Endothelium-derived contraction in a model of rheumatoid arthritis is mediated via angiotensin II type 1 receptors

Hamilton, Kayleigh; Dunning, Lynette; Ferrell, William R. ; Lockhart, John C.; MacKenzie, Andrew

Published in:
Vascular Pharmacology

DOI:
10.1016/j.vph.2017.11.001

Published: 31/01/2018

Document Version
Peer reviewed version

Endothelium-Derived Contraction in a Model of Rheumatoid Arthritis is mediated via Angiotensin II Type 1 Receptors

Kayleigh Hamilton[a], Lynette Dunning[a], William R Ferrell[b], John C Lockhart[a], Andrew MacKenzie[a,*]

[a] Centre of Musculoskeletal Science, Institute for Biomedical and Environmental Health Research, School of Science & Sport, University of the West of Scotland, Paisley, UK

[b] Institute of Infection, Immunity & Inflammation, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow, UK

*Corresponding author at:

Centre of Musculoskeletal Science
Institute for Biomedical and Environmental Health Research
School of Science & Sport
University of the West of Scotland
Paisley Campus
Paisley PA1 2BE, UK
Tel: +44 (0)141 848 3117
Fax: + 44 (0)141 848 3663
E-mail: andrew.mackenzie@uws.ac.uk

Abstract

A role for endothelium-derived constricting factors (EDCF), and the angiotensin II type 1 receptor (AT1R) pathway, in the vascular impairment found in the rat Freund's complete adjuvant (FCA)-model of rheumatoid arthritis (RA) was examined. FCA arthritis was induced in rats ± losartan. Vehicle-treated rats served as controls. Knee-joint swelling and red blood cell (RBC) aggregation were measured as indicators of inflammation and endothelium reactivity assessed by response to acetylcholine (ACh) on aortic rings. Results show that knee-joint swelling and RBC aggregation were elevated in the FCA+vehicle group and restored to control levels in the FCA+losartan-treated animals. ACh-induced relaxation of aortic rings taken from FCA+vehicle animals was significantly impaired compared to vehicle-controls and this vasoreactivity was restored to control levels in the FCA+losartan-treated group. Further examination of aorta from the FCA+vehicle animals revealed an EDCF that was reliant on cyclooxygenase-2 (but not cyclooxygenase-1), generation of superoxide anion generation (but not hydrogen peroxide) and activation of thromboxane-prostanoid receptor. Losartan administration *in vivo* or *ex vivo* (to aortic rings) prevented the generation of the EDCF. In summary, this is the first evidence of an EDCF in a model of RA and identifies this mechanism as potentially significant in the cardiovascular disorder associated with the disease.

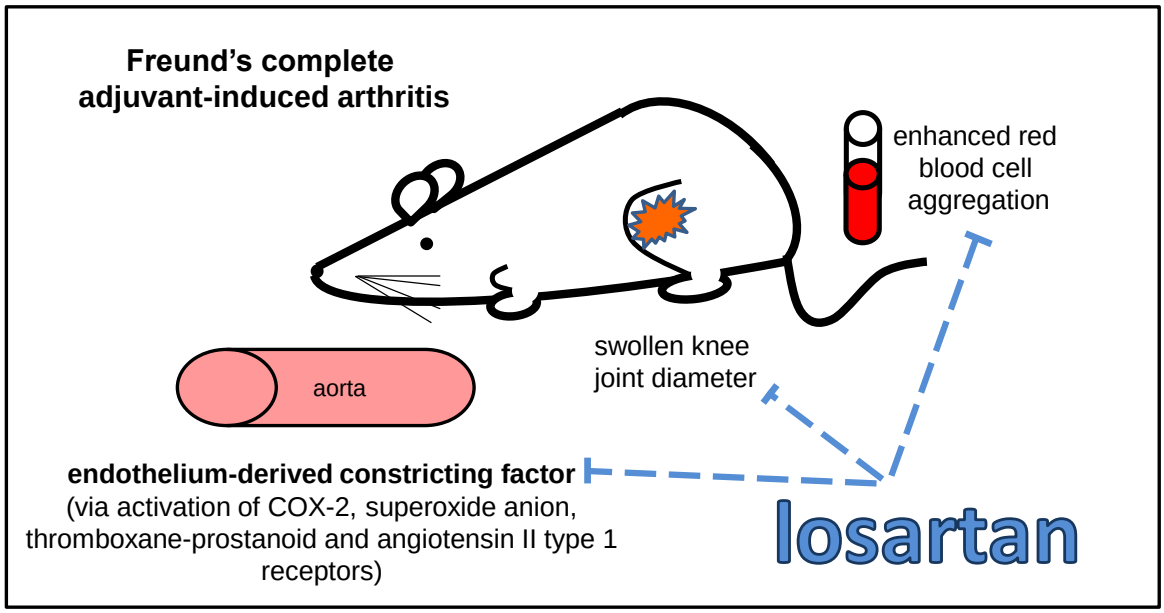
Keywords:

endothelium-derived constricting factor
angiotensin II type 1 receptor
rheumatoid arthritis
losartan
inflammation

Abbreviations:

acetylcholine (ACh); angiotensin II (Ang II); angiotensin II type 1 receptor (AT1R); cyclooxygenase (COX) ;endothelial cell (EC); endothelium-derived constricting factors (EDCF); Freund's complete adjuvant (FCA); knee joint diameter (KJD); nitric oxide (NO); reactive oxygen species (ROS); red blood cell (RBC); rheumatoid arthritis (RA); superoxide dismutase (SOD); thromboxane-prostanoid (TP); vascular smooth muscle cells (VSMC).

Graphical Abstract



1. Introduction

Rheumatoid arthritis (RA) is a chronic, progressive and disabling disease characterized by both articular and extra-articular indicators, including a high cardiovascular mortality beyond that found in the general population [1, 2]. Indeed vascular incident is around 4-times more common in people with RA meaning the condition is comparable to type 2 diabetes as an independent risk factor for the development of atherosclerosis and cardiovascular disease [3-5]. The causes of RA and atherosclerosis are not fully understood and likely to be multifactorial in nature but both diseases are recognized to have a strong inflammatory component with suggestion that inflammatory pathways may be common to both disorders [6-8]. Of these, the renin-angiotensin system, and angiotensin II (Ang II) in particular, has received recent attention [9]. While most often recognized for its vasoconstrictor action, Ang II is now understood to be a significant mediator of inflammation through stimulation of the Ang II-type 1 receptor (AT1R) [10, 11]. Ang II and AT1R stimulation have been reported to be pro-inflammatory through mechanisms including the generation of reactive oxygen species (ROS); activation of nuclear factor kappa B; promotion of cytokines like tumor necrosis factor and interleukin-6; elevation of adhesion molecules and stimulation of leukocyte proliferation and migration [12-15].

Within the context of RA, we have demonstrated that blockade of the AT1R with the antagonist losartan reduces knee joint swelling (a cardinal sign of RA) by more than half in a Freund's complete adjuvant (FCA)-model of arthritis in rats [16, 17]. Moreover losartan has been reported to ameliorate pain and edema found in both mouse and rat models of RA [18]. Further support for a role of AT1R in the physical manifestation of arthritis was shown through demonstration that another AT1R receptor blocker (olmesartan) reduced the arthritis score and joint destruction found in a collagen-induced model of RA in mice [19]. Within human RA, AT1R expression is elevated in chondrocytes [20] and both we and others have reported raised synovial expression of the receptor [16, 21]. Moreover, both the finding that plasma renin activity is elevated in normotensive patients with RA [22], and that inhibition of angiotensin-converting enzyme improved vascular reactivity in patients [23], suggest that renin-angiotensin system involvement in RA is systemic and not localized to inflamed joints.

