a,d). Rosuvastatin attenuated AGE accumulation by approximately 50% in plaques ($p < 0.05$; Fig. 2f), while candesartan reduced CML abundance to those seen in control mice ($p < 0.01$; Fig. 2e). The combination of rosuvastatin and candesartan was associated with a further decrease in vascular CML accumulation ($p < 0.01$; Fig. 2g). Similarly, diabetes induced a sixfold increase in CML deposition in the vascular wall, this being significantly attenuated only in the combination treatment group ($p < 0.01$ versus diabetes and candesartan, $p < 0.05$ versus rosuvastatin; Fig. 2b).

Fig. 2 Accumulation of the AGE CML in the plaque (a) and vessel wall (b). Representative images of vessels of control (c), diabetic (d), diabetic + candesartan (e), diabetic + rosuvastatin (f) and diabetic + candesartan/rosuvastatin (g) mice. Data are expressed as mean $\pm$ SEM. ** $p < 0.01$ vs control; † $p < 0.05$, †† $p < 0.01$ vs diabetic. Magnification $\times 430$. C, control; D, diabetic; D + C, diabetic + candesartan; D + R, diabetic + rosuvastatin; D + C/R, diabetic + candesartan + rosuvastatin

The changes in abundance of the AGE receptor (RAGE) were similar to those seen for CML in plaques. Indeed, all three treatments were effective at attenuating the diabetes-associated upregulation of RAGE in the plaques ($p < 0.01$; Fig. 3a,c–g). In the vessel wall, however, RAGE protein tended to be decreased in the rosuvastatin group, with a further decrease in the combination group. These changes, however, did not reach statistical significance (Fig. 3b).

Oxidative stress Nitrotyrosine, a downstream product of nitric oxide and superoxide, is considered to reflect vascular oxidative stress; it was markedly upregulated in the plaques of diabetic mice ($p < 0.01$; Fig. 4). Moreover, all three treatments decreased staining of this molecule ($p < 0.01$). A similar pattern was seen in the adjacent vascular wall with all three treatments being effective at attenuating the diabetes-associated upregulation of nitrotyrosine and the combination treatment being the most effective ($p < 0.01$ versus rosuvastatin).

Fig. 3 Immunostaining of RAGE in the plaque (a) and vessel wall (b). Representative images of vessels of control (c), diabetic (d), diabetic + candesartan (e), diabetic + rosuvastatin (f) and diabetic + candesartan/rosuvastatin (g) mice. Data are expressed as mean $\pm$ SEM. ** $p < 0.01$ vs control; †† $p < 0.01$ vs diabetic. Magnification $\times 430$. C, control; D, diabetic; D + C, diabetic + candesartan; D + R, diabetic + rosuvastatin; D + C/R, diabetic + candesartan + rosuvastatin

Diabetes was associated with increased production of the NAD(P)H subunit, p47phox, in plaques of *Apoe*^{-/-} mice ($p < 0.05$), an increase attenuated by rosuvastatin ($p < 0.05$; Fig. 5a) and candesartan ($p < 0.01$). As seen in the plaque, there was a marked increase in p47phox expression in the vascular wall, which was normalised by all three treatments ($p < 0.01$; Fig. 5b).

Abundance of the NAD(P)H oxidase subunit, gp91phox, in plaques of *Apoe*^{-/-} tended to be increased by diabetes, but none of the treatments studied significantly reduced this parameter (ESM Fig. 1). In the adjacent vessel wall, however, diabetes was associated with an upregulation of gp91phox, which was attenuated by rosuvastatin ($p < 0.01$) and candesartan ($p < 0.05$); no significant effect was seen for combination therapy.

Ras-related C3 botulinum toxin substrate 1 (RAC-1), which is also a subunit of NAD(P)H oxidase, tended to be

increased in aortas of diabetic *Apoe*^{-/-} mice and was mildly reduced in the plaques by all three treatment groups, although changes were not statistically significant (ESM Fig. 2). In the adjacent vessel wall, RAC-1 was significantly upregulated in association with diabetes ($p < 0.01$) and was attenuated to control levels by rosuvastatin ($p < 0.01$) and dual therapy ($p < 0.01$).

Smooth muscle cell infiltration Diabetes was associated with an approximately sixfold increase in smooth muscle cell accumulation within plaques ($p < 0.05$) as assessed by α -smooth muscle cell actin abundance (ESM Fig. 3).

