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Angiotensin receptor 1 blockade reduces secretion of inflammation associated cytokines from cultured human carotid atheroma and vascular cells in association with reduced extracellular signal regulated kinase expression and activation

Paula Clancy[a], Simon A. Koblar[b], Jonathan Golledge[c,d]

[a]Health practitioners And Researchers Together-Blood, Endothelium And Tissue (HART-BEAT), Australian Institute for Tropical Health and Medicine (AITHM), School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Queensland, Australia;

[b]Stroke Research Programme, School of Medicine, The Queen Elizabeth Hospital (TQEH) campus, Adelaide University, Woodville South, South Australia, Australia;

[c]The Vascular Biology Unit, Queensland Research Centre for Peripheral Vascular Disease, School of Medicine and Dentistry, James Cook University, Townsville, Queensland, Australia;

[d]Department of Vascular and Endovascular Surgery, The Townsville Hospital, Townsville, QLD, Australia

Correspondence and reprint requests to:

Dr Paula Clancy,

HART-BEAT,

AITHM,

School of Veterinary and Biomedical Sciences,

James Cook University,

Townsville,

Queensland,

Australia 4811

Tel: +61 7 4781 3295

Email: paula.clancy@jcu.edu.au

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Abstract

Background: A number of studies have suggested that angiotensin II (AII) receptor type 1 (ATR1) blocking drugs (ARBs) have anti-inflammatory effects however the mechanisms responsible are poorly investigated.

Objective: To determine the role of extracellular signal regulated kinase (ERK)1/2 in ARB induced anti-inflammatory effects within human carotid atherosclerosis.

Methods: Atheroma samples obtained from patients undergoing carotid endarterectomy were cultured with and without ATR1 (irbesartan), ERK1/2 (PD98059), AII ([Sar^1, Ile^8]-AII) and angiotensin converting enzyme (ACE)2 (DX600) blockade. The *in vitro* effects of ATR1 and ERK1/2 blockade and exogenous AII on serum stimulated healthy, primary vascular cells were also investigated. Outcome was assessed by measuring cytokine, (interleukin (IL)-6, IL-8, C-C motif chemokine (CCL)2, C-X-C motif chemokine (CXCL)5, osteoprotegerin (OPG), osteopontin (OPN), CXCL16), concentrations in supernatants and phosphorylated ERK1/2 in the tissue lysates using ELISA. ERK1/2 expression in the tissue was assessed using Western blotting.

Results: Irbesartan reduced concentrations of IL-6, IL-8, CCL2, CXCL5, OPG, OPN and CXCL16 in both atheroma and primary vascular cell culture supernatants. The reduction in cytokine levels in the atheroma supernatant was correlated to a reduction in ERK1/2 expression in the tissue. Inhibition of ERK1/2 downregulated IL-6, IL-8 and CXCL5 in both atheroma and cell culture supernatants. AII and ACE2 blockade had no impact on cytokine or active ERK1/2 levels in the atheroma culture.

Conclusion: Our findings suggest that ATR1 blockade downregulates atheroma tissue ERK1/2 expression leading to a reduction in cytokine production and that a non-AII agonist ATR1 signalling response may induce expression of these inflammation associated cytokines in the atheroma.

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1. Introduction

The renin-angiotensin signalling pathway plays a key role in regulating the cardiovascular system. It is widely accepted that angiotensin II (AII) signalling through angiotensin receptor (ATR)1 (Fig. 1) promotes cardiovascular events. Treatment with ATR1 blocking drugs (ARBs) has been associated with a reduction in cardiovascular events, such as stroke, in a number of clinical trials [1-3]. These studies suggest ARBs have an impact beyond their anti-hypertensive actions [1,3] and can directly reduce inflammation associated with cardiovascular disease (CVD) [4-5] with reductions in levels of a range of inflammatory cytokines in patients' receiving ARB therapy [6-15]. AII/ATR1 signalling also leads to the activation of several downstream kinase pathways, such as extracellular signal regulated kinases (ERK)1/2 [16], which are also associated with inflammatory cytokine upregulation [17-18].

Examining the cellular signalling responses that lead to the anti-inflammatory effects of ATR1 blockade in patients is not straight forward. One approach is the use of tissue explant culture [19-22]. This culture system has been demonstrated to simulate the *in vivo* AII response to ARB [6,23-24] with an increase in AII levels in the atheroma explant culture supernatant with ATR1 blockade [19].

In this study we aimed to investigate the impact of ATR1 and ERK1/2 blockade on inflammatory cytokine production *in vitro* using two different culture systems. The first system utilised cultured, diseased, human carotid atheroma tissue [19-22]. The second system used healthy, primary vascular cells stimulated by serum [22]. Previous studies have demonstrated that exposure of smooth muscle cells (SMC) or fibroblasts to blood initiates cell signalling responses, such as inflammatory cytokine release, within the vasculature [25].

There are a number of studies reporting that the ATR1 can be stimulated by non-AII agonists, which are also inhibited by ARBs [26-29]. A secondary element of this study was to investigate the role of AII in promoting inflammatory cytokine secretion from atheroma. The effect

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of an AII peptide antagonist [30-35] and an angiotensin converting enzyme (ACE)2 inhibitor on inflammatory cytokine secretion were also investigated. ACE2 is the carboxypeptidase which converts AII to Ang-(1-7). The authors demonstrated previously that blockade of ACE2 activity in atheroma increased AII levels ~10-fold *in vitro* [19].

The aims of this study were threefold: Firstly, to clarify the role of ERK1/2 in the ability of ATR1 blockade to limit inflammation in human carotid atheroma, secondly to investigate the role of AII in stimulating inflammation in human carotid atheroma and thirdly, to determine if ATR1 and ERK1/2 blockade had anti-inflammatory effects in human, primary vascular cells stimulated by serum.