Vascular endothelial cell (EC) dysfunction represents the earliest stage of atherosclerosis and is central to the pathogenesis found in a variety of cardiovascular disorders [24-26]. EC dysfunction is also a hallmark of human RA, as well as numerous animal models of the disorder [27, 28], and is thought to be fundamental to the elevated cardiovascular risk of the disease [8, 29, 30]. At a functional level, impaired EC activity is typically defined by a reduction in the ability of the

endothelium to mediate the relaxation of neighboring vascular smooth muscle cells (VSMC). Impaired EC in RA has classically been characterized by reduced nitric oxide (NO) bioavailability; however, our group was the first to demonstrate impaired EC activity mediated by the reduced influence of endothelium-derived hyperpolarizing factor [17]. In that study, conducted in isolated saphenous arteries of the FCA rat model, we demonstrated that the impaired endothelium-derived hyperpolarizing factor activity and EC function was restored by prophylactic treatment with losartan. As such we highlighted that that disordered endothelium in a model of RA involves mechanisms beyond that of impaired NO activity and also provided further support for the role of AT1R activation in EC dysfunction.

Importantly, impaired EC activity is not just defined by a reduction in endothelium-derived relaxing factors but also but also by elevation in endothelium-derived contracting factors (EDCF). Such EDCF-mediated contractions are augmented in arteries from both human and animal models of aging, hypertension, type 2 diabetes mellitus and atherosclerosis [31-34] and are considered to be central to the vascular pathophysiology and subsequent cardiovascular events associated with these disorders. EDCF is not mediated by a universal cellular pathway but rather several mechanisms are reported to contribute the contractile response and the nature of the EDCF can vary in different disorders. Frequently reported pathways underlying EDCF include that of generation of ROS (such as superoxide anion and hydrogen peroxide); enhanced cyclooxygenase (COX) activity and stimulation of thromboxane-prostanoid (TP) receptors on VSMC [35].

In this study we provide the first evidence of an EDCF in a model of RA and demonstrate that the impaired EC response is altered in an AT1R-senstive manner. Furthermore, we show AT1R blockade also provides protection against non-vascular inflammatory parameters induced by the model. As such we highlight that renin-angiotensin system and AT1R activation is likely to be key to the development of the clinical manifestations of RA and is identified as a means of therapeutic intervention to reduce, slow or even prevent the consequences of the disease.

2. Materials and Methods

2.1. Chemicals and solution

All drugs and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) with the exception of L655 (L665,240; selective TP receptor antagonist, Tocris Bioscience, Abingdon, UK). Drugs were prepared in distilled water and frozen in stock solutions with the exception indomethacin (10 mM stock) which was dissolved in Na_2CO_3 (0.4 mg ml^{-1}). Control studies demonstrated that the concentration of solvents was without influence on vascular reactivity. Serial dilutions were made in isotonic saline.

2.2. Animals

A total of 43 adult male Sprague Dawley rats (bred in-house, weighing 380 - 420 g) were used in the experiments described here. Animals were housed in a centralized animal facility in standard cages, with food and water available *ad libitum*. The rats were maintained in a thermoneutral environment, with a 12-hour light/dark cycle. All procedures were performed in accordance with UK Home Office regulations described in compliance with the ARRIVE guidelines for reporting of experiments involving animals [36, 37].

2.3. Experimental Groups and Induction of Inflammation

FCA-mediated inflammation, as a model of chronic monoarthritis, was induced by intra-articular (200 µl x 2) injection of FCA, supplemented with 10 mg ml^{-1} of heat-killed *Mycobacterium tuberculosis*, into one (the left) knee, with the animal under general anesthesia (O_2/N_2O/2% halothane), as described previously [17, 38].

In one group, losartan (15 mg kg^{-1}), an AT1R antagonist, was dissolved in saline and administered subcutaneously 1 hour prior to FCA induction and maintenance doses administered every 48 hours thereafter. This regime has been shown to substantially reduce knee joint swelling in rats with adjuvant arthritis [16, 17]. Control animals (i.e. receiving neither FCA nor losartan) were administered with a saline vehicle.

Rats were euthanized with carbon dioxide and dislocation of the neck 21 days following the induction of FCA inflammation ± losartan or vehicle control.

2.4. Assessment of Inflammation

Joint swelling, a key characteristic of inflammation, was assessed by measurement of knee joint diameter (KJD, using modified Vernier calipers) at set time points over the 21 days and expressed

as the percentage of the pre-induction diameter. Repeated measurements of normal knees on 5 successive days are reproducible with a coefficient of variation of ≤2%.

RBC aggregation in whole blood is associated with inflammatory conditions [39] and is used in the clinical evaluation of RA [40]. Immediately following cull, whole blood (~2 ml) was collected via cardiac puncture and placed in a heparin-containing tube. The sample was then analyzed photometrically at room temperature using a Myrenne MA1 cone-plate aggregometer (Myrenne, Roetgen, Germany). After a period of high shear rate disaggregation, the aggregation index was determined from the change in light intensity over a 5 second period at a zero shear rate.

2.5. Vessels for the study

The thoracic aorta was carefully removed from the animal, placed in ice-cold Krebs' solution of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11 gassed with 95% O_2/ 5% CO_2, pH 7.4; cleaned of adhering connective tissue and sliced in to 2 mm length ring preparations. In some rings the EC were removed mechanically by gentle abrasion of the lumen with the tip of small forceps. The rings were mounted for isometric tension recording in 10 ml tissue baths (maintained at 37°C and gassed with 95% O_2/ 5% CO_2), force was measured by TBM4 bridge amplifiers (World Precision Instruments) and data collected via Data-Trax2 acquisition system. The aortic preparations were stretched to a 1g resting tension and allowed to equilibrate for 60 minutes adjusting the tension as necessary. Following equilibration, tissues were exposed twice to KCl (80 mM).

2.5.1. Characterization of vascular responses

Following sub-maximal (~70% maximal) pre-contraction of the vessels with phenylephrine, responses to acetylcholine (ACh, 1nM-30μM) were measured. In some experiments the role of NO in endothelium-dependent relaxation was examined using the NO synthase inhibitor N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 100 μM).

2.5.2. Characterization of the nature of EDCF

As is demonstrated in Results, ACh was able to produce contraction in aortic segments (already pre-contracted with phenylephrine) taken from FCA+vehicle rats. These ACh-mediated contractions became apparent when the opposing relaxant influence of ACh-stimulation of NO synthase was removed (i.e. in the presence of L-NAME) and was lost following removal of the endothelium. Such observations are characteristic of the presence of an EDCF and consequently a series of experiments were performed to determine the nature of the phenomenon. In these experiments L-NAME was applied throughout, to remove the opposing relaxant component of

EC, and a series of pharmacological inhibitors were employed to determine the identity of the EDCF(s). The general influence of COX was determined by incubation of the aortic rings with indomethacin (10 μM) while selective inhibitors of COX-1 (nimesulide, 10 μM) and COX-2 (ketoprofen, 10 μM) were used to define any role of specific COX isoforms. The influence of superoxide anion and hydrogen peroxide were determined by the use of superoxide dismutase (SOD; 200 U ml^{-1}) and catalase (2000 U ml^{-1}), respectively. Any role for AT1R was determined by *ex vivo* application (i.e. direct to the bathing medium of the tissue bath) of losartan (300 μM) and the influence of TP receptor was determined by use of the selective antagonist L655 (100 μM).

2.6. Data analysis

Data are expressed as mean ± s.e. mean; *n* values represent the number of animals from which measurements were made or tissue harvested. Responses to ACh are represented as a percentage of the vasoconstrictor tone generated by sub-maximal contraction to phenylephrine (immediately prior to application of ACh), expressed either as reduction (relaxation) or enhancement (contraction) to this tone. Concentration-response curves were generated by and statistical comparisons (one or two-way ANOVA followed by a Bonferroni post hoc test) made with the aid of GraphPad Prism (GraphPad, San Diego, CA, USA). $P<0.05$ was considered significant.

