Diabetologia (2010) 53:584–592
DOI 10.1007/s00125-009-1619-x

ARTICLE

Angiotensin II subtype 2 receptor blockade and deficiency attenuate the development of atherosclerosis in an apolipoprotein E-deficient mouse model of diabetes

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Received: 18 August 2009 / Accepted: 5 November 2009 / Published online: 3 December 2009

Abstract
Aims/hypothesis Most of the known actions of angiotensin II have been considered primarily to be the result of angiotensin II subtype 1 receptor activation. However, recent data suggest that the angiotensin II subtype 2 receptor (AT_2R) may modulate key processes linked to atherosclerosis. The aim of this study was to investigate the role of AT_2R in diabetes-associated atherosclerosis using pharmacological blockade and genetic deficiency.
Methods Aortic plaque deposition was assessed in streptozotocin-induced diabetic apolipoprotein E (*Apoe*) knockout (KO) and *At_2r* (also known as *Agtr2*)/*Apoe* double-KO (DKO) mice. Control and diabetic *Apoe*-KO mice received an AT_2R antagonist PD123319 (5 mg kg^{-1} day^{-1}) via osmotic minipump for 20 weeks (n=7–8 per group).
Results Diabetes was associated with a sixfold increase in plaque area (diabetic *Apoe*-KO: 12.7±1.4% vs control *Apoe*-KO: 2.3±0.4%, $p<0.001$) as well as a significant increase in aortic expression of the gene *At_2r* (also known as *Agtr2*). The increase in plaque area with diabetes was attenuated in AT_2R antagonist-treated diabetic *Apoe*-KO mice (7.1±0.5%, $p<0.05$) and in diabetic *At_2r*/*Apoe* DKO mice (9.2±1.3%, $p<0.05$). These benefits occurred independently of glycaemic control or BP, and were associated with downregulation of a range of pro-inflammatory cytokines, adhesion molecules, chemokines and various extracellular matrix proteins.
Conclusions/interpretation This study provides evidence for AT_2R playing a role in the development of diabetes-associated atherosclerosis. These findings suggest a potential utility of AT_2R blockers in the prevention and treatment of diabetic macrovascular complications.

Keywords *Apoe*-KO mouse · Atherosclerosis · AT_2R · Diabetes

Abbreviations

ACEi	ACE inhibitors
AII	Angiotensin II
AT_1R	Angiotensin II subtype 1 receptor
AT_2R	Angiotensin II subtype 2 receptor
AT_2RB	Angiotensin II subtype 2 receptor blocker
DKO	Double knockout
KO	Knockout
MCP-1	Monocyte chemoattractant protein-1

Electronic supplementary material The online version of this article (doi:10.1007/s00125-009-1619-x) contains supplementary material, which is available to authorised users.

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NF-κB	Nuclear factor-kappa B
RAS	Renin–angiotensin system
α-SMA	Alpha-smooth muscle actin
VCAM-1	Vascular cell adhesion molecule-1

Introduction

Atherosclerosis is a major complication of diabetes, representing the predominant cause of morbidity and mortality in both type 1 and type 2 diabetic patients [1]. The renin–angiotensin system (RAS), and in particular its effector molecule, angiotensin II (AII), participates in the development of atherosclerosis, through the regulation of at least two key processes, inflammation and fibrosis [2]. As a result, pharmacological inhibition of the RAS has been proposed as a strategy for reducing atherosclerosis, beyond BP reduction. Pharmacological agents include ACE inhibitors (ACEi), which block the conversion of the prohormone angiotensin I to the active hormone AII, and selective AII subtype 1 receptor blockers (ARBs), which competitively inhibit the action of AII at the AT_1 receptor subtype (AT_1R) [3–6]. Both classes of compounds are widely used for reducing BP in patients with type 1 and type 2 diabetes. Furthermore, it has been suggested that there is a potentially important BP-independent protective effect on the risk of CHD for ACEi that has not been demonstrated for ARBs [7], although this possibility remains controversial [8].

AII binds with similar affinity to two major receptor subtypes, AT_1R and the type 2 (AT_2R) receptor [9]. While most of the well-known effects of AII are mediated via AT_1R, there is growing interest in effects mediated via AT_2R. The AT_2R is ubiquitously produced in fetal tissues, but its production declines after birth [10]. In adults, AT_2R production is detectable in the pancreas, heart, adrenals, brain, kidney and vasculature [9, 11, 12]. AT_2R is overproduced in pathological situations involving tissue remodelling or inflammation, including kidney damage [13–15] and atherosclerosis [16, 17]. However, the role of AT_2R in diabetes-associated atherosclerosis is not completely understood. It has been postulated that AT_1R and AT_2R have opposing actions on proliferation and apoptosis. The proliferative properties of AII have generally been considered to be associated with AT_1R, whereas AT_2R is viewed as promoting apoptosis and inhibiting cell growth [18]. However, the effects of AT_2R activation are not always uniform and reproducible. Indeed, there is currently no consensus on the role of AT_2R in atherosclerosis, with many studies, albeit in the non-diabetic setting, suggesting either a neutral or anti-atherosclerotic effect of this AII receptor subtype [17, 19–23]. In addition, recent data suggest that AT_2R may promote cell growth and inflammation [24–26].