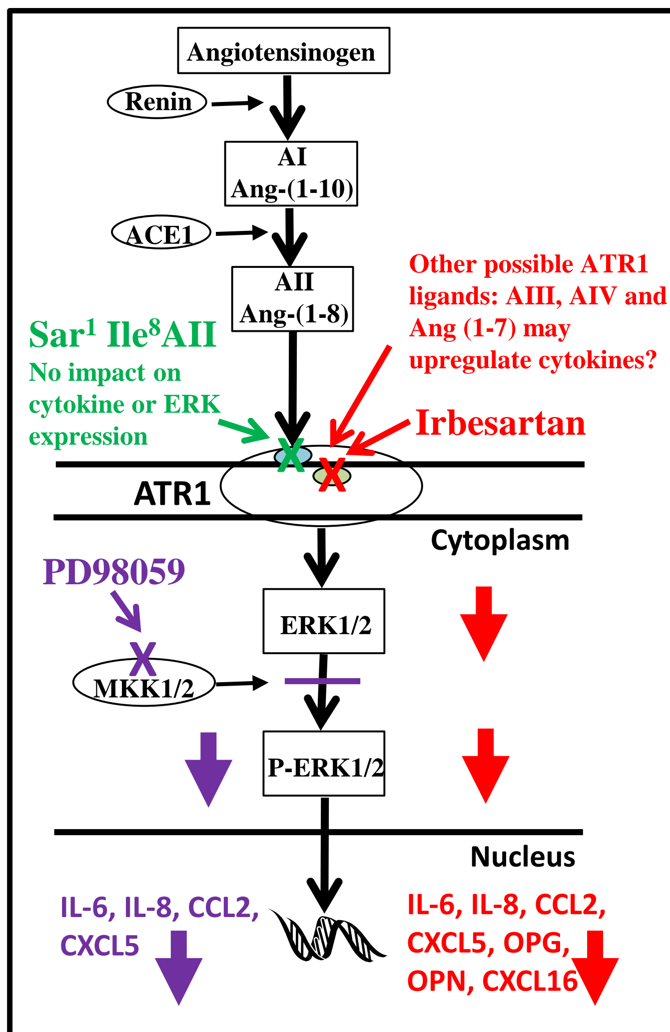

Fig. 1. The angiotensin II/ angiotensin type 1 receptor signalling pathway. Presented is a simplified pathway by which ATR1 activation potentially leads to inflammatory cytokine production. ATR1 has two different, distinct binding sites, an extracellular pocket for the angiotensin peptides (blue) and a separate membrane binding site (green) for the non-peptide diphenylimidazole antagonists, such as irbesartan (red cross). AII binding was blocked by the specific peptide antagonist, [Sar^{1}, Ile^{8}]-AII (green cross), which binds directly to the peptide binding pocket (blue). Activation of ERK1/2 was blocked by PD98059, which binds to MKK1/2, inactivating the enzymes capacity to phosphorylate ERK1/2 (purple cross).

2. Methods

2.1. Patients

Patients were recruited from those selected to undergo primary carotid endarterectomy at The Townsville and Mater Hospitals, Queensland, Australia between Jul 2011 and Feb 2013. Inclusion criteria included: 1) verbal and written informed consent; 2) carotid stenosis of ≥ 70%

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identified on duplex imaging using criteria previously described [20]; and 3) no previous carotid artery intervention. Ethics approval was provided by the appropriate committees (Townsville Health Service District, 61/05). Patients with a history of transient ischemic attack (TIA), stroke with good recovery and those with an asymptomatic stenosis were included. Please refer to Appendix A online for the patient characteristics (http://atherosclerosis-journal.com).

2.2. Explant Culture

Explant culture was carried out as previously described [19-22]. Two macroscopically similar, disease matched, atheroma biopsy pairs were obtained from each patient. The interventional agent concentrations were based on circulating concentrations measured in patients receiving therapeutic levels or published effective *in vitro* concentrations. Therapeutic circulating concentrations of irbesartan are reported to be between ~1-10 mmol/L [36-37]. The MKK1/2 inhibitor, PD98059, binds directly to MKK1/2 blocking it's capacity to phosphorylate ERK1/2, consequently blocking ERK1/2 activation [38]. When used at a concentration of 20 μmol/L, PD98059, abolished AII induced upregulation of C-reactive protein from vascular SMCs [39]. The AII peptide antagonist, [Sar^1, Ile^8]-AII, has been used in culture at a variety of concentrations ranging from 0.1 μmol/L [30-31], 1 μmol/L [32-34] to 10 μmol/L [35].

The following interventions were assessed: a) ATR1 (Irbesartan; 2 mmol/L; Sigma #I2286); b) ERK1/2 (PD98059; 20 μmol/L; Sigma #P215); c) AII ([Sar^1, Ile^8]-AII; 10 μmol/L; Auspep #2153); d) ACE2 (DX600; 1 μmol/L; Anaspec #62337) [19].

2.3. Primary vascular cell culture

Due to the nature of the carotid endarterectomy procedure the types of vascular cells present in the explant tissue include: endothelial cells (ECs), SMCs, inflammatory cells which have infiltrated the atheroma from the blood and red blood cells (RBCs) on the tissue surface. A

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previously developed mixed, healthy, primary vascular cell technique [22] was used to assess the impact of ATR1 and ERK1/2 activity blockade, and increasing levels of AII on inflammatory cytokine production from vascular cells stimulated by serum. Therapeutic circulating concentrations of the ARB, losartan, range from ~0.1-1.5 μmol/L [40-41] and the normal level of AII found in the blood ranges from 18.4-47.6 pg/mL [6].

The following interventions were assessed: a) Irbesartan at 0, 0.144, 0.575, 2.3 and 4.6 mmol/L, b) Losartan at 0, 40.6, 162.5, 650 and 1300 nmol/L, c) PD98059 at 0, 1.25, 5, 20 and 40 μmol/L and d) AII at 0, 0.2, 2, 20 and 200 pmol/L in triplicate. Details of the culturing conditions are presented online in Appendix A.

2.4 Assessment of supernatant inflammatory cytokine and AII concentrations

Commercially available ELISA kits were used to measure the expression of interleukin (IL)-6, IL-8, C-C motif ligand (CCL)2, C-X-C motif ligand (CXCL)5, osteoprotegerin (OPG), osteopontin (OPN) and CXCL16 in the culture supernatants (R&D Systems) following the manufacturer's instructions. AII levels were assessed as previously reported [19]. Details of each ELISA and the assay conditions used are presented online in Appendix A.

2.5 Assessment of atheroma protein concentrations

Protein was extracted from the cultured atheroma and quantified as previously described [19]. Total protein content of each explant sample was used to normalise between the samples cultured with and without intervention. Atheroma concentrations of the phosphorylated, activated forms of ERK1 and ERK2 (ERK1-P and ERK2-P), were measured in the explant tissues by ELISA (R&D Systems). This ELISA system utilises antibodies that specifically detect the phosphorylated residues of T202/T204 and T185/T187 for ERK1 and ERK2, respectively. This means the ELISAs will detect changes arising from either a change in overall expression of ERK1/2 or the degree of

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phosphorylation. ERK1 and ERK2 levels were determined using Western blotting. Details of each ELISA and the Western blotting assay conditions are presented online in Appendix A.

2.6. Data Analysis

The normalised amount of inflammatory cytokines, ERK and ERK-P proteins from atheroma exposed to interventions were presented as mean ± SEM of the relative ratios in paired, experimental and control samples [19-22]. Differences between experimental and control samples were assessed using Wilcoxon's paired test. Values for experimental samples <1 demonstrate the intervention reduced expression while values =1 demonstrate the intervention had no effect. Correlations of total well expression of atheroma proteins were assessed using Spearman's rho correlation. GraphPad Prism 6 software was used for all the explant sample analyses. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Heterogeneity of human carotid atheroma samples from different patients

Concentration of IL-6, IL-8, CCL2, CXCL5, OPG, OPN and CXCL16 present in the control atheroma supernatant from all of the studies combined (n=42 samples from 21 patients) and ERK1-P and ERK2-P in the control atheroma tissue (n=34 samples from 17 patients) were measured using ELISA. The mean normalised amount of expressed protein ± SEM (ng/mg) in the control cultures were as follows: IL-6, 347.1±58.4 ranging from 3.3-13511; IL-8, 59.8±10.4 ranging from 2.8-265; CCL2, 97.1±21.8 ranging from 4.9-917.9; CXCL5, 32.2±5.5 ranging from 0.8-134.7; OPG, 224.3±35.8 ranging from 14.7-1333; OPN, 772±123.9 ranging from 35.4-3706; CXCL16, 4.0±0.7 ranging from 0.2-24.7; ERK1-P, 0.95±0.11 ranging from 0.02-2.13; ERK2-P, 0.23±0.03 ranging from 0.003-0.72 (Fig. 2A-I).