3. Results

3.1. Assessment of inflammatory parameters

Induction of FCA resulted in a substantial knee joint swelling within a 24 hour period which was sustained over the next 21 days such that KJD was 72.1 ± 1.9 % greater than the pre-induction diameter at the time of cull (Figure 1A). FCA+vehicle rats administered prophylactically with the AT1R antagonist losartan show a significant attenuation to knee joint swelling such that KJD was 13.9 ± 2.2 % greater than pre-induction diameter at day 21, i.e. a reduction in swelling by around 80% compared to the FCA+vehicle group, $P<0.001$).

RBC aggregation was significantly greater ($P<0.001$) in FCA+vehicle rats compared vehicle controls (Figure 1B). Aggregation levels fell dramatically in the FCA+losartan-treated group ($P<0.001$) compared to FCA alone although remained elevated ($P<0.05$) when compared blood taken from vehicle controls.

Fig 1A

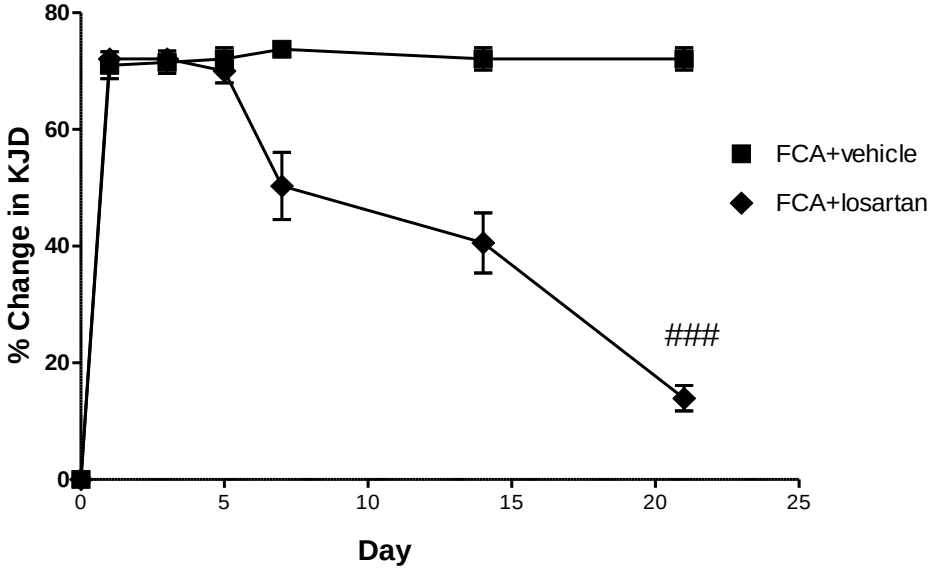

80
60
40
20
0
% Change in KJD
0
5
10
15
20
25
Day
FCA+vehicle
FCA+losartan
###

Fig 1B

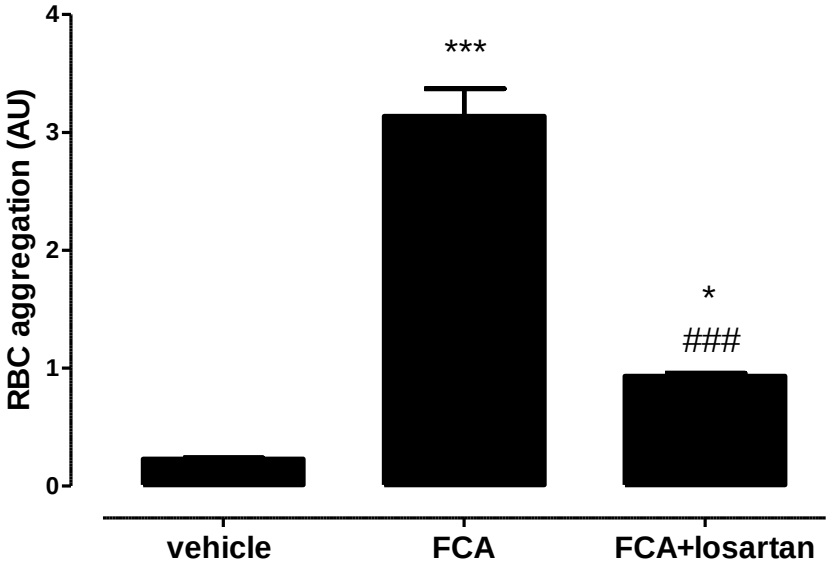

4
3
2
1
0
RBC aggregation (AU)
***
*
###
vehicle
FCA
FCA+losartan

Fig 1. Assessment of inflammatory parameters in FCA+vehicle rats and of the prophylactic influence of AT1R blockade with losartan. **A**. Change in knee joint diameter (KJD) over a 21 day period following induction of adjuvant arthritis (FCA+vehicle) was substantially reduced in rats treated with losartan (FCA+losartan). Data expressed as the percentage of the pre-induction diameter; *n=8-14.* **B.** Red blood cell (RBC) aggregation (arbitrary units, AU) was elevated in FCA+vehicle rats compared to vehicle controls while those of the FCA+losartan group were reduced compared to FCA but remain raised in comparison to control; *n=5-7*. **P*<0.05 and ***P*<0.001 indicate a significant difference from vehicle controls. ###P<0.001 indicates a significant difference compared to FCA+vehicle rats.

3.2. Vascular responses

No differences were observed in the ability of KCl or phenylephrine to induce contraction in aortic rings taken from either the vehicle-control, FCA+vehicle or FCA+losartan-treated groups (data not shown). Tissue segments from all groups demonstrated an increase in sensitivity to phenylephrine when pre-treated with L-NAME; consequently the concentration of this spazmogen was altered accordingly to maintain consistency in the level of generated tone i.e. level of contraction prior to the addition of ACh.

3.2.1. Endothelium-dependent responses in experiential groups

Following generation of submaximal tone with phenylephrine, ACh (1 nM - 30 µM) produced a concentration-dependent relaxation of the aortic rings harvested from vehicle control rats (relaxation was 87.4 ± 1.5 % at 30 µM ACh, Figure 2A). In FCA+vehicle rats, the relaxation generated by low concentrations of ACh (< 300 nM) was no different to that observed in vehicle rats, however, when ACh was applied at concentrations greater than 1 µM, the relaxation produced was significantly different from that found in controls such that the response to ACh was markedly reduced (58.6 ± 5.0% at 30 µM ACh; $P<0.01$). In response to ACh, the relaxation generated in rings from the FCA+losartan-treated rats was free of the marked reduction in vasodilation observed in FCA+vehicle rats, such that the relaxation in the FCA+losartan group (84.5 ± 3.2 % at 30 µM ACh, Figure 2A) was substantially greater than that found in FCA+vehicle animals ($P<0.01$) and indistinguishable from that of the vehicle controls ($P>0.05$).

The relaxation induced by ACh in rings from either the control group or animals treated with FCA+losartan was abolished ($P<0.001$) following treatment with L-NAME (Figure 2A) or by the mechanical removal of EC (data not shown), indicating that the relaxation was both endothelium-dependent and NO-mediated. In contrast, treatment of aortic rings from the FCA+vehicle group with L-NAME did not simply abolish ACh-induced relaxation but rather led to a further contraction of tissue (by around 15%) beyond that mediated by the initial contraction induced by phenylephrine. This ACh-mediated contraction was absent in rings in which the EC had been removed (Fig 2B) confirming that the endothelium was the source of the contractile influence i.e. indicative of an EDCF.