The present study examined the role of AT_2R in the context of long-term diabetes in a well-characterised and widely used model of diabetic atherosclerosis, the streptozotocin-induced diabetic apolipoprotein E knockout (*Apoe*-KO) mouse [27–30]. The validity of this model has recently been confirmed as appropriate for the study of diabetes-associated atherosclerosis by the National Institutes of Health (NIH)/Juvenile Diabetes Research Foundation (JDRF)-supported Animal Models of Diabetic Complications Consortium [29]. Our aim was to determine whether disruption of the AT_2R would protect these animals from developing vascular lesions in the presence of diabetes. Thus, two different approaches were employed: first, a pharmacological strategy using an AT_2R antagonist, PD123319, and second, a genetic strategy involving the use of *At₂r*-deficient rodents.

Methods

Experimental model Six-week-old male *Apoe*-KO mice (backcrossed 20 times onto a C57BL6/J background; Animal Resource Centre, Canning Vale, WA, Australia) and *At₂r* (also known as *Agtr2*) double-KO (DKO) mice, confirmed by PCR genotyping and generated by backcrossing *At₂r*-KO mice [31] on the C57BL6/J background (had ≥98% C57BL6/J background, genome scan by Jackson Laboratory; www.jax.org) into *Apoe*-KO mice for ten generations were housed at the Precinct Animal Centre (Baker IDI Heart and Diabetes Institute, Melbourne, VIC, Australia). The protocols followed for animal handling and experimentation were in accordance with ethical guidelines of the Alfred Medical Research and Education Precinct animal ethics committee and the National Health and Medical Research Council of Australia guidelines.

Mice were randomised to have diabetes induced via five daily i.p. injections of streptozotocin at 55 mg kg^{-1} day^{-1} (Boehringer, Mannheim, Germany) [2, 28] or sham injected with vehicle (citrate buffer, pH 4.5). On the sixth day, blood glucose was tested using Accu-Chek Advantage II test strips (Roche Diagnostics, Mannheim, Germany), and only mice with blood glucose >15 mmol/l were included as diabetic in the study (>90% of injected mice).

Control and diabetic *Apoe*-KO mice were then randomised to treatment with PD123319 (5 mg kg^{-1} day^{-1}; Sigma-Aldrich, St Louis, MO, USA) [13, 32] or to no treatment for 20 weeks. The PD123319 drug and the vehicle (sterile Milli-Q water) were administrated s.c. via osmotic minipumps (Model 2004; Alzet, Cupertino, CA, USA), and these minipumps were replaced every 28 days. Animals were allowed access to standard mouse chow and

water ad libitum. After 20 weeks, excised aortas (n=15–20 per group) were placed in 10% neutral buffered formalin (10%, vol./vol.) and quantified for lesion area (n=7–8 per group) before being embedded in paraffin for immunohistochemical analysis. In the remaining mice (n=8–12 per group) aortas were snap-frozen in liquid nitrogen and stored at −80°C for subsequent RNA extraction [2, 28].

Metabolic variables and BP After 20 weeks, blood was collected from the left ventricle for the measurement of glycated haemoglobin (HbA_{1c}) [33], fasting glucose, total cholesterol and triacylglycerol [34].

Systolic BP was measured at week 19 of the study period by a computerised, non-invasive tail cuff system in conscious prewarmed mice [35].

Plaque area quantification Aortas removed from mice were cleaned of excess fat under a dissecting microscope and subsequently stained with Sudan IV-Herxheimer's solution (0.5% wt/vol.) (Gurr; BDH, Poole, UK) as described previously [2, 28]. The aortas were divided into arch, thoracic and abdominal sections and then cut longitudinally. After pinning en face onto wax, images were acquired with a dissecting microscope (Olympus SZX10, Olympus Optical, Tokyo, Japan) with a video-camera (Q-capture Pro Version 5.1, Burnaby, BC, Canada). Total plaque area was quantified as a percentage area of the whole aorta stained by Sudan IV (Adobe Photoshop version 6.0). Aortas were subsequently embedded in paraffin and sections cut for cross-sectional analysis.

Real-time RT-PCR Total RNA was extracted from the whole aorta by homogenising and treated with DNAse as described previously [2, 28]. cDNA was synthesised by reverse transcription (Pierce, Rockford, IL, USA). Quantitative real-time RT-PCR was performed using the Taqman System on an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) and analysed using Sequence Detection Software (SDS version 1.9; Applied Biosystems, Foster City, CA, USA). Gene expression was normalised to 18S rRNA (Applied Biosystems). Detailed information on probes is provided in the Electronic supplementary material (ESM) Table 1.

