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Title

Losartan protects endothelium-dependent relaxation \textit{in vivo} in a murine model of rheumatoid arthritis

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Abstract

Angiotensin II-type 1 receptor stimulation is recognised to promote inflammation, a state central to the development and maintenance of rheumatoid arthritis. Herein we examined the use of losartan, an angiotensin II-type 1 receptor antagonist, on vascular reactivity, knee joint diameter and behavioural assessment of pain in a Freund’s complete adjuvant (FCA) mouse model of joint inflammation.

Monoarthritis was induced via FCA in the presence or absence of losartan with naive mice serving as controls. Knee joint swelling, joint pain (assessed by dynamic weight bearing of limb use), knee joint artery reactivity (assessed ex vivo) and blood perfusion of the knee joint (assessed in vivo) were determined.

FCA mediated a significant increase in knee joint diameter and reduced weight-bearing (a surrogate for pain sensation) of the affected limb. Notably, these phenomena were substantially reduced when mice were prophylactically treated with losartan. Assessment of arterial relaxation and blood perfusion with acetylcholine stimulation revealed that FCA resulted in significant vascular dysfunction, which was resolved to naïve levels with losartan treatment.

Through the actions of losartan, these findings indicate that the angiotensin II-type 1 receptor is a likely therapeutic target of importance in the development of the physical changes, pain sensation and vascular dysfunction found in inflammatory arthritis.

Key words

rheumatoid arthritis, losartan, angiotensin II-type 1 receptor, endothelium
**Ethics statement animal experimentation**

All procedures described were performed in accordance with regulations of the UK Home Office (project licence 60/4328) and received ethical approval by both the Universities of the West of Scotland and Glasgow.

**Credit Author Statement**

**Moanna Villaluz:** Methodology, Formal analysis, Investigation, Writing - Original Draft.

**Lynette Dunning:** Methodology, Formal analysis, Investigation, Writing - Review & Editing.

**Carl S Goodyear:** Project administration, Writing - Review & Editing. **William R Ferrell:** Conceptualization, Project administration, Methodology, Formal analysis, Writing - Review & Editing. **John C Lockhart:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - Review & Editing. **Andrew MacKenzie:** Conceptualization, Methodology, Formal analysis, Writing - Original Draft.

**Declaration of Competing Interest**

The authors declare no conflicts of interest.

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1. Introduction

Rheumatoid arthritis is a disorder characterised by joint destruction, restricted movement, pain and cardiovascular dysfunction. Indeed, patients with rheumatoid arthritis are around twice as likely to suffer vascular incident than the general population making the disorder comparable to diabetes mellitus as an independent risk factor for the development of atherosclerosis and cardiovascular disease (Avina-Zubieta et al., 2008; Sattar et al., 2003; Zou et al., 2017). Furthermore, blood flow to the affected joint is typically compromised such that intra-articular hypoxia is a common feature of both animal models of joint inflammation (Najafipour and Ferrell, 1995) and human rheumatoid joint (Harilainen et al., 1989), and this likely exacerbates joint injury and worsens the effects of arthritis.

The mechanistic links between rheumatoid arthritis and atherosclerosis are likely to be complex in nature and remain to be fully clarified, however, given that both conditions have an inflammatory origin it is not surprising that much focus has been given to mediators of inflammation as a probable connection between the diseases. Notably, components of the renin-angiotensin system, including angiotensin II-type 1 receptor, are recognised to be pro-inflammatory and are found in a variety of tissues including synovium (Price et al., 2007; Walsh et al., 2000) and vascular endothelium (MacKenzie, 2011). Indeed, our group has previously shown that blockade of angiotensin II-type 1 receptor with losartan reduces the associated knee joint swelling (Price et al., 2007) and protects vascular reactivity (Hamilton et al., 2018; Mackenzie et al., 2013) against the dysfunction generated in a rat model of rheumatoid arthritis. These latter vascular studies examined isolated artery segments ex vivo and, while providing important proof-of-concept, did not address whether vascular reactivity and blood perfusion of the joint are similarly protected by angiotensin II-type 1
receptor antagonism in *in vivo*. Given that we have previously demonstrated blood perfusion in the knee joint (as determined *in vivo* using a laser Doppler-based method) was impaired in the rat model of rheumatoid arthritis (Egan et al., 2004), the present study was designed to investigate if losartan can offer protection against such vascular dysfunction assessed both *in vivo* and *ex vivo*.

