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Associations of inflammatory markers and vascular cell adhesion molecule-1 with endothelial dysfunction in collagen-induced arthritis

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ABSTRACT

We determined the role of high-grade inflammation on endothelial function and its association with biomarkers of endothelial dysfunction in collagen-induced arthritis. Sprague-Dawley rats were divided into a control (n = 12) or collagen-induced arthritis (CIA; n = 21) group. To induce arthritis, Bovine-type-II collagen emulsified in incomplete Freund's adjuvant was injected at the base of the tail. Nine-weeks after the primary immunisation, vascular reactivity in mesenteric and saphenous arteries was assessed using a wire-myograph. Serum concentrations of inflammatory markers (tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β), C-reactive protein (CRP)) and biomarkers of endothelial dysfunction (vascular cell adhesion molecule-1 (VCAM-1) and asymmetric dimethylarginine (ADMA)) were measured by ELISA. Acetylcholine-induced relaxation in mesenteric and saphenous arteries was impaired in CIA compared to controls ($P < 0.05$). Responses to sodium nitroprusside were similar between controls and CIA in mesenteric arteries and marginally impaired in saphenous arteries of CIA rats. Compared to controls, TNF-α, IL-6, IL-1β, CRP (all $P < 0.00001$) and VCAM-1 ($P = 0.02$) were elevated in CIA. TNF-α (std β(SE) = 0.39(0.16); $P = 0.03$), IL-6 (std β(SE) = 0.37(0.17); $P = 0.03$), IL-1β (std β(SE) = 0.41(0.16); $P = 0.02$) and CRP (std β(SE) = 0.36(0.17); $P = 0.04$) were associated with VCAM-1. Associations between inflammatory markers and the maximal relaxation (E_{max}) to acetylcholine in mesenteric arteries were no longer significant after adjusting for VCAM-1 (except for IL-1β). VCAM-1 was inversely associated with the E_{max} to acetylcholine in mesenteric (std β(SE) = -0.49(0.16); $P = 0.01$) but not in saphenous arteries (std β(SE) = -0.06(0.18); $P = 0.76$). In conclusion, exposure to high-grade inflammation impairs endothelial-dependent relaxation. The inflammation-induced increase in VCAM-1 concentrations may contribute to the impaired endothelium-dependent relaxation in mesenteric arteries of CIA rats.

1. Introduction

Endothelial dysfunction is a common feature in rheumatoid arthritis (RA) (Di Minno et al., 2015). In RA, chronically elevated pro-inflammatory cytokine concentrations are believed to cause functional and structural changes in endothelial cells, resulting in endothelial dysfunction (Steyers and Miller, 2014). Indeed, inflammatory markers have been associated with endothelial dysfunction in RA patients (Arosio et al., 2007; Dessein et al., 2005; Galarraga et al., 2008; Klimiuk et al., 2002; Vaudo et al., 2004; Yki-Järvinen et al., 2003). Furthermore, treatment with anti-inflammatory drugs improves endothelial function (Dessein and Joffe, 2006; Kotani et al., 2016; Ruiz-Limón et al., 2017; Ursini et al., 2017). However, as a result of confounding factors and the cross-sectional nature of majority of the aforementioned studies, the independent contribution of inflammation to impaired endothelial function is uncertain. In this regard, several animal models of RA have been developed to understand the mechanisms of inflammation-induced vascular dysfunction in RA.

Inflammation-induced endothelial dysfunction has been reported in adjuvant-induced arthritis (Totoson et al., 2015, 2016). However, collagen-induced arthritis (CIA) is the most widely used arthritis model as it shares clinical and pathological features most similar to human RA

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compared to other experimental models (Bendele, 2001; Brand et al., 2007; Hegen et al., 2008). To date, the findings of studies investigating endothelial function in CIA are controversial (Dooley et al., 2015; He et al., 2013; Palma Zochio Tozzato et al., 2016; Reynolds et al., 2012). Impaired endothelial function has been reported in mice aorta (He et al., 2013) and coronary or digital arteries of sheep (Dooley et al., 2015), while others have showed unaltered endothelial function in mice aorta (Palma Zochio Tozzato et al., 2016; Reynolds et al., 2012). These conflicting results emphasises the need to elucidate the mechanistic role of inflammation in endothelial function in CIA.

Increased circulating concentrations of soluble intercellular adhesion molecules and asymmetric dimethylarginine (ADMA) are biomarkers of endothelial dysfunction (Dessein et al., 2005; Wållberg-Jonsson et al., 2002). Moreover, these biomarkers of endothelial dysfunction lend insight to the possible mechanisms responsible for the endothelial dysfunction. Expression of the cell surface protein vascular adhesion molecule 1 (VCAM-1) has been implicated in the recruitment of lymphocytes which mediate vascular remodelling (Liao, 2013). Similarly, ADMA, an endogenous inhibitor of nitric oxide (NO) production causes reduction in nitric oxide synthase (eNOS) which contributes to endothelial dysfunction (Sibal et al., 2010). However, reports of biomarkers of endothelial function and their relation to endothelial dysfunction in pre-clinical studies are limited. Therefore, the present study aimed to determine the impact of high-grade inflammation on endothelial function and the relationship between systemic inflammatory markers, biomarkers of endothelial dysfunction and vascular reactivity in a model of CIA.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC number: 2017/03/21C) and conducted in compliance with the Guide for the Care and Use of Laboratory Animals. Thirty-three, three-month-old, male Sprague Dawley rats (480–510 g) were studied. The current study was conducted in adult rats to reflect RA in adult patients. Rats were housed individually and maintained under a 12-h light-dark cycle under controlled temperature conditions (23 ± 2 °C). Commercial rat chow and water were given to the animals ad libitum. Rats were habituated to the housing conditions for two weeks; blood pressure was measured twice a week and body weight, paw thickness and articular index scores were measured once a week. After two weeks habituation to the laboratory conditions, rats were randomly allocated to either the control (n = 12) or the CIA (n = 21) group.