Immunostaining Serial 4 μm paraffin aortic cross-sections were stained with haematoxylin and eosin to evaluate the atherosclerotic lesion complexity, or with Picrosirius-Red with polarisation microscopy to detect collagen content [36, 37]. Serial 4 μm paraffin aortic cross-sections were immunostained for alpha-smooth muscle actin (α-SMA) (1:500; Serotec, Oxford, UK), vascular cell adhesion molecule-1 (VCAM-1) (1:50; Pharmingen, San Diego, CA, USA), monocyte chemotactic protein-1 (MCP-1) (1:500; R&D Systems, Minneapolis, MN, USA) and the macrophage marker F4/80 (1:50; Serotec) as previously described [34]. Results were quantified as per cent of positively stained tissue. Image analysis was performed using ImageJ (http://rsb.info.nih.gov/ij, accessed 3 March 2008) and ImagePro 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

Statistical analysis Data were analysed by ANOVA using Statview (version 5.0) and post hoc analysis of group means was performed by Fisher's least significant difference method. Data are expressed as means±SEM unless otherwise specified. $p<0.05$ was considered to be statistically significant.

Results

Metabolic variables and BP The induction of diabetes in both *Apoe*-KO and *At₂r*/*Apoe*-DKO mice resulted in increased plasma glucose and HbA_{1c} concentrations, which were comparable between the diabetic groups. Plasma total cholesterol concentrations were also significantly increased in both groups following the induction of diabetes. BP was not altered after 20 weeks of diabetes in either *Apoe*-KO or *At₂r*/*Apoe*-DKO groups compared with the non-diabetic groups. PD123319 treatment in the diabetic *Apoe*-KO mice did not alter any of these variables (Table 1).

Atherosclerotic plaque area The induction of diabetes in *Apoe*-KO mice led to an approximately sixfold increase in atherosclerotic plaque area of the whole aorta compared with non-diabetic *Apoe*-KO mice (Fig. 1). In these diabetic animals lesions were predominantly complex fibrous plaques (Fig. 2). AT_2R blocker (AT_2RB) treatment significantly attenuated the extent and complexity of plaques in diabetic *Apoe*-KO mice. Plaque area was also significantly reduced in diabetic *At₂r*/*Apoe*-DKO mice (Fig. 1). By contrast, no effect was detected in control *Apoe*-KO mice treated with AT_2RB, nor did control *At₂r*/*Apoe*-DKO mice show any difference in plaque area compared with control *Apoe*-KO mice alone.

At₂r expression In diabetic *Apoe*-KO mice, aortic mRNA levels of the *At₂r* gene were increased compared with control *Apoe*-KO mice and this increase in gene expression was decreased by AT_2RB treatment (Fig. 3).

Inflammation Inflammation plays a central role in the development and progression of atherosclerotic lesions. Thus, we assessed the regulation of nuclear factor-kappa B

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

Variable	Control *Apoe*-KO	AT_2RB-treated control *Apoe*-KO	Control *At₂r*/*Apoe*-DKO	Diabetic *Apoe*-KO	Diabetic *Apoe*-KO+vehicle	AT_2RB-treated diabetic *Apoe*-KO	Diabetic *At₂r*/*Apoe*-DKO
Body weight (g)	29.2±0.6	31.4±0.6	33.4±1.2*	24.0±0.9*	26.1±0.8*	24.2±0.6*	23.6±1.2*
Systolic BP (mmHg)	94±4	88±5	89±4	106±9	107±2	103±3	106±6
Plasma glucose (mmol/l)	10.2±0.8	9.8±1.3	12.1±0.8	30.7±2.7*	29.1±0.8*	32.5±1.7*	31.3±2.3*
HbA_{1c} (%)	3.6±0.3	3.2±0.9	3.3±0.2	13.1±1.3*	14.7±1.1*	13.4±1.0*	13.4±0.6*
Total cholesterol (mmol/l)	11.2±0.7	10.1±0.9	9.7±0.5	19.6±3.3*	24.2±2.7*	20.2±0.9*	18.7±1.0*
Triacylglycerol (mmol/l)	1.4±0.2	0.9±0.2	1.4±0.2	2.0±0.4	2.0±0.3	1.9±0.4	1.6±0.3

Means±SEM

*$p<0.05$ vs control *Apoe*-KO (n=7–10 per group)

(NF-κB), a key mediator of inflammation, by measuring aortic expression of the gene encoding NF-κB subunit p65. Diabetes induced a greater than threefold increase in the expression in *Apoe*-KO aorta. This upregulation was significantly attenuated by both AT_2RB treatment and *At₂r* gene deletion (Table 2). MCP-1, an NF-κB-dependent chemokine, is considered a key mediator in AII-induced progression of atherosclerosis [38]. Indeed, in this study, diabetes was associated with a significant increase in levels of this chemokine. AT_2RB treatment and *At₂r* gene deletion significantly decreased the aortic production of MCP-1 (Table 2, Figs 4 and 5) as well as suppressing the induction of pro-inflammatory cytokines such as TNF-α (Table 2) and cell adhesion molecules such as VCAM-1 (Table 2, Figs 4 and 5). Furthermore, macrophage accumulation, as detected by staining with the F4/80 marker, was increased in diabetic *Apoe*-KO mice compared with control *Apoe*-KO mice and also reduced by both AT_2RB treatment and *At₂r* gene deletion (Fig. 5). Macrophage staining was detected in both the media and the fibrous cap of the atherosclerotic lesions, particularly in diabetic *Apoe*-KO mice (Fig. 5).