Accordingly, this study investigated the action of angiotensin II-type 1 receptor blockade with losartan on vascular reactivity, knee joint diameter, movement behaviour (as a reporter of joint impediment and pain) and serum cytokine levels in a mouse model of joint inflammation with many similarities to rheumatoid arthritis in humans.
2. Materials and methods

2.1. Experimental animals

Adult male C57Bl/6J mice were obtained from Envigo Company, UK. The animals were housed in a centralised animal facility in standard cages, with food and water available ad libitum and maintained in a thermoneutral environment, with a 12h light/dark cycle. Mice were euthanised by CO₂ inhalation, weighing 22-26 g at time of cull. All procedures described were performed in accordance with regulations of the UK Home Office (project licence 60/4328) and received ethical approval by both the Universities of the West of Scotland and Glasgow. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath and Lilley, 2015).

2.2. Experimental groups

Mice were randomly assigned to three principal experimental groups: Control (no procedure), Freund’s complete adjuvant (FCA) + isotonic saline vehicle (inflammatory arthritis group, induction detailed below, referred to as FCA group), and FCA+losartan (inflammatory arthritis group prophylactically treated with losartan, detailed below). A separate group was also generated to assess the influence of losartan on control mice (control+losartan). To maintain consistency of induction across experimental groups and in the measurement of recorded variables, one group member was responsible for all induction procedures and data collection. Consequently, experimental blinding was not possible in this study. Experimental protocols were determined in advance and data analysed by the team.

2.3. Induction procedures
FCA supplemented with heat-killed *mycobacterium tuberculosis* (10 mg/ml) was used to induce chronic monoarthritis. This model has been well characterised by our group previously (Hamilton et al., 2018; Kelso et al., 2007; Mackenzie et al., 2013; Price et al., 2007) and is considered to feature key aspects of human arthritis (Asquith et al., 2009).

Mice were held under general anaesthesia (O$_2$/N$_2$O/2% isofluorane mixture) throughout the induction of arthritis and placed in a recovery cage after the procedure. Administration of FCA was performed to the right (ipsilateral) knee by intraarticular (20 μl) and periarticular (80 μl, divided into four different sites) injection. In some groups (FCA+losartan), prophylactic treatment of losartan (30 mg/ml) was first administered via i.p. injections 1 h prior to FCA induction and every 48 h thereafter. Similarly, losartan was administered in the control+losartan group via intraperitoneal injection every 48 h. FCA+losartan, control+losartan and control groups were all maintained and monitored in an identical manner to the FCA group and culled 28 days following initial induction. The 30 mg/ml of losartan utilised in this study was determined through a series of preliminary experiments in our laboratory (losartan assessed from 15 – 60 mg/ml) to ascertain the dose which afforded best protection against the influence of FCA.

2.4. Measurement of physical and behavioural parameters

Knee joint swelling, a cardinal sign of inflammation and a characteristic of the FCA model, was assessed by measurement of the knee joint diameter at set time points (with the aid of Vernier callipers) and expressed as percentage of pre-FCA diameter. Knee joint diameter was measured daily until day 7 and every 48 h thereafter. Body weight was also monitored as an index of the general health of the animal.
As a surrogate marker of joint pain, animal movement was determined at day 28 by placing animals in a dynamic weight bearing apparatus (Bio-Seb In vivo Research Instruments, USA) equipped with a mechanosensory footpad (added to the floor of the chamber) and a camera (to record the event). Mice were allowed to acclimatise to these surroundings before animal movement was recorded for a 5 min period. This protocol determined limb use (as a percentage of time any limb was elevated compared to time placed on chamber floor) and how much load was applied to each limb (as a percentage of total weight) i.e. the apparatus was used to assess gait characteristics of the animals. Dynamic weight bearing methodology is a well-established means of assessing gait alteration and nociceptive behaviours in experimental arthritis (Abdullah et al., 2016; Bagi et al., 2015).

2.5 Serum cytokine analysis

Blood was removed from animals following the day 28 cull. After centrifugation, serum was collected and analysed for Interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) using enzyme-linked immunosorbent assay (ELISA; eBioscience, Inc. UK) according to the manufacturer’s instructions. Samples were read at 450 nm wavelength using a plate reader LT-4500 (Labtech International Ltd., UK).

