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RESEARCH PAPER

Urocortin promotes the development of vasculitis in a rat model of thromboangiitis obliterans via corticotrophin-releasing factor type 1 receptors

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Background and purpose: Urocortin is a locally expressed pro-inflammatory peptide. Here we have examined the effects of urocortin on sodium laurate-induced peripheral arterial vasculitis in rats, modelling the mechanisms of thromboangiitis obliterans (TAO).

Experimental approach: Peripheral vasculitis in rats was induced by sodium laurate and graded by gross appearance on the 12th day after injection. Histological changes in rat femoral arteries were assessed by histopathology and transmission electron microscopy. Blood cell counts, blood rheology, blood coagulation and plasma urocortin, thromboxane B_2, prostaglandin E_2 and soluble intercellular adhesion molecule-1 levels were measured. Expression of urocortin, corticotrophin-releasing factor ($CRF_{1/2}$) receptors, cyclooxygenase (COX)-2 and intercellular adhesion molecule-1 (ICAM-1) at both mRNA and protein levels were determined by RT-PCR and Western blot.

Key results: Rats showed grossly visible signs and symptoms of TAO on the 12th day after sodium laurate injection. In these rats, blood was in a hypercoagulable state; plasma urocortin, prostaglandin E_2 and soluble intercellular adhesion molecule-1 levels were elevated; and the expression of urocortin, CRF_1 and $CRF_{1\alpha}$-receptors, COX-2 and ICAM-1 in rat femoral arteries were markedly increased. Exogenous urocortin, given for 12 days after sodium laurate, exacerbated the hypercoagulable state and augmented expression of $CRF_{1\alpha}$-receptors, COX-2 and ICAM-1. These effects were abolished by a CRF_1-receptor antagonist, NBI-27914, or a non-selective CRF-receptor antagonist, astressin, but not by the CRF_2-receptor antagonist, antisauvagine-30, given with exogenous urocortin.

Conclusion and implications: Urocortin exacerbated the hypercoagulable state and vasculitis in a model of TAO induced by sodium laurate in rats, via CRF_1-receptors. COX-2 and ICAM-1 might also have contributed to this exacerbation.
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Keywords: Urocortin; thromboangiitis obliterans; vasculitis; CRF_1-receptor; CRF_2- receptor

Abbreviations: CRF, corticotrophin-releasing factor; CRF_1 receptor, corticotrophin-releasing factor type 1 receptor; CRF_2 receptor, corticotrophin-releasing factor type 2 receptor; ICAM-1, intercellular adhesion molecule-1; PG, prostaglandin; TAO, thromboangiitis obliterans; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2

Introduction

Vasculitis is a pathological process characterized by inflammatory damage to blood vessels, which may be induced by genetic abnormalities, autoimmune or physical damage. Thromboangiitis obliterans (TAO) is a non-atherosclerotic, segmental, inflammatory disease that most commonly affects the small- and medium-sized arteries, veins and nerves of the extremities (Olin, 2000). In the characteristic acute-phase lesion, in association with occlusive cellular thrombosis, the acute inflammation that involves all layers of the vessel wall leads TAO to be classified as a vasculitis (Puéchal and Fiessinger, 2007). However, the exact aetiology for TAO is not yet well defined (Olin, 2000). In sodium laurate-induced arterial occlusive disease of rats, the injected sodium laurate is supposed to cause endothelial cell damage that may lead to the aggregation of platelets in peripheral vascular beds

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(Ashida *et al.*, 1980). The progression of the disease in this model of vasculitis resembles that reported in the patients with TAO (Nakata *et al.*, 1976; Nielubowicz *et al.*, 1980).

Urocortin, a peptide of the corticotrophin-releasing factor (CRF) family (Vaughan *et al.*, 1995), is expressed in both central and peripheral tissues (Fekete and Zorrilla, 2007). Besides its cardiovascular protective property (Okosi *et al.*, 1998; Oki and Sasano, 2004), urocortin is now considered to be a potent immunomodulatory factor that participates in immune responses (Fekete and Zorrilla, 2007). In contrast to its systemic indirect immunosuppressive effects on the hypothalamic–pituitary adrenal axis, urocortin acts as a locally expressed, autocrine or paracrine, pro-inflammatory factor in a series of inflammatory diseases, such as rheumatoid arthritis and osteoarthritis (Kohno *et al.*, 2001) and ulcerative colitis (Saruta *et al.*, 2004). Moreover, it can stimulate the release of pro-inflammatory mediators under inflammatory conditions (Saruta *et al.*, 2004; Theoharides *et al.*, 2004). Our previous study demonstrated the overexpression of urocortin in lung tissues of rats with allergic asthma and inhalation of aerosolized urocortin increased pulmonary vascular permeability via mast cell infiltration and activation (Wu *et al.*, 2006). This process was mediated by CRF receptors (CRF_1 and CRF_2) (Singh *et al.*, 1999; nomenclature follows Alexander *et al.*, 2008). We demonstrated for the first time that mast cell degranulation induced by urocortin was mediated by increasing intracellular calcium concentration via CRF_1-receptors (Wu *et al.*, 2008). Moreover, our data showed that CRF played a significant role in promoting the development of atherosclerosis (Wu *et al.*, 2009), which also exhibits features of vasculitis (Cipollone and Fazia, 2006). All these results suggested that endogenous urocortin might participate in the pathophysiology of many inflammatory conditions, as a pro-inflammatory mediator.