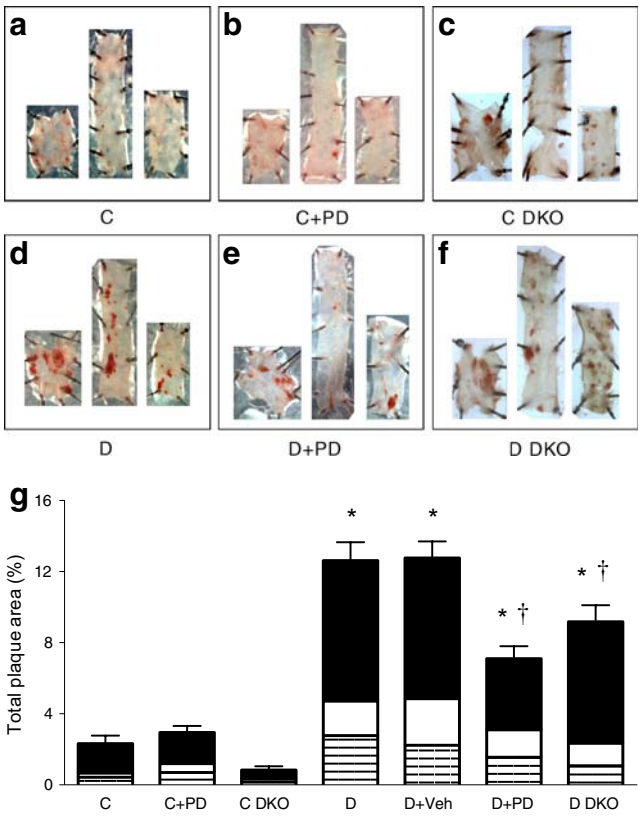

Fig. 1 **a–f** Representative en face photomicrographs of aortas with each box divided into aortic arch (left), thoracic (centre) and abdominal aorta (right) segments. **g** Total aortic plaque area expressed as the percentage of aortic area staining for Sudan IV Red (black, arch; white, thoracic; horizontal lines, abdominal). C, control *Apoe*-KO; C+PD, AT_2RB-treated control *Apoe*-KO; C DKO, control *At₂r*/*Apoe*-DKO; D, diabetic *Apoe*-KO; D+Veh, vehicle-treated diabetic *Apoe*-KO; D+PD, AT_2RB-treated diabetic *Apoe*-KO; D DKO, diabetic *At₂r*/*Apoe*-DKO. Means ± SEM. *$p<0.05$ vs control *Apoe*-KO; †$p<0.05$ vs diabetic *Apoe*-KO (n=7–8 per group)

Smooth muscle cell recruitment and extracellular matrix accumulation Atherosclerosis in diabetic *Apoe*-KO mice involves the proliferation and migration of medial vascular

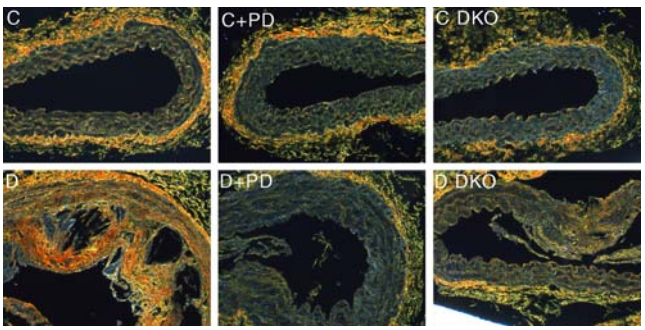

Fig. 2 Representative thoracic aortic sections stained with Picrosirius Red with polarisation microscopy to evaluate the presence of collagen I and III in control *Apoe*-KO (C), AT_2RB-treated control *Apoe*-KO (C+PD), control *At₂r*/*Apoe*-DKO (C DKO), diabetic *Apoe*-KO (D), AT_2RB-treated diabetic *Apoe*-KO (D+PD) and diabetic *At₂r*/*Apoe*-DKO (D DKO). Magnification ×200