2.6 Vascular assessment

2.6.1. Measurement of reactivity in vivo: In vivo measurement of synovial perfusion was performed by laser Speckle contrast imaging. The laser speckle contrast imager (moorFLPI-2 blood flow imager from Moor Instruments, UK) delivers real-time, high-resolution blood
perfusion images by using laser light that illuminates a diffuse object producing a random interference pattern. This apparatus was used to monitor relative changes in blood perfusion in the synovial microcirculation using methodology adapted from our previous work (Egan et al., 2001). Perfusion was monitored during the application of acetylcholine and sodium nitroprusside (SNP), delivered topically via pipette to the joint (exposed by excision of overlying skin).

In areas of increased blood perfusion, the intensity of the speckle fluctuates rapidly thus a blur pattern is captured by the charge-coupled device. In these experiments each frame was set to a high speed with low resolution, and an exposure time of 10 ms. The images collected from the experiment were later analysed by dedicated software (Moor Instruments, UK) to obtain a median flux value over the knee joint region. Animals were culled immediately following this protocol.

The arterial pressure was monitored via a cannula inserted into the left common carotid artery connected to a pressure transducer (Elcomatic EM-751, UK) linked to a chart recorder (Lectromed Ltd., UK) to give a continuous blood pressure trace. Mean arterial pressure was calculated and preliminary experiments demonstrated that application of either acetylcholine or SNP did not alter mean arterial pressure. Consequently, any changes to knee joint blood perfusion induced by the addition of acetylcholine or SNP can be attributed to direct action of these agents on the vasculature of the knee joint and not due to any systemic alteration in blood pressure.

**2.6.2. Calculation of Area under the Curve and Log10 transformation:** The data acquired from direct comparison of change in blood perfusion, measured as a percentage by the laser
Speckle contrast imaging technique, were further analysed by calculation of area under the curve. The area under the curve was calculated by taking consecutive points (6-11 points determined by the number of images captured prior the peak and one post peak) using Prism 5, GraphPad, San Diego, California, USA over the period of time following the addition of stimulating agent (acetylcholine or SNP). This analysis has the advantage of providing an insight on blood flow over time by representing “global” rather than “peak” perfusion at the knee joint. The change in blood perfusion and area under the curve data were normalised by log transformation, an approach used in previous studies (Mullan et al., 2016; Opazo Saez et al., 2005; Tibiriçá et al., 2011)
2.6.3. Measurement of reactivity ex vivo: Immediately following cull, the femoral artery directly upstream from the knee joint under investigation was carefully removed and placed in ice-cold Kreb’s solution composed of (mM): NaCl 119, KCl 4.7, CaCl$_2$ 2.5, MgSO$_4$ 1.2, KH$_2$SO$_4$ 1.2, NaHCO$_3$ 25 and glucose 5mM, pH 7.4, gassed with 5% CO$_2$ in O$_2$. The vessel was cleaned of adhering connective tissue and kept at 4°C overnight. The following day, the vessel was sliced into transverse rings and mounted onto two-40μm diameter stainless steel wires in a 4-channel myograph (Danish Myo Technology M610, Aarhus, Denmark) for isometric tension recording (captured by LabChart 7, ADInstruments Ltd). The vessels were allowed to equilibrate for up to 30 minutes before step-wise changes in the passive tension-internal circumference were performed in order to achieve an internal circumference equivalent to 90% of the circumference of the artery under a transmural pressure of 100 mmHg, as previously described (Mulvany and Halpern, 1977).

Ex vivo characterisation of vascular reactivity was initiated with a challenge of KCl (80 mM) to assess viability of the vessels. Rings were then pre-contracted with the thromboxane A2 receptor agonist U46619 before application of either acetylcholine (1nM - 1µM) or SNP (1nM - 10µM) to assess endothelium and endothelium-independent relaxation, respectively. The level of U46619-induced constriction was matched between groups to a level of around 70% of the maximum produced by the spazmogen.

