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Quinapril Treatment Abolishes Diabetes-Associated Atherosclerosis in RAGE/Apolipoprotein E Double Knockout Mice

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Abstract:

Objective/Rationale: Both the renin-angiotensin system (RAS) and the receptor for advanced glycation end products (RAGE) potentiate diabetes-associated atherosclerosis (DAA). We assessed the effectiveness of concomitant RAS and RAGE inhibition on DAA.

Methods: Diabetic (5x 55mg/kg streptozotocin daily) and non-diabetic male RAGE/apolipoprotein E double knockout (RAGE/apoE DKO) mice were treated with quinapril (30mg/kg/day) for 20 weeks. At the end of the study aortic plaques were assessed.

Results: Diabetic RAGE/apoE DKO showed significantly less plaque area than diabetic apoE KO mice. Plaque deposition was almost abolished in quinapril treated diabetic RAGE/apoE DKOs, with significant attenuation of vascular collagen deposition, nitrotyrosine staining, and reduced macrophage infiltration. Expression of the advanced glycation end product receptor 3 (galectin 3) was also significantly reduced.

Conclusion: Concomitant inhibition of RAS and RAGE signalling almost completely inhibited the development of experimental DAA. A dual therapeutic approach may be a superior strategy for the treatment of diabetic macrovascular disease.

Introduction:

Diabetes-associated atherosclerosis is a leading cause of vascular disease in the aging population and is generally considered to be more inflammatory than atherosclerosis in the non-diabetic setting^{1,2}. Whilst inhibitors of the renin-angiotensin system (RAS) attenuate the development of both clinical and experimental diabetes-associated atherosclerosis, they do not completely prevent it. With the dramatic increase in the incidence of diabetes world-wide^{3,4}, more effective therapies are urgently needed to alleviate the burden of diabetes-related vascular disease.

Production of advanced glycation end products (AGEs) is significantly increased in diabetes with altered protein structure and function directly contributing to inflammation via several receptor dependent and independent pathways⁵. In diabetes the main pro-inflammatory AGE-receptor is the receptor for AGEs (RAGE)⁵. Previously we have shown that atherosclerotic apolipoprotein E knockout (apoE KO) mice which lack RAGE develop significantly less diabetes-associated aortic plaques than wild type diabetic apoE KO mice. This was associated with reduced vascular inflammation⁶. Additionally, we have shown that even in established atherosclerosis, targeting the RAS can attenuate the development of experimental diabetes-associated atherosclerosis⁷. In diabetic nephropathy, a microvascular complication of diabetes, we demonstrated that ACE inhibition and concomitant inhibition of AGE-RAGE signalling exerted renoprotective effects^{8,9}. Thus we postulate that a combination of ACE inhibition and concomitant inhibition of AGE-RAGE signalling represents a potential superior anti-atherosclerotic therapeutic approach than current strategies. Therefore we assessed the effect of quinapril on the development of diabetes-associated atherosclerosis in the diabetic RAGE/apoE double KO mouse.

Methods:

Male apoE KO mice (Animal Resource Centre, Canning Vale, Western Australia) and RAGE/apoE DKO mice (University of Heidelberg, Heidelberg, Germany^{6, 10}) were housed at the Precinct Animal Centre, Baker IDI Heart and Diabetes Research Institute and studied according to National Health and Medical Research Council guidelines in line with international standards (Alfred Medical Research and Education Precinct Animal Ethics Committee approval#E/0556/2006, n= 20/group).

Diabetes was induced at six weeks of age by 5 daily injections of streptozotocin, 55mg/kg i.p. (Sigma-Aldrich, St Louis, MO, USA) in citrate buffer. Animals had unrestricted access to water and standard mouse chow (Specialty Feeds, Glen Forrest, WA, Australia) and were maintained on a 12 h light–12 h darkness cycle. After induction of diabetes, animals were either untreated, or treated with quinapril 30mg/kg/day in drinking water (Sigma-Aldrich, St Louis, MO, USA). Systolic blood pressure was assessed by a non-invasive tail cuff system (ADInstruments, Bella Vista, NSW, Australia) as described previously¹¹.