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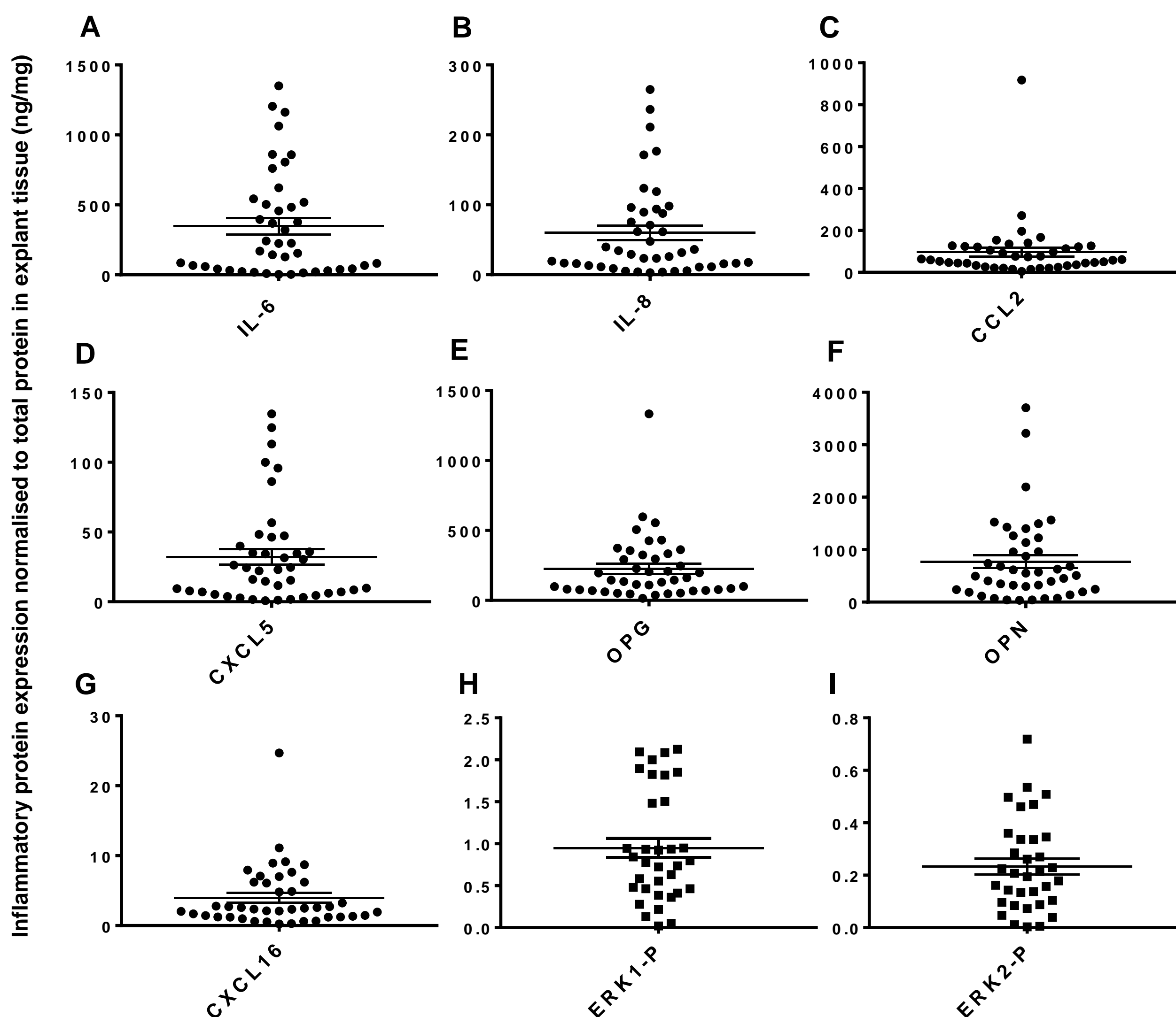

Fig. 2. Variation in inflammatory cytokine and activated ERK levels in cultured carotid atheroma samples. Mean ± SEM of (**A**) IL-6, (**B**) IL-8, (**C**) CCL2, (**D**) CXCL5, (**E**) OPG, (**F**) OPN, (**G**) CXCL16, (**H**) ERK1-P and (**I**) ERK2-P detected in the control culture supernatant and tissue, normalised to the total amount of protein present in the explant tissue (n=42 samples for inflammatory cytokines and n=34 samples for ERK-Ps). Protein expression was measured by ELISA. Black circles indicate individual supernatant results and squares indicate individual tissue results.

3.2. Effect of ATR1 blockade on inflammatory protein expression in human carotid atheroma culture

Incubation of human carotid atheroma samples with irbesartan (ATR1 specific inhibitor, 2.3 mmol/L, n=12 pairs) for 4d significantly reduced expression of IL-6 (~0.31-fold change, P=0.0037), IL-8 (~0.34-fold change, P=0.0022), CCL2 (~0.03-fold change, P=0.0022), CXCL5 (~0.02-fold change, P=0.0022), OPG (~0.33-fold change, P=0.0029), OPN (~0.13-fold change, P=0.0022) and CXCL16 (~0.06-fold change, P=0.0022) in the culture supernatant and ERK1-P (~0.21-fold change,

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P=0.0022), ERK2-P (~0.18-fold change, P=0.0029) and ERK1 (~0.45-fold change, P=0.0022) in the atheroma tissue. ERK2 was not consistently detectable in the tissue. Irbesartan reduced ERK2 expression at borderline significance (~0.52-fold change, P=0.0679) (Fig. 3A).