2.7. Data and Statistical Analysis

Data are expressed as mean ± standard error of mean; n values represent the number of animals used; one vessel segment per protocol per animal for ex vivo vascular experiments. In vivo data are log10 transformed (described above). For ex vivo vascular experimental
data, relaxations to SNP and acetylcholine are expressed as percentage of the vasoconstrictor tone generated by U46619. Concentration-response curves were generated, and statistical comparisons made, with the aid of a computer programme Prism 5 (GraphPad, San Diego, CA, USA).

Data provided in figures have a sample size of n≥5 for each experimental group. Statistical comparisons were evaluated by one- or two-way analysis of variance followed by a Bonferroni post-hoc test. A P < 0.05 was classed as statistically significant. Data was analysed using Prism 5 (GraphPad, San Diego, CA, USA).

2.8. Materials

All drugs and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) except for U46619 (9,11-Dideoxy-9a,11a-methaneoepoxy prostaglandin F2α; Tocris Bioscience, Abingdon, UK). Drugs were prepared in distilled water and frozen in stock solutions with the exception of U46619 (1 mM stock in 50% ethanol). Control studies have demonstrated that the concentration of this solvent did not influence ex vivo vascular reactivity when applied at equal volumes to that of drug serial dilutions. Serial dilutions were made in isotonic saline.
3. Results

At the time of cull, no difference in body weight was found between the three experimental groups (*data not shown*). No observable differences were apparent between the control and control+losartan groups in body weight, knee joint diameter and *in vivo* or *ex vivo* vascular reactivity response to acetylcholine or SNP (*data not shown*).

3.1. Knee Joint Diameter

Induction of FCA resulted in rapid increase in joint swelling after 24 h, which was maintained until cull on day 28 (Fig. 1), with joint diameter clearly elevated compared to the control group. Prophylactic treatment with losartan resulted in a knee joint diameter that was significantly reduced (by about 50%) compared to the FCA group although the joint swelling remained elevated from that found in control animals.

3.2. Dynamic Weight Bearing

In control mice, limb load bearing was equally distributed between left- and right-hand sides with the majority of the load being borne by the rear limbs (Fig. 2A). Mice of the FCA group altered their movement pattern such that load bearing shifted substantially towards both front limbs while the weight burden on the ipsilateral rear limb was significantly reduced i.e. less body weight was applied to the limb that received the FCA injection. Furthermore, the time spent with the rear ipsilateral limb elevated was considerably longer for the FCA group compared to controls (Fig. 2B). The FCA+losartan mice had a limb use pattern that was similar to control mice except for a reduced employment of the rear ipsilateral limb, which was similar to that found in FCA mice (Fig. 2A). The time spent with the rear ipsilateral limb elevated for the FCA+losartan group was significantly less than that found in FCA mice and not different to that found in control mice (Fig. 2B).
3.3. Serum cytokine levels

At day 28, serum levels of IL-6 appeared to be elevated in the FCA-treated animals compared to controls, with a reduced level found in FCA+losartan mice. However, no statistical difference was found (Table 1). Similarly, no statistical difference was found in levels of TNF-α between the groups.

Table 1 Serum levels of IL-6 and TNF-α in experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FCA</th>
<th>FCA+losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg.ml⁻¹)</td>
<td>41.38 ± 2.07</td>
<td>99.24 ± 39.34</td>
<td>7.10 ± 3.00</td>
</tr>
<tr>
<td>TNF-α (pg.ml⁻¹)</td>
<td>61.60 ± 3.30</td>
<td>77.76 ± 22.12</td>
<td>117.10 ± 26.60</td>
</tr>
</tbody>
</table>

Serum samples taken at day 28. P > 0.05 between groups for both IL-6 and TNF-α.

3.4. Vascular reactivity in vivo

The acetylcholine-induced increased perfusion of the knee joint in FCA-treated mice was substantially reduced compared to control animals when the data was analysed as either log of blood perfusion or area under the curve (giving an indication of peak perfusion and perfusion volume over time; Fig. 3A and B, respectively). The reduction in blood flow found in the FCA-treated group in response to acetylcholine appeared to be mediated by impaired endothelial cell activity rather than smooth muscle responsiveness since endothelium-independent relaxation induced by SNP was, albeit depressed, not statistically different in the FCA group compared to controls (0.86 ± 0.41 vs 1.50 ± 0.25, respectively, P>0.05, percentage change in peak perfusion). Prophylactic treatment with losartan protected vascular reactivity in vivo such that the acetylcholine mediated change to blood perfusion
was substantially greater than that found in the FCA-treated mice and similar in magnitude to that found in controls (when analysed as either change to blood perfusion or area under the curve).