We therefore hypothesized that urocortin, which is synthesized and secreted by the systemic vasculature, may be a potent, locally expressed, pro-inflammatory factor in TAO, which is classified as a vasculitis with endothelial dysfunction (Joras *et al.*, 2006). In this study, we assessed the role of urocortin in a rat model of TAO induced by the intravascular injection of sodium laurate. We also investigated the type of CRF receptors involved in these effects of urocortin.

Methods

Animals

All animal care and this investigation conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996), and were approved by the Ethical Review Board of Nanjing Medical University. Male Wistar rats (200–250 g) were purchased from Shanghai laboratory animal centre. Food and water were given *ad libitum* unless otherwise noted.

Experimental protocol

Male Wistar rats were randomly divided into seven groups (eight animals per group) as follows: (i) normal group; (ii) sham operated group; (iii) vasculitis (TAO model) group; (iv) urocortin group; (v) urocortin + NBI-27914 group; (vi) urocortin + antisauvagine-30 group; and (vii) urocortin + astressin group. Rats with sodium laurate-induced vasculitis were prepared according to the method described previously (Ashida *et al.*, 1980). Briefly, rats were anaesthetized with 10% (w/v) chloral hydrate (3.5 $mL \cdot kg^{-1}$, i.p.). The left hind leg was shaved and the femoral artery was exposed by surgical incision and retraction of muscles. Sodium laurate solution (10 $mg \cdot mL^{-1}$ in normal saline, adjusted to pH 8.0, 0.1 mL per animal) was injected into the left femoral artery while the sham operated group were treated with normal saline as vehicle. All groups were subjected to the surgical procedure except for the normal group. Urocortin (3 $nmol \cdot kg^{-1}$) and $CRF_{1/2}$ receptor antagonists (15 $nmol \cdot kg^{-1}$) (Li *et al.*, 2008) were given s.c., 2 h after sodium laurate injection and once a day for the following 11 days.

The gross appearance of the rat hind legs was checked daily after the operation. The degree of the disease on the 12th day was graded according to the system developed by Murakami *et al.* (1995), as follows: 0, normal appearance; 1, change in nail colour; 2, change in digit colour; 3, gangrene of digit; 4, loss or mummification of digit.

At the end of the experiment, rats were anaesthetized with 10% chloral hydrate. A blood sample was drawn from the common carotid artery and anti-coagulated with heparin, sodium citrate or EDTA, depending on the variable measured. Plasma was frozen at –80°C for biochemical analysis. Left femoral arteries were collected and divided into two: one was frozen at –80°C until used to provide a homogenate; the other was taken for histological examination.

Assessment of blood cell counts, blood coagulation and blood rheology

Blood cell counts, blood coagulation and blood rheology were measured by the clinical laboratory in the First Affiliated Hospital of Nanjing Medical University.

Plasma urocortin, thromboxane B_2, prostaglandin E_2 and soluble intercellular adhesion molecule-1 assays

Plasma urocortin, prostaglandin (PG)E_2 and soluble intercellular adhesion molecule (sICAM)-1 were determined by ELISA Kits (purchased from Phoenix Pharmaceuticals, USA; R&D Systems, USA; Boster Biological Technology, China respectively). Plasma thromboxane (TX)B_2 was measured with an enzyme immunoassay kit (Cayman Chemical, USA) according to the manufacturer's protocol.

Assessment by light and transmission electron microscopy

For light microscopy, samples of rat femoral artery were fixed in 10% neutral formalin for at least 24 h. After being embedded in paraffin, the tissues were cut into 5-μm-thick sections and stained with haematoxylin and eosin before examination.