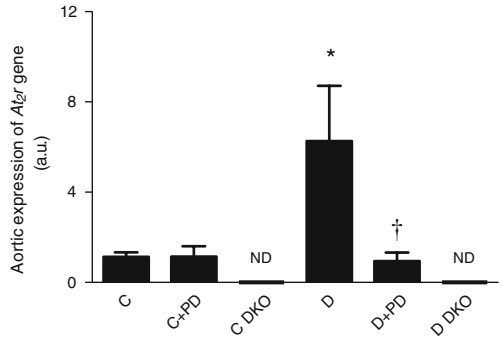

Fig. 3 At_2r gene expression quantified by real-time RT-PCR at the conclusion of the 20 week study in the whole aorta from the six experimental groups. C, control *Apoe*-KO; C+PD, AT_2RB-treated control *Apoe*-KO; C DKO, control At_2r/*Apoe*-DKO; D, diabetic *Apoe*-KO; D+PD, AT_2RB-treated diabetic *Apoe*-KO; D DKO, diabetic At_2r/*Apoe*-DKO. Means±SEM. *$p<0.05$ vs control *Apoe*-KO; †$p<0.05$ vs diabetic *Apoe*-KO. a.u., arbitrary units; ND, not detected

smooth muscle cells into the vessel intima, as demonstrated by increased gene and protein expression of α-SMA compared with control *Apoe*-KO mice. The atherosclerotic plaque in AT_2RB-treated diabetic *Apoe*-KO mice and diabetic At_2r/*Apoe*-DKO mice was significantly less complex, with reduced α-SMA production compared with diabetic *Apoe*-KO mice (Table 2, Fig. 4).

Extracellular matrix accumulation is a dominant feature of the diabetic plaque. Aortic expression of genes encoding fibronectin (*Fn1*), collagen I (*Col1a1*) and collagen III (*Col3a1*) were significantly increased in diabetic *Apoe*-KO mice compared with non-diabetic *Apoe*-KO mice. mRNA levels for these variables were decreased in AT_2RB-treated diabetic *Apoe*-KO mice and in diabetic At_2/*Apoe*-DKO mice compared with diabetic *Apoe*-KO mice (Table 2). Aortic fibrillar collagen content was also assessed by summing the percentage of orange-red (type I collagen) and yellow-green (type III collagen) fibres with polarisation microscopy. Collagen content was significantly increased in diabetic *Apoe*-KO mice compared with non-diabetic *Apoe*-KO mice. AT_2RB treatment and At_2r gene deletion significantly reduced collagen content compared with untreated diabetic *Apoe*-KO mice (Fig. 2).

Discussion

The current study has demonstrated in vivo that both AT_2R blockade using a pharmacological approach and At_2r deficiency induced by gene deletion attenuated to a similar degree diabetes-associated atherosclerosis. This occurred despite similar HbA_{1c} and BP levels between diabetic mice with and without interruption of AT_2R. Furthermore, this attenuation of diabetes-associated atherosclerosis, as a result of modulation of AT_2R, was associated with reduced levels of pro-inflammatory and profibrotic molecules.

It remains controversial as to what is the best model to study diabetes-associated atherosclerosis. As recently reviewed [29], there are strengths and limitations of the various models. Multiple injections of low-dose streptozotocin has been used to induce diabetes-associated atherosclerosis in *Apoe*-KO mouse, and this is one of the more popular approaches to be employed by investigators [27, 28, 30]. One of the potentially confounding factors of

Table 2 Aortic gene expression quantified by real time RT-PCR (arbitrary units) at the conclusion of the 20 week study

Gene	Official gene symbol	Control *Apoe*-KO	AT_2RB-treated control *Apoe*-KO	Control At_2r/*Apoe*-DKO	Diabetic *Apoe*-KO	AT_2RB-treated diabetic *Apoe*-KO	Diabetic At_2r/*Apoe*-DKO
Inflammatory markers							
Vcam-1	*Vcam1*	1.0±0.2	1.1±0.9	0.8±0.1	20.2±6.7*	6.3±3.0†	6.1±2.9†
Mcp-1	*Ccl2*	1.0±0.1	0.7±0.1	0.9±0.1	22.2±4.2*	4.7±0.8†	5.2±1.1†
Tnf-α	*Tnf*	1.0±0.1	0.6±0.2	1.2±0.3	19.3±3.3*	4.0±0.9†	6.8±1.3*†
Nf-κb p65	*Rela*	1.0±0.2	0.4±0.5	1.6±0.5	7.8±1.9*	0.8±0.4†	2.5±0.8†
Growth factors and fibrosis							
Tgf-β	*Tgfb1*	1.0±0.1	0.6±0.2	1.2±0.3	18.3±3.3*	3.0±0.9†	3.8±1.3*†
Ctgf	*Ctgf*	1.0±0.3	0.1±0.1	2.4±0.7	8.1±2.0*	1.0±0.4†	3.9±0.9*†
Extracellular matrix accumulation							
Collagen I	*Col1a1*	1.0±0.2	0.2±0.1	1.4±0.3	11.4±2.9*	2.2±0.3†	5.6±1.6†
Collagen III	*Col3a1*	1.0±0.2	0.2±0.1	1.7±0.2	16.7±5.0*	2.0±0.4†	4.6±1.1†
Fibronectin	*Fn1*	1.0±0.2	0.1±0.1	1.3±0.1	4.3±1.1*	1.3±0.2†	1.2±0.4†
α-Sma	*Acta2*	1.0±0.2	0.2±0.1	1.5±0.1	7.6±1.3*	0.5±0.2†	1.7±0.5†