3.5. Vascular reactivity *ex vivo*

Both endothelium-independent (assessed by SNP, Fig. 4A) and endothelium-dependent (assessed by acetylcholine, Fig. 4B) relaxation found in femoral artery rings of FCA-treated mice were impaired compared to that found in vessel from control animals. The magnitude of impairment was different between initiating relaxants such that the SNP-induced relaxation was reduced by around 30% (with respect to maximum SNP relaxation) while the response to acetylcholine was diminished by around 70%, thus suggesting that the endothelial cells are more sensitive than smooth muscle to the dysfunction induced by the FCA protocol. The relaxation produced in response to acetylcholine in both groups was abolished by Nω-Nitro-L-arginine methyl ester hydrochloride suggesting that nitric oxide as the sole mediator of relaxation (*data not shown*). Treatment with losartan enhanced relaxation to both SNP and acetylcholine such that vascular response in FCA+losartan group was not different to that found in control mice (Fig. 4A & 4B).
4. Discussion

The key findings of this study are that losartan, a recognised angiotensin II-type 1 receptor antagonist, preserves vascular reactivity when assessed *in vivo* or *ex vivo*, reduces knee joint swelling and attenuates the altered gait characteristics generated in a model of inflammatory arthritis. We believe this is the first demonstration *in vivo* of a preserved vascular response against an FCA-induced arthritis. Furthermore, we also provide the first presentation of the protective action of losartan against knee joint swelling and movement behaviour found in the mouse model of FCA-induced arthritis. These actions of losartan highlight the angiotensin II-type 1 receptor as a likely therapeutic target of importance in the development of both the physical and vascular impairment observed in inflammatory arthritis.

The *in vivo* assessment of blood perfusion in the knee is an important measure of the local vascular response that determines the nutrient state of the knee joint. The acetylcholine-induced increase of joint blood perfusion has been previously shown to be reduced in an adjuvant-induced arthritis model in the rat (McDougall et al., 1998). In this latter study, the ability of SNP to elevate blood perfusion in the knee joint was also impaired, indicating an endothelial and vascular smooth muscle cell dysfunction in this model. The present study demonstrated an FCA-induced impairment in the blood perfusion response to acetylcholine but not SNP. This suggests the FCA-induced impairment in blood perfusion response in the mouse knee joint is largely a consequence of endothelial, rather than smooth muscle, cell dysfunction. The significant restoration of the vascular response to acetylcholine on treatment of FCA mice with losartan is, we believe, the first demonstration that prophylactic
treatment of losartan provides substantial protection against FCA-induced impairment in vascular reactivity in vivo.

This study also demonstrates a vascular dysfunction in the primary feeder artery of the knee joint when examined ex vivo. Segments of the saphenous artery showed an impaired endothelium-dependent and -independent relaxation (as determined via acetylcholine and SNP, respectively) in the FCA group compared to controls. Previous studies have demonstrated an impaired responsiveness to acetylcholine in models of arthritis while that to nitric oxide donors remained unchanged (He et al., 2013; Prati et al., 2011). In these studies, the vascular tissue investigated was aorta, a vessel both distant from the site of inflammation and considerably larger than the artery that feeds the knee joint. Therefore, the finding from the current study may provide a more representative insight into the nature of impairment (i.e. both endothelial and smooth muscle cell-driven) occurring in the blood vessels close to the site of joint inflammation. Importantly, we found that administration of losartan in the FCA-treated animals produced an SNP and acetylcholine-induced reactivity that was not different from that found in controls. Therefore, the FCA-induced vascular dysfunction in a feeder artery of the knee is afforded complete protection by losartan. As such, we demonstrate that the ability of losartan to prevent the FCA-mediated impairment of endothelial cell activity is consistent across both in vivo and ex vivo assessment.

The endothelium-derived relaxation examined ex vivo is entirely mediated by nitric oxide generation and therefore losartan affords protection to this relaxant pathway. Previously, we have shown in the rat that FCA induced an endothelium dysfunction that was mediated via a reduction in endothelium-derived hyperpolarising factor-mediated relaxation
(saphenous artery, Mackenzie, Dunning, Ferrell & Lockhart, 2013) and elevation of endothelium-derived constricting factor (aorta, Hamilton, Dunning, Ferrell, Lockhart & MacKenzie, 2018). As such the protective action of losartan on endothelial cell function is found across different vasoactive pathways, artery types and species.