For electron microscopy, samples of arteries were immediately placed in sodium acetate-buffered 2.5% glutaraldehyde solution and left overnight. The subsequent procedures were kindly performed by the Key Laboratory of Antibody

Table 1 Summary of the RT-PCR primer sequences used to amplify GAPDH, urocortin, CRF_1-receptors, CRF_2-receptors, COX-2 and ICAM-1 from rat tissues

		Sequences	*Product size (bp)*	*Annealing T(C)*
GAPDH	Sense	TCCCAGAGCTGAACGGGAAGCTCACTG	339	68.1
	Antisense	TGGAGGCCATGTAGGCCATGAGGTCCA		
Urocortin	Sense	GCTACGCTCCTGGTAGCGTTGCTGCTTCTG	356	68.1
	Antisense	GCCGATCACTTGCCCACCGAGTCGAATATG		
CRF_1	Sense	AAGGCGGGATCCAGGCAGTAGAGA	508	60.8
	Antisense	TCCCGGTAGCCATTGTTTGTCGTG		
$CRF_{1\alpha}$	Sense	TCCTACGCAACGCCAC	147	58.8
	Antisense	AGCAGCCCTCACCGAAC		
CRF_2	Sense	CTGGTGGCTGCTTTCCTGCTTTTC	425	58.1
	Antisense	ATGGGGCCCTGGTAGATGTAGTCC		
COX-2	Sense	TTCACCAGACAGATTGCTGGC	530	63.5
	Antisense	AGTCTGGAGTGGGAGGCACTTG		
ICAM-1	Sense	AGAAGGACTGCTTGGGGAA	332	58.1
	Antisense	CCTCTGGCGGTAATAGGTG		

CRF, corticotrophin-releasing factor; COX, cyclooxygenase; ICAM-1, intercellular adhesion molecule-1.

Technique, Ministry of Health, Nanjing Medical University. Artery specimens were then cut into 0.5-μm-thick sections, the sections stained with uranyl acetate and lead citrate and examined in a JEOL 2000FX transmission electron microscope operated at 80 kV.

Semi-quantitative RT-PCR analysis

Total RNAs were extracted from femoral arteries, using TRIzol (Invitrogen) according to the manufacturer's protocol. For cDNA synthesis, Moloney murine leukemia virus (Invitrogen) was applied as the reverse transcriptase. For PCR reaction, Taq DNA polymerase (Promega) was used in the reaction system. Primers applied in the experiment were synthesized from published sequences (Baigent and Lowry, 2000; Ohnaka *et al.*, 2000; Taal *et al.*, 2000; Brar *et al.*, 2004) (Table 1). The products were analysed by electrophoresis in 2.0% agarose gel containing 0.5 $\mu g \cdot mL^{-1}$ ethidium bromide. Specific genes were verified by their predicted sizes. GAPDH was set as the internal control.

Western blot analysis

The protein samples were separated on a 10% SDS-polyacrylamide gel and electrophoretically transferred to PVDF membranes in Tris-glycine transfer buffer. Then membranes were blocked in 5% (wt/vol) instant non-fat dried milk for 2 h at room temperature, and incubated with primary antibody for CRF_1-receptor (1:400), CRF_2-receptor (1:400), COX-2 (1:1000), ICAM-1 (1:500) or β-actin (1:200, Boster Biological Technology) at 4°C overnight. After three washes with TBST (50 $mmol \cdot L^{-1}$ Tris-HCl, pH 7.5, 150 $mmol \cdot L^{-1}$ NaCl, 0.05% Tween 20), the membranes were incubated with secondary horseradish peroxidase-conjugated IgG for 2 h at room temperature. Immunoreactive proteins were visualized by LumiGLO chemiluminescent reagent and peroxide (Cell Signaling Technology). The light-emitting bands were detected with X-ray films (Kodak, Rochester, NY).

Statistical analysis

Data were expressed as means ± SEM. The significance for the difference among groups was analysed with SPSS 11.0 by one-way ANOVA with Student–Newman–Keuls multiple comparison methods. The Kruskal–Wallis *H* test was used to compare the gross appearance of the vasculitis among groups. $P < 0.05$ was considered statistically significant.

Materials

Rat urocortin, CRF_1-receptor antagonist NBI-27914, CRF_2-receptor antagonist, antisauvagine-30 and the non-selective CRF-receptor antagonist, astressin were purchased from Sigma (Missouri, USA). Sodium laurate was provided by International Laboratory (California, USA). Polyclonal urocortin and antibodies to CRF_1- and CRF_2-receptors were obtained from Santa Cruz Biotechnology (California, USA). Polyclonal COX-2 and monoclonal ICAM-1 antibodies were from Abcam (Cambridge, UK). All other reagents used were derived from commercial sources.