After 20 weeks (26 weeks of age) animals were anaesthetised (i.p. injection sodium pentobarbitone 100 mg/kg body weight; Euthatal, Delvet Limited, Seven Hills, NSW, Australia). Blood was collected and plasma and glycated haemoglobin assessed as described previously⁸. Aorta were taken for *en face* analysis as described previously¹² before being embedded in paraffin for immunohistochemistry.

Quantitative RT-PCR

Extraction and analysis of total RNA from aorta with trizol was performed and cDNA generated as described previously⁶. See supplementary data *Igals3* (AGE-R3) probe and primers.

Immunohistochemistry and Staining

Staining using picosirius red was performed as described previously¹³.

Immunohistochemistry was performed to detect nitrotyrosine and F4/80 (1:50; monoclonal rat anti-mouse F4/80, Serotec, Oxford, UK) as described previously^{6, 12}. See supplemental methods for AGE-R3 immunostaining. Image capture and analysis was conducted as previously described¹¹ (see also supplementary methods).

Statistical analysis

Data were analysed by one-way ANOVA with comparisons of group means being performed by Fisher's least-significant different method (SPSS v17.0). Data are shown as means \pm SEM. $p \leq 0.05$ was considered statistically significant.

Results:

Diabetic Milieu and BP

Levels of plasma glucose were significantly increased in diabetic mice (apoE control (C) 10.5 ± 0.7 , apoE diabetic (D) 35.0 ± 2.0 $p < 0.001$; RAGE/apoE C 12.6 ± 0.6 mmol/L, RAGE/apoE C+Q 13.0 ± 1.1 mmol/L, RAGE/apoE D 35.0 ± 2.0 mmol/L, RAGE/apoE D+Q 32.1 ± 2.6 mmol/L, $p < 0.001$ D vs C, $n = 10-15$), as was glycated haemoglobin, and total cholesterol (Supplemental table 1). These values were not significantly different in quinapril treated animals. As expected, quinapril treatment of control and diabetic RAGE/apoE DKO mice reduced BP (RAGE/apoE C 122 ± 5 mmHg, RAGE/apoE 109 ± 3 C+Q mmHg, $p = 0.018$; RAGE/apoE D 117 ± 5 mmHg, RAGE/apoE D+Q 105 ± 3 mmHg, $p = 0.035$, $n = 13-20$).

Aortic Plaque Area

Both diabetic apoE KO (Figure 1a) and diabetic RAGE/apoE DKO (Figure 1b) had significantly more aortic atherosclerotic plaque compared to their respective non-diabetic controls, as assessed by *en face* analysis (Figure 1d, 1e respectively). RAGE deletion attenuated plaque formation in diabetic mice and quinapril treatment resulted in a further significant decrease in plaque area in both diabetic and control RAGE/apoE DKO animals, with plaque deposition being essentially abolished in the whole aorta (Figure 1c; RAGE/apoE D $6.9 \pm 1.4\%$, RAGE/apoE D+Q $0.6 \pm 0.1\%$, $p < 0.001$), including the aortic arch ($p < 0.001$) and thoracic aorta ($p = 0.021$)(abdominal aorta n.s.).

Fibrotic and Inflammatory Markers

Assessment of picrosirius red staining of the aortas under polarised light showed that diabetic RAGE/apoE KO mice had significantly greater accumulation of collagen I, II and III staining (Figure 2c) than non-diabetic control RAGE/apoE KO mice ($p = 0.005$, Figure 2a

respectively). Quinapril treatment significantly reduced staining for collagen I, II, and III in the aorta of diabetic animals compared to non-diabetic controls ($p=0.05$, Figure 2e). The marker of oxidative stress and nitration (peroxynitrite), nitrotyrosine, was significantly elevated in diabetic RAGE/apoE DKO mice compared to non-diabetic mice ($p=0.048$, Figure 2f-j). Quinapril significantly reduced this staining in the aorta of diabetic animals, suggesting a decrease in oxidative stress in diabetic treated animals ($p=0.017$, Figure 2j). As expected, staining for the macrophage marker F4/80 was significantly greater in diabetic compared to non-diabetic RAGE/apoE KO animals ($p=0.002$, Figure 2k-o) and was significantly reduced in diabetic quinapril treated RAGE/apoE DKO animals ($p=0.015$, Figure 2o), suggesting significantly less macrophage infiltration and accumulation in treated animals.