The dynamic weight bearing system gives insight into changes in movement behaviour that reflect the influence and severity of joint impediment and pain found in several models of arthritis (Griffioen et al., 2015; Pitzer et al., 2016). Moreover, this methodology has been shown to correlate the level of arthritic nociception with changes in levels of cytokines and pain promoting glial cells (Quadros et al., 2015). The present study gives the first demonstration of a protective influence of losartan on an animal’s mobility following a chronic inflammation. Specifically, losartan modified both the front ipsilateral load bearing and the time spent with the effected limb elevated to produce values no different to that found in control mice. However, the load applied to the rear ipsilateral limb (the limb injected with the inflammatory adjuvant) remained similar to that observed with the FCA group. Taken together these findings suggest that the behaviour modification of limb use induced by FCA is largely, although not completely, prevented by prophylactic treatment with losartan. The excessive knee joint swelling in the human form of arthritis results in a reduction in mobility of patients due to difficulties in moving the affected limb and pain generation (Hammond, 2004). Therefore, the significant shift in movement behaviour induced by losartan towards that found in control mice likely reflects both the reduced level of joint swelling observed and reduction in pain sensation. Indeed, angiotensin II has been shown to promote nociceptive behaviour (Nemoto et al., 2013), while losartan reduces inflammatory-mediated pain (Costa et al., 2014) in mice, thus highlighting the angiotensin II-type 1 receptor as an important modulator of nociceptive sensation.
Joint swelling is a cardinal sign of inflammation. This study has shown that in the FCA murine model of arthritis, knee joint diameter rapidly increased in the days following induction and continued to increase at a reduced rate before reaching a plateau by the day 28 cull time point. This pattern of change is very similar to that previous published by our group using the same model in mice (Kelso et al., 2007). In the current study we demonstrate that prophylactic treatment with losartan offers a protective influence against the knee joint swelling induced by FCA. This is the first study to demonstrate this action of losartan in mouse FCA model and corroborates our previous findings with the same angiotensin II-type 1 receptor antagonist used with the FCA model in the rat (Hamilton et al., 2018; Mackenzie et al., 2013; Price et al., 2007), albeit over a different time scale and losartan dose (21 days and 15 mg/kg, respectively). In both this and the earlier studies losartan offered substantial, although not complete, protection since knee joint swelling remained elevated in the FCA+losartan group compared to control animals (although reduced by about 50% in comparison to the FCA-treated mice). The incomplete blockade of angiotensin II-type 1 receptor by the dose of losartan utilised seems unlikely since we have shown that elevated doses in rat (50 mg/kg; Price et al., 2007) and mice (60 mg/kg; unpublished observations from our laboratory) offered no additional protection to joint swelling induced by FCA. The implication here being that joint swelling induced by FCA is substantially, although not entirely, mediated via an angiotensin II-type 1 receptor-sensitive pathway.

Serum levels of IL-6 followed a pattern suggestive of an elevation in the FCA group compared to controls with a reduction in FCA+losartan animals, yet no statistical difference was found. Furthermore, levels of TNF-α were found to be statistically unaltered between the three groups suggesting that these cytokines play no role in the changes found in our FCA model. However, care should be taken with interpretation of our cytokine results because of
variation within the data set and that they are derived from end-point samples. i.e. we have no indication of earlier timepoint changes to cytokine level. Indeed, a study utilising the rat FCA model demonstrates an enhanced serum level of IL-6 and TNF-α (as well as rheumatoid factor) at a 2 week time point following FCA induction (Abdel El-Gaphar et al., 2018). The elevation of these factors was reduced with losartan treatment, highlighting the immunomodulatory action of this compound. Species and model differences aside, these data alongside those presented in our study could indicate that a losartan-sensitive change in cytokine level occurs within a 2-week timespan which normalises by week 4.