Means±SEM

*$p<0.05$ vs control *Apoe*-KO; †$p<0.05$ vs diabetic *Apoe*-KO (n=6–12 per group)

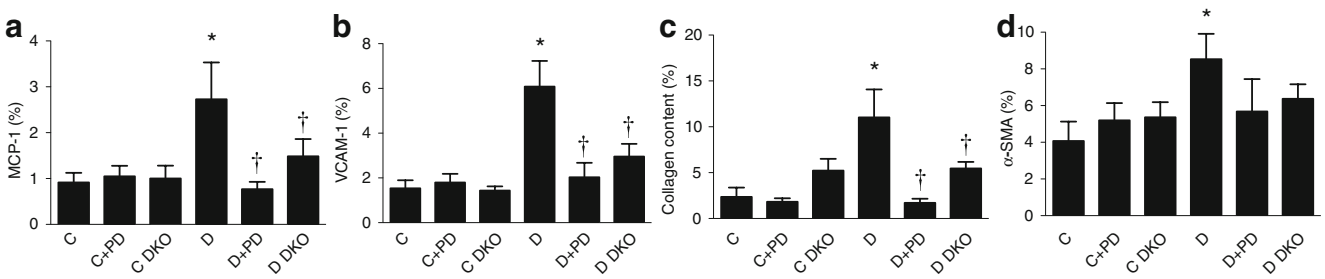

Fig. 4 Aortic protein abundance quantified by immunohistochemistry (% stained area) for MCP-1 (**a**), VCAM-1 (**b**), collagen (**c**) and α-SMA (**d**) at the conclusion of the 20 week study in control *Apoe*-KO (C), AT_2RB-treated control *Apoe*-KO (C+PD), control *At₂r*/*Apoe*-DKO (C DKO), diabetic *Apoe*-KO (D), AT_2RB-treated diabetic *Apoe*-KO (D+PD) and diabetic *At₂r*/*Apoe*-DKO (D DKO). Means±SEM. *$p<0.05$ vs control *Apoe*-KO; †$p<0.05$ vs diabetic *Apoe*-KO

this model is the increase in lipids seen in the diabetic mice. However, importantly in this study, despite diabetic *Apoe*-KO mice having elevated cholesterol levels, the diabetic *At₂r*/*Apoe*-DKO mice and AT_2RB-treated diabetic *Apoe*-KO mice had similar elevations in serum cholesterol, yet demonstrated reduced plaque accumulation.

AT_2R is produced at very low levels in the cardiovascular system of the adult. Its rate of production changes according to age [39], vessel type and the presence of pathophysiological states associated with tissue remodelling or inflammation, including diabetes. In several models of renal and vascular damage characterised by an inflammatory response, AT_2R overproduction has been described [13–15]. In this study, we have reported that *At₂r* gene expression is indeed increased in the aorta from diabetic *Apoe*-KO mice.

The role of AT_2R in the vasculature is not well defined, but it has been conventionally considered to act in an opposite manner with respect to the trophic responses mediated by the AT_1R subtype. The vasodilator, antigrowth