Fig 2A

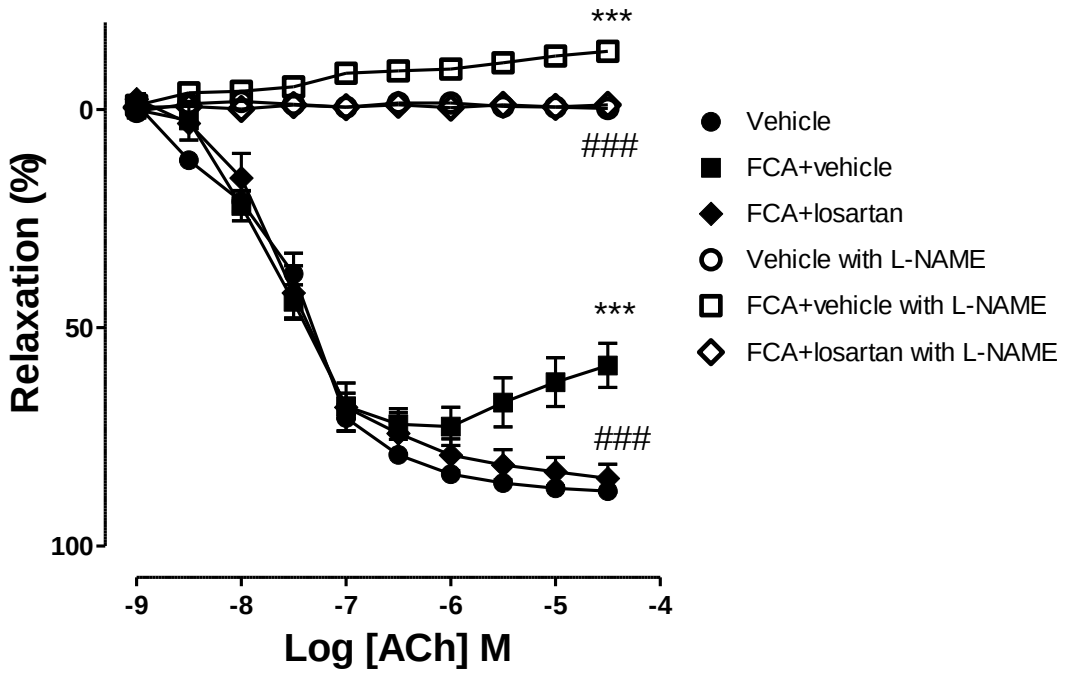

***
0
###
***
50
###
100
Relaxation (%)
-9
-8
-7
-6
-5
-4
Log [ACh] M
Vehicle
FCA+vehicle
FCA+losartan
Vehicle with L-NAME
FCA+vehicle with L-NAME
FCA+losartan with L-NAME

Fig 2B

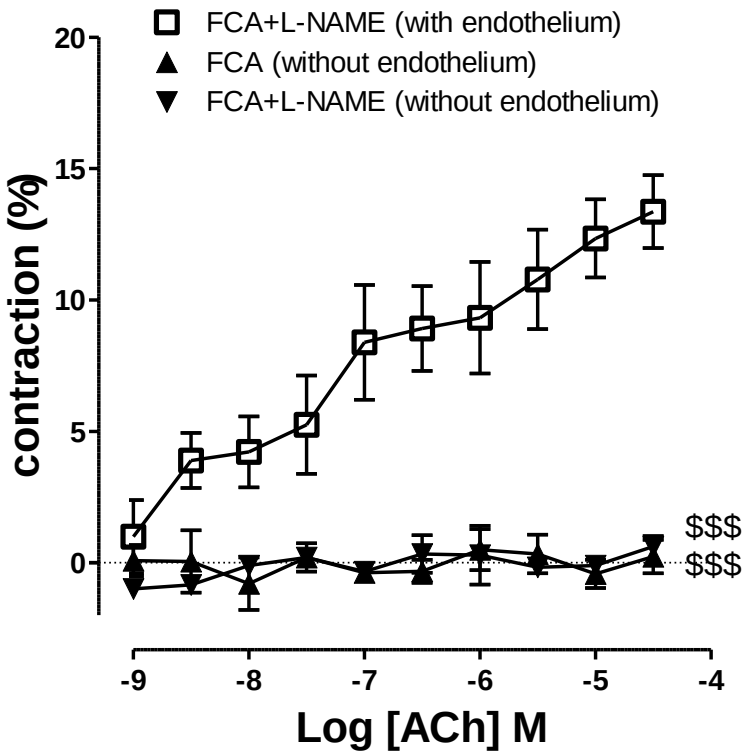

FCA+L-NAME (with endothelium)
FCA (without endothelium)
FCA+L-NAME (without endothelium)
contraction (%)
20
15
10
5
0
$$$
$$$
-9
-8
-7
-6
-5
-4
Log [ACh] M

Fig 2. Cumulative concentration-response curves showing the response to acetylcholine (ACh) in aortic segments isolated from vehicle control, FCA+vehicle and FCA+losartan groups.

A) ACh-induced relaxation was impaired in FCA+vehicle rats when compared to the vehicle controls and this attenuation to relaxation was absent in the FCA+losartan group. ACh induced relaxation was abolished in all groups following the administration of L-NAME *ex vivo* albeit the response found in the FCA+vehicle group became negative in nature (i.e. a contraction); *n=6-11.*

B) In aortic rings taken from the FCA+vehicle group, the ACh mediated contraction found in endothelium-containing segments administered with L-NAME was abolished by the removal of the endothelium; *n=6-10.* ****P*<0.001 indicates a significant difference from equivalent vehicle controls. ###*P*<0.001 indicates a significant difference from equivalent FCA+vehicle rats. $$$P<0.001 indicates a significant difference from FCA+L-NAME (with endothelium).

3.3. Nature of the endothelium-dependent contraction in the aorta of FCA+vehicle animals

A series of experiments were performed on aortic rings taken from FCA+vehicle rats to identify the nature of the EDCF underlying the ACh-mediated contraction. All data generated here was produced in endothelium-containing rings treated with L-NAME and pre-contracted with phenylephrine.

3.3.1. Role of cyclooxygenase

ACh-induced contractions were abolished ($P<0.001$) following treatment with either the general COX inhibitor indomethacin or the selective COX-2 inhibitor nimesulide (Figure 3A). Incubation of aortic rings with the selective COX-1 inhibitor ketoprofen was without influence ($P>0.05$) on the contraction, suggesting that the ACh-mediated contractions are dependent on COX-2 activity.

3.3.2. Reactive oxygen species

EDCF responses were unaltered ($P>0.05$) following application of catalase but eliminated ($P<0.001$) in the presence of SOD suggesting that the endothelium-derived contraction is not mediated by hydrogen peroxide but is dependent on superoxide anion (Figure 3B).

3.3.3. Blockade of thromboxane and AT1R receptors

ACh-mediated contractions were abolished ($P<0.001$) following treatment by L655 highlighting that TP receptors play a key role in the EDCF response (Figure 3C). Exogenous application of losartan directly to the bathing medium of aortic rings (i.e. not administered to animals) also obliterated ($P<0.001$) the contraction suggesting that AT1R receptors are critical to the EDCF mechanism.