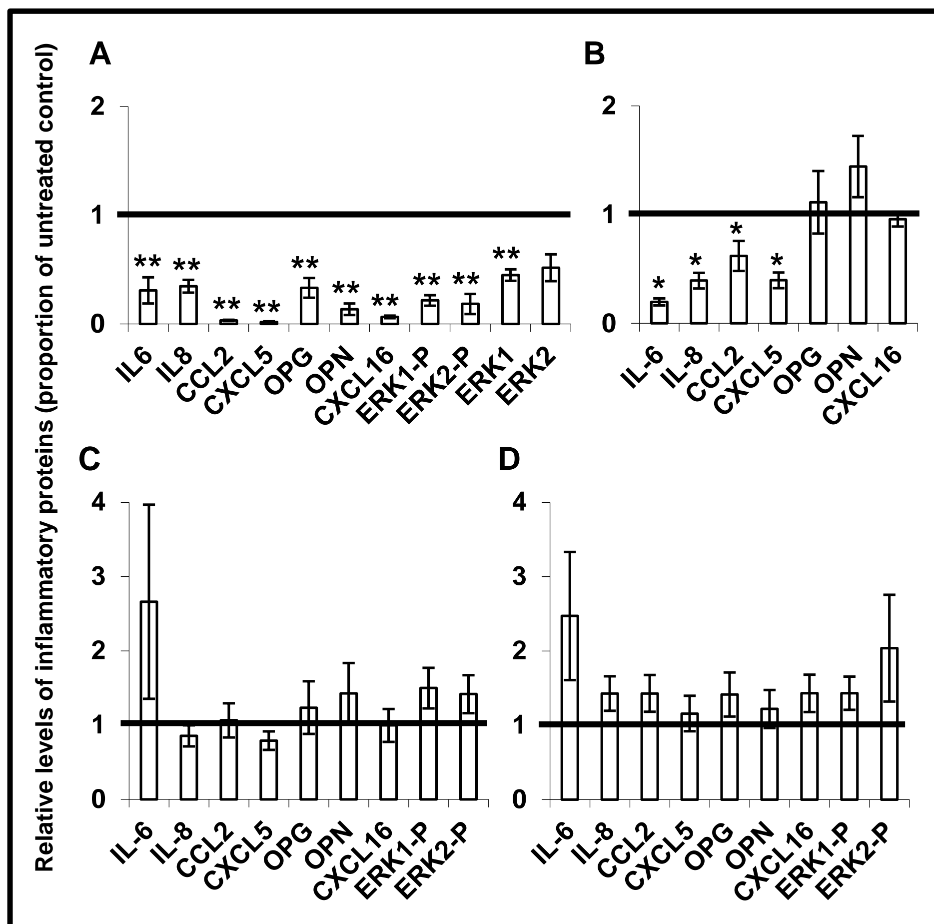

Fig. 3. Changes in inflammatory response in carotid atheroma samples cultured with different interventions. Relative expression of inflammatory cytokines in the supernatant and ERKs in the tissue after 4d culture with and without (**A**) ATR1 (irbesartan, 2.3 mmol/L, n=12 pairs); (**B**) ERK1/2 (PD98059, 20 µmol/L, n=8 pairs), (**C**) AII ([Sar[1], Ile[8]]-AII, 10 µmol/L, n=10 pairs) and (**D**) ACE2 (DX600, 1 µmol/L, n=12 pairs) blockade. Shown are the mean values ± SEM of the ratio of inflammatory cytokine or signalling protein (normalised to total protein) expressed by paired atheroma samples incubated with intervention relative to control. Protein expression was measured by ELISA for all except ERK1 and ERK2, which were determined by Western blotting. **P<0.01; *P<0.05 using Wilcoxon's paired test. (1=no change-denoted by horizontal line, <1 decreased with blockade)

3.3. Effect of blockade of ERK1/2 activation on inflammatory cytokine expression in human carotid atheroma supernatant

Incubation of human carotid atheroma samples with PD98059 (ERK1/2 specific inhibitor, 20 µmol/L, n=8 pairs) for 4d significantly reduced expression of IL-6 (~0.20-fold change, P=0.0117), IL-8 (~0.39-fold change, P=0.0117), CCL2 (~0.62-fold change, P=0.0357) and CXCL5 (~0.40-fold change, P=0.0117) in the culture supernatant. There was no significant changes in expression of any of the other proteins assessed (p>0.05) (Fig. 3B).

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3.4. Effect of AII peptide and ACE2 activity blockade on inflammatory protein expression in human carotid atheroma culture

Incubation of the atheroma samples with [Sar^1, Ile^8]-AII (AII peptide specific inhibitor, 10 μmol/L, n=10 pairs) for 4d caused no significant changes in expression of any of the proteins assessed ($p>0.05$) (Fig. 3C). Equally, incubation of the atheroma samples with DX600 (ACE2 inhibitor, 1 μmol/L, n=12 pairs) for 4d caused no significant changes in expression of any of the proteins assessed ($p>0.05$) (Fig. 3D).

3.5. Correlation between cytokines and the ERK signalling proteins in the experiment assessing the effect of ATR1 blockade on cultured human carotid atheroma

Unlike the heterogeneity study that only used the control samples, this study assessed the correlation between the secreted and cellular proteins in the matched irbesartan cultured (2.3 mmol/L; n=12 samples) and control samples (n=12 samples). The aim was to determine if there was a direct association between the reduction in ERK1/2 expression in the tissue and the reduced inflammatory cytokine levels in the supernatant.

Expression of ERK1 (n=24 samples from 12 matched pairs) in the atheroma tissue was strongly correlated to the level of its activated, phosphorylated form, ERK1-P (Spearman's rho=0.6887, P=0.0002), and expression of IL-6, IL-8, CCL2, CXCL5, OPG, OPN and CXCL16 in the supernatant (Spearman's rho=0.6878, P=0.0002; Spearman's rho=0.6313, P=0.0009; Spearman's rho=0.6703, P=0.0003; Spearman's rho=0.7106, P<0.0001; Spearman's rho=0.6626, P=0.0004; Spearman's rho=0.5984, P=0.0020; Spearman's rho=0.6915, P=0.0002) (Table 1).

ERK2 was not consistently detectable in the tissue. Expression of ERK2 (n=8 samples) in the atheroma tissue was correlated to the level of its activated, phosphorylated form, ERK2-P (Spearman's rho=0.7381, P=0.0458), and expression of IL-6, CCL2, OPG and CXCL16 in the supernatant (Spearman's rho=0.8333, P=0.0154; Spearman's rho=0.8503, P=0.0115; Spearman's

rho=0.9762, P=0.0004; Spearman's rho=0.7619, P=0368, respectively) (Table 1). ERK2 demonstrated a correlation with IL-8, CXCL5 and OPN at borderline significance (Spearman's rho=0.6667, P=0.0831; Spearman's rho=0.7143, P=0.0576; Spearman's rho=0.6826, P=0.0729, respectively) (Table 1).

Table 1 Correlation of ERK proteins and inflammatory cytokines in the study examining the effect of ATR1 blockade on human carotid atheroma

	Tissue ERK1		**Tissue ERK2**	
	rho	**P value**	**rho**	**P value**
IL-6	**0.6878*****	0.0002	**0.8333***	0.0154
IL-8	**0.6313*****	0.0009	0.6667	0.0831
CCL2	**0.6703*****	0.0003	**0.8503***	0.0115
CXCL5	**0.7106******	<0.0001	0.7143	0.0576
OPG	**0.6626*****	0.0004	**0.9762*****	0.0004
OPN	**0.5984****	0.0020	0.6826	0.0729
CXCL16	**0.6915*****	0.0002	**0.7619***	0.0368
ERK1-P	**0.6887*****	0.0002	**0.7619***	0.0368
ERK2-P	**0.6765*****	0.0003	**0.7381***	0.0458

Spearman's rho correlations between the secreted inflammatory cytokines in the supernatant and the ERK signalling proteins in the matched atheroma tissue samples incubated with and without irbesartan (2.3 mmol/L) were performed using the total amount of each protein in the samples (n=24 for ERK1 and n=8 for ERK2). As the explant culture system is a closed system the correlations were determined using the total amounts of ERKs and inflammatory cytokines present within each culture well.