Gene expression of *Igals3* encoding the AGE receptor AGE-R3 (galectin 3) was significantly increased in the aortas of diabetic RAGE/apoE DKO aorta compared to control RAGE/apoE DKO. This was significantly attenuated in quinapril treated animals (see supplemental figure 1).

The majority of immunostaining for AGE-R3 was seen in the atherosclerotic plaque (Figure 2r, s). Thus, as plaque formation was almost abolished in quinapril treated diabetic RAGE/apoE DKO mice, quantification of AGE-R3 staining in the aorta of treated diabetic mice treated was negligible ($0.3 \pm 0.1\%$) (vs. RAGE/apoE D $3.3 \pm 1.1\%$)(Figure 2t).

Discussion:

For the first time we show that concurrent inhibition of the RAS together with deletion of RAGE effectively abolishes the development of experimental diabetes-associated atherosclerosis and significantly attenuates diabetes-associated increases in fibrosis and inflammation. This suggests that the AGE-RAGE axis appears to act synergistically with the RAS in the development of diabetes-associated macrovascular disease.

We have previously shown that RAGE deletion in diabetic apoE KO mice significantly reduces diabetes associated atherosclerosis by 70-75% and resulted in a decrease in inflammatory markers⁶. We have also previously shown that quinapril treatment of diabetic apoE KO mice resulted in a decrease in plaque area of approximately 70-75% with the reduction in blood pressure being similar to that seen in this study¹². Whilst the reduction in blood pressure with quinapril treatment could potentially contribute to the decrease in plaque area observed, lower blood pressure is not likely to be solely responsible for the dramatic decrease seen in plaque area in the quinapril treated RAGE/apoE mice. Previous studies by our group have shown that the calcium channel blocker amlodipine also reduced BP by an average of 25 mmHg, however this drug failed to reduce aortic plaque area¹⁴.

We demonstrated a significant attenuation of the diabetes induced increase in levels of AGE-R3 (also known as galectin-3) at both the gene and protein level when comparing quinapril treated and untreated diabetic RAGE/apoE DKO mice. AGE-R3 is known to promote LDL including oxidated-LDL and AGE uptake¹⁵, is up-regulated during monocyte-macrophage differentiation¹⁶ and promotes macrophage chemotaxis¹⁷. AGE-R3 has been found in both in plaques from apoE KO mice and human atherosclerotic plaques^{18, 19}. While apoE KO mice lacking AGE-R3 have reduced aortic atherosclerosis on a standard chow diet¹⁹, high fat feeding for 8 months resulted in significantly more atherosclerosis in the aortic sinus²⁰. More recently, AGE-R3 (galectin 3) has been suggested as a useful

biomarker for macrovascular disease²¹. Our data would support this suggestion, at least in the setting of experimental diabetes.

Previously we have demonstrated attenuation of diabetic nephropathy in RAGE/apoE DKO animals treated with quinapril when compared to untreated diabetic DKO counterparts⁸. Although diabetic nephropathy was attenuated, it was not completely prevented. Thus, it appears that in the apoE KO mouse model of diabetes induced atherosclerosis and nephropathy, inhibition of AGE-RAGE signalling and angiotensin II formation is more effective in reducing diabetes-associated macrovascular than renal microvascular disease.

Whilst several anti-AGE therapies have been trialled clinically with mixed results²² beneficial cardiovascular effects have been reported, in particular for arterial compliance²³. To our knowledge there is no data available on clinical inhibition of RAGE itself in diabetes, although clinical trials are in progress in relation to Alzheimer's disease²⁴.

The results of this study strongly suggest that combined inhibition of AGE-RAGE signalling and the RAS holds therapeutic potential for the treatment and prevention of diabetes-associated macrovascular disease, however further clinical investigation is warranted to validate this dual therapy.

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Conflict of Interest: The authors have nothing to disclose.