Fig 3A

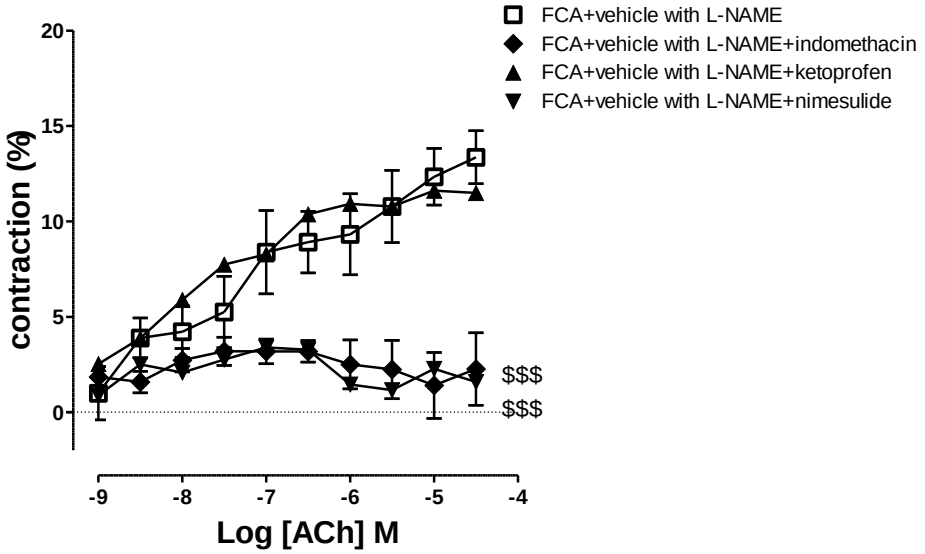


Fig 3B

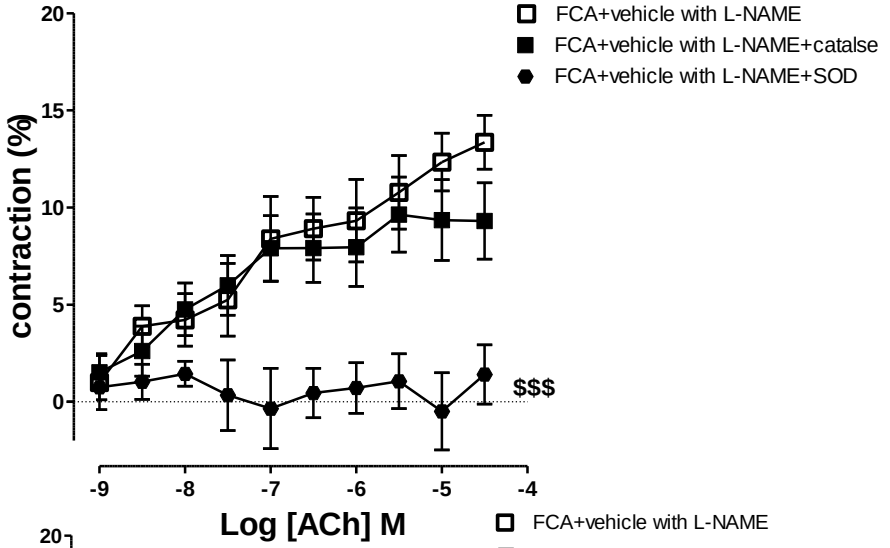


Figure 3C

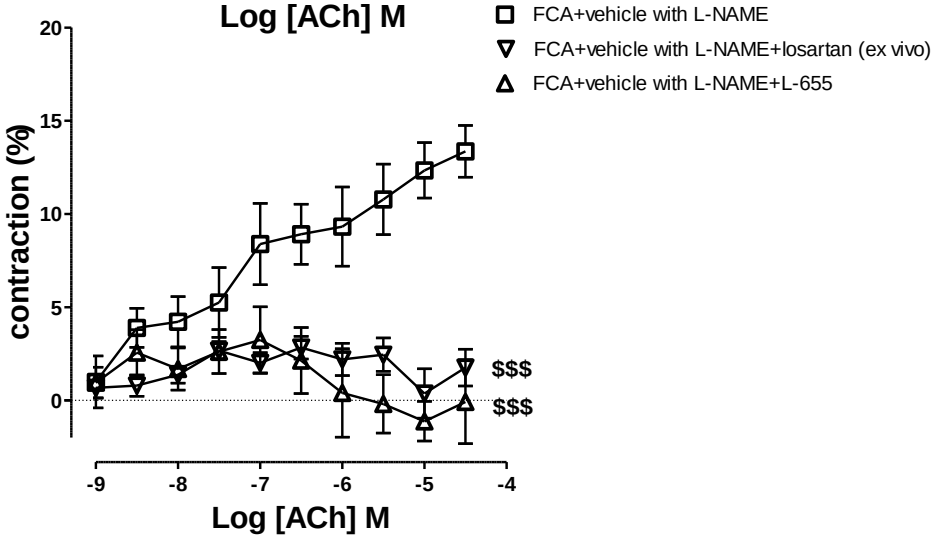


Fig 3. Cumulative concentration-response curves showing the endothelium-derived contraction (EDCF) induced by acetylcholine (ACh) in aortic rings administered with L-NAME taken from FCA+vehicle rats and the influence of a series of pharmacological inhibitors. **A)** The non-selective COX antagonist indomethacin and selective COX-2 inhibitor nimesulide obliterated the action of EDCF but the COX-1 antagonist ketoprofen was without influence; *n=5-7.* **B)** Treatment with the superoxide anion scavenger superoxide dismutase (SOD) but not catalase (which decomposes hydrogen peroxide) eliminated the contractile action of EDCF; *n=6-8.* **C**) Both inactivation of the TP receptor by addition of L655 and the *ex vivo* application (direct to bathing medium of aortic rings) of losartan to antagonize AT1R prevented the ability of ACh to induce contraction; *n=5-7.* $$$*P*<0.001 indicates a significant difference from FCA+vehicle with L-NAME.

4. Discussion

Extending our previous finding that AT1R blockade reduces joint inflammation in the rat FCA model [16, 17], the present study is the first to report the presence of an EDCF in a model of inflammatory arthritis and highlights the potential for this phenomenon to contribute to the cardiovascular dysfunction associated with human RA. Furthermore we demonstrate that prophylactic treatment with losartan prevents the EDCF phenomenon thereby identifying AT1R as key to the disrupted EC reactivity. Moreover, given the substantial protection afforded by losartan to the elevation of both knee joint swelling and RBC aggregation induced by the FCA model, the AT1R pathway appears likely to contribute to the inflammatory-induced changes observed in the joint, blood and vascular tissue.

ACh-induced relaxation has been shown to be impaired in RA patients [41], a variety of experimental models of RA [27], and reflects the disturbed reactivity of the endothelium underling the elevated cardiovascular risk of the disease. As with these studies, we also found a compromised response to ACh in our FCA model of arthritis. Specifically, we found that relaxation produced by high concentrations of ACh was significantly impaired compared to controls (such that the maximum relaxation was reduced by around a third). Treatment of the EC-containing aortic rings from the FCA+vehicle rats with L-NAME abolished the relaxation to ACh (as was the case in tissue from control animals) but crucially ACh mediated a contraction beyond that induced by the initial application of phenylephrine. This contractile action of ACh was not found in L-NAME-treated rings from control rats and such observations strongly implicate the presence of an EDCF in the FCA+vehicle animals.

From the data we cannot determine if the reduced relaxation found in the FCA+vehicle group is mediated by a reduced NO activity or by enhanced influence of EDCF. It seems feasible though that ACh (in tissues not treated with L-NAME) enhances NO synthase activation to promote a relaxation while simultaneously activating the ECDF mechanism i.e. ACh-induced NO-derived relaxation vs. ACh-mediated contractile action of EDCF. The sum influence of these two forces is a net relaxation but one that is considerably impaired with respect to relaxation found in tissue from control rats.

The relaxation induced by ACh in pre-contracted aortic rings from FCA+losartan-treated rats was comparable to that found in the control group. As such the response found in the FCA+losartan rats is substantially different from that found in the FCA+vehicle animals when assessed by two methods. Firstly, the reduced relaxation found in response to high concentrations of ACh in the FCA+vehicle group was reversed in the FCA+losartan-treated rats. Secondly, the EDCF activity

induced by ACh in the L-NAME-treated rings of the FCA+vehicle group was absent in the FCA+losartan group. Consequently administration of losartan affords complete protection to the impaired EC reactivity in this model.