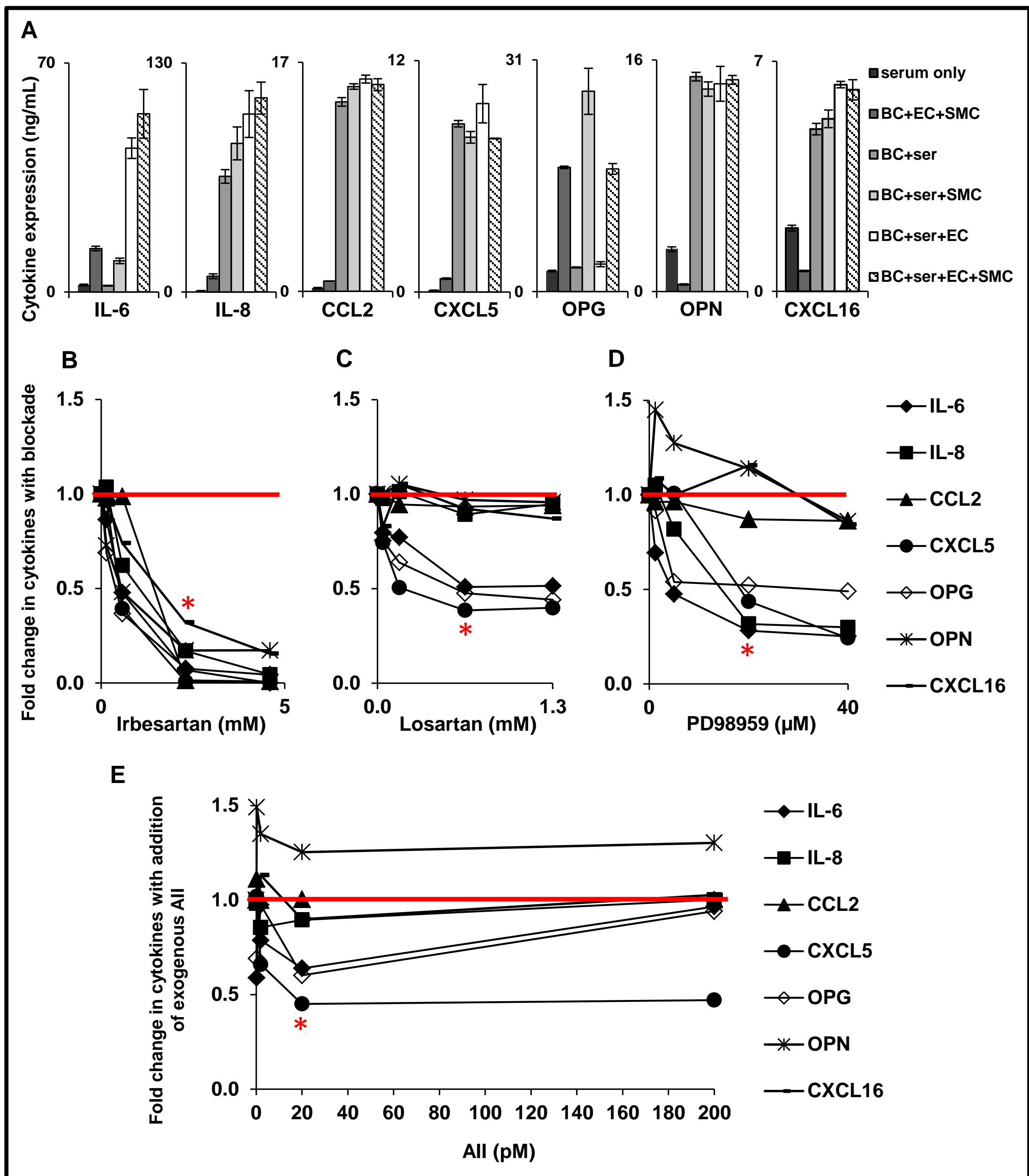

Fig. 4. Changes in inflammatory response in mixed, healthy, primary vascular cell culture with different interventions. Expression of inflammatory cytokines in the supernatant of healthy, primary vascular cells (ECs, SMCs, BCs) stimulated by serum (4d culture). Inflammatory cytokine expression was measured by ELISA. (**A**) Various serum and cell combinations demonstrating an inflammatory response to the serum. Shown are the mean values of triplicates ± SEM. Relative expression of inflammatory cytokines after 4 days culture with titrated (**B**) irbesartan (ATR1 blockade), (**C**) losartan (ATR1 blockade), (**D**) PD98059 (ERK1/2 blockade) and (**E**) exogenous AII. SEMs were too small to present. The asterisked concentration was used in the atheroma intervention studies and is the level of AII found in normal blood [6] (1= no change-denoted by horizontal line, <1 decreased with blockade)

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3.6. Effect of ATR1 and ERK1/2 blockade on inflammatory cytokine expression in mixed, healthy, primary vascular cells stimulated by human serum in vitro

The level of the inflammatory cytokines, IL-6, IL-8, CCL2, CXCL5, OPG, OPN and CXCL16, secreted by healthy, primary vascular cells increased with addition of normal human serum suggesting an inflammatory response by the cells to the serum (Fig. 4A). Within the mixed culture IL-6 appears to be predominantly secreted by ECs, OPG by SMCs and IL-8, CCL2, CXCL5, OPN and CXCL16 by cells derived from the blood (Fig. 4A). Titration of irbesartan in the mixed culture (EC+SMC+BC+serum) downregulated secretion of IL-6, IL-8, CCL2, CXCL5, OPG, OPN and CXCL16 (Fig. 4B). Titration of another ARB, losartan, also downregulated secretion of IL-6, CXCL5 and OPG (Fig. 4C). Titration of PD98959 downregulated secretion of IL-6, IL-8, CXCL5 and OPG (Fig. 4D) in a dose dependent manner. Titration of AII in the mixed culture had no impact on any of the inflammatory cytokines measured, except CXCL5, which showed a reduction in secretion with increasing AII (Fig. 4E).

4. Discussion

In the present study, using human atheroma explant culture we demonstrated, that ATR1 blockade reduces expression of a range of inflammatory cytokines and the ERK1/2 signalling proteins. It is widely accepted that AII has a role in atherosclerosis and that therapies which block AII binding to ATR1 are beneficial to cardiovascular patients in reducing the incidence of cardiovascular events, such as stroke [1-3]. A fundamental aim of this study was to determine the influence of the AII/ATR1/ERK axis (Fig. 1) on inflammatory cytokine secretion within human carotid atherosclerosis.

Blockade of ATR1 with irbesartan had an anti-inflammatory impact on the atheroma reducing expression of all the inflammatory cytokines measured, which included IL-6, IL-8, CCL2, CXCL5, OPG, OPN and CXCL16. This response was in keeping with many studies investigating

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the *in vivo* response to ARB therapy. Circulating IL-6 has been demonstrated to drop with a range of ARBs, including irbesartan [7-11]. The other inflammatory proteins investigated in this study are not as well studied, but ARB therapy has also been shown to decrease IL-8, MCP-1 and OPN levels *in vivo* [6,10-15].