2.2. Arthritis induction and evaluation

After the two-week habituation period, systemic arthritis was induced as previously described (Trentham et al., 1977). Briefly, the arthritis-inducing emulsion was prepared by dissolving bovine type-II collagen (Chondrex cat. #20021, Redmond, WA, USA) in 0.05M acetic acid at 4 °C. Equal amounts of dissolved bovine type-II collagen (2 mg/ml) and incomplete Freund's adjuvant (Chondrex cat. #7002, Redmond, WA, USA) were mixed using an electric homogenizer. The emulsion was prepared immediately before immunisation. Rats were immunised with 0.2 ml (200 μg) of the emulsion by a subcutaneous injection at the base of the tail. Seven days after the first immunisation, rats received a 0.1 ml (100 μg) booster injection of the bovine type-II collagen emulsified in incomplete Freund's adjuvant to ensure a high incidence and severity of arthritis. Control rats received a subcutaneous injection of 0.1 ml (100 μg) of 0.05M acetic acid at the base of the tail. All procedures were performed under general anaesthesia (isoflurane).

Arthritis scoring was performed on the hind paws once a week as previously described (Hu et al., 2017). Arthritis was scored as follows: 0 = no swelling or focal redness (normal); 1 = slight swelling and/or focal redness; 2 = low-to-moderate oedema, 3 = pronounced oedema with reduced paw function; 4 = excessive oedema with deformity and joint rigidity. The cumulative score for the two hind paws of each rat (maximum score of 8) were used to represent the overall disease severity and progression. As the rat paw arthritis score only provides a subjective quantification of the inflammation, hind paw thickness at the tarsometatarsal and ankle joints were measured once a week using a digital calliper.

2.3. Blood pressure

Systolic and diastolic blood pressure was measured every week using a tail cuff technique which employs non-invasive blood pressure amplifiers (Biopac Systems, Santa Barbara, CA, USA).

2.4. Blood glucose and triglyceride concentrations

Three days before termination, after an overnight fast, a blood droplet was obtained from the tail vein using a pinprick to determine blood glucose and triglyceride concentrations. Glucose concentrations were determined using a calibrated glucometer (Ascensia EliteTM Blood glucose meter, Bayer Corporation, Mishawaka, USA) and triglycerides were determined using a calibrated triglyceride analyzer (Accutrend Roche, Mannheim, Germany).

2.5. Vascular reactivity

Nine-weeks after the first immunisation, rats were anaesthetised with ketamine (100 mg/kg) and xylazine (5 mg/kg) and euthanised by thoracotomy. The intestines and the saphenous arteries were removed and placed in a cold modified Krebs-Ringer bicarbonate (control) solution with the following composition (mmol/l: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11 glucose). Second branches of mesenteric arteries and the saphenous branch of the femoral arteries were cleaned of fat and connective tissue and the arteries were cut into rings (2 mm length) and suspended into a wire myograph (model 610M; Danish Myo Technology, Aahrus, Denmark). The rings were suspended between 2 stainless steel wires with a diameter of 40 mm in an organ chamber filled with control solution which was continuously bubbled with 95% O_2 and 5% CO_2 and kept at a constant temperature of 37 °C. One of the wires was connected to a movable holder supporting a tension transducer (Grass Instrument Co, Quincy, MA) to collect the isometric force measurements using a data acquisition system (PowerLab 4SP; ADInstruments). The rings were placed under an optimal resting tension as previously described (Mokotedi et al., 2018). After a 60-min stabilisation period, the arteries were exposed to potassium chloride (KCl, 80 mM), which produced a maximal contraction response and served as the reference contraction. Preparations were exposed to increasing concentration of KCl (1–120 mM) or phenylephrine [Phe, 1 nM to 0.1 mM (in a half-log increment)]. Vascular contraction was expressed as the percentage of KCl (80 mM)-induced increases in tension. The relaxation responses induced by acetylcholine, an endothelium-dependent vasodilator (ACh, 0.1 nM to 10 mM), or sodium nitroprusside, an endothelium-independent vasodilator (SNP, 1 nM to 100 mM) were assessed in half-log increments during phenylephrine (Phe, 10 μM)-induced contractions. Vascular relaxation was expressed as a percentage of the baseline tension during contractions induced by Phe (10 μM). KCl, Phe, SNP and ACh were purchased from Sigma (St. Louis, MO, USA).

2.6. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology Co. Ltd, Wuhan, China) were used to measure serum levels of tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6),

interleukin 1β (IL-1β), C-reactive protein (CRP), vascular cell adhesion molecule-1 (VCAM-1) and asymmetric dimethylarginine (ADMA) according to the instructions of the manufacturer. Each sample was tested in duplicate. The lower detection limit for TNF-α, IL-6, IL-1β, CRP, VCAM-1 and ADMA were 78 pg/ml, 62 pg/ml, 31 pg/ml, 0.3 ng/ml, 12 pg/ml and 1.5 ng/ml respectively, all with coefficients of variation of < 10%.

2.7. Statistical analysis

Statistical analysis was performed using the SAS software, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). Data are expressed as means ± S.E.M. Differences in arthritis scores between the control and CIA groups were determined using the non-parametric Mann-Whitney U-test. Differences in body weight, paw thickness and the vascular reactivity dose-response curves between the control and CIA groups were evaluated using repeated-measures analysis of variance (ANOVA) followed by Tukey post-hoc tests. The sensitivity (EC_{50}) and the maximal (E_{max}) responses were determined from regression analysis logistic sigmoid function curves (GraphPad Software Inc, San Diego, CA). Unpaired, two-tailed t-tests were performed to determine differences in inflammatory markers and the EC_{50} and E_{max} responses between the control and CIA groups. Associations between biomarkers of endothelial dysfunction and the sensitivity (EC_{50}) and maximal (E_{max}) to ACh-induced relaxation were determined using regression analysis. To determine the relative contribution of inflammatory markers to vascular reactivity, linear regression analysis was performed. In order to determine whether the relationships between inflammatory markers and vascular reactivity was mediated by circulating biomarkers of endothelial function, VCAM-1 and ADMA were included as confounders in separate models in the relationship between inflammatory markers and E_{max}. $P < 0.05$ was considered statistically significant.

