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Role of the angiotensin converting enzyme 1/angiotensin II/angiotensin receptor 1 axis in interstitial collagenase expression in human carotid atheroma

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Abstract

Background and Aims: Angiotensin II (AII) receptor 1 (ATR1) and angiotensin converting enzyme 1 (ACE1) blockers have been shown to reduce acute cardiovascular events in patients, improve plaque stability and modify matrix metalloproteinase (MMP) expression. However, the role of the ACE1/AII/ATR1 axis in interstitial collagenase regulation has not been fully explored. In this study, we investigated the effect of ATR1 and ACE1 blockade on the expression and activity of MMP-1, -8 and -13 in human carotid atheroma.

Methods: Atheroma samples (n=24) were obtained from patients undergoing carotid endarterectomy. The effects of ATR1 (irbesartan), ACE1 (quinapril), ACE2 (DX600) and MMP (GM6001) blockade on the expression of AII, the interstitial collagenases and soluble elastin fragments were investigated in explant culture supernatants. Paired atheroma samples were incubated with intervention or media control for 4 days. Protein levels (AII, MMP-1, -8, -13 and soluble elastin) were determined by ELISA.

Results: ATR1, but not ACE1, blockade significantly reduced MMP-1 and -8 concentrations in atheroma supernatants. ACE2 blockade significantly increased MMP-1 and -8 concentrations in atheroma supernatants. AII concentration in atheroma supernatants significantly increased after ATR1, ACE1 and ACE2 blockade. Release of soluble elastin fragments increased after ATR1 and ACE1 blockade, but was not changed by an MMP inhibitor.

Conclusions: Our findings suggest that ATR1 blockade alters AII, MMP-1, MMP-8 expression and a marker of elastin degradation in human atheroma, but that the elastin degradation response is not MMP driven. This data contributes to the recognised ability of ATR1 blockade to modify plaque stability.

1. Introduction

Rupture of the fibrous cap lining the atherosclerotic plaque is thought to be instrumental in most ischemic syndromes, including stroke. Extracellular matrix (ECM) proteins, such as fibrillar collagens, which provide structural support to the fibrous cap [1] can be degraded by matrix metalloproteinases (MMPs), in particular the interstitial collagenase subfamily, MMP-1, -8 and -13 [2]. Degradation of the ECM protein, elastin, is also a feature of atherosclerosis development with levels of the soluble degradation product elevated in the serum of atherosclerotic patients [3].

MMP-1 is expressed in the atherosclerotic plaque, predominantly in the vulnerable shoulder region [4] and implicated in the degradation of fibrous cap ECM potentially promoting plaque rupture [2]. Inducible MMP-8 has also been demonstrated in atheroma tissue in association with endothelial cells, smooth muscle cells and mononuclear phagocytes. MMP-8 is also called neutrophil elastase, and is usually constitutively associated with neutrophils [5]. MMP-13 is also found in atheroma tissue co-localising with macrophages [2]. Both MMP-1 and -13 also co-localise with collagen specifically degraded by MMP activity within the plaque [2].

Angiotensin II (AII) (Fig 1A) has been implicated in the progression of atherosclerosis [6] and associated with MMP regulation [7]. The signalling pathway involving ACE/AII/ATR1 (also called the pressor axis) is the focus of this study (Fig 1B). Several clinical trials have demonstrated the benefits of blockade of this pathway in reducing acute cardiovascular events, including stroke, independent of blood pressure reduction [8]. The AII receptor type 1 (ATR1), angiotensin converting enzyme (ACE)1 and ACE2 enzyme are all present in atheroma [9,10,11].
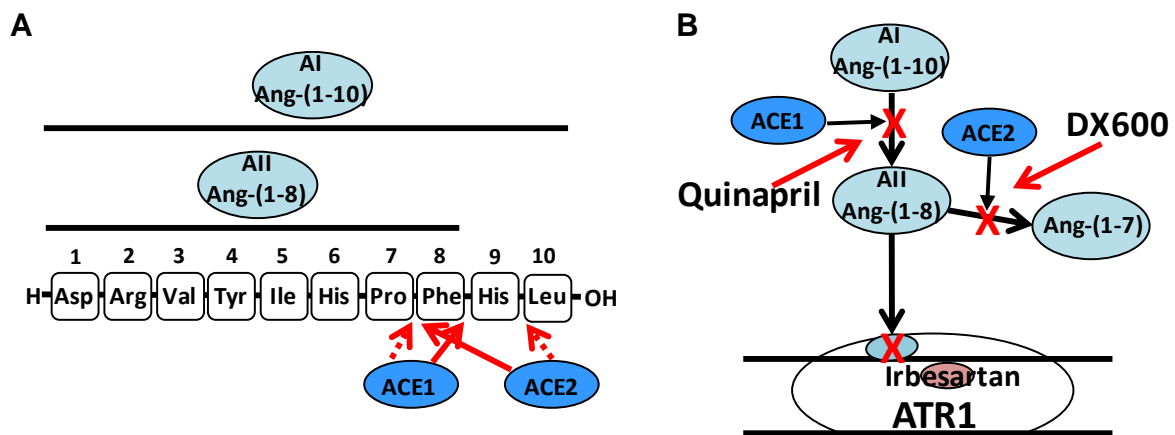
Fig. 1. ACE1/AII/ATR1 signalling axis. (**A**) Angiotensin I and II peptide sequences with known cleavage sites for the carboxypeptidases, ACE1 and ACE2. The bold arrows demonstrate the more favoured reactions from *in vitro* activity studies. (**B**) ACE1/AII/ATR1 axis demonstrating the blockade points for this study. It would be expected that blockade of ACE1 would decrease the level of AII and blockade of ACE2 increase the level of AII.