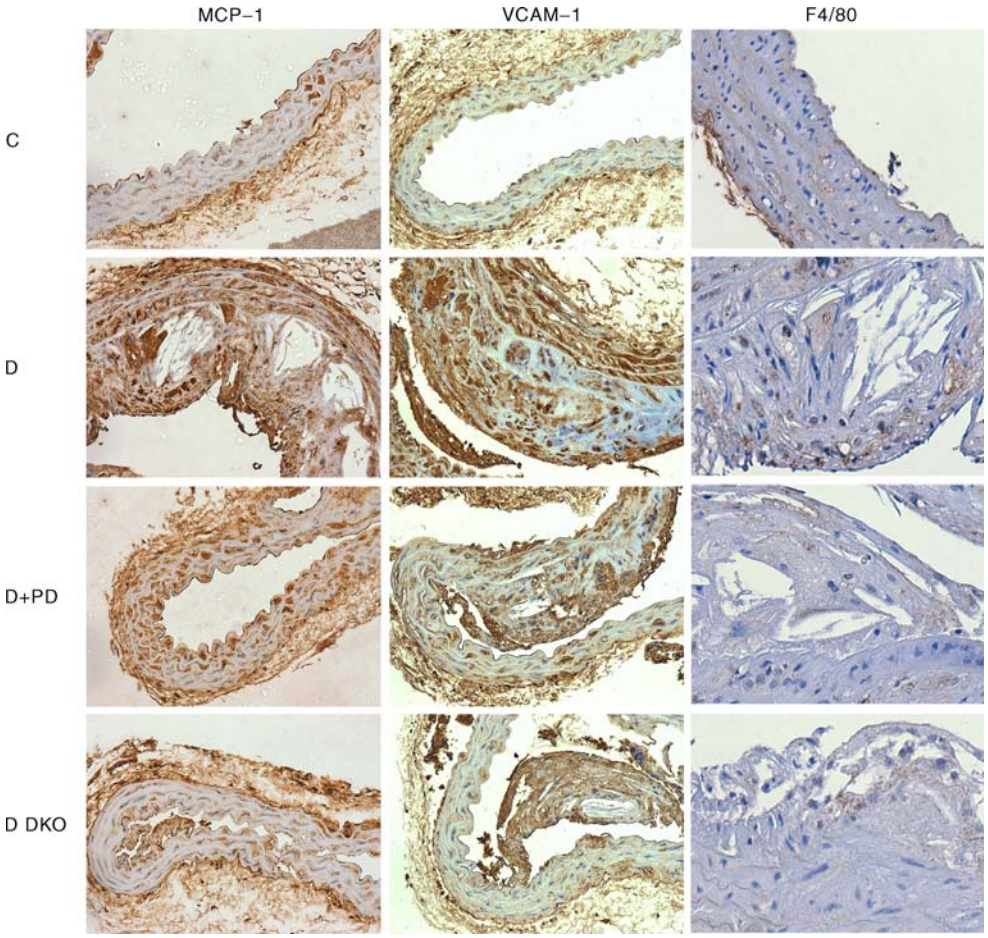

Fig. 5 Representative aortic sections stained for MCP-1, VCAM-1 and F4/80 in control *Apoe*-KO (C), diabetic *Apoe*-KO (D), AT_2RB-treated diabetic *Apoe*-KO (D+PD) and diabetic *At₂r*/*Apoe*-DKO (D DKO). Magnification ×200 for MCP-1 and VCAM-1; ×400 for F4/80

and apoptotic actions of AT_2R are postulated to act in a counter-regulatory manner to those conferred by AT_1R. Thus, the effects of stimulation of AT_2R on the cardiovascular system have been viewed as beneficial and thus no harm has been assumed as a result of increased activation of these receptors. However, there is recent evidence to suggest that activation of AT_2R could, in certain contexts, exert growth stimulatory and pro-inflammatory effects that result in complementary rather than opposite effects to AT_1R activation [22]. In the context of diabetes, our findings suggest that AT_2R may have deleterious effects in the development and progression of atherosclerosis. We have used two disparate approaches to interrupt AT_2R-dependent pathways, first a pharmacological blocker and second the use of *At₂r*/*Apoe*-DKO diabetic mice. While no clear anti-atherosclerotic effects of AT_2RB treatment or *At₂r* deficiency were observed in control animals, as reported previously by other groups [17, 20], interrupting AT_2R showed clear anti-atherosclerotic effects in diabetic mice. This is consistent with the view that AT_2R activation may enhance vascular injury in certain contexts, particularly in pathological conditions such as diabetes.

Our group has previously shown that the atherosclerotic plaques observed in these animals resemble the complex morphology seen in patients with diabetes, including enhanced accumulation of macrophages, foam cells and cholesterol clefts within the fatty atheroma in association with increased levels of inflammatory markers, pro-inflammatory cytokines and chemokines [2, 40, 41]. In line with these findings, Levy et al. observed that chronic AT_2R inhibition by PD123319 in AII-induced hypertensive rats was associated with a reduction in aortic collagen accumulation, hypertrophy and fibrosis [42]. Although the findings from the present study suggest that AT_2R plays a pro-atherogenic role in the diabetic setting, this has not been a universal finding. For example, in AII-infused or non-diabetic high-fat fed mice, two different models of atherosclerosis, blockade of AT_2R action, either using an antagonist such as PD123319 [20, 43] or deletion of this receptor subtype [17, 19], showed no effect on atherosclerosis, or possibly even an increase.

It remains unexplained why in the context of diabetes we observed a potential pro-atherogenic role for AT_2R in this study. Other studies in the atherosclerotic setting were performed in different contexts such as AII infusion [43] and high-fat diet [17] and therefore it is possible that AT_2R plays a different role in the various experimental settings that have been examined. However, in keeping with these previously reported findings, the atherosclerosis seen in non-diabetic *Apoe*-KO mice was not significantly influenced by AT_2R blockade or deletion in this study. Indeed, the mechanisms examined in this study that may be responsible for the development of atherosclerosis in the context of high-fat diet and diabetes may be different, although this has not been extensively investigated. This issue clearly needs to be fully clarified with the advent of AT_2R agonists such as C21, which has been shown to improve cardiac function in post-myocardial infarction [44]. However, the role of such an agonist has not been assessed in models of atherosclerosis, particularly with concomitant diabetes.