3. Results

3.1. CIA model

Inflammation developed within 21–28 days after the first immunisation in hind paws of CIA rats and showed clear signs of joint swelling and oedema compared to control rats (Fig. 1A). The arthritis score was significantly higher in the CIA group compared to the control group from week four to week nine after the primary immunisation (Fig. 1B). The hind paw thickness at the tarsometatarsal joint (Fig. 1C) was significantly increased in the CIA group compared to the control group from week five to week nine and at the ankle joint (Fig. 1D) from week four until week nine after the primary immunisation. There were no significant differences in body weight between the control and CIA groups throughout the study intervention (Fig. 1E).

There were no differences in systolic (control: 133 ± 1 mm Hg; CIA: 137 ± 3 mm Hg) or diastolic (control: 92 ± 2 mm Hg; CIA: 94 ± 1 mm Hg) blood pressure between the control and CIA groups. No differences were observed in blood glucose (control: 4.0 ± 0.6 mmol/L; CIA: 4.1 ± 0.6 mmol/L; P = 0.91) or blood triglyceride concentrations (control: 1.5 ± 0.3 mmol/L; CIA: 1.5 ± 0.1 mmol/L; P = 0.97) between the control and CIA groups.

3.2. Vascular reactivity differences in control and CIA groups

The maximal contractile response to KCl was similar between the experimental groups in mesenteric arteries (control: 14 ± 1 mN; CIA: 15 ± 1 mN; P = 0.36) and saphenous arteries (control: 15 ± 1 mN; CIA: 15 ± 1 mN; P = 0.87). Fig. 2 shows the vascular contractile responses to KCl and Phe in mesenteric and saphenous arteries. The sensitivity (EC_{50}) and maximal (E_{max}) contraction to KCl and Phe were similar between the control and CIA groups in both the mesenteric and saphenous arteries (Fig. 2; $P > 0.05$).

Phe (10 μM)-induced contraction were similar in the mesenteric and saphenous arteries before addition of ACh (mesenteric arteries: control: 24 ± 1.6 mN; CIA: 23 ± 1.5 mN; P = 0.62 and saphenous arteries: control: 19 ± 1.9 mN; CIA: 18 ± 1.2 mN; P = 0.61) or SNP (mesenteric arteries: control: 21 ± 1.9 mN; CIA: 20 ± 1.3 mN; P = 0.89 and saphenous arteries: control: 17 ± 1.6 mN; CIA: 18 ± 1.6 mN; P = 0.74). Fig. 3 shows the concentration-response curves and Table 1 shows the EC_{50} and E_{max} to ACh or SNP exposure in mesenteric and saphenous arteries of control and CIA groups. In mesenteric arteries, the ACh-induced vasodilation at $10^{-6.5}$ to 10^{-4} M was significantly impaired in the CIA group compared to controls ($P < 0.01$; Fig. 3A). Similarly, the EC_{50} was significantly increased (P = 0.02) and the E_{max} significantly decreased (P = 0.01) in the CIA group compared to controls (Table 1). In saphenous arteries, the ACh-induced vasodilation at $10^{-6.5}$ to $10^{-4.5}$ M was significantly impaired in the CIA group compared to controls (Fig. 3B). The EC_{50} were significantly increased in the CIA group compared to controls (P = 0.04) but the E_{max} were similar between the experimental groups (P = 0.18; Table 1). In mesenteric arteries, the SNP-induced vasodilation was similar between the control and the CIA groups ($P > 0.05$; Fig. 3C). In saphenous arteries, the SNP-induced vasodilation at 10^{-5} to $10^{-4.5}$ M was significantly impaired in the CIA group compared to controls ($P < 0.05$; Fig. 3D). However, the EC_{50} and the E_{max} were similar between the two groups ($P > 0.05$; Table 1).

3.3. Systemic inflammatory markers and biomarkers of endothelial dysfunction

Fig. 4 shows systemic inflammatory markers and biomarkers of endothelial function in control and CIA groups. The serum concentrations of TNF-α (control: 278 ± 25 pg/ml; CIA: 646 ± 29 pg/ml), IL-6 (control: 100 ± 21 pg/ml; CIA: 378 ± 21 pg/ml), IL-1β (control: 106 ± 16 pg/ml; CIA: 246 ± 12 pg/ml) and CRP (control: 0.2 ± 0.1 ng/ml; CIA; 1.0 ± 0.1 ng/ml) were significantly greater in the CIA group compared to controls (all $P < 0.0001$). Serum concentrations of VCAM-1 were significantly greater in CIA rats compared to controls (control: 68 ± 11 pg/ml; CIA: 111 ± 12 pg/ml; P = 0.02); no differences were observed in serum concentrations of ADMA between the experimental groups (11 ± 2 ng/ml; 11 ± 2 ng/ml; P = 0.83).

3.4. Systemic inflammatory markers associated with biomarkers of endothelial dysfunction

Table 2 shows the associations between systemic inflammatory markers and biomarkers of endothelial dysfunction. Greater serum concentrations of TNF-α (std β (SE) = 0.39 (0.16), $R^2 = 0.15$, P = 0.03); IL-6 (std β (SE) = 0.37 (0.17), $R^2 = 0.14$, P = 0.04); IL-1β (std β (SE) = 0.41 (0.16), $R^2 = 0.17$, P = 0.02) and CRP (std β (SE) = 0.36 (0.17), $R^2 = 0.13$, P = 0.04) were associated with greater serum concentrations of VCAM-1. Serum concentrations of TNF-α (std β (SE) = -0.06 (0.18), $R^2 = 0.004$, P = 0.76); IL-6 (std β (SE) = -0.06 (0.17), $R^2 = 0.004$, P = 0.76); IL-1β (std β (SE) = -0.11 (0.17), $R^2 = 0.01$, P = 0.61) and CRP (std β (SE) = -0.11 (0.18), $R^2 = 0.01$, P = 0.56) were not related to ADMA concentrations.