The underlying regulatory mechanism behind the benefits of blockade of the pressor axis in atherosclerosis has not been fully investigated. In this study we aimed to clarify the roles of ACE1, AII and ATR1 in modifying interstitial collagenase expression in carotid atheroma. Using paired human carotid atheroma explant culture [12,13] and antagonists specific to ACE1 and ATR1 we investigated expression of MMP-1, -8, and -13 in the culture supernatant and tissue. An ACE2 antagonist was also used to further determine the role of AII. Soluble elastin fragments were measured in the culture supernatants as a marker of elastin degradation and the role of the MMPs was evaluated using a general MMP activity blocker.

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 Sept. 2012 and Jan. 2013. Inclusion criteria included: 1) verbal and written informed consent; 2) carotid stenosis of $\geq 70\%$ identified on duplex imaging using criteria previously described [12]; and 3) no previous carotid artery intervention. Ethics approval was provided by the appropriate committees. Patients with a history of transient ischemic attack, 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. Specimens

A conventional endarterectomy was performed, developing a deep medial plane and removing the specimen *en bloc [12]*. Specimens were placed in chilled culture medium immediately after excision, and transferred chilled to the laboratory for processing [12]. Specimens were dissected aseptically for explant culture. Culture medium components are presented online in Appendix A.

2.3. Explant Culture

Explant culture was carried out as described previously [12,13]. Two disease matched pairs were obtained from each of the six patients selected. The concentration of interventional agents used were based on circulating concentrations measured in patients receiving therapeutic levels of the drug or published effective *in vitro* concentrations. Therapeutic circulating concentrations of irbesartan are reported to be between ~1-10 mmol/L [14,15] and ~0.5-2 mmol/L for quinapril [16]. Pharmacokinetic studies for DX600 demonstrated 99% inhibition when used at 100 µmol/L in *in vitro* ACE2 activity assessment with a K_i <1 µmol/L [17]. The broad spectrum MMP specific activity blocker which inhibits most MMPs, including MMP-1 (K_i 0.4nM), MMP-2 (K_i 0.5nM), MMP-3 (K_i 27nM), MMP-8 (K_i 0.1nM), MMP-9 (K_i 0.2nM), MMP-12 (K_i 3.6nM) and MMP-13 was used at the concentration recommended by the manufacturer. The following interventions were assessed using this paired test and control explant culture approach: a) ATR1 blocker (irbesartan; 2 mmol/L; Sigma #I2286); b) ACE1 blocker (quinapril; 1 mmol/L; Sigma #Q0632); c) ACE2 blocker (DX600; 1 µmol/L; Anaspec #62337); d) Broad spectrum MMP specific activity blocker (GM6001; 25 µmol/L; Sigma #M5939). A detailed description of the culture method is presented online in Appendix A.

2.4. ELISA

Commercially available ELISA kits were used to measure expression of the interstitial collagenases in the explant culture supernatant and tissue, and AII and collagen neo-epitope fragments in the supernatant following the manufacturer's instructions. The level of soluble elastin fragments in the culture supernatant was measured using a previously developed competitive ELISA [18]. Details of each ELISA and the assay conditions used are presented online in Appendix A.

2.5. Protein quantification

Following completion of the explant studies atheroma tissue was processed to determine the total protein content of each cultured sample for normalising purposes. These extracts were also used in the tissue assessments. A detailed description of the protein extraction procedure is presented online in Appendix A.

2.6. Data Analysis

MMP-1, -8, -13, AII, and soluble elastin fragment levels in the paired explant samples are presented as mean±SEM for the relative ratios of paired experimental and control samples (n=12 pairs) [12]. Differences between experimental and control samples were assessed using Wilcoxon's paired test in Graphpad Prism 5 software. Values for experimental samples >1 implied the intervention enhanced expression while values <1 implied the intervention reduced expression. A p value of <0.05 was considered statistically significant. The mean±SEM protein levels across all the untreated control samples were also presented to demonstrate the inherent heterogeneity between the individual patients.