Fig. 4 Immunostaining of nitrotyrosine in the plaque (a) and vessel wall (b). Representative images of vessels of control (c), diabetic (d), diabetic + candesartan (e), diabetic + rosuvastatin (f) and diabetic + candesartan/rosuvastatin (g) mice. Data are expressed as mean $\pm$ SEM. ** $p < 0.01$ vs control; †† $p < 0.01$ vs diabetic. Magnification $\times 430$. C, control; D, diabetic; D + C, diabetic + candesartan; D + R, diabetic + rosuvastatin; D + C/R, diabetic + candesartan + rosuvastatin

Fig. 5 Immunostaining of the NAD(P)H oxidase subunit p47phox in the plaque (a) and vessel wall (b). Representative images of vessels of control (c), diabetic (d), diabetic + candesartan (e), diabetic + rosuvastatin (f) and diabetic + candesartan/rosuvastatin (g) mice. Data are expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs control; † $p < 0.05$, †† $p < 0.01$ vs diabetic. Magnification $\times 430$. C, control; D, diabetic; D + C, diabetic + candesartan; D + R, diabetic + rosuvastatin; D + C/R, diabetic + candesartan + rosuvastatin

Rosuvastatin ($p<0.01$), candesartan ($p<0.05$) and dual therapy ($p<0.01$) were all effective at attenuating smooth muscle cell infiltration. With respect to the vessel wall, the induction of diabetes was associated with an increase in α -smooth muscle actin staining, which was attenuated by rosuvastatin ($p<0.05$) and dual therapy ($p<0.05$); candesartan also had an effect, albeit more modest, on this parameter.

Markers of fibrosis The plaques of diabetic mice tended to exhibit increased abundance of collagen I compared with control mice; however, this did not reach significance (Table 2). Candesartan was most effective at attenuating collagen I abundance in the plaques ($p<0.05$). In the adjacent vessel wall, there was a threefold increase in collagen I abundance in diabetic mice. Interestingly, rosuvastatin was the most effective treatment for attenuating collagen I abundance in the vessel wall adjacent to the plaque ($p<0.05$).

A similar pattern was seen with collagen IV in plaques, with diabetic mice displaying increased collagen IV compared with control mice, although this did not reach statistical significance (Table 2). None of the treatments significantly altered collagen IV abundance in the plaques. The diabetes-associated upregulation of collagen IV within the vessel wall was more marked ($p<0.01$). All three treatments were equally effective at attenuating collagen IV production in the vessel wall ($p<0.01$).

Connective tissue growth factor (CTGF) abundance was markedly increased in plaques of diabetic mice compared with control mice ($p<0.01$; ESM Fig. 4). All three treatments were effective at attenuating CTGF expression to control levels (rosuvastatin, $p<0.01$) or lower (candesartan and combination), with the combination therapy being the most effective. In the vascular wall, the level of CTGF was increased in diabetes and tended to be reduced by rosuvastatin and in particular by combination treatment, but changes were not statistically significant.

Discussion

Diabetic animals demonstrated significantly increased plaque areas after 20 weeks of untreated diabetes. Plaque area in the diabetic *Apoe*^{-/-} mouse was significantly reduced by treatment with the HMG-CoA reductase inhibitor rosuvastatin, the AT1 receptor blocker candesartan and by a combination of both drugs. The anti-atherosclerotic effect conferred by rosuvastatin was independent of effects on lipids and was comparable to the anti-atherosclerotic effect conferred by candesartan. The combination treatment was also effective at decreasing plaque area, but was not

superior to candesartan or rosuvastatin therapy alone. The anti-atherosclerotic effects conferred by the three treatment regimens were independent of effects on plasma lipids or glycaemic control.

Previously we have demonstrated, using the diabetic *Apoe*^{-/-} mouse, that the renin–angiotensin system plays a significant role in diabetes-accelerated atherosclerosis [2, 3]. The ACE inhibitor perindopril and the AT1 receptor blocker irbesartan were able to reduce plaque area after 20 weeks of diabetes in this model [2, 3]. Moreover, despite similar effects on blood pressure in that study, irbesartan was more effective than amlodipine in reducing plaque area, suggesting that blood pressure-lowering per se did not have a major effect on plaque reduction [3].

This is the first report to demonstrate that, in a model of diabetes-accelerated atherosclerosis, the diabetic *Apoe*^{-/-} mouse, a statin reduces plaque area independently of its effects on lipids, suggesting that these vasculoprotective effects are related to the pleiotropic effects of rosuvastatin. Furthermore, it should be noted that rosuvastatin attenuated many pro-atherosclerotic pathways, similar to effects seen with the AT1 receptor blocker, a treatment known to have anti-atherosclerotic effects in this model by directly interfering with the local renin–angiotensin system in the vascular wall.