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Figure 1. Sudan IV stained apolipoprotein E knockout (apoE- **a, d**) and RAGE/apoE double (DKO) mouse aorta (**b, c, e, f**), both diabetic (D; **a, b, c**) and non-diabetic controls (C; **d, e, f**) and RAGE/apoE DKO mice with quinapril treatment (+Q; **c, f**). En face analysis of intimal aortic plaque area (**g, h, i, j**) shows D animals had significantly increased plaque area in both strains compared to C. Treatment of RAGE/apoE mice with quinapril (Q) virtually abolished plaque area. n= 5-8, p<0.05: * vs apoE D, † vs RAGE D, ** vs apoE C, ‡ vs RAGE C+Q. Abdominal aorta (**j**):

Figure 2 Aorta from RAGE/apoE double KO mice stained for collagen I, II and II with picrosirius red (under polarised light) (**a-e**), the oxidative stress marker nitrotyrosine (**f-j**), the macrophage marker F4/80 (**k-o**) and advanced glycation receptor 3 (AGE-R3, **p-t**). AGE-R3 staining was most intense in the atherosclerotic plaque, with only a small amount of staining also seen in the endothelium and media of the adjacent vessel wall of diabetic animals.

Untreated non-diabetic control (C) animals (**a, f, k, p**), quinapril treated (Q) control animals (**b, g, l, q**), untreated diabetic (D) animals (**c, h, m, r**) and quinapril treated diabetic animals (**d, i, n, s**). Digital quantification of staining (**e, j, o, t**) shows that diabetic mice treated with quinapril have staining levels equivalent to that seen in non-diabetic control mice. Scale bars = 50µm. n=5-8, p<0.05 † vs RAGE D.