3. Results

3.1. Secretion of AII by carotid atheroma tissue and the effect of pressor axis angiotensin signalling

blockers

AII was detectable in the culture supernatant by ELISA. The mean normalised concentration in the untreated control culture supernatants was 5.84±0.77 pg/mg protein in explant tissue, with a range from 0.43-24.82 pg/mg (n=36) (Fig. 2A). There was a significant increase in the concentration of AII in the culture supernatant after blockade of the ACE/AII/ATR1 axis. Irbesartan (ATR1 specific inhibitor, 2 mmol/L), quinapril (ACE1 specific inhibitor, 1 mmol/L) and DX600 (ACE2 specific inhibitor, 1 μmol/L) each significantly increased generation of AII although this was most marked for the ACE2 inhibitor (n=12 pairs; P=0.0269, P=0.0020 and P=0.0024, respectively) (Fig. 2B).
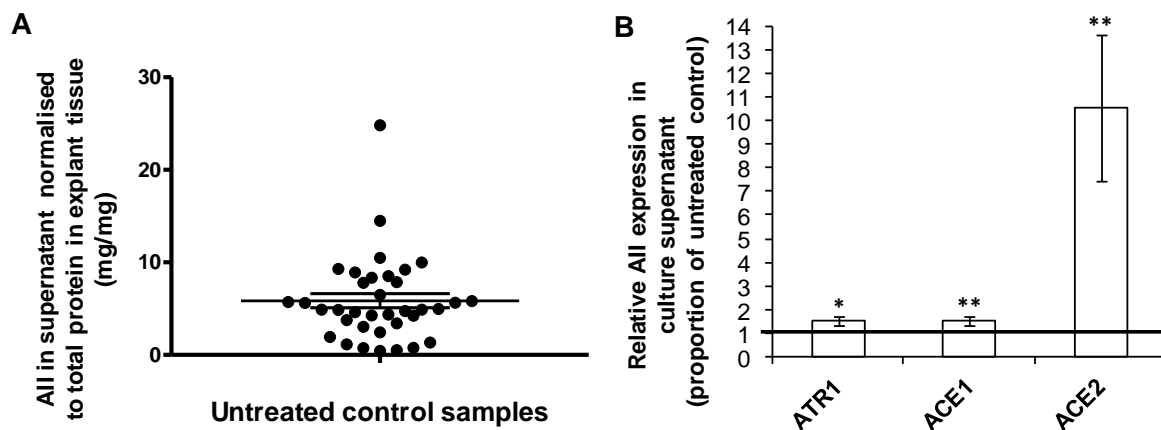

Fig. 2. AII expression in atheroma explant supernatant. (**A**) Mean ± SEM of AII detected in the untreated control culture supernatant, normalised to the amount of protein present in the explant tissue (n=36). (B) Relative expression of AII in human carotid atheroma supernatant after 4d culture with and without ATR1 (irbesartan, 2 mmol/L, n=12 pairs); ACE1 (quinapril, 1 mmol/L, n=12 pairs) and ACE2 (DX600, 1 μmol/L, n=12 pairs) blockade. Shown are the mean values ± SEM of the ratio of peptide (normalised to total protein) secreted by paired atheroma samples incubated with intervention relative to control. AII levels were determined using ELISA. ***P<0.001; **P<0.01; *P<0.05 using Wilcoxon's paired test. (1=no change, >1 increased, <1 decreased with treatment)

3.2 Effect of pressor axis angiotensin signalling blockers on interstitial collagenase expression in human carotid atheroma supernatants

Concentration of MMP-1, -8, and -13 present in the atheroma supernatant were measured using ELISA. All the interstitial collagenases were detectable in the culture supernatant. The mean normalised amount of protein in the untreated control culture supernatants were as follows: MMP-1,

7.85±1.41 ranging from 0.59-35.32 ng/mg (n=36); MMP-8, 1.13±0.23 ranging from 0.02-6.20

ng/mg (n=36); MMP-13, 12.37±3.42 ranging from 0.63-78.8 ng/mg (n=36) (Fig. 3A-C). Irbesartan

(2 mmol/L) significantly downregulated the concentration of MMP-1 (n=12 pairs; P=0.0005) and

downregulated the concentration of MMP-8 with borderline significance (n=12 pairs; P=0.0537)

(Fig. 3D). DX600 (1 µmol/L) caused a significant upregulation in MMP-1 and MMP-8

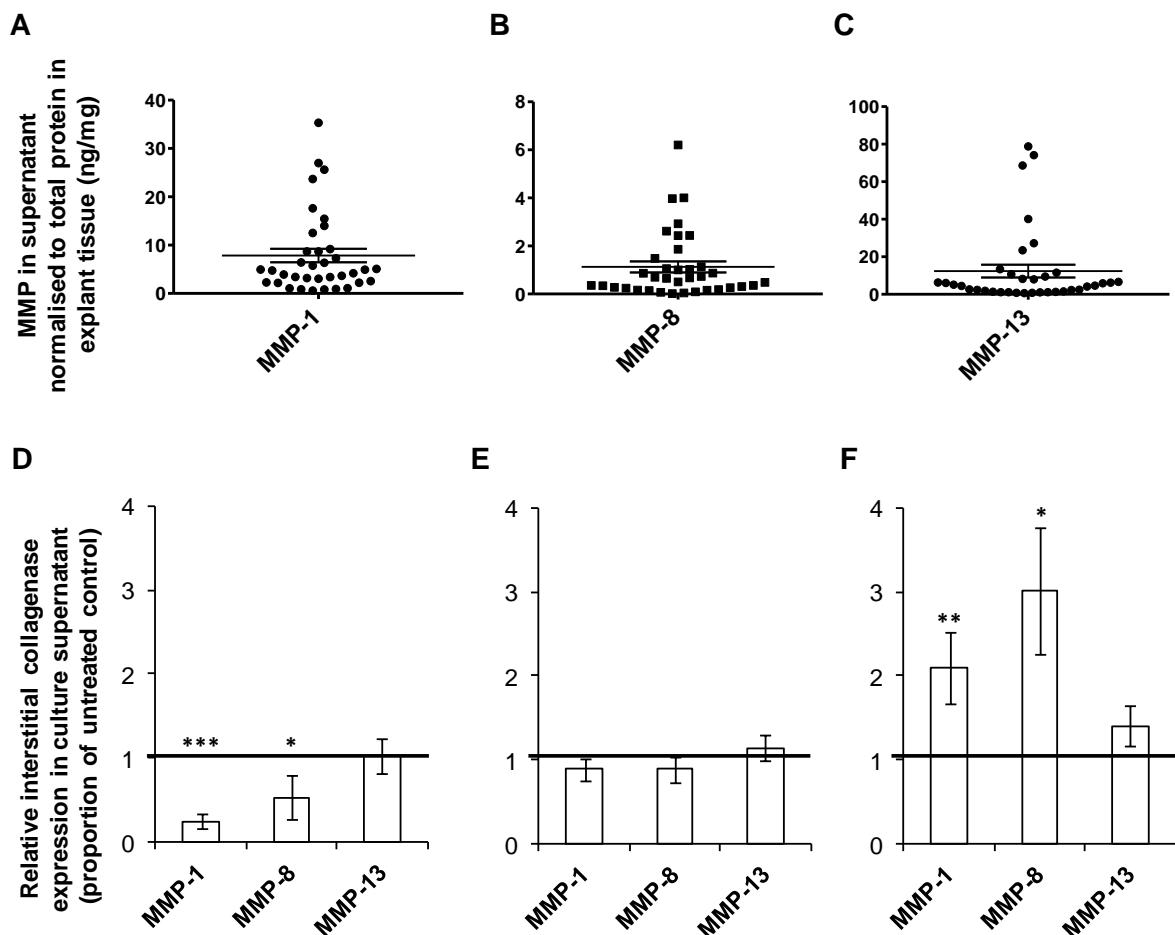concentrations (n=12 pairs; P=0.0034 and P=0.0210, respectively) (Fig. 3F).