There are several studies in the clinical and pre-clinical setting investigating the effect of an ARB and a statin, although none in the context of diabetes. Chen et al treated *Apoe*^{-/-} mice fed a high-cholesterol diet with candesartan and rosuvastatin for 12 weeks and demonstrated that simultaneous administration of these agents reduced atherosclerosis to a greater extent than candesartan or rosuvastatin alone [36]. This was associated with a reduction in CD40 and matrix metalloproteases. Similarly, in another model of high-fat-induced atherosclerosis, treatment with valsartan and fluvastatin for 10 weeks decreased atherosclerotic lesions in *Apoe*^{-/-} mice fed a high-cholesterol diet [37]. Changes in oxidative stress and inflammatory parameters were also observed. Administration of olmesartan and pravastatin together to Watanabe heritable hyperlipidaemic rabbits resulted in a greater anti-atherogenic effect than monotherapy [38]. In contrast, *Apoe*^{-/-} mice fed a high-fat diet for 12 weeks demonstrated that combination therapy with telmisartan and atorvastatin was not superior to telmisartan alone [39]. However, an additive effect was seen with respect to plasma inflammatory markers such as IL-10.

A number of small clinical studies have also demonstrated varying effects with combination therapies. Hussein et al demonstrated an additive antioxidant effect when valsartan was co-administered for 2 months to seven hypercholesterolaemic, hypertensive patients taking fluvastatin [40]. The Endothelial Protection, AT1 Blockade and

Cholesterol-Dependent Oxidative Stress (EPAS) Trial demonstrated that treatment with pravastatin (40 mg/day), in 60 patients with stable coronary artery disease prior to elective coronary artery bypass grafting, was associated with an increase in the anti-atherosclerotic endothelial expression quotient Q, including mRNA expression (endothelial nitric oxide synthase and CNP divided by lectin-like oxidised LDL receptor-1, and gp91phox in left internal mammary arteries) [41]. Treatment with irbesartan (150 mg/day) had no significant effect. However, when combined with pravastatin, it further increased lnQ, but a putative interaction of both therapies on lnQ was not significant [41]. Finally, the Simvastatin/Enalapril Coronary Atherosclerosis Trial (SCAT) examined the effect of simvastatin treatment in 460 normocholesterolaemic patients [42]. This study demonstrated beneficial effects on quantitative coronary angiographic measurements with simvastatin treatment, an effect not seen with enalapril. Moreover, there was no additive effect of these drugs on the parameters measured. However, it should be noted that in this study interruption of the renin–angiotensin system was with an ACE inhibitor, rather than with an ARB. While these studies show varying effects of combined statin and ARB therapy on cardiovascular disease, none of these studies was carried out in the setting of diabetes.

In the current study, there appeared to be differences in the mechanisms leading to the anti-atherosclerotic effects observed with each drug. Whereas candesartan was effective at reducing inflammation as evidenced by a decrease in macrophage infiltration and MCP-1 levels in the plaque, rosuvastatin did not have such a marked effect on these parameters. Previous studies have shown reduced inflammation in plaques from people with and without type 2 diabetes in response to various statins [17, 43].

Both drugs were effective in reducing AGE accumulation and RAGE accumulation in the plaque. Although such an effect on the AGE/RAGE axis has previously been observed in cell culture experiments [44] and postulated to be important in type 2 diabetic patients for plaque stabilisation [43], this is the first study to demonstrate effects of a statin on the AGE/RAGE pathway in diabetes-accelerated atherosclerosis. Indeed, our study suggests that statins may reduce AGE accumulation and thereby reduce activation of NAD(P)H oxidase, thus linking oxidant stress to altered gene expression via RAGE, as previously suggested *in vitro* and in gp91phox-deficient mice [45]. Furthermore, this is the first report to describe superior effects on the AGE/RAGE pathway, particularly in the vascular wall, by a combination regimen consisting of candesartan and rosuvastatin.