The phenomenon of EDCF has long been established in variety of diseases but until now has neither been reported in human RA nor in an animal model of RA. A number of mediators have been described to underlie the EDCF effect and here we examined lead candidates as potential contributors to the ACh-induced contractile response found in this study.

Prophylactic treatment of FCA+vehicle rats with losartan (i.e. *in vivo* administration) prevents the influence of EDCF suggesting that AT1R activation *in vivo* is a critical step in the generation of the contractile response. However the EDCF is also abolished when losartan is added exogenously to tissue from FCA+vehicle rats i.e. the action of losartan when administered *ex vivo* in tissue taken from animals with FCA treatment alone (i.e. with no losartan administered *in vivo*). This evidences a key role of AT1R activation in the generation of the EDCF but also suggests that the EDCF can be inhibited locally in blood vessels (i.e. in tissue isolated from the rest of the body). These data suggest that ACh leads to the local generation (i.e. within the blood vessel) of Ang II, activation of AT1R and subsequent contraction of VSMC. Indeed there is abundant evidence that all components of the renin-angiotensin system machinery exist locally in the vascular wall and can be elevated by conditions of disrupted homeostasis [42].

Perhaps the most common pathway reported to underlie EDCF in disease states is that of COX activation. Application of indomethacin removes the ACh-induced contractions thus highlighting a role for COX involvement in the generation of EDCF. Further analysis shows that ketoprofen (at a pharmacological concentration reported to produce effective inhibition [43, 44]) was without influence on the contraction induced by ACh, suggesting that COX-1 has no involvement. However nimesulide abolished the contraction to ACh suggesting that COX-2 stimulation is central to the contraction induced by ACh. TP receptor activation is a common downstream effector of COX stimulation and here we demonstrate that selective inhibition of the TP receptor abolishes the ACh-mediated contraction thus highlighting a role for this pathway in the EDCF mechanism. A role of COX-2/TP activity in the vascular dysfunction found in an experimental model of RA (as is reported here) is supported by other studies who report that: the enhanced COX-2 expression found in the femoral artery of a rabbit model of atherosclerosis combined with adjuvant arthritis was reduced following COX-2 inhibition [45]; and that, the impaired ACh-induced vasodilation in a rat model was improved when aortic rings were incubated with selective inhibitors of COX-2 and thromboxane synthase [46].

ROS have been reported to play a role in the EDCF phenomenon and here we investigated action of the enzymes SOD and catalase (as inhibitors of superoxide anion and hydrogen peroxide, respectively). Incubation of aortic rings from FCA+vehicle rats with SOD abolished the contraction to ACh but catalase (at a concentration shown to be effective [47]) was without influence suggesting the involvement of superoxide anion, but not hydrogen peroxide, in the EDCF pathway. Previously, superoxide anion has been reported to underlie the EDCF in models of hypertension [48] and type 2 diabetes mellitus [49].

Key mechanistic roles for AT1R, COX-2, TP receptor and superoxide anion have been identified in the novel EDCF phenomenon demonstrated in this FCA study. These pathways are liable to be linked since if they were separate they would likely lead to a summative effect on the total influence of EDCF (i.e. the individual inhibitors would produce only partial inhibition of the EDCF rather than the elimination observed here).

Interactions between COX-2 and TP receptor activation have been well established in inflammation and most likely involve generation of isoprostanes [50]. However COX-2 activation of TP has also been reported to be mediated by superoxide anion with suggestion that ROS can be formed simultaneously during COX activation [48, 51, 52]. Furthermore, ROS has been reported to have a circuitous relationship with COX-2 (in hypertensive mice at least) such that the COX-2/TP receptor axis both produces and is stimulated by superoxide anion [53]. From the current data we cannot determine if superoxide anion plays a role upstream or downstream from COX-2 or TP receptor activation in the EDCF phenomenon found in this study.

Losartan administration has been shown to reduce the influence of EDCF in this study and also in the mesenteric vessel of a model of type 2 diabetes mellitus type 2 (EDCF mechanism involving prostaglandin E2 and perhaps superoxide anion; [49]) and the aorta of spontaneously hypertensive rats (EDCF mechanism involving TP receptor activation; [54]) suggesting, within the confines of these models, some commonality in the mechanisms of endothelium dysfunction across disease states. Within the context of arthritis, losartan has been shown to restore the impaired ACh-induced relaxation found in aortic rings taken from (angiotensin-induced) hypertensive rats with concomitant adjuvant-induced arthritis [55]. This model is shown to be quite different form the one used in our current study in that the impairment to relaxation was mediated by a NAD(P)H oxidase–derived elevation of superoxide anion and subsequent impairment of NO bioavailability. Interestingly this study found, like we did, that the beneficial effect of losartan was present whether it was administered *in vivo* or to vascular tissue *ex vivo* highlighting the importance of locally-located renin-angiotensin system in the vascular dysfunction.

In this study, KJD increased by over 70% in the days following FCA-induction and stabilized close to this value until the time of the cull suggesting that joint inflammation was consistent throughout the 21 day period. This is in agreement with previous studies from our group using a similar induction protocol in the rat [38, 56]. Furthermore, the current data confirms our earlier finding [16, 17] that losartan administration substantially inhibits the development of adjuvant-induced knee joint swelling in the rat, which again highlights a central role of AT1R in this cardinal indicator of RA. Moreover, RBC aggregation (as a measure of systemic inflammation) was significantly elevated in the FCA+vehicle group and as such reflect a key clinical aspect of human RA [39, 57]. Importantly, treatment with losartan significantly reduced the aggregation indicating that the AT1R antagonist provided anti-inflammatory protection in the model. Losartan has also been reported to prevent platelet aggregation in rats [54, 58] and humans [59-61] and therefore our study gives further support to the antithrombotic influence of this agent and therapeutic potential beyond its classical blood pressure lowering action.

5. Conclusions

This study demonstrates that AT1R antagonism protects against the manifestations of inflammation as assessed through changes in the joint, RBC aggregation and vascular reactivity. Importantly we identify, for the first time, an EDCF phenomenon in a model of chronic monoathritis that shares many features with human RA. The EDCF was dependent on pathways involving AT1R, COX-2, ROS and TP receptor activation. Furthermore, AT1R antagonism with losartan defends against the generation of EDCF and protects the functional reactivity of the endothelium. We also provide evidence that losartan administration reduces joint swelling and RBC aggregation, both cardinal signs of RA, suggesting that AT1R blockade provides protection beyond the damage inflicted to vascular tissue alone. Indeed, this is supported by the recent demonstration that losartan reduced the enhanced leukocyte-endothelium interaction and joint hypernociception found in two separate experimental models of RA [18]. Given the importance of cardiovascular events in RA, new insight into the mechanisms underlying endothelial dysfunction, as with this EDCF, is vital to improving treatment.

Funding sources: this work was supported by the Institute for Biomedical and Environmental Health Research, University of the West of Scotland.