Fig. 3. Changes in interstitial collagenase expression following angiotensin signalling blockade. Mean ± SEM of (**A**) MMP-1, (**B**) MMP-8 and (**C**) MMP-13 detected in the untreated control culture supernatant, normalised to the amount of protein present in the explant tissue (n=36). Relative expression of MMP-1, -8 and -13 in human carotid atheroma supernatant after 4d culture with and without (**D**) ATR1 (irbesartan, 2 mmol/L, n=12 pairs); (**E**) ACE1 (quinapril, 1 mmol/L, n=12 pairs) and (**F**) ACE2 (DX600, 1 µmol/L, n=12 pairs) blockade. Shown are the mean values ± SEM of the ratio of enzyme (normalised to total protein) secreted by paired atheroma samples incubated with intervention relative to control. MMP expression was determined using ELISA. ***P<0.001; **P<0.01; *P<0.05 using Wilcoxon's paired test. (1=no change, >1 increased, <1 decreased with treatment)
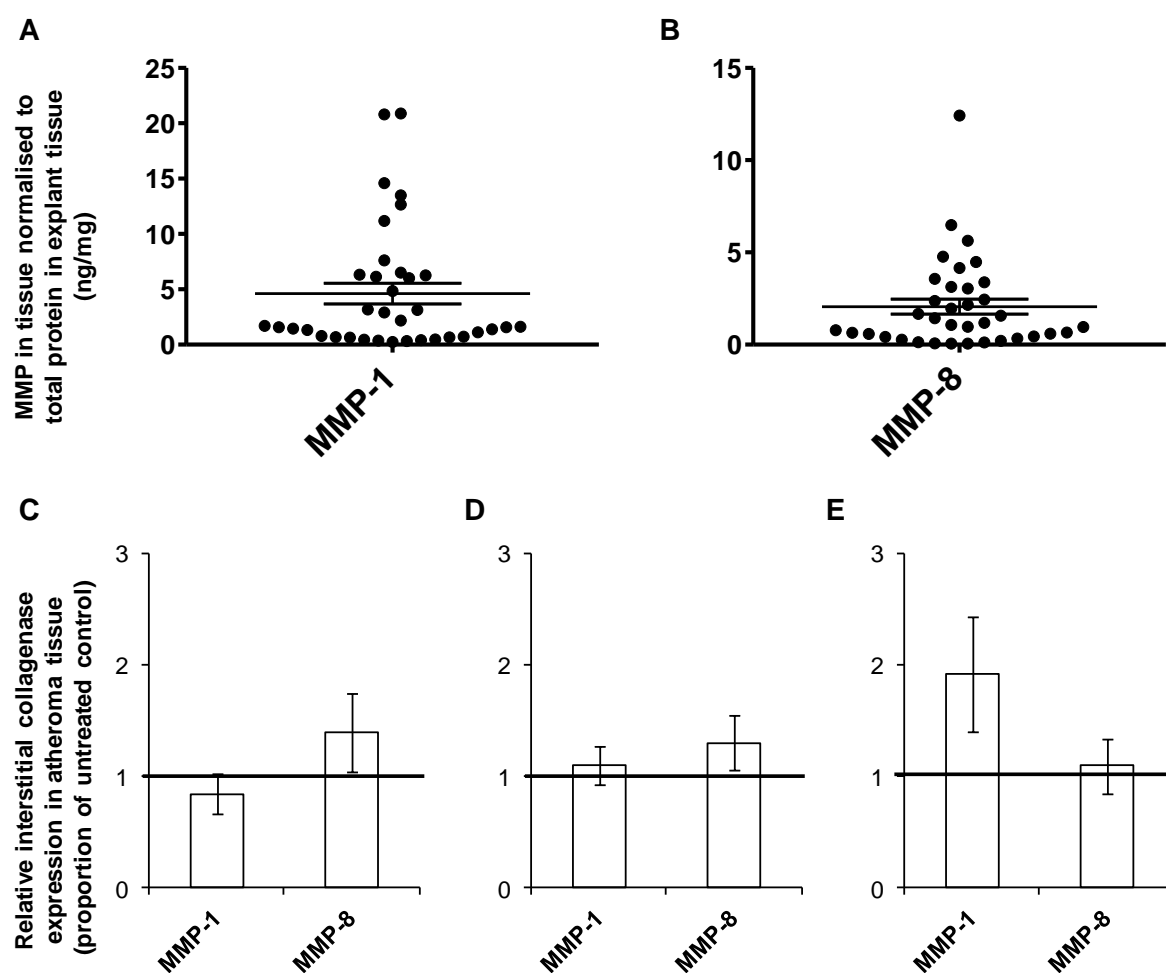
Fig. 4. Changes in interstitial collagenase tissue expression following angiotensin signalling blockade. Mean ± SEM of (**A**) MMP-1 and (**B**) MMP-8 detected in the untreated control explant tissue, normalised to the amount of protein present in the explant tissue (n=36). Relative expression of MMP-1 and -8 in human carotid atheroma tissue after 4d culture with and without (**C**) ATR1 (irbesartan, 2 mmol/L, n=12 pairs); (**D**) ACE1 (quinapril, 1 mmol/L, n=12 pairs) and (**E**) ACE2 (DX600, 1 μmol/L, n=12 pairs) blockade. Shown are the mean values ± SEM of the ratio of enzyme (normalised to total protein) expressed in paired atheroma samples incubated with intervention relative to control. MMP expression was determined using ELISA. ***$P<0.001$; **$P<0.01$; *$P<0.05$ using Wilcoxon's paired test. (1=no change, >1 increased, <1 decreased with treatment)