Results

Rats showed gross signs and symptoms of TAO and histological changes after sodium laurate injection

The rat model of TAO, induced by sodium laurate, has been widely accepted. In our study, as reported previously (Nakata *et al.*, 1976; Nielubowicz *et al.*, 1980), the whole left hind paw went pale 2 or 3 min after injection of sodium laurate. On the 12th day, in sham operated and normal rats (Figure 1A and B), no ischaemic signs were found; while rats in the vasculitis (TAO model) group showed typical signs and symptoms of TAO (Figure 1C), with four rats displaying obvious signs of gangrene, and two with loss or mummification of paws.

Histological changes were also observed in sections of the femoral artery examined by light microscopy (Figure 2A and B). The sections from the TAO model rats (Figure 2B), stained with haematoxylin and eosin, showed obvious thrombi, which resulted in the narrowing or complete occlusion of the vessel lumen, and some showed recanalization of the artery. To investigate ultrastructural changes in the arterial tissue, sections were examined by transmission electron microscopy.

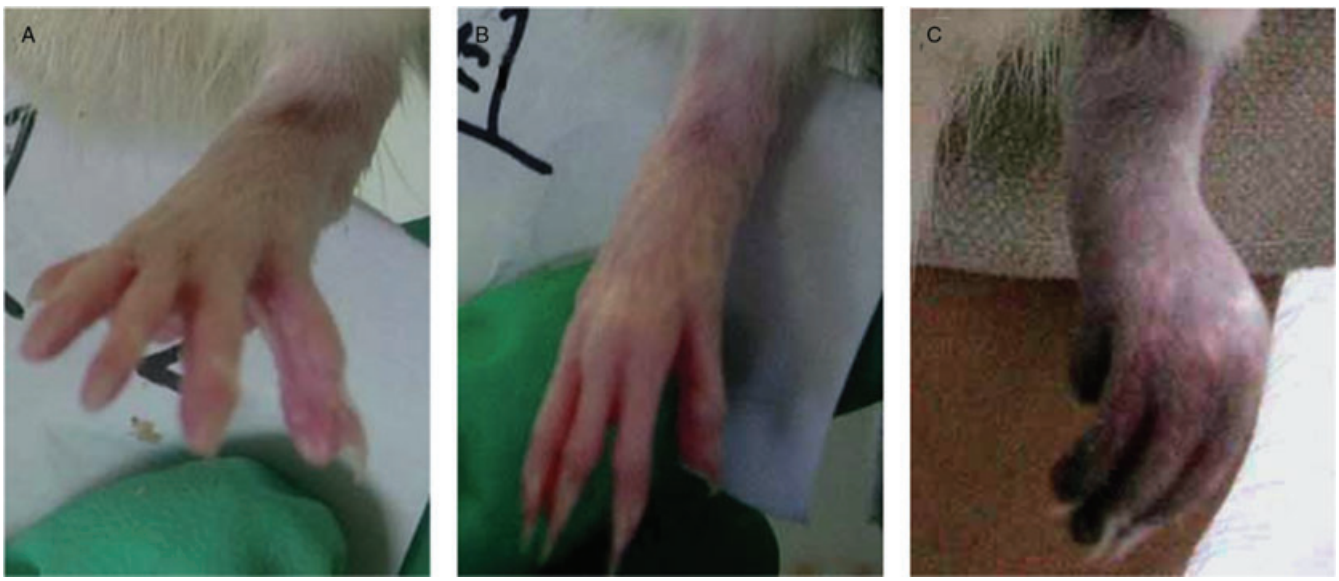

Figure 1 Gross appearance of rat paws in normal, sham operated and thromboangiitis obliterans (TAO) model rats on the 12th day after sodium laurate injection. Paws in normal (A), sham operated (B) and TAO model (C) rats.

The endothelium in sections from normal rats looked intact with regular arrangement of the endothelial cells [Figure 2C (a)]; while in arteries from the TAO model rats, the artery intima was modified with gaps in the endothelial sheet and desquamation in many places, and proliferation and hypertrophy of endothelium were also extensively found [Figure 2C (b)]. Under the intima, smooth muscle cells were in a regular order in normal arteries [Figure 2C (c)]; while in arteries from the TAO group, muscle cells were disordered and most showed multiformity and degeneration [Figure 2C (d)]. These findings were comparable with those found in TAO patients (Cui Pan Cui *et al*., 2000).

Urocortin and CRF_1-receptors were increased in TAO model rats

As shown in Figure 3, plasma urocortin was increased markedly in the TAO model group, compared with the levels in the sham operated or normal groups ($P < 0.05$). There was no difference between normal and sham operated group.

RT-PCR analysis (Figure