3.5. VCAM-1 associated with impaired endothelium-dependent relaxation in mesenteric but not in saphenous arteries

Fig. 5 shows the associations between serum concentrations of VCAM-1 or ADMA and the E_{max} of ACh-induced relaxation in mesenteric arteries. Greater serum concentrations of VCAM-1 were associated with lower E_{max} of ACh-induced relaxation in mesenteric arteries (std β (95% CI) = -0.49(-0.72 to −0.17), $R^2 = 0.24$, P = 0.01; Fig. 5A). ADMA concentrations were not associated with the E_{max} ACh-induced relaxation in mesenteric arteries (std β (95% CI) = -0.09(-0.42 to 0.27),

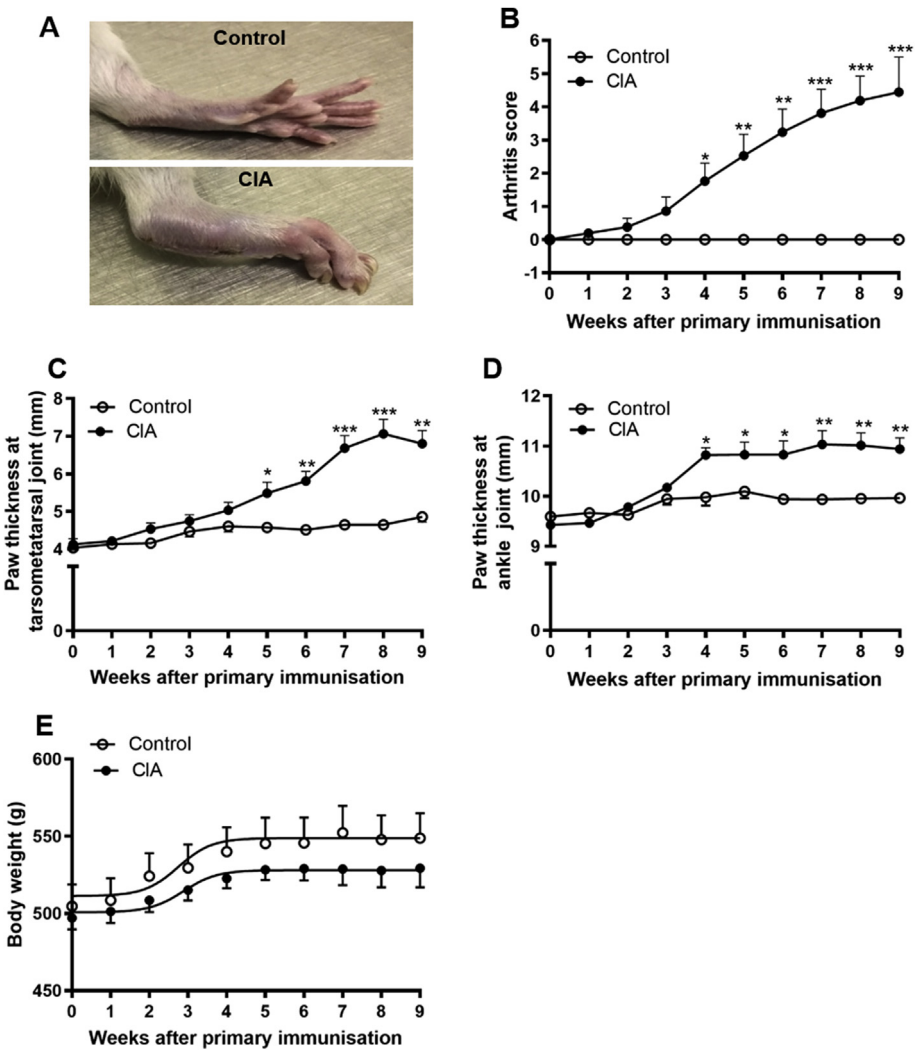

Fig. 1. Changes in **(A)** hind limbs, **(B)** arthritis scores, **(C)** paw thickness at the tarsometatarsal joint, **(D)** paw thickness at the ankle joint and **(E)** body weights in control (n = 12) and CIA (n = 21) rats nine weeks after the primary immunisation. Data expressed as means ± S.E.M. *P < 0.05 compared to control group; **P < 0.01 compared to control group; ***P < 0.0001 compared to control group, using non-parametric Mann-Whitney U-test (B) and repeated-measures ANOVA followed by Tukey post-hoc tests (A, C and D).

R^2 = 0.01, P = 0.65; Fig. 5B). No associations were observed between VCAM-1(std β (95% CI) = 0.34(-0.02 to 0.62), R^2 = 0.11, P = 0.08) or ADMA (std β (95% CI) = -0.02 (−0.38 to 0.34), R^2 = 0.006, P = 0.92) concentrations and the EC_{50} of ACh-induced relaxation in mesenteric arteries. No associations were found between biomarkers of endothelial dysfunction and the E_{max} (VCAM-1: std β (95% CI) = -0.06 (−0.40 to 0.31), R^2 = 0.003, P = 0.76; ADMA: std β (95% CI) = 0.28(-0.07 to 0.58), R^2 = 0.08, P = 0.13) or EC_{50} (VCAM-1: std β (95% CI) = -0.07 (−0.41 to 0.29), R^2 = 0.005, P = 0.70; ADMA: std β (95% CI) = 0.35(-0.02 to 0.61), R^2 = 0.12, P = 0.07) of ACh-induced relaxation in saphenous arteries.

3.6. Systemic inflammatory markers associated with impaired endothelium-dependent relaxation in mesenteric but not in saphenous arteries

Table 3 shows the relative contribution of systemic inflammatory markers to the maximal relaxation (E_{max}) to acetylcholine in mesenteric arteries. Greater serum concentrations of TNF-α (std β (SE) = -0.49 (0.16), R^2 = 0.24, P = 0.01), IL-6 (std β (SE) = -0.51 (0.16), R^2 = 0.26, P = 0.004), IL-1β (std β (SE) = -0.55 (0.15), R^2 = 0.31, P = 0.001) and CRP (std β (SE) = -0.48 (0.18), R^2 = 0.23, P = 0.01) were associated with impaired ACh-induced relaxation (lower E_{max}) in mesenteric arteries.