3.3. Effect of pressor axis angiotensin signalling blockers on interstitial collagenase expression in human carotid atheroma tissue

MMP-1 and -8 were detectable in the explant tissue by ELISA. The mean normalised amounts of protein in the untreated control culture tissue were as follows: MMP-1, 4.61±0.94 ranging from 0.23-20.89 ng/mg (n=36); MMP-8, 2.06±0.41 ranging from 0.05-12.42 ng/mg (n=36) (Fig. 4A-B). There was no significant change in expression of either MMP-1 or MMP-8 in the

tissue using irbesartan (2 mmol/L), quinapril (1 mmol/L) or DX600 (1 µmol/L) (Fig. 4C-E).

3.4. Effect of pressor axis angiotensin signalling and MMP activity blockers on extracellular matrix fragments in human carotid atheroma supernatants

The effect of angiotensin signalling pathway blockade on degradation of collagen and elastin was assessed by measuring the expression of collagen fragments containing an MMP derived neo-epitope and soluble elastin fragments in the atheroma explant supernatant using ELISA. The collagen fragment was detectable in 15 of the 36 untreated control supernatants measured with a mean of 27.03±6.07 ranging from 1.09-70.71 ng/mL/mg (Fig. 5A). The mean normalised amount of elastin fragments in the untreated control culture supernatants was 140.5±15.6 mg/mg protein of explant tissue, with a range from 6.3-369.4 mg/mg (n=48) (Fig. 5B). Irbesartan (2 mmol/L) and quinapril (1 mmol/L) significantly increased generation of soluble elastin fragments (n=12 pairs; P=0.0425 and P=0.0049, respectively) (Fig. 5C).
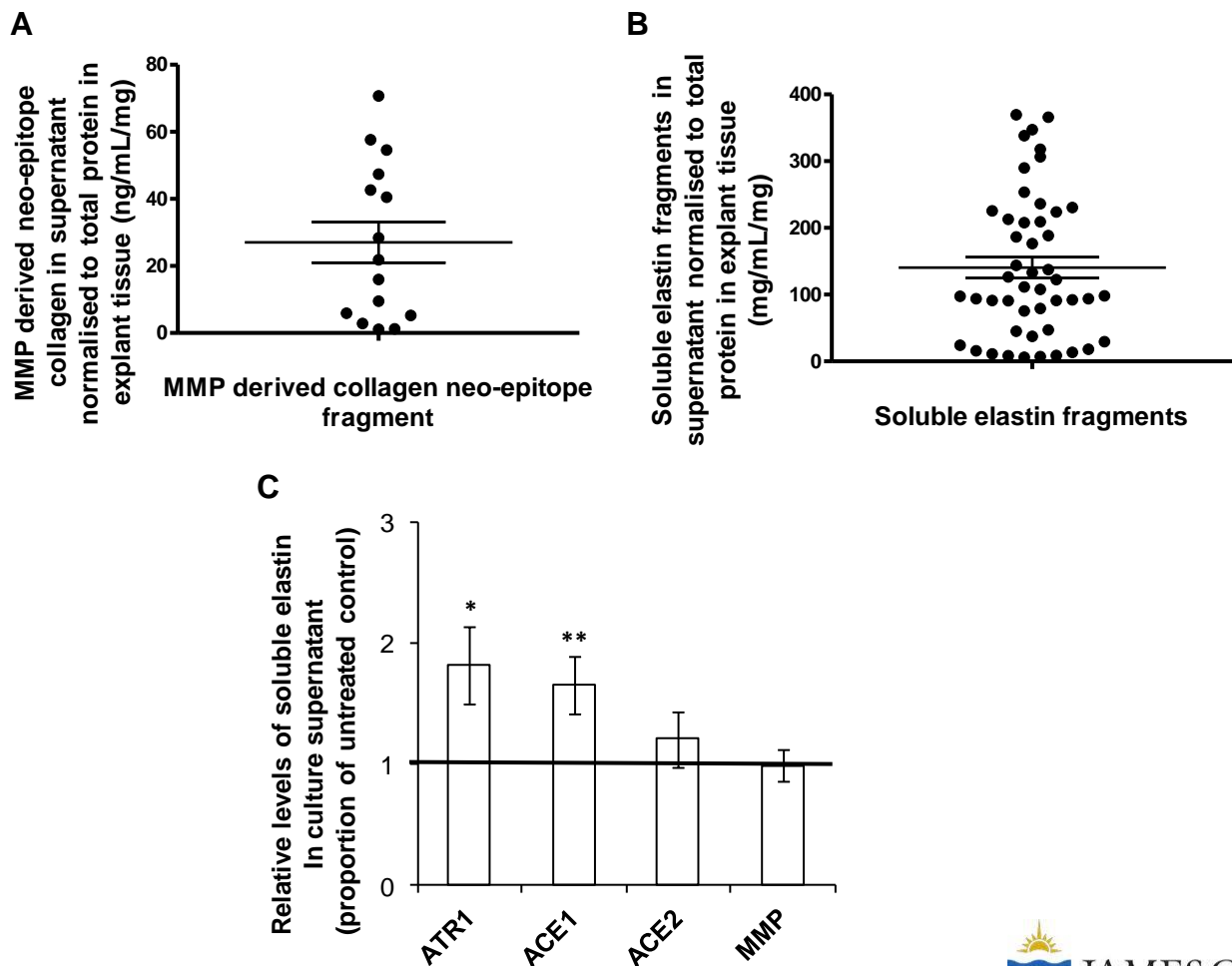
Fig. 5. Changes in extracellular matrix degradation fragment levels following angiotensin signalling and MMP activity blockade. Mean ± SEM of (**A**) MMP derived collagen neo-epitope fragments (n=36) and (**B**) soluble elastin fragments (n=48) detected in the untreated control culture supernatant, normalised to the amount of protein present in the explant tissue. (**C**) Relative expression of soluble elastin fragments in human carotid atheroma supernatant after 4d culture with and without ATR1 (irbesartan, 2 mmol/L, n=12 pairs); ACE1 (quinapril, 1 mmol/L, n=12 pairs); ACE2 (DX600, 1 μmol/L, n=12 pairs) and general MMP activity (GM6001, 25 μmol/L, n=12 pairs) blockade. Shown are the mean values ± SEM of the ratio of cleaved elastin (normalised to total protein) generated by paired atheroma samples incubated with intervention relative to control. Collagen and elastin fragment expression was determined using ELISA. ***P<0.001; **P<0.01; *P<0.05 using Wilcoxon's paired test. (1=no change, >1 increased, <1 decreased with treatment)