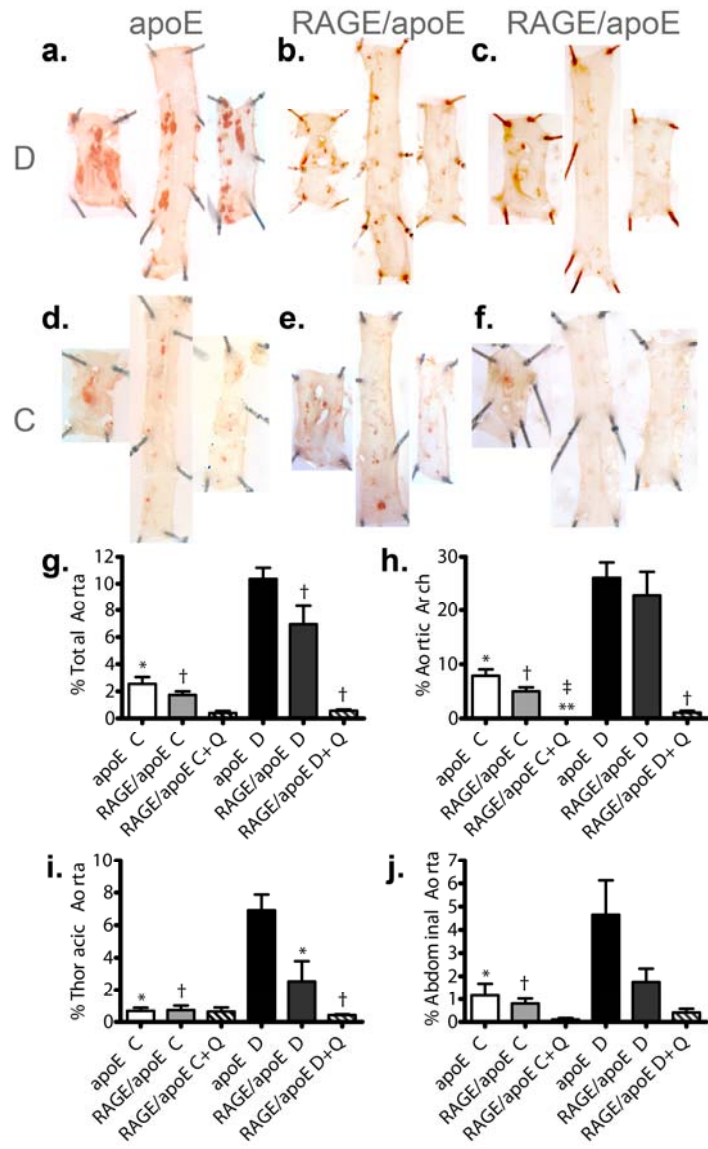


Figure 1

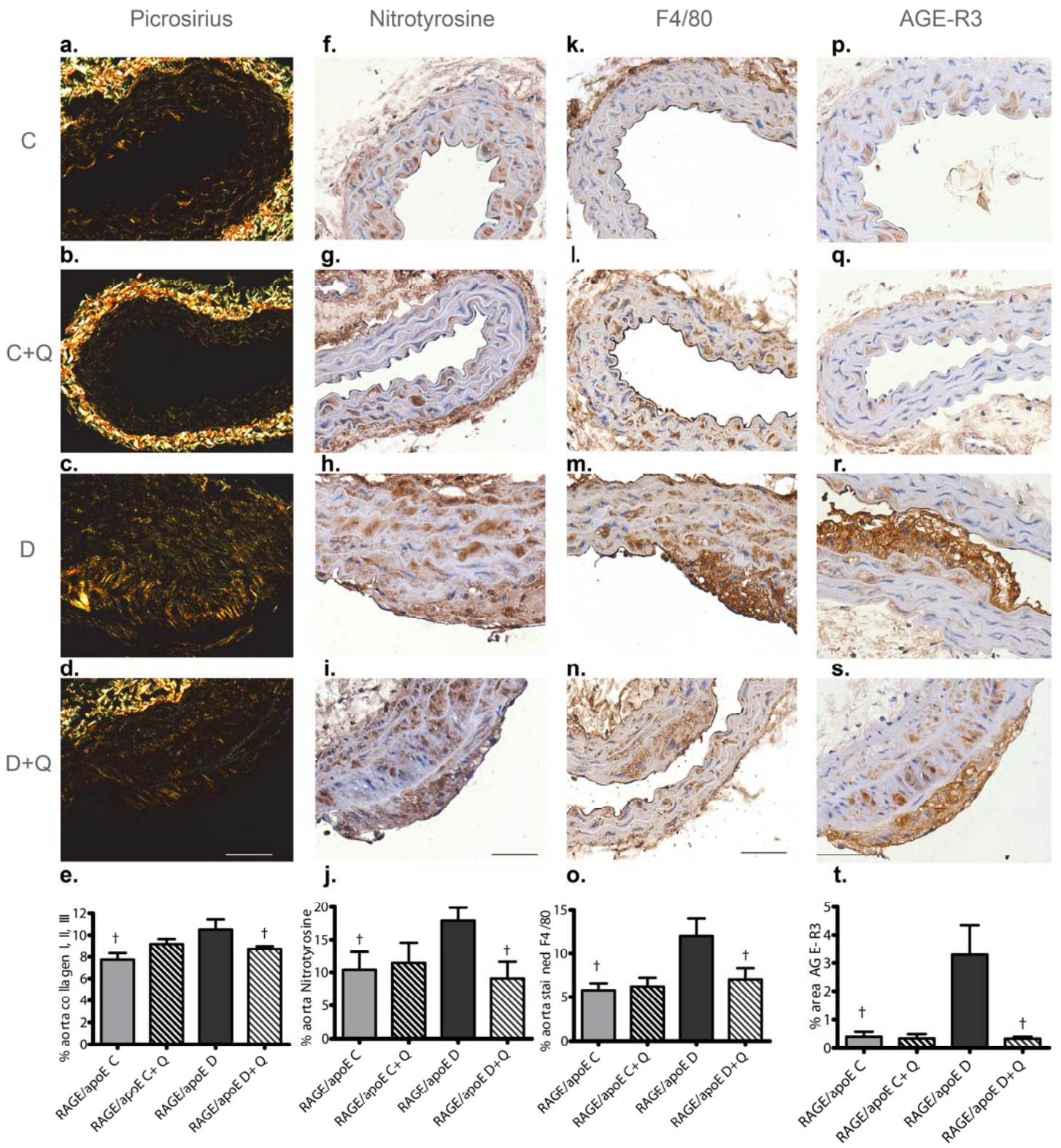


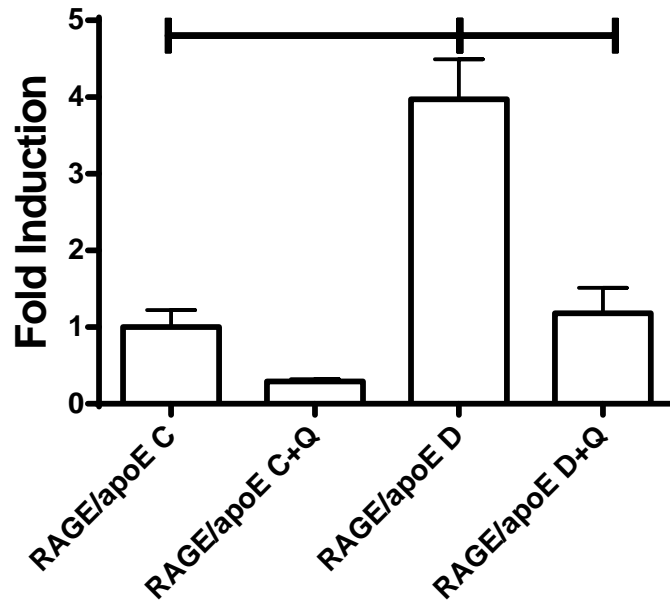
Figure 2

Supplementary Data Table 1: Metabolic parameters and blood pressure as assessed by tail cuff. Data are presented as mean±SEM. *

p<0.05 vs relative control ; # p< 0.05 vs relative control or diabetic ApoE. n=10-21

Animal Groups	CONTROL			DIABETES		
	ApoE KO	RAGE/ApoE DKO	RAGE/apoE DKO + Quinapril	ApoE KO	RAGE/ApoE DKO	RAGE/apoE DKO + Quinapril
Glycated Hb (%)	4.3±0.1	4.1±0.2	4.0±0.1	14.3±0.4*	13.7±0.5*	13.8±0.3*
Cholesterol (mM/L)	10.5±0.7	11.4±0.5	11.5±0.7	21.2 ±1.2*	16.9±1.2*#	18.3±1.8*

Supplemental Figure 1



Supplementary Figure 1. Gene expression of *Igals3*, encoding AGE-R3 (or galectin 3) is significantly increased in diabetic (D) RAGE/apoE mouse aorta, as compared to non-diabetic control (C) ($p < 0.001$). Quinapril treated animals (+Q) had significantly less *Igals* expression than untreated diabetic animals ($p < 0.001$). $n = 6-8$.

Supplemental Methods:

Igals3 Probes and Primers

Igals3 (receptor of advanced glycation end products-R3) probe and primers. used the following primer and probe sequence run on the Taqman system (ABI Prism 7500; Perkin-Elmer, Foster City, CA, USA) (probe: CTGGCTCTGGAAACC , F: CAGACAGCTTTTCGCTTAACGA , R: CCATGCACCCGGATATCC, GeneBank X16834). Expression was normalised to the housekeeping gene ribosomal subunit 18S and expressed relative to untreated non-diabetic control RAGE/apoE aorta.