Increasingly, it is appreciated that the biological actions of AT_2R involve the participation of AT_2R-interacting proteins and promyelocytic zinc finger proteins, but the status of these proteins in the various contexts have not yet been fully examined [45]. Other possibilities for the different results among the various studies include the differences in mouse strains from which the *At₂r*/*Apoe*-DKO mice were generated [31, 46]. For example, in a recent study of *At₂r*/*Apoe*-DKO mice, the *At₂r*-KO mice used were on a predominantly FVB background, which is a different background from that of the *Apoe*-KO mouse [17]. By contrast, in the present study the *At₂r*-KO mice were on a C57BL6/J background, the same background as the *Apoe*-KO mice that were used to generate the *At₂r*/*Apoe*-DKO mice. Finally, in previous reports PD123319 was employed at the lower dose of 3 mg kg^{-1} day^{-1} rather than the dose of 5 mg kg^{-1} day^{-1} used in the present study and this lower dose could explain the neutral effects seen with the AT_2R antagonist in these other studies [20, 43]. The dose chosen in the present study was based on pilot studies and previous experiments using in vitro autoradiography to define a dose effectively inhibiting AT_2R without influencing AT_1R [13, 32].

AT_2R activation is involved not only in the acceleration of atherosclerotic lesion formation but also in promoting pro-inflammatory pathways considered to play an important role in diabetes-accelerated atherosclerosis. Indeed, the diabetic plaque is characterised by increased production of inflammatory cytokines, chemokines and adhesion molecules that promote leucocyte infiltration and foam cell accumulation. Gene and protein expression of VCAM-1 were upregulated in aortas from diabetic *Apoe*-KO mice and these variables were attenuated in both diabetic AT_2RB-treated and diabetic *At₂r*/*Apoe*-DKO mice. In addition, although activation of NF-κB by AII has been reported to be AT_1R dependent, there is also a significant body of literature implicating the AT_2R subtype in AII-induced NF-κB activation, as reported by Ruiz-Ortega et al. in vascular smooth muscle cells [25]. In the present study, there was upregulation of expression of genes encoding the pivotal NF-κB subunit p65 in association with upregulation of the well-characterised NF-κB-dependent chemokine, MCP-1. Production of both of these proteins was attenuated in diabetic *Apoe*-KO mice with either gene deletion of *At₂r* or with treatment with PD123319. Furthermore, other

proteins such as TNF-α that are also pro-inflammatory and linked to NF-κB activation were also modified by AT_2R blockade. This prominent inflammatory phenotype previously reported by several groups in diabetic *Apoe*-KO mice [34] remains to be fully investigated, but it appears that these changes, including macrophage infiltration, may be more prominent in the diabetic setting than in various other pro-atherosclerotic contexts such as high-fat feeding. It is hoped that ultimately more comprehensive elucidation of diabetes-specific mechanisms of atherosclerosis with a particular emphasis on inflammation may lead to a more targeted approach to reduce cardiovascular burden in diabetes.

In this study there was a reduction in extracellular matrix accumulation with blockade or deletion of *At2r*. It remains to be determined if this is a truly beneficial effect with the possibility, albeit unproven in experimental models, that a reduction in collagen content may lead to enhanced plaque rupture. Various other approaches have been reported to reduce matrix accumulation, including blockade of the RAS [2, 28] and molecular and pharmacological strategies to interrupt the advanced glycation pathway [30, 34, 41]. Indeed, some of these strategies that reduce vascular matrix accumulation, when adapted to the clinical context, have been shown to reduce cardiovascular events [47].

The potential clinical significance of AT_2R as a target for reducing atherosclerosis in diabetic patients currently remains controversial. One must be very cautious in extrapolating these positive findings linking AT_2R to diabetes-associated atherosclerosis to the clinical context. Nevertheless, a recent meta-analysis has suggested the possibility of superiority of an ACEi over an AT_1R blocker in reducing cardiovascular events [7]. However, the recent ONTARGET study [8] suggested no advantage of one approach to interrupt the RAS over the other, albeit that that study was performed predominantly in non-diabetic individuals who already had established cardiovascular disease at the time of commencement of the study. Thus, given that currently no AT_2RBs are available for clinical use, it remains to be fully clarified whether blockade of AT_2R could be a beneficial therapeutic strategy in diabetic patients, particularly since they are at a high risk of premature cardiovascular disease as a result of accelerated atherosclerosis.

Acknowledgements We thank K. Sheehy, M. Arnstein and K. Gilbert for excellent technical assistance. This work was supported by the Juvenile Diabetes Research Foundation (JDRF), the National Institutes of Health (RO1HL083452) and the National Heart Foundation of Australia. A. Koïtka was supported by an Association de Langue Française pour l'Etude du Diabète et des Maladies Métaboliques fellowship and the Société Française d'Hypertension Artérielle. A. M. D. Watson is supported by a National Health and Medical Research Council (NHMRC) of Australia Postdoctoral Biomedical Fellowship. K. A. M. Jandeleit-Dahm and T. J. Allen are recipients of NHMRC Senior Research Fellowships. M. E. Cooper is a recipient of an NHMRC Australia Fellowship and a JDRF Scholars Award.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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