Associations between inflammatory markers and the E_{max} of ACh-induced relaxation in mesenteric arteries were no longer significant after adjusting for VCAM-1 (TNF-α: std β (SE) = -0.31 (0.19), R^2 = 0.27, P = 0.11; IL-6: std β (SE) = -0.34 (0.18), R^2 = 0.29, P = 0.08; CRP: std β (SE) = -0.27 (0.19), R^2 = 0.26, P = 0.17) except for IL-1β (std β (SE) = -0.44 (0.17), R^2 = 0.36, P = 0.02). Associations between inflammatory markers and the E_{max} of ACh-induced relaxation in mesenteric arteries remained significant after adjusting for ADMA (TNF-α: std β (SE) = -0.49 (0.17), R^2 = 0.20, P = 0.01; IL-6: std β (SE) = -0.51 (0.16), R^2 = 0.22, P = 0.01; IL-1β: std β (SE) = -0.58 (0.15), R^2 = 0.31, P = 0.001; CRP: std β (SE) = -0.46 (0.18), R^2 = 0.17, P = 0.01).

No significant associations were found between TNF-α (std β (SE) = 0.31 (0.18), R^2 = 0.17, P = 0.06), IL-6 (std β (SE) = 0.31 (0.17), R^2 = 0.17, P = 0.07), IL-1β (std β (SE) = 0.33 (0.17), R^2 = 0.11, P = 0.07) and CRP (std β (SE) = 0.34 (0.19), R^2 = 0.11, P = 0.11) and the EC_{50} of ACh-induced relaxation in mesenteric arteries. No associations were found between inflammatory makers and the E_{max} of ACh-induced relaxation in saphenous arteries (TNF-α: std β (SE) = 0.32 (0.18), R^2 = 0.10, P = 0.09; IL-6: std β (SE) = 0.25 (0.19), R^2 = 0.06, P = 0.17; IL-1β: std β (SE) = 0.18 (0.19), R^2 = 0.04, P = 0.35; CRP: std β (SE) = 0.19 (0.20), R^2 = 0.03, P = 0.34) or EC_{50} (TNF-α: std β (SE) = 0.37 (0.18), R^2 = 0.14, P = 0.05; IL-6: std β

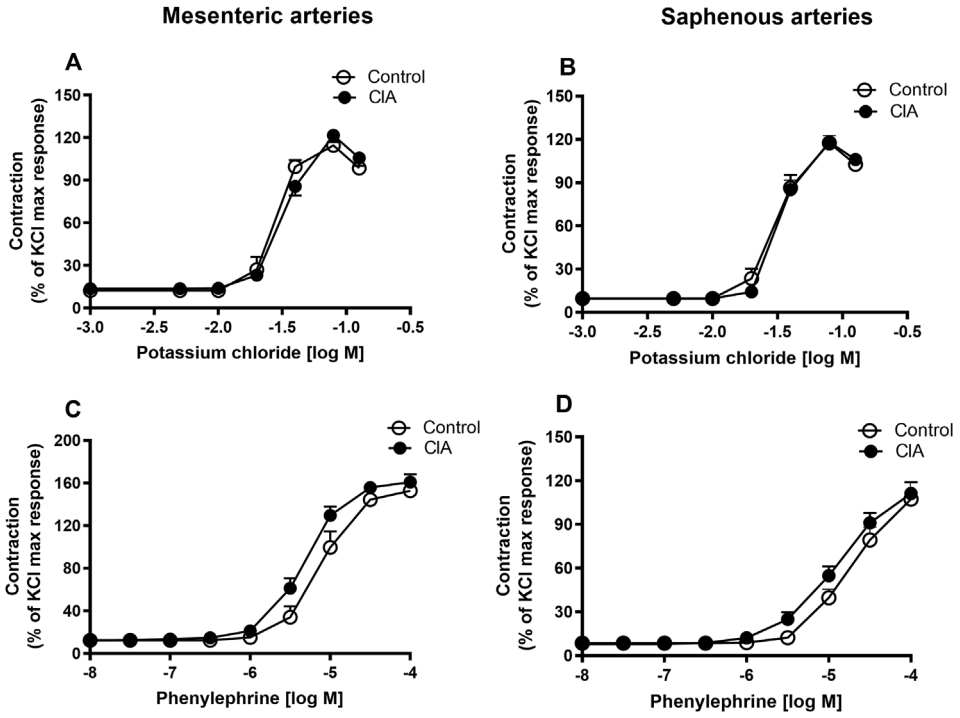

Fig. 2. Cumulative dose-response curves to (**A** and **B**) potassium chloride and (**C** and **D**) phenylephrine in the second branch of mesenteric arteries (left panel) and saphenous arteries (right panel) of control (n = 11) and CIA (n = 19) rats. Contractile responses are expressed as a percentage of the KCl maximal response. Data expressed as means ± S.E.M., using repeated-measures ANOVA followed by Tukey post-hoc tests. Open circles represent the control group and closed circles represent the collagen-induced arthritis (CIA) group.