4. Discussion

In the present study, using human atheroma explant culture we demonstrated, for the first time, that ATR1 blockade reduces MMP-1 and MMP-8 expression, but increases elastin degradation. It is widely accepted that AII has a role in atherosclerosis, impacting on inflammation, vascular remodelling and thrombosis and that therapies that disrupt the ACE/AII/ATR1 axis are beneficial to cardiovascular patients in reducing the incidence of stroke [8]. A fundamental aim of this study was to determine the influence of the ACE1/AII/ATR1 axis on interstitial collagenase regulation within human carotid atherosclerosis.

The cultured atheroma tissue actively secreted AII showing significant increases with all three blockades (ACE1, ATR1, ACE2). A number of *in vivo* studies have demonstrated increases in AII levels in the blood with ATR1 blockade using several different non-peptide antagonists [15,19,20]. Of particular relevance to this work is the study that demonstrated an oral dose of 100mg of irbesartan daily led to a bloodstream irbesartan concentration of 3.2 mmol/L and a 1.7-fold increase in the concentration of plasma AII. The increase in AII was detectable within 24h [15]. In our explant culture system a 2 mmol/L concentration of irbesartan over 96h led to a 1.5-fold increase in AII concentration in the culture supernatant, which is comparable with the responses measured *in vivo*. Our findings of an increase of AII concentration in culture supernatant after ACE1 inhibition might appear surprising. However, oral administration of ACE1 inhibition has

been reported to induce a decrease in plasma AII for less than 24 hours after which levels come back to baseline and subsequently increase with long term usage. This phenomemon is referred to as *ACE escape* and has been blamed for an attenuation of the efficacy of ACE1 inhibitors in chronic heart failure patients after 1 year of treatment [21]. This response was observed in the ACE1 blockade explant culture with an increase in AII demonstrated after 96h of culture. The increase in the AII level following ACE2 blockade was as expected since inhibition of the conversion of AII to Ang-(1-7) (Fig 1B) should lead to a higher concentration of AII confirming the efficacy of the ACE2 blocking agent.

The ACE based blockers have a different mode of action to that of the receptor based, ATR1 blocker. As their role in the signalling pathway is to generate or process the AII ligand any downstream impact they have should be reflected in the level of the AII ligand itself. Consequently, the impact of the ACE1 and ACE2 blockers on collagenase concentrations should be linked with the concentration of AII if it is the ligand involved in their upregulation.