It is not easy to determine the cellular signalling responses that lead to the *in vivo* inflammatory cytokine responses to ARB therapy in patients, but it is possible to measure the response to ATR1 blockade in cultured atheroma tissue [19-22]. ATR1 blockade, with irbesartan, also led to a decrease in expression of the tissue signalling proteins, ERK1 and ERK2. The reduction in the ERK signalling proteins correlated to the reduction in the expression of the inflammatory cytokines in the supernatant and the levels of their respective activated, phosphorylated forms suggesting that the reduction in active ERK1/2 with ARB may be a direct result of downregulation of expression of the proteins rather than a reduction in the degree of phosphorylation. A previous study using left ventricles from spontaneously hypertensive rats also demonstrated a downregulation of ERK1 expression in the diseased ventricle tissue with irbesartan administration that correlated to the downregulation of activated levels [42].

The role of ERK1/2 activation in inflammatory cytokine secretion from the atheroma was investigated using the specific MKK1/2 blocking agent, PD98059, which blocks activation of ERK1/2. Blockade of ERK1/2 activation led to a significant decrease in the expression of IL-6, IL-8, CCL2 and CXCL5. In combination these results suggest ERK activation occurs in the signalling response that leads to the expression of these inflammatory cytokines. However, OPG, OPN and CXCL16 expression were also correlated to the expression of the ERKs, but not downregulated with ERK activity blockade, suggesting these inflammatory cytokines are upregulated in conjunction with the ERKs not as a result of their activation.

The anti-inflammatory impact of ATR1 and ERK1/2 blockade was also assessed using a culture of mixed, healthy, primary vascular cells (ECs, SMCs and BCs) stimulated with serum.

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Titration of irbesartan in these cultures led to a dose dependent decrease in expression of IL-6, IL-8, CCL2, CXCL5, OPG, OPN and CXCL16, and titration of PD98059 led to a decrease in expression of IL-6, IL-8, CXCL5 and OPG. Irbesartan downregulated the same inflammatory cytokines and PD98059 reduced production of IL-6, IL-8 and CXCL5 in both culture systems. These results suggest that ATR1 and ERK1/2 are involved in upregulation of inflammatory cytokines from sites of atherosclerotic disease and healthy sites in the vasculature. ARBs would therefore be expected to reduce inflammatory cytokine production throughout the vasculature.

Another ARB, losartan, was also titrated in the mixed, healthy, primary vascular cell cultures. Titration of losartan led to a dose dependent decrease in secretion of IL-6, CXCL5 and OPG demonstrating that individual ARBs have different anti-inflammatory cytokine responses in the primary vascular cell culture system. Our findings highlight the need for further investigation of the anti-inflammatory impact of ARBs on patients, such as those with diabetes, who have a range of secondary inflammatory complications, including CVD, impaired wound healing and impaired responses to infection. Defining the anti-inflammatory response to different ARBs is potentially valuable since agents that downregulate chronic inflammation, such as that present in atherosclerosis, but maintain inflammation associated with wound healing or infection may be of therapeutic value.

An AII peptide antagonist that has been demonstrated previously to inhibit AII binding [30-35] and the ACE2 activity inhibitor, DX600, which has been demonstrated to increase AII levels in atheroma culture [19], were used to investigate the role of AII in the upregulation of the inflammatory cytokines from the atheroma. Both AII peptide blockade and ACE2 blockade had no impact on the expression of any of the inflammatory cytokines measured or atheroma ERK1/2 activity. Further to this result, titration of AII in mixed, healthy, primary vascular cell cultures did not have an impact on secretion of the inflammatory cytokines. In combination the atheroma and vascular cell culture results suggest the possibility that the anti-inflammatory ARB response is

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driven by blockade of another ligand capable of binding to ATR1. The angiotensin peptides, AIII (similar binding affinity to AII), Ang-(1-7) (binds weakly) [43] and AIV (low potency generation of inositol phosphate) [44] can also bind to ATR1. As AII can be converted to all of these peptides *in vivo* it is difficult to determine the individual role of each peptide without further extensive blockade studies. Further investigation is needed to determine if other angiotensin peptides might be involved in the anti-inflammatory response to ARBs within the vasculature.

A number of studies have demonstrated that activation of the ATR1 (for example to induce mechanical stretch), can be stimulated by non-AII agonists that are inhibited by ARBs [26-29]. Of particular relevance to this current work is a study that investigated the roles of activated, phosphorylated ERK and AII in the inhibition of the *in vitro* mechanical stretch activation of cells by candesartan. Human embryonic kidney (HEK)293 and COS7 cells (these cells do not express the AII precursor, angiotensinogen, or ATR1) and HEK293 or COS7 cells transfected to express ATR1 were used. Neither, mechanical stretch or addition of AII (10 µmol/L) induced an increase in ERK-P in either wild type cell, whereas in the ATR1 transfected cells ERK-P levels increased in response to both stimuli. In this culture system candesartan reduced ERK-P levels stimulated by both mechanical stretching and AII, but [Sar^{1}, Ile^{8}]-AII only reduced the AII stimulated ERK activation [29]. The cultured atheroma tissue in this study demonstrated a similar non-AII agonist driven ERK-P response to ARB as the cells undergoing mechanical stretch in the previous studies.

5. Conclusion

This study presents a number of significant findings. Firstly, ATR1 blockade within diseased, human carotid atheroma *in vitro* decreased secretion of a range of inflammatory cytokines in conjunction with the expression of ERK signalling proteins in the tissue. Secondly, neither direct blockade of AII with a peptide antagonist, or blockade of ACE2 activity had any impact on either expression of the inflammatory cytokines or the level of ERK-Ps in atheroma culture. Thirdly,

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ATR1 and ERK1/2 blockade *in vitro* with mixed, healthy, primary vascular cells stimulated by serum also decreased expression of the same inflammatory cytokines as in the atheroma culture, whilst exogenous AII had no impact. In sum, this suggests that ARBs have an anti-inflammatory impact in the vasculature which potentially occurs via a non-AII agonist/ATR1/ERK driven mechanism.

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Conflict of interest

The authors have no conflicts of interest.

References

[1] Dahlof B, et al. Cardiovascular morbidity and mortality in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. Lancet 2002;359:995-1003.

[2] Lithell H, et al. The Study on Cognition and Prognosis in the Elderly (SCOPE): principal results of a randomised double-blind intervention trial. J Hypertens 2003;21:875-86..

[3] Schrader J, et al. Morbidity and Mortality After Stroke, Eprosartan Compared With Nitrendipine for Secondary Prevention: Principal results of a Prospective Randomised Controlled Study (MOSES). 2005;36:1218-24.

[4] Di Raimondo D, et al. Effects of ACE-inhibitors and angiotensin receptor blockers on inflammation. Curr Pharm Des 2012;18:4385-413.

[5] Takagi H, et al. Effects of telmisartan therapy on interleukin-6 and tumour necrosis factor-alpha levels: a meta-analysis of randomised controlled trials Hypertens Res 2013;36:368-73.

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[6] Xie Q-Y, et al. Effects of valsartan and indapamide on plasma cytokines in essential hypertension. J Cent South Univ (Med Sci) 2006;31:629-34.