Conflicts of interest: none

References

[1] S. Wallberg-Jonsson, M.L. Ohman, S.R. Dahlqvist, Cardiovascular morbidity and mortality in patients with seropositive rheumatoid arthritis in Northern Sweden, J. Rheumatol 24(3) (1997) 445-451.
[2] F. Wolfe, D.M. Mitchell, J.T. Sibley, J.F. Fries, D.A. Bloch, C.A. Williams, P.W. Spitz, M. Haga, S.M. Kleinheksel, M.A. Cathey, The mortality of rheumatoid arthritis, Arthritis Rheum 37(4) (1994) 481-494.
[3] N. Sattar, D.W. McCarey, H. Capell, I.B. McInnes, Explaining how "high-grade" systemic inflammation accelerates vascular risk in rheumatoid arthritis, Circulation 108(24) (2003) 2957-2963.
[4] M.J. Peters, V.P. van Halm, A.E. Voskuyl, Y.M. Smulders, M. Boers, W.F. Lems, M. Visser, C.D. Stehouwer, J.M. Dekker, G. Nijpels, R. Heine, B.A. Dijkmans, M.T. Nurmohamed, Does rheumatoid arthritis equal diabetes mellitus as an independent risk factor for cardiovascular disease? A prospective study, Arthritis Rheum 61(11) (2009) 1571-9.
[5] D.H. Solomon, N.J. Goodson, J.N. Katz, M.E. Weinblatt, J. Avorn, S. Setoguchi, C. Canning, S. Schneeweiss, Patterns of cardiovascular risk in rheumatoid arthritis, Ann Rheum Dis 65(12) (2006) 1608-12.
[6] M. Mahmoudi, S. Aslani, R. Fadaei, A.R. Jamshidi, New insights to the mechanisms underlying atherosclerosis in rheumatoid arthritis, Int J Rheum Dis (2017).
[7] S. Skeoch, I.N. Bruce, Atherosclerosis in rheumatoid arthritis: is it all about inflammation?, Nat Rev Rheumatol 11(7) (2015) 390-400.
[8] I.A. Ku, J.B. Imboden, P.Y. Hsue, P. Ganz, Rheumatoid arthritis: model of systemic inflammation driving atherosclerosis, Circ. J 73(6) (2009) 977-985.
[9] Y. Chang, W. Wei, Angiotensin II in inflammation, immunity and rheumatoid arthritis, Clin Exp Immunol 179(2) (2015) 137-45.
[10] Z.J. Cheng, H. Vapaatalo, E. Mervaala, Angiotensin II and vascular inflammation, Med. Sci. Monit 11(6) (2005) RA194-RA205.
[11] C. Marchesi, P. Paradis, E.L. Schiffrin, Role of the renin-angiotensin system in vascular inflammation, Trends Pharmacol. Sci 29(7) (2008) 367-374.
[12] M. Ruiz-Ortega, O. Lorenzo, Y. Suzuki, M. Ruperez, J. Egido, Proinflammatory actions of angiotensins, Curr. Opin. Nephrol. Hypertens 10(3) (2001) 321-329.
[13] K. Husain, W. Hernandez, R.A. Ansari, L. Ferder, Inflammation, oxidative stress and renin angiotensin system in atherosclerosis, World J Biol Chem 6(3) (2015) 209-17.
[14] A. MacKenzie, Endothelium-derived vasoactive agents, AT1 receptors and inflammation, Pharmacol. Ther 131(2) (2011) 187-203.
[15] M. Pacurari, R. Kafoury, P.B. Tchounwou, K. Ndebele, The Renin-Angiotensin-aldosterone system in vascular inflammation and remodeling, Int J Inflam 2014 (2014) 689360.
[16] A. Price, J.C. Lockhart, W.R. Ferrell, W. Gsell, S. McLean, R.D. Sturrock, Angiotensin II type 1 receptor as a novel therapeutic target in rheumatoid arthritis: in vivo analyses in rodent models of arthritis and ex vivo analyses in human inflammatory synovitis, Arthritis Rheum 56(2) (2007) 441-447.
[17] A. Mackenzie, L. Dunning, W.R. Ferrell, J.C. Lockhart, Angiotensin II Type 1 receptor blockade protects endothelium-derived hyperpolarising factor-mediated relaxation in a rat model of monoarthritis, Life Sci 92(23) (2013) 1131-7.
[18] K.D. Silveira, F.M. Coelho, A.T. Vieira, L.C. Barroso, C.M. Queiroz-Junior, V.V. Costa, L.F. Sousa, M.L. Oliveira, M. Bader, T.A. Silva, R.A. Santos, A.C. Silva, M.M. Teixeira, Mechanisms of the anti-inflammatory actions of the angiotensin type 1 receptor antagonist losartan in experimental models of arthritis, Peptides 46 (2013) 53-63.
[19] K. Sagawa, K. Nagatani, Y. Komagata, K. Yamamoto, Angiotensin receptor blockers suppress antigen-specific T cell responses and ameliorate collagen-induced arthritis in mice, Arthritis Rheum 52(6) (2005) 1920-8.

[20] Y. Kawakami, K. Matsuo, M. Murata, K. Yudoh, H. Nakamura, H. Shimizu, M. Beppu, Y. Inaba, T. Saito, T. Kato, K. Masuko, Expression of Angiotensin II Receptor-1 in Human Articular Chondrocytes, Arthritis 2012 (2012) 648537.
[21] D.A. Walsh, T. Suzuki, G.A. Knock, D.R. Blake, J.M. Polak, J. Wharton, AT1 receptor characteristics of angiotensin analogue binding in human synovium, Br. J. Pharmacol 112(2) (1994) 435-442.
[22] M.E. Mavrikakis, G. Vaiopoulos, B. Papantoniou, L.G. Antoniades, C. Kostopoulos, S. Papazoglou, E.A. Lianos, Plasma renin activity as a marker of renovascular injury in patients with rheumatoid arthritis, Clin Exp Rheumatol 14(6) (1996) 613-7.
[23] A.J. Flammer, I. Sudano, F. Hermann, S. Gay, A. Forster, M. Neidhart, P. Kunzler, F. Enseleit, D. Periat, M. Hermann, J. Nussberger, T.F. Luscher, R. Corti, G. Noll, F. Ruschitzka, Angiotensin-converting enzyme inhibition improves vascular function in rheumatoid arthritis, Circulation 117(17) (2008) 2262-9.
[24] P. Libby, P.M. Ridker, G.K. Hansson, Inflammation in atherosclerosis: from pathophysiology to practice, J. Am. Coll. Cardiol 54(23) (2009) 2129-2138.
[25] J. Herrmann, A. Lerman, The endothelium - the cardiovascular health barometer, Herz 33(5) (2008) 343-53.
[26] M. Toborek, S. Kaiser, Endothelial cell functions. Relationship to atherogenesis, Basic Res. Cardiol 94(5) (1999) 295-314.
[27] P. Totoson, K. Maguin-Gate, C. Prati, D. Wendling, C. Demougeot, Mechanisms of endothelial dysfunction in rheumatoid arthritis: lessons from animal studies, Arthritis Res Ther 16(1) (2014) 202.
[28] P. Libby, Role of inflammation in atherosclerosis associated with rheumatoid arthritis, Am. J. Med 121(10 Suppl 1) (2008) S21-S31.
[29] H. Maradit-Kremers, P.J. Nicola, C.S. Crowson, K.V. Ballman, S.E. Gabriel, Cardiovascular death in rheumatoid arthritis: a population-based study, Arthritis Rheum 52(3) (2005) 722-732.
[30] G. Vaudo, S. Marchesi, R. Gerli, R. Allegrucci, A. Giordano, D. Siepi, M. Pirro, Y. Shoenfeld, G. Schillaci, E. Mannarino, Endothelial dysfunction in young patients with rheumatoid arthritis and low disease activity, Ann. Rheum. Dis 63(1) (2004) 31-35.
[31] A. Virdis, L. Ghiadoni, S. Taddei, Human endothelial dysfunction: EDCFs, Pflugers Arch 459(6) (2010) 1015-23.
[32] M.S. Wong, P.M. Vanhoutte, COX-mediated endothelium-dependent contractions: from the past to recent discoveries, Acta Pharmacol Sin 31(9) (2010) 1095-102.
[33] Y. Shi, P.M. Vanhoutte, Reactive oxygen-derived free radicals are key to the endothelial dysfunction of diabetes, J Diabetes 1(3) (2009) 151-62.
[34] S. Taddei, A. Virdis, L. Ghiadoni, D. Versari, A. Salvetti, Endothelium, aging, and hypertension, Curr Hypertens Rep 8(1) (2006) 84-9.
[35] P.M. Vanhoutte, E.H. Tang, Endothelium-dependent contractions: when a good guy turns bad!, J. Physiol 586(Pt 22) (2008) 5295-5304.
[36] C. Kilkenny, W. Browne, I.C. Cuthill, M. Emerson, D.G. Altman, N.C.R.R.G.W. Group, Animal research: reporting in vivo experiments: the ARRIVE guidelines, Br J Pharmacol 160(7) (2010) 1577-9.
[37] J.C. McGrath, G.B. Drummond, E.M. McLachlan, C. Kilkenny, C.L. Wainwright, Guidelines for reporting experiments involving animals: the ARRIVE guidelines, Br J Pharmacol 160(7) (2010) 1573-6.
[38] S.M. Day, J.C. Lockhart, W.R. Ferrell, J.S. McLean, Divergent roles of nitrergic and prostanoid pathways in chronic joint inflammation, Ann. Rheum. Dis 63(12) (2004) 1564-1570.
[39] A. Luquita, L. Urli, M.J. Svetaz, A.M. Gennaro, R. Volpintesta, S. Palatnik, M. Rasia, Erythrocyte aggregation in rheumatoid arthritis: Cell and plasma factor's role, Clinical hemorheology and microcirculation 41(1) (2009) 49-56.
[40] C.S. Crowson, M.U. Rahman, E.L. Matteson, Which measure of inflammation to use? A comparison of erythrocyte sedimentation rate and C-reactive protein measurements from randomized clinical trials of golimumab in rheumatoid arthritis, J Rheumatol 36(8) (2009) 1606-10.