The levels of secreted MMP-1 and 8 in the supernatant decreased with ATR1 blockade and increased with ACE2 blockade. As the level of AII increased in line with the MMPs in the ACE2 blockade it is possible AII via ATR1 could be driving the upregulation of these proteases in the supernatant, particularly as the increase in AII was marked at around 10-fold. However, the levels of MMP-1 and -8 did not increase in the ACE1 blockade experiment, despite increases in AII (although this was only around 1.5-fold). It is therefore possible that other mechanisms are involved. Another angiotensin peptide which is also known to have a high affinity for ATR1 is AIII [22], further studies would be needed to determine if AII or AIII is the key peptide involved in the secretion of MMP-1 and -8 via ATR1 from atheroma. A previous study treating hypercholesterolemic rabbits with the ATR1 blocker, losartan, also demonstrated a reduction in MMP-1 expression [23]. MMP-8 is usually a constitutive component of neutrophils, however MMP-8 was demonstrated to be upregulated in endothelial cells, smooth muscle cells and

mononuclear cells in the atheroma [5], this study suggests that ATR1 is involved in MMP-8 upregulation in the atheroma (possibly via AII binding) as ATR1 blockade lowered MMP-8 expression.

The levels of MMP-1 and -8 altered in the supernatant, but were unchanged in the tissue, regardless of the blockade used. Previous studies have demonstrated a similar phenomenon for AII generation in tissue and solution. One study for example compared the capacity of serum and heart tissue to generate AII with and without ACE1 blockade. Whilst the serum showed complete inhibition of AII generating capacity (the incubation time for the assay was 30min) the tissue only showed an 11% decrease, these workers determined that AII in the tissue was generated predominantly by a soybean trypsin inhibitor sensitive enzyme [24]. Chymase and cathepsin G are other enzymes present in human atheroma tissue, which are capable of generating AII and implicated in progression of atherosclerosis [25,26].

As rupture of the fibrous cap lining the atherosclerotic plaque arises from degradation of the ECM proteins, which provide structural support to the fibrous cap [1] collagen and elastin degradation products were also measured in the culture supernatants. Unfortunately, the assay for collagen fragments specifically degraded by the interstitial collagenases did not consistently detect the fragments so the blockade results could not be included, but soluble elastin fragments were consistently detectable. The level of elastin fragments in the culture supernatant increased with both ATR1 and ACE1 blockade, but there was no change with ACE2 blockade suggesting this effect is not mediated by AII. Several studies have measured a decrease in *in vivo* levels of structural elastin in rats after ATR1 blockade [27] and ACE1 blockade [28]. These results are in keeping with this study, where increased levels of soluble elastin fragments in the supernatant would arise from increased elastin degradation and a subsequent decrease in levels of structural elastin in the tissue. Blockade of general MMP activity in this culture system did not impact on the extent of elastin degradation suggesting that the enzyme responsible for elastin degradation in the atheroma is not an

MMP. Cathepsin K is another elastinolytic enzyme, which has been associated with atherosclerosis, with cathepsin K deficiency leading to less elastic lamina fragmentation in atheroma in low-density lipoprotein receptor deficient mice [29].

Measuring signalling pathways using explant culture has the potential to be more representative of what happens in the patient than cell monocultures or animal models. Using fresh human tissue from an active disease state rather than animals is a major advantage in the application of findings to treatments in humans. Explant culture also has several advantages over cell monocultures: 1. All the cell types that occur in the diseased tissue are present in the culture giving a signalling outcome potentially more representative of the disease with contributions from all the types of cells normally present in the plaque; 2. The cells present in the atheroma remain in close relationship to other cells in the plaque which could influence any intervention under study, e.g. by the production of cytokines; 3. The cells present are in contact with elements such as fat or calcium deposits which may also impact on their signalling behaviour. The results of this study suggest that the elements of the pressor axis are behaving in a manner representative of the *in vivo* situation.

The main limitation of this technique is that the circulating factors derived from specialist organs within the vasculature in the *in vivo* context are missing so their impact cannot be assessed, only human trials can address this issue. Another potential limitation is the heterogeneity of the cultured tissue and the patients themselves. The patients included in these studies are heterogenous in terms of co-morbidites and medical therapies. In order to minimise this effect we have developed a system of assessing paired biopsies from adjacent sites within macroscopically severe atheroma and including a 24h preincubation step before the intervention study itself. We have previously shown this technique minimised variation between the matched control and blockade pair [12,13].

In evidence-based medicine it is accepted that ATR1 blocking drugs are beneficial for cardiovascular patients, and effective at reducing the incidence of stroke [8]. These studies suggest one of the plaque stabilising mechanisms of these medications could be through a reduction in the

initiation of ECM degradation by MMP-1 and or MMP-8 [2]. However, the soluble elastin fragment studies suggest the MMPs are not involved in elastin degradation in the atheroma. Further studies determining the functional roles of all the proteases capable of degrading ECM, in atherosclerosis are needed.

This study further elucidates the underlying molecular mechanism of action in the treatment of the atherosclerotic human plaque and studies of this nature are vital to targeting therapeutics in this major disease.

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Disclosures

None.

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Appendix A: Supplementary material

A1. Methods

A1.1. Culture medium

4.5g glucose DMEM medium, 4 mmol/L L-glutamine, 2.5 µg/mL amphotericin, 100 U/mL penicillin G, 100 U/mL streptomycin, 0.05 mg/mL gentamicin, 1x non-essential amino acids, pH 7.2, 20 mmol/L HEPES pH 7.5, 10 µmol/L β-mercaptoethanol, 2.4 µg/mL sodium bicarbonate, 10 mmol/L sodium pyruvate. 20% foetal bovine serum (FBS) was added to the medium for transfer from the hospital to the laboratory and 10% FBS was added to the experimental culture medium.