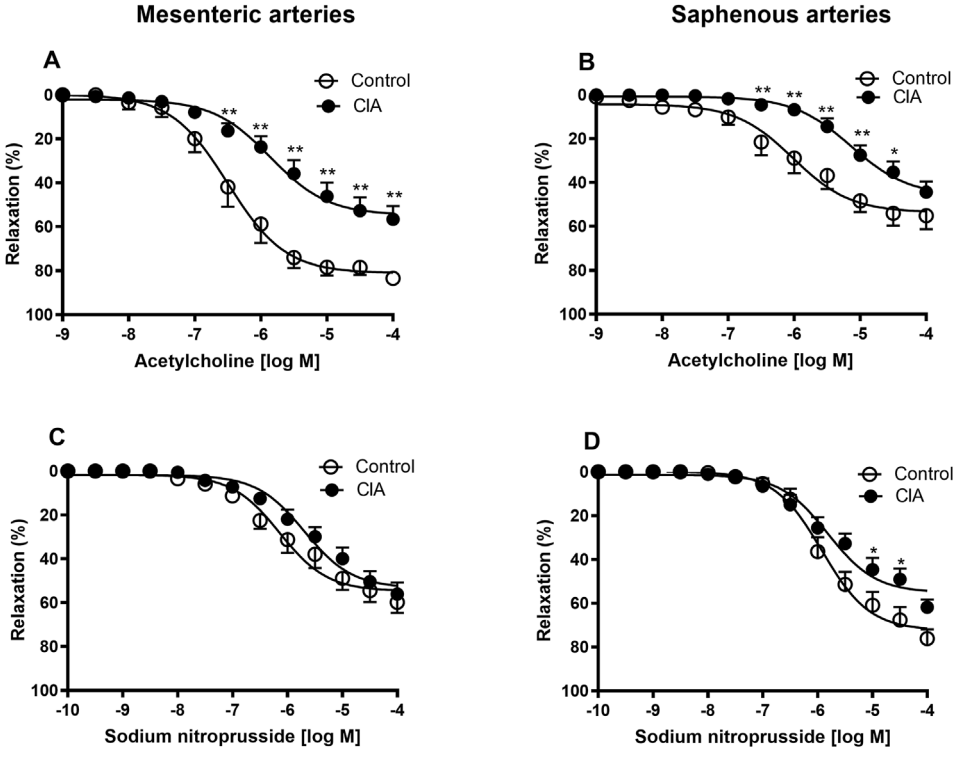

Fig. 3. Cumulative dose-response curves to **(A** and **B)** acetylcholine and **(C** and **D)** sodium nitroprusside in the second branch of mesenteric arteries (left panel) and saphenous arteries (right panel) of control (n = 11) and CIA (n = 19) rats. Relaxation responses are expressed as a percentage of the Phe (10 μM)-induced contraction. Data expressed as means ± S.E.M. *P < 0.05 compared to control group. **P < 0.01 compared to control group, using repeated-measures ANOVA followed by Tukey post-hoc tests. Open circles represent the control group and closed circles represent the collagen-induced arthritis (CIA) group.

(SE) = 0.33 (0.19), R^2 = 0.10, P = 0.10; IL-1β: std β (SE) = 0.21 (0.19), R^2 = 0.04, P = 0.29; CRP: std β (SE) = 0.29 (0.18), R^2 = 0.09, P = 0.14).

4. Discussion

The present study demonstrated associations between systemic inflammatory markers, circulating VCAM-1 concentrations and impaired endothelium-dependent relaxation in a model of CIA. Exposure to inflammation impaired ACh-induced relaxation in mesenteric and saphenous arteries. However, the SNP-induced relaxation remained unaltered in mesenteric arteries and was marginally impaired in saphenous arteries, suggesting an endothelial nature of the dysfunction induced by inflammation. The impaired endothelium-dependent relaxation was not accompanied by concomitant contractile dysfunction in mesenteric and saphenous arteries. Moreover, high-grade inflammation resulted in increased circulating VCAM-1 concentrations but unaltered ADMA concentrations. Finally, only circulating VCAM-1 concentrations were associated with impaired endothelium-dependent relaxation in mesenteric arteries. These results enhance our

Table 1
The sensitivity (EC_{50}) and maximum responses (E_{max}) to acetylcholine (ACh) and sodium nitroprusside (SNP) in mesenteric and saphenous arteries of control and CIA rats.

		Mesenteric arteries		Saphenous arteries	
		Control (n = 11)	CIA (n = 19)	Control (n = 11)	CIA (n = 19)
ACh	EC_{50} (μM)	6.6 ± 0.2	32 ± 0.9[a]	0.3 ± 0.1	3.1 ± 0.8[a]
	E_{max}(mN)	83 ± 3.5	56 ± 6.3[a]	55 ± 6.2	44 ± 4.8
SNP	EC_{50} (μM)	1.8 ± 0.9	7.3 ± 0.1	1.3 ± 0.9	9.15 ± 1.5
	E_{max}(mN)	58 ± 5.7	56 ± 5.7	76 ± 5.7	61 ± 4.8

Data expressed as means ± S.E.M. [a] P < 0.05 compared to the control group. Data analyzed using unpaired t-tests. CIA, collagen-induced arthritis.

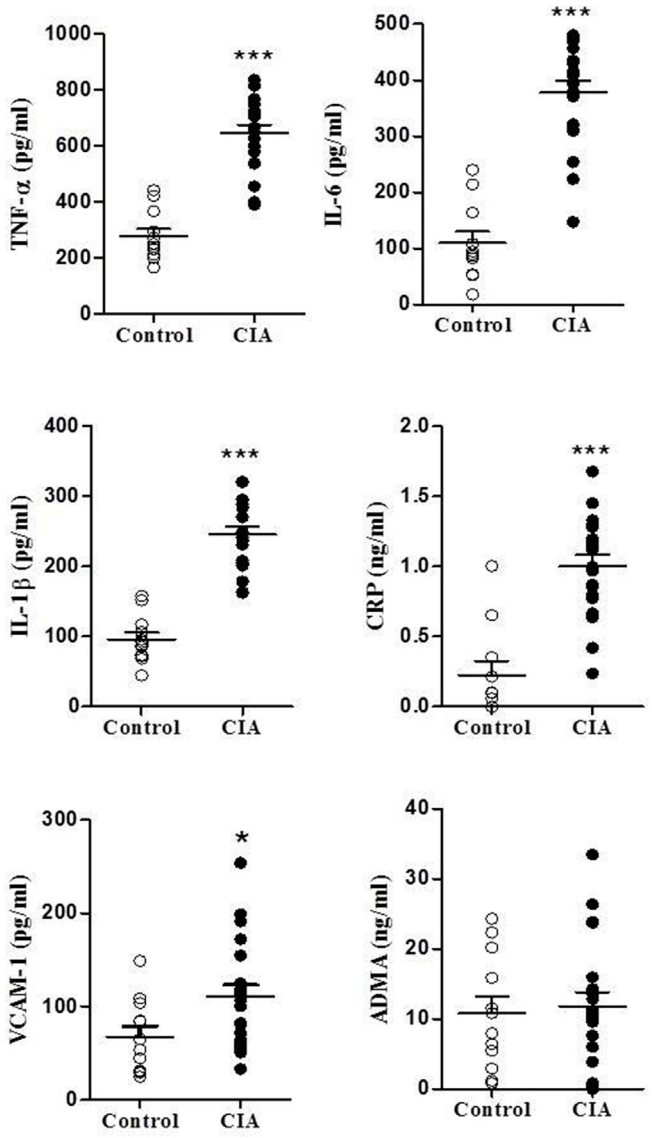

Fig. 4. Serum levels of tumour necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), interleukin 1β, (IL-1β), C-reactive protein (CRP), vascular adhesion modecule-1 (VCAM-1) and asymmetric dimethylarginine (ADMA) in control (n = 11) and CIA (n = 20) groups nine weeks after the primary immunisation. Data expressed as means ± S.E.M. *P < 0.05 compared to control group. ***P < 0.0001 compared to control group, using unpaired t-tests.