Overall, our data are supportive of a growing body of evidence demonstrating a beneficial influence of losartan in inflammatory arthritis. Indeed, the angiotensin II-type 1 receptor antagonist decreased serum total cholesterol and low-density lipoprotein while elevating high density lipoprotein in the FCA rat (Abdel El-Gaphar et al., 2018), suggesting suppression of oxidative changes linked with rheumatoid arthritis. Furthermore, in a collagen-induced model of arthritis in the rat, losartan has been shown to reduce levels of the pro-inflammatory cytokines TNF-α and IFN-γ as well as diminish phosphorylation of p38, ERK, and p65, p50 nuclear transposition in T cells and B cells (Wang et al., 2019). Moreover, in blood taken from humans with rheumatoid arthritis, losartan reduced the inflammatory activity of peripheral blood mononuclear cells as demonstrated by the reduced production of factors such as IL-22, IL-6 and others (Cardoso et al., 2018). Interestingly, this study also demonstrated that the anti-inflammatory action of the angiotensin II-type 1 receptor antagonist valsartan and the angiotensin converting enzyme inhibitor enalapril were both considerably weaker than that found with losartan, perhaps suggesting that losartan provides an off-target immunomodulatory action.
There are some limitations to our study. The effect of losartan on blood pressure was not assessed in any animal group. Moreover, we did not assess changes to serum angiotensin II levels nor tissue angiotensin II-type 1 receptor expression in either FCA- or FCA+losartan-treated animals. Consequently, we cannot eliminate an influence of blood pressure on our results nor can we categorically state that elevation of angiotensin II is the mediator driving the FCA-induced changes mitigated by losartan. There was wide variation in our ELISA data which likely would have been reduced with a larger sample size.

5. Conclusions

The findings of this study demonstrate that prophylactic treatment with losartan alleviates the vascular impairment and dramatically reduces the knee joint pain and swelling found in a mouse model of rheumatoid arthritis. Consequently, losartan may well be a viable therapy of likely significance in the prevention of the physical changes, pain sensation and vascular dysfunction found in human inflammatory arthritis.
Figure legends

**Fig. 1.** Changes to knee joint diameter over a 28-day period in mice following treatment with FCA. The augmented knee diameter was moderated by prophylactic treatment with losartan, although knee joint diameter remained enhanced compared to control mice. n=5-11; # P < 0.05, significant effect of losartan compared to FCA-treated group.

**Fig. 2 (A)** Load bearing distribution of each limb from control, FCA-treated and FCA+losartan treated mice. FCA-treated animals reduced their rear ipsilateral limb use (limb with inflamed knee joint) by re-distributing weight to both front limbs. In the FCA+losartan group, use of rear ipsilateral limb remained reduced but front limb use was comparable that found in control mice. **(B)** The time spent with rear ipsilateral limb elevated was considerably enhanced in the FCA-treated group compared to control. Losartan administration reduced this such that limb elevation in FCA+losartan treated mice did not significantly differ from the control group. n= 5-8; * P < 0.05, significant compared to control group; # P < 0.05, significant compared to FCA-treated group.

**Fig. 3.** *In vivo* assessment of acetylcholine (100 nM)-induced change in knee joint blood perfusion calculated as **(A)** the log10 percentage change in blood perfusion or **(B)** log10 area under the curve. Both forms of analysis demonstrate an impairment to blood perfusion in the FCA group with respect to control animals, while joint perfusion in the FCA+losartan-treated animals was restored towards control levels. n= 5-8; * P < 0.05, significant effect
compared to control group. # P < 0.05, significant effect of losartan compared to FCA-treated group.

**Fig. 4.** *Ex vivo* assessment of isolated femoral artery segments in response to SNP (A) and acetylcholine (B) in control, FCA and FCA+losartan groups. Both SNP- and acetylcholine-induced relaxation was impaired in FCA-treated mice compared to controls. This impaired relaxation (to both SNP and acetylcholine) was reversed in the FCA+losartan group, levels being no different from the control mice. n= 6-9; * P < 0.05, significant effect compared to control group. # P < 0.05, significant effect of losartan compared to FCA-treated group.
References


McGrath, J.C., Lilley, E., 2015. Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Br J Pharmacol 172, 3189-3193.


Losartan protects endothelium-dependent relaxation \textit{in vivo} in a murine model of rheumatoid arthritis
Fig 2.
Fig 3.

A

B

Log10 change in blood perfusion

Log10 area under curve

control  FCA  FCA+losartan

control  FCA  FCA+losartan

*  #
Fig 4.