Both therapies alone and in combination were effective at reducing the abundance of the various NAD(P)H oxidase subunits in the plaque and vessel wall. It is well established

that angiotensin II can increase superoxide production via NAD(P)H oxidase-dependent pathways and has been shown to modulate NAD(P)H oxidase subunit abundance [46]. Indeed, AT1 blockade has been associated with a reduction in superoxide production [47]. Withdrawal of statins has also been shown to increase superoxide production in vessels of mice [48]. This effect was absent in *gp91phox* (also known as *Cybb*)^{−/−} mice. Furthermore, withdrawal of statins from human umbilical vein endothelial cells resulted in translocation of the NAD(P)H oxidase subunit, RAC-1, to the membrane and an increase in NAD(P)H-induced lucigenin chemiluminescence. It appears that both drugs, candesartan and rosuvastatin, act on this pathway in a similar manner, modulating NAD(P)H oxidase subunit abundance. Furthermore, interruption of the NAD(P)H oxidase pathway results in reduced superoxide production, which could explain the reduction in nitrotyrosine present in the plaques and adjacent vessel wall, as seen with both therapies alone and in combination. Moreover, a reduction in ROS may, in part, be responsible for the attenuation in AGE accumulation as well as for reduced abundance of the pro-inflammatory RAGE, as was seen with both therapies since AGE such as CML are indeed glycoxidation products [49].

Interestingly, while the treatments were effective at attenuating plaque deposition in the aortic arch and abdominal segment, there was no significant effect in the thoracic region. This may partially be due to the lower deposition of plaque within this region. Moreover, it may be due to the specific nature of the effect of the treatment on the vessel wall. For example, treatment of diabetic *Apoe*^{−/−} mice with inhibitors of AGE formation resulted in an attenuation of atherosclerosis in the thoracic and abdominal regions with no effect seen in the aortic arch, whereas treatment with the peroxisome proliferator-activated receptor- γ agonist rosiglitazone, postulated to have direct effects on the vessel wall, was associated with most marked effects within the aortic arch [4, 31, 32].

Diabetes is associated with an upregulation of the renin–angiotensin system [2, 3] in particular within the vascular wall. In addition, at sites of diabetes-related end-organ injury there is increased oxidative stress and inflammation. Thus, it was anticipated that therapies targeting these pathways would confer superior vascular protection in the setting of diabetes. However, in the current study, the combination treatment with candesartan and rosuvastatin did not confer additional anti-atherosclerotic effects as assessed by aortic plaque area. There are several potential explanations for these findings. It is possible that the anti-atherosclerotic effect achieved with candesartan was already maximal, as the plaque areas in the candesartan treated diabetic mice were similar to those observed in non-diabetic *Apoe*^{−/−} mice.

However, the combination treatment clearly exerted superior effects on key mediators of atherosclerosis such as AGE accumulation and RAGE accumulation, as well as on macrophage accumulation and abundance of the chemokine MCP-1, and on the profibrotic cytokine CTGF. Although this superior effect on key mediators of atherosclerosis did not translate into a further reduction of plaque area in the time course of this study, it may be speculated that the more effective suppression of the AGE/RAGE pathway and oxidative stress parameters by the combination regimen might lead to superior vasculoprotection including improved plaque composition and stability, if studied for longer.

In conclusion, the HMG-CoA reductase inhibitor rosuvastatin and the ARB candesartan, both well-known treatments used in diabetic patients at high risk of vascular disease, conferred similar anti-atherosclerotic effects in a model of diabetes-accelerated atherosclerosis, the diabetic *Apoe*^{-/-} mouse, via effects on the AGE/RAGE axis and oxidative stress. These direct pleiotropic vasculoprotective effects were independent of effects on metabolic control and lipids. The anti-atherosclerotic effect conferred by rosuvastatin was comparable to that observed by treatment with the AT1 receptor blocker candesartan, a drug type known to be anti-atherosclerotic in this model, via a mechanism that interrupts the local vascular renin–angiotensin system. The combination of candesartan and rosuvastatin conferred superior effects on the AGE/RAGE pathway, inflammation and oxidative stress.

These studies provide strong evidence for direct beneficial vascular effects of statins and ARBs in diabetes-associated atherosclerosis. Moreover, the vasculoprotection observed is independent of effects on metabolic control, lipids and blood pressure. In particular, the combination treatment of diabetic *Apoe*^{-/-} mice with the statin rosuvastatin and the ARB candesartan was associated with more effective suppression of AGE accumulation and RAGE accumulation, macrophage accumulation, MCP-1 abundance and oxidative stress parameters, all well-known key mediators in atherosclerosis. Therefore, the combination regimen of both agents has the potential to exert superior long-term vasculoprotection, in particular in the high-risk diabetic patient.

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