[7] Ceriello A, et al. Effect of atorvastatin and irbesartan, alone and in combination, on postprandial endothelial dysfunction, oxidative stress, and inflammation in type 2 diabetic patients. Circulation 2005;111:2518-24.

[8] Tsutamoto T, et al. Angiotensin II type 1 receptor antagonist decreases plasma levels of tumor necrosis factor alpha, interleukin-6 and soluble adhesion molecules in patients with chronic heart failure. J Am Coll Cardiol 2000;35:714-21.

[9] Manabe S, et al. Effects of angiotensin II receptor blockade with valsartan on pro-inflammatory cytokines in patients with essential hypertension. J Cardiovasc Pharmacol 2005;46:735-9.

[10] Martinez-Martin FJ, et al. Olmesartan/amlodipine vs olmesartan/hydrochlorothiazide in hypertensive patients with metabolic syndrome: the OLAS study. J Hum Hypertens 2011;25:346-53.

[11] Fliser D, et al. Antiinflammatory effects of angiotensin II subtype 1 receptor blockade in hypertensive patients with microinflammation. Circulation 2004;110:1103-7.

[12] Koh KK, et al. Pleiotropic effects of angiotensin II receptor blocker in hypertensive patients. J Am Coll Cardiol 2003;42:905-10.

[13] Koh KK, et al. Additive beneficial effects of losartan combined with simvastatin in the treatment of hypercholesterolemic, hypertensive patients. Circulation 2004;110:3687-92.

[14] Kurata M, et al. Angiotensin II receptor blockade with valsartan decreases plasma osteopontin levels in patients with essential hypertension. J Hum Hypertens 2011;25:334-9.

[15] Lorenzen JM, et al. Angiotensin II receptor blocker and statins lower elevated levels of osteopontin in essential hypertension-results from the EUTOPIA trial. Atherosclerosis 2010;209:184-8.

[16] Mehta PK, Griendling KK. Angiotensin II cell signalling: physiological and pathological effects in the cardiovascular system. Am J Physiol Cell Physiol 2007;292:C82-C97.

[17] Roux PP, et al. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 2004;68:320-44.

[18] Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy-from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta 2005;1754:253-62.

[19] Clancy P, et al. Role of the angiotensin converting enzyme 1/angiotensin II/angiotensin receptor 1 axis in interstitial collagenase expression in human carotid atheroma. Atherosclerosis 2013;29:331-7.

[20] Golledge J, et al. Effects of peroxisome proliferator-activated receptor ligands in modulating tissue factor and tissue factor pathway inhibitor in acutely symptomatic carotid atheromas. Stroke 2007;38:1501-8.

[21] Mangan S, et al. Modulation of endothelial cell thrombomodulin by PPAR ligands- variation according to environment. Thromb Res 2007;121:827-34.

[22] Clancy, P, et al. Tenascin-C is increased in atherothrombotic stroke patients and has an anti-inflammatory effect in the human carotid artery. BioFactors 2014;40:448-57.

[23] Christen Y, et al. Oral administration of DuP 753, a specific angiotensin II receptor antagonist, to normal male volunteers. Inhibition of pressor response to exogenous angiotensin I and II. Circulation 1991;83:1333-42.

[24] Seva Pessoa B, et al. Key developments in renin-angiotensin-aldosterone system inhibition. Nat Rev Nephrol 2013;9:26-36.

[25] Rao LVM, et al. Tissue factor-factor VIIa signalling. ATVB 2005;25:47-56.

[26] Thway M, et al. Antibodies From Preeclamptic Patients Stimulate Increased Intracellular CA^{2+} Mobilisation Through Angiotensin Receptor Activation. Circulation 2004;110:1612-19.

[27] Dragun D, et al. Angiotensin II Type 1-Receptor Activating Antibodies in Renal-Allograft Rejection. N Engl J Med 2005;352:558-69.

[28] Yatabe J, et al. Angiotensin II type 1 receptor blocker attenuates the activation of ERK and NADPH oxidase by mechanical strain in mesangial cells in the absence of angiotensin II. Am J Physiol Renal Physiol. 2009;296:F1052-F1060.

[29] Zou Y, et al. Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. Nat Cell Biol 2004;6:499-506.

[30] Chiu AT, et al. Inhibition of induced aldosterone biosynthesis with a specific antagonist of angiotensin II. PNAS 1974;71(2):341-4.

[31] Goodfriend TL, et al. Angiotensin III: (Des-Aspartic Acid[1])-Angiotensin II Evidence and speculation for its role as an important agonist in the renin angiotensin system. Circ Res 1975;36(suppl):I-38-40.

[32] Ray PE, et al. Angiotensin II receptor-mediated proliferation of cultured human fetal mesangial cells. Kid Internat 1991;40:764-71.

[33] Ibrahim J, et al. Action of angiotensin II on DNA synthesis by human saphenous vein in organ culture. Hypertension 2000;36:917-21.

[34] Feener EP, et al. Angiotensin II induces plasminogen activator inhibitor-1 and-2 expression in vascular endothelial and smooth muscle cells. J Clin Invest 1995;95:1353-62.

[35] Olson Jr JA, et al. Angiotensin II induces secretion of plasminogen activator inhibitor 1 and a tissue metalloprotease inhibitor-related protein from rat brain astrocytes. PNAS 1991;88:1928-32.

[36] Bae SK, et al. HPLC determination of irbesartan in human plasma: its application to pharmacokinetic studies. Biomed Chromatogr 2009;23:568-72.

[37] Pool JL, et al. Dose-related antihypertensive effects of Irbesartan in patients with mild-to-

moderate hypertension. Am J Hypertens 1998;11:462-70.

[38] Alessi DR, et al. PD 098059 Is a Specific Inhibitor of the Activation of Mitogen-activated Protein Kinase Kinase *in Vitro* and *in Vivo*. J Biol Chem 1995;270:27489-94.

[39] Peng N, et al. Angiotensin II-induced C-reactive protein generation: Inflammatory role of vascular smooth muscle cells in atherosclerosis. Atherosclerosis 2007;193:292-8.

[40] Lo M-W, et al. Pharmacokinetics of losartan, an angiotensin II receptor antagonist, and its active metabolite EXP3174 in humans. Clin Pharmacol Ther 1995;58:641-9.

[41] Kazierad DJ, et al. Effect of fluconazole on the pharmacokinetics of eprosartan and losartan in healthy male volunteers. Clin Pharmacol Ther 1997;62:417-25.

[42] Wang J-M, et al. Diverse effects of long-term treatment with imidapril and irbesartan on cell growth signal, apoptosis and collagen type I expression in the left ventricle of spontaneously hypertensive rats. Life Sci 2004;75:407-20.

[43] Bosnyak S, et al. Relative affinity of angiotensin peptides and novel ligands at AT_1 and AT_2 receptors. Clin Sci 2011;121:297-303.

[44] Le MT, et al. Angiotensin IV is a potent agonist for constitutive active human AT1 receptors: distinct roles of the C-terminal residues of angiotensin II during AT1 receptor activation. J Biol Chem 2002;277:23107-11.