AGE-R3 immunostaining

AGE-R3 immunostaining (1:100, rabbit anti-galectin-3, Santa Cruz Biotechnology, Biolab, Clayton, Vic, Australia) was performed on paraffin sections (4 µm thick) which had endogenous peroxide activity quenched (3% H₂O₂ 10 min) were incubated in 'protein blocking agent' (Thermo Electron, Pittsburgh, PA, USA, 30min) then primary antibody (4°C overnight) followed by. biotinylated anti-rabbit (Vector Laboratories Inc., Burlingame, CA, USA, 10min), avidin-biotin horseradish peroxidase complex (Vectastain ABC ELITE kit, Vector Laboratories, 30min) and visualised with 3-3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St.Louis, CO, USA) in 0.08% H₂O₂. Sections were counterstained with Mayer's hemotoxylin and Scott's tap water.

Quantification of Immunohistochemistry

In a blinded manner aorta sections were captured as photo-micrographs (Olympus BX-50, Olympus Optical; Q-imaging MicroPublisher 3.3 RTV camera, Surrey, BC, Canada) under identical light conditions and the percentage area of the aorta stained (excluding adventitia) digitally quantitated based on red, green and blue channels (Image Pro-Plus ver. 6.0; Media Cybernetics, Silver Spring, MD, USA).