Table 2
Associations between systemic inflammatory markers and biomarkers of endothelial dysfunction in the control and CIA groups.

	Std β (SE)	R^2	P-value
VCAM-1			
TNF-α	0.39 (0.16)	0.15	0.03
IL-6	0.37 (0.17)	0.14	0.04
IL-1β	0.41 (0.16)	0.17	0.02
CRP	0.36 (0.17)	0.13	0.04
ADMA			
TNF-α	−0.06 (0.18)	0.004	0.76
IL-6	−0.06 (0.17)	0.004	0.76
IL-1β	−0.11 (0.17)	0.01	0.61
CRP	0.11 (0.18)	0.01	0.56

Data analyzed using linear regression analysis. TNF-α, tumor necrosis factor-alpha; IL-6, interleukin 6; IL-1β, interleukin 1-β; CRP, C-reactive protein; VCAM-1, vascular cell adhesion molecule-1; ADMA, asymmetric dimethylarginine; CIA, collagen-induced arthritis.

understanding of the mechanisms whereby inflammation impairs vascular function.

In the present study, endothelial dysfunction was impaired in both mesenteric and saphenous arteries. Indeed endothelial dysfunction is observed in small resistance arteries and large conduit arteries in RA patients (Arosio et al., 2007; Dessein et al., 2005; Galarraga et al., 2008; Klimiuk et al., 2002; Vaudo et al., 2004; Yki-Järvinen et al., 2003) and in animal models of RA (Dooley et al., 2015; He et al., 2013; Palma Zochio Tozzato et al., 2016; Reynolds et al., 2012; Totoson et al., 2015, 2016).

Moreover, endothelial dysfunction was associated with inflammatory markers. These results are in accordance with previous cross-sectional studies in RA that reported impaired endothelium-dependent relaxation in patients with adverse inflammatory profiles (Arosio et al., 2007; Galarraga et al., 2008; Vaudo et al., 2004). Associations between circulating inflammatory markers and impaired ACh-induced relaxation have previously been shown in an animal study using adjuvant-induced arthritis (Totoson et al., 2016). Although systemic inflammation-endothelial dysfunction relations in cross-sectional RA studies have been adjusted for potential confounders (Arosio et al., 2007; Galarraga et al., 2008; Mäki-Petäjä et al., 2008), traditional risk factor profiles are different in RA compared to the general population (Zegkos et al., 2016). Traditional risk factors contribute to the heightened cardiovascular mortality in RA (Baghdadi et al., 2015). Recent findings indicate that traditional risk factors such as obesity, dyslipidemia and hypertension, are independently associated with endothelial dysfunction (Erre et al., 2018; Rojas-Villarraga et al., 2008; Sandoo et al., 2012; Vaudo et al., 2004). Consistent with previously reported findings in animal models of RA, our results confirm that endothelial dysfunction was not explained by metabolic (He et al., 2013) or blood pressure-dependent mechanisms (Totoson et al., 2015). Indeed, evidence suggests that inflammatory cytokines may modify the phenotype of endothelial cells, thereby leading to endothelial dysfunction (Dessein and Joffe, 2006; Sattar et al., 2003; Sprague and Khalil, 2009).

Similar to one previously reported study in CIA (Chen et al., 2013), the present study showed no differences in circulating ADMA concentrations between the control and CIA groups. In the adjuvant-induced arthritis model, one study reported similar ADMA levels in rats exposed to inflammation compared to rats receiving anti-inflammatory drugs (Verhoeven et al., 2017). However, the absence of a control group in the aforementioned study limits the interpretation of these results. In contrast, clinical studies (Dimitroulas et al., 2017; Şentürk et al., 2016; Spasovski et al., 2013) frequently report increased circulating ADMA concentrations in RA patients and is believed to be a marker endothelial dysfunction (Dimitroulas et al., 2017; Şentürk et al., 2016) and a promising cardiovascular disease risk biomarker. ADMA acts as an eNOS inhibitor by competitive inhibition of cellular L-arginine uptake by

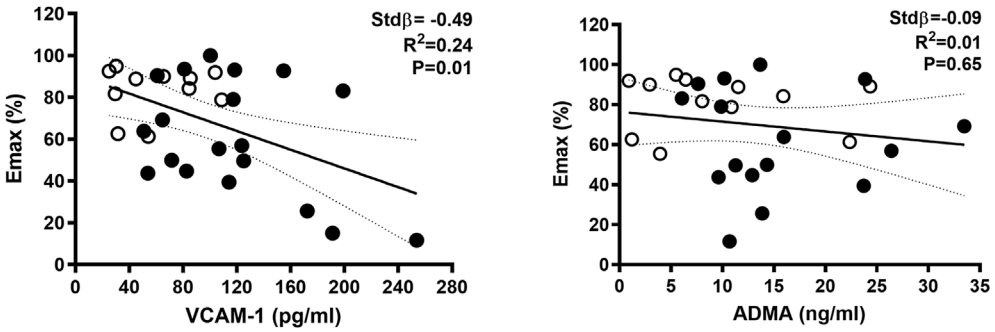

Fig. 5. Regression analysis showing the association between **(A)** vascular adhesion modelcule-1 (VCAM-1) and **(B)** asymmetric dimethylarginine (ADMA) with the maximal relaxation (E_{max}) to acetylcholine in the mesenteric arteries of the control and CIA groups. Open circles represent the control group (n = 11) and closed circles represent the CIA group (n = 19). The solid line represents the best-fit linear regression line and dashed lines represent the 95% confidence interval limits (CI).