[41] R. Bergholm, M. Leirisalo-Repo, S. Vehkavaara, S. Makimattila, M.R. Taskinen, H. Yki-Jarvinen, Impaired responsiveness to NO in newly diagnosed patients with rheumatoid arthritis, Arterioscler. Thromb. Vasc. Biol 22(10) (2002) 1637-1641.
[42] M. Paul, M.A. Poyan, R. Kreutz, Physiology of local renin-angiotensin systems, Physiol Rev 86(3) (2006) 747-803.
[43] A. De Angelis, B. Rinaldi, A. Capuano, F. Rossi, A. Filippelli, Indomethacin potentiates acetylcholine-induced vasodilation by increasing free radical production, British Journal of Pharmacology 142(8) (2004) 1233-1240.
[44] X. Ji, T. Nishihashi, C.C. Trandafir, A. Wang, Y. Shimizu, K. Kurahashi, Pharmacological nature of nicotine-induced contraction in the rat basilar artery: Involvement of arachidonic acid metabolites, European Journal of Pharmacology 577(1–3) (2007) 109-114.
[45] F.I. Romero, M.J. Martinez-Calatrava, O. Sanchez-Pernaute, O. Gualillo, R. Largo, G. Herrero-Beaumont, Pharmacological modulation by celecoxib of cachexia associated with experimental arthritis and atherosclerosis in rabbits, Br J Pharmacol 161(5) (2010) 1012-22.
[46] C. Prati, A. Berthelot, B. Kantelip, D. Wendling, C. Demougeot, Treatment with the arginase inhibitor Nw-hydroxy-nor-L-arginine restores endothelial function in rat adjuvant-induced arthritis, Arthritis Res. Ther 14(3) (2012) R130.
[47] Y.J. Gao, R.M. Lee, Hydrogen peroxide is an endothelium-dependent contracting factor in rat renal artery, Br J Pharmacol 146(8) (2005) 1061-8.
[48] D. Yang, M. Feletou, C.M. Boulanger, H.F. Wu, N. Levens, J.N. Zhang, P.M. Vanhoutte, Oxygen-derived free radicals mediate endothelium-dependent contractions to acetylcholine in aortas from spontaneously hypertensive rats, Br J Pharmacol 136(1) (2002) 104-10.
[49] T. Matsumoto, K. Ishida, N. Nakayama, K. Taguchi, T. Kobayashi, K. Kamata, Mechanisms underlying the losartan treatment-induced improvement in the endothelial dysfunction seen in mesenteric arteries from type 2 diabetic rats, Pharmacol. Res (2010).
[50] J. Bauer, A. Ripperger, S. Frantz, S. Ergun, E. Schwedhelm, R.A. Benndorf, Pathophysiology of isoprostanes in the cardiovascular system: implications of isoprostane-mediated thromboxane A2 receptor activation, Br J Pharmacol 171(13) (2014) 3115-31.
[51] E.H. Tang, P.M. Vanhoutte, Prostanoids and reactive oxygen species: team players in endothelium-dependent contractions, Pharmacol. Ther 122(2) (2009) 140-149.
[52] E.H. Tang, P.M. Vanhoutte, Endothelial dysfunction: a strategic target in the treatment of hypertension?, Pflugers Arch 459(6) (2010) 995-1004.
[53] S. Martinez-Revelles, M.S. Avendano, A.B. Garcia-Redondo, Y. Alvarez, A. Aguado, J.V. Perez-Giron, L. Garcia-Redondo, V. Esteban, J.M. Redondo, M.J. Alonso, A.M. Briones, M. Salaices, Reciprocal relationship between reactive oxygen species and cyclooxygenase-2 and vascular dysfunction in hypertension, Antioxid Redox Signal 18(1) (2013) 51-65.
[54] P. Li, C.M. Ferrario, K.B. Brosnihan, Losartan inhibits thromboxane A2-induced platelet aggregation and vascular constriction in spontaneously hypertensive rats, J Cardiovasc Pharmacol 32(2) (1998) 198-205.
[55] T. Sakuta, Y. Morita, M. Satoh, D.A. Fox, N. Kashihara, Involvement of the renin-angiotensin system in the development of vascular damage in a rat model of arthritis: effect of angiotensin receptor blockers, Arthritis Rheum 62(5) (2010) 1319-1328.
[56] C.G. Egan, J.C. Lockhart, W.R. Ferrell, Pathophysiology of vascular dysfunction in a rat model of chronic joint inflammation, J. Physiol 557(Pt 2) (2004) 635-643.
[57] L. Nielung, R. Christensen, B. Danneskiold-Samsoe, H. Bliddal, C.C. Holm, K. Ellegaard, H. Slott Jensen, E.M. Bartels, Validity and Agreement between the 28-Joint Disease Activity Score Based on C-Reactive Protein and Erythrocyte Sedimentation Rate in Patients with Rheumatoid Arthritis, Arthritis 2015 (2015) 401690.

[58] W. Buczko, T. Matys, R. Pawlak, I. Kucharewicz, E. Chabielska, Studies on the antithrombotic action of AT1 receptor antagonists, Med Sci Monit 7(4) (2001) 600-5.
[59] J.I. Guerra-Cuesta, M. Monton, J.A. Rodriguez-Feo, A.M. Jimenez, F. Gonzalez-Fernandez, L.A. Rico, R. Garcia, J. Gomez, J. Farre, S. Casado, A. Lopez-Farre, Effect of losartan on human platelet activation, J Hypertens 17(3) (1999) 447-52.
[60] T. Sakamoto, T. Kudoh, K. Sakamoto, K. Matsui, H. Ogawa, Antithrombotic effects of losartan in patients with hypertension complicated by atrial fibrillation: 4A (Angiotensin II Antagonist of platelet Aggregation in patients with Atrial fibrillation), a pilot study, Hypertens Res 37(6) (2014) 513-8.
[61] A. Suresh, N. Sanji, P.M. Kamath, S.L. Devendrappa, S.G. Hanumanthareddy, I. Maniyar, S.S. Rudrappa, A Pilot Study on the Effect of Angiotensin Receptor Blockers on Platelet Aggregation in Hypertensive Patients- A Prospective Observational Study, J Clin Diagn Res 10(11) (2016) FC14-FC16.