APPENDIX A

Appendix A: Supplementary material

A1. Methods

Table 1: Patient characteristics for explant interventions

Characteristic	ATR1 blockade	ERK1/2 blockade	AII blockade	ACE2 blockade
Number of patients, n	6	4	5	6
Age, y, mean±SD	69.9±6.7	66.1±4.7	66.4±9.9	68.5±9.1
Male, n	4	4	4	4
TIA, n	2	2	4	4
Stroke, n	1	1	1	1
Diabetes, n	0	0	2	2
Hypertension, n	6	3	5	5
IHD, n	1	1	2	2
Non-smoker, n	1	0	1	0

TIA, Transient Ischaemic Attack; IHD, Ischaemic Heart Disease; AII, Angiotensin II; ATR, AII receptor; ACE, AII Converting Enzyme; ERK, extracellular signal regulated kinase

A1.1. Mixed, healthy, primary vascular cell culture

Using a method determined previously [1]. Mixed, healthy, primary vascular cell cultures

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comprising human aortic endothelial cells (HAEC; Lonza; #CC-2535; seeded at $2.6x10^2$ cells/mL) and human aortic smooth muscle cells (HASMC; Lonza; #CC-2571; seeded at $1.5x10^4$ cells/mL) were established in triplicate the day before and allowed to adhere overnight. Total blood derived cells (BCs), including inflammatory cells and red blood cells (RBCs), (isolated from heparin plasma and rinsed 3 times with Hanks Balanced Salt Solution to remove intrinsic clotting factors) and donor matched serum were added to the healthy, primary EC/SMC with titrated intervention agents (0, 0.0625, 0.25, 1 and 2 times explant intervention concentration) and cultured at 37°C in a humidified 5% CO_2 atmosphere for 4 days. At the end of the culture period the RBCs flowed freely when pipetted and there was no formation of a bolus. Conditioned media was harvested (30,000g, 30min, 4°C) and stored at -80°C until assayed. The blood was collected from four healthy, male volunteers and pooled. Verbal and written, informed consent was received. Ethics approval for these control samples was provided by the appropriate committee (James Cook University, H4109).

A1.2. ELISA

Explant conditioned supernatant (ES) and serum stimulated mixed, healthy, primary vascular cell conditioned supernatant (VS) from the various intervention studies were assessed for the secreted levels of interleukin (IL)-6, IL-8, C-C motif ligand (CCL)2, C-X-C motif ligand (CXCL)5, osteoprotegerin (OPG), osteopontin (OPN) and CXCL16 and tissue from the explant studies was assessed for signalling proteins, ERK1-P and ERK2-P, using commercially available ELISA kits following manufacturer's instructions (R&D Systyems). The appropriate conditions for each assay were determined by sample titration. Based on these preliminary studies the following volumes and dilutions of sample were assayed: IL-6 (ES: 8μL of 1/100, VS: 30μL 1/100; #DY206), IL-8 (ES: 30μL of 1/100, VS: 10μL 1/10; #DY208), CCL2 (ES: 35μL of 1/100, VS: 50μL 1/10; #DY279), CXCL5 (ES: 16μL of 1/10, VS: 30μL neat; #DY254), OPG (ES: 15μL of 1/10, VS: 50μL neat; #DY805), OPN (ES: 13μL of 1/100, VS: 15μL neat; #DY1433), CXCL16 (ES: 80μL of 1/10, VS: 10μL neat; #DY1164), ERK1-P (17μL of lysate; #DYC1825) and ERK2-P (17μL of lysate; #DYC1483).

As both the culture systems are closed systems the total amount of each protein expressed was determined for each sample and used in the subsequent analyses.

A1.3. Western blotting

The ERK signalling proteins were extracted from the atheroma tissue as described previously [2]. The proteins were separated under denaturing conditions on a 12% SDS polyacrylamide gel [3]. The ECL Advance Western Blotting Detection kit (Amersham, #RPN2135) was used for visualisation, as per the manufacturer's instructions. Briefly, the separated proteins were transferred onto PVDF membrane (Biorad, #162-0177) and probed with 0.1μg/mL rabbit polyclonal antibody against ERK1 and ERK2 (R&D Systems, #AF1575 and #AF1230, respectively) and 1/2000 goat anti-rabbit HRP secondary (Dako, #P0448). Bands migrating at ~42 and 44kD were digitally captured using a Chemidoc XRS (Biorad) and band volume (intensity units*mm^2) determined using Quantity One software (Biorad). Raw blots are shown in Fig A1.

A2. Results

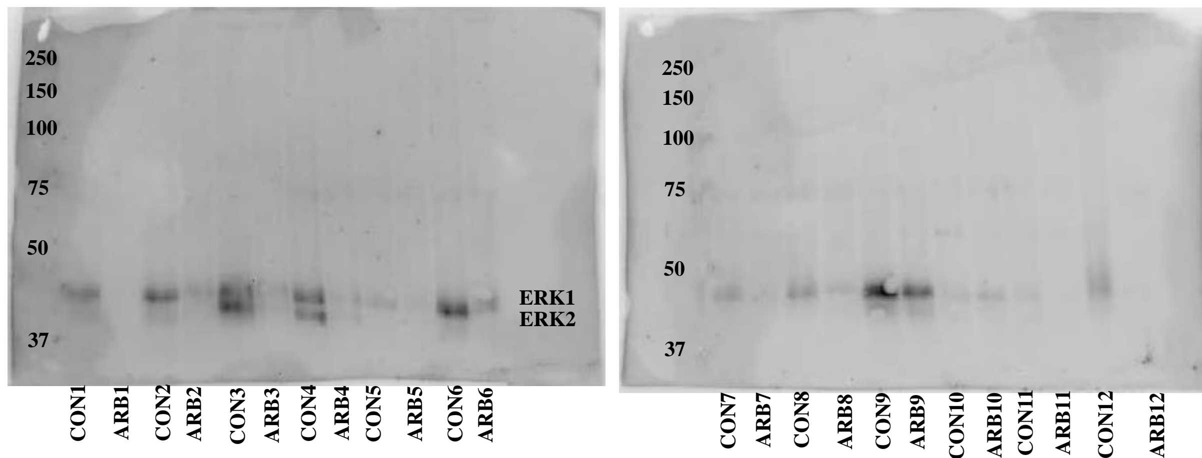

Fig. A1. Raw western blots for ERK1 and ERK2 protein expression determination. Macroscopically, disease matched carotid atheroma tissue samples were treated with and without irbesartan (2.3 mmol/L, n=12 matched pairs, 4 d in culture). Untreated control samples are assigned "CON" and irbesartan treated samples are assigned "ARB".

Supplemental References

[1] Clancy P, et al. Tenascin-C is increased in atherothrombotic stroke patients and has an anti-inflammatory effect in the human carotid artery. [published online May 14th, 2014] BioFactors, 2014.

[2] Clancy P, et al. Role of the angiotensin converting enzyme 1/angiotensin II/angiotensin receptor 1 axis in interstitial collagenase expression in human carotid atheroma. Atherosclerosis 2013;29:331-7.

[3] Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 1970;227:680-5.

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