Table 3
The relative impact of inflammatory markers on the maximal relaxation (E_{max}) to acetylcholine in mesenteric arteries before and after adjusting for endothelial activation markers.

	Std β (SE)	R^2	P-value
Unadjusted			
TNF-α	−0.49 (0.16)	0.24	0.01
IL-6	−0.51 (0.16)	0.26	0.004
IL-1β	−0.55 (0.15)	0.31	0.001
CRP	−0.48 (0.18)	0.23	0.01
Adjusted for VCAM-1			
TNF-α	−0.31 (0.19)	0.27	0.11
IL-6	−0.34 (0.18)	0.29	0.08
IL-1β	−0.44 (0.17)	0.36	0.02
CRP	−0.27 (0.19)	0.26	0.17
Adjusted for ADMA			
TNF-α	−0.49 (0.17)	0.20	0.01
IL-6	−0.51 (0.16)	0.22	0.01
IL-1β	−0.58 (0.15)	0.31	0.001
CRP	−0.46 (0.18)	0.17	0.01

Data analyzed using linear regression analysis. TNF-α, tumor necrosis factor-alpha; IL-6, interleukin 6; IL-1β, interleukin 1-beta; CRP, C-reactive protein; VCAM-1, vascular cell adhesion molecule-1; ADMA, asymmetric dimethylarginine.

endothelial cells or by increasing superoxide oxide production, which further decreases NO bioavailability leading to endothelial dysfunction (Antoniades et al., 2009; Sibal et al., 2010; Veresh et al., 2008). The lack of associations between ADMA concentrations and systemic inflammatory markers and impaired endothelium-dependent relaxation of mesenteric arteries in our study suggests that early inflammation-induced endothelial dysfunction may not be a result of eNOS inhibition by ADMA. Although ADMA is a promising biomarker of endothelial dysfunction in patients with RA, ADMA may not be an early biomarker of endothelial dysfunction in CIA. ADMA concentrations following longer duration exposure to systemic inflammation in the CIA model needs further investigation.

Similar to described findings in RA patients (Dessein and Joffe, 2006; Klimiuk et al., 2002; Wållberg-Jonsson et al., 2008), the present study showed increased circulating VCAM-1 concentrations in the CIA group which were associated with systemic inflammatory markers and endothelial dysfunction. Previously, in the adjuvant-induced arthritis model, impaired endothelium-dependent relaxation of mesenteric arteries have been reported in the presence of increased circulating intercellular adhesion molecule 1 (ICAM-1) concentration but not VCAM-1 concentration (Totoson et al., 2015). However, no association between ICAM-1 or VCAM-1 and endothelial dysfunction was demonstrated in the aforementioned study (Totoson et al., 2015). These discrepancies may be explained by the different protocols used to induce arthritis in the aforementioned studies. To the best of our knowledge, the current study is the first to demonstrate associations between VCAM-1 and impaired endothelial-dependent relaxation in an animal model of RA.

VCAM-1, a marker of endothelial activation, is expressed by endothelial cells and leukocytes in response to endothelial cell injury, often as a results of increased circulating inflammatory cytokines (Liao, 2013; Zhang, 2008). Endothelial activation increases the production of reactive oxygen species inside the endothelium which inactivate and reduce intracellular concentrations of NO (Gryglewski et al., 1986; Yoshizumi et al., 1993). Our study confirms that exposure to systemic inflammation causes endothelial activation. Importantly our results showed that the relationship between inflammatory markers and impaired endothelial dependent relaxation was abrogated when adjusted for VCAM-1 concentrations. This suggest that VCAM-1 may in part explain the associations of inflammatory markers with impaired endothelial function.

ACh-induced relaxation in larger arteries, such as the saphenous branch of the femoral artery, is largely NO-dependent, whereas in smaller resistance arteries, such as the mesenteric arteries, endothelium derived hyperpolarizing factor (EDHF) plays a greater role (Wigg et al., 2001; Zygmunt et al., 1995). Hence, the impaired E_{max} to ACh-induced relaxation in mesenteric arteries of CIA rats is likely due to an impaired EDHF-mediated response. In the present study, VCAM-1 was correlated to endothelial dysfunction only in mesenteric arteries, suggesting that the role of VCAM-1 as a possible mediator of inflammation-induced microvascular endothelial dysfunction extends beyond altered NO activity.

The present study has further limitations. Although CIA is most closely related to human RA (Bendele, 2001; Brand et al., 2007; Hegen et al., 2008), the inflammatory processes involved in endothelial dysfunction and atherogenesis may differ from the complexities of the human condition. Endothelial dysfunction and inflammatory markers were assessed at a single time point, nine weeks after the primary immunisation. Future studies should investigate the time course of inflammation and endothelial function in CIA. The current study investigated only one biomarker of endothelial activation, future studies should determine the role of other biomarkers of endothelial activation including ICAM-1 and E-selectin. Although we have demonstrated the effect of inflammation on both VCAM-1 and impaired endothelial function, the direct casual effect of VCAM-1 on impaired endothelial function can only be implied. Hence, future studies should investigate whether VCAM-1 neutralization (using monoclonal anti-VCAM-1 antibody treatment) restores the inflammation-induced endothelium dysfunction in CIA rats. Furthermore, other mechanisms such as the overproduction of cyclo-oxygenase-2 (COX-2) activity and upregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which could account for the effect of inflammation on endothelial function should be investigated.

In conclusion, the present study showed that exposure to high-grade inflammation *in vivo* impairs endothelium-dependent relaxation of mesenteric and saphenous arteries. VCAM-1 but not ADMA was associated with systemic inflammation and microvascular endothelial dysfunction in CIA. These findings add to the evidence that systemic inflammation impairs endothelial function that may lead to cardiovascular disease in RA. Future studies should elucidate the

mechanisms whereby inflammation impairs microvascular relaxation.

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Ethical approval

This investigation was approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand (AESC number: 2017/03/21C) and conducted in compliance with the Guide for the Care and Use of Laboratory Animals. This study does not contain any studies with human participants performed by any of the authors.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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