Table: Patient characteristics

Characteristic	ATR1 blockade	ACE1 blockade	ACE2 blockade	Broad spectrum MMP specific activity blockade
Number of patients, n	6	6	6	6
Age, y, mean±SD	69.9±6.7	73.0±8.4	68.5±9.1	73.2±8.4
Male, n	4	4	4	5
TIA, n	2	2	4	1
Stroke, n	1	2	1	1
Diabetes, n	0	0	2	0
Hypertension, n	6	6	5	6
IHD, n	1	2	2	1
Non-smoker, n	1	0	0	1
Ex-smoker, n	2	4	6	2
Current smoker, n	3	2	0	3
ATR1i, n	0	2	3	1
ACEi, n	4	4	2	3

TIA indicates Transient Ischaemic Attack; IHD, Ischaemic Heart Disease; ATR1i, Angiotensin II receptor 1 inhibitor; ACEi, Angiotensin Converting Enzyme inhibitor

A1.2. Explant culture
There are few studies investigating intracellular signalling pathways in atherosclerosis with cell isolation from plaques being problematic [1] and the heterogeneity of the plaque making correlation with *in vitro* monocultures of limited value. An *ex vivo* system is required for the study of intracellular pathways in the plaque [2]. In previous studies, we have established that: i) Carotid endarterectomy samples remain viable in culture for at least 8d, as assessed by histology, immunohistochemistry, cytokine release, and measurement of tissue ATP [3]; ii) Adjacent paired biopsies have similar features of plaque instability *in vitro*. TF concentration and activity were similar in adjacent biopsies with a coefficient of variation for TF activity <5% (n=6) [4]. The latter was particularly important to establish given the heterogeneity of atherosclerosis and the need to have comparable baseline outcomes in order to test the effect of potential plaque stabilising interventions. Paired adjacent biopsies (5-10mm^2) were matched by disease at the macroscopic level and placed in tissue-culture wells, intima up, in 1mL of experimental culture medium, and incubated at 37°C in a humidified 5% CO_2 atmosphere. The initial 24h culture was in media alone to allow the tissue to stabilise and be free from the impact of any medications the patient may have been taking when the intervention was introduced. Subsequent incubations (48h) were replenished such that one of the paired samples was cultured with media containing the intervention and the matched control sample had media alone. Conditioned media was harvested (30,000g, 30min, 4°C), at every replenishment and stored at -80°C until assayed. The explant tissue was stored in media + 10% DMSO at -80°C until processed.

A1.3. ELISA
Explant conditioned supernatant removed from atheroma exposed to interventional agent for 4 days and appropriate controls were assessed for the secreted levels of MMP-1, -8 (R&D Systems), -13 (USCN), collagen fragment containing a neo-epitope generated by MMP cleavage (Ibex) and AII (Bachem, this kit does show some cross-reactivity with AIII) using commercially available kits following manufacturer's instructions. Each of the MMP kits measured both latent and active forms combined. 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: MMP-1

(10μL supernatant; 8μL tissue extract; #DY901), MMP-8 (5μL supernatant; 2.5μL tissue extract; #DY902), MMP-13 (25μL supernatant; #E90099Hu), collagen neo-epitope fragment (50μL supernatant; #60-1002-001) and AII (50μL supernatant; #S-1133).

Briefly, the format for the soluble elastin ELISA was as follows:

1. Coat plate overnight with 150μL/well of 1.25 µg/mL soluble human aortic elastin (Elastin Products Company #HA587) in carbonate coating buffer pH 9.6 at 4°C. Wash 4 times with wash buffer (PBS+0.05% tween 20).

2. Pre-incubate 100μL sample or standard (1 µg/mL soluble elastin as upper point) for 2h at room temperature with 50uL 1/1000 detection antibody (Elastin Products Company #PR533) in reagent buffer (PBS+0.05% tween 20+7%BSA)

3. Transfer to washed, coated plate and incubate for 1h at room temperature. Wash 4 times.

4. Add 150μL/well of 1/2000 goat α rabbit-HRP (Dako #P0448) in reagent buffer and incubate 2h at room temperature. Wash 4 times with wash buffer.

5. Add 150μL/well TMB substrate and incubate 5min at room temperature. Use 75μL 1 mol/L HCl as stop mix.

As the explant culture system is a closed system the total amount of each protein expressed was determined for each sample and used in the subsequent analyses.

A1.4. Protein extraction and quantification
Initially the tissue was rinsed in chilled PBS to remove the residual storage medium then chopped into small pieces (~2mm^3) with a scalpel and added to a snap-lock microfuge tube containing lysis buffer (2x µL volume/mg weight) and 3x the tissue weight of 1.4mm stainless steel beads (Next Advance; #SSB14B). The tube of tissue/buffer/beads was then incubated at 4°C on ice to be processed the next day. The sample underwent 3x liquid nitrogen freeze, thaw and vortex cycles and was then processed in a Bullet Blender (Next Advance) on speed 10 (5min, 3 times). The sample was clarified (30,000g, 30min, 4°C) and 5μL 1/10 lysate used to measure protein content of the extract in a Bradford Assay as per manufacturer's instructions (Biorad; #500-0006). The total protein content for each explant sample was determined using the extract concentration and the lysis buffer volume. The lysis buffer comprised: 1 mmol/L EDTA, 0.5% Triton-X 100, 5 mmol/L NaF, 6M urea, 2.5 mmol/L Na pyrophosphate, 1 mmol/L Na orthovanadate, 1x Protease inhibitor tablet (Roche; #04693116001) in PBS, pH 7.4.

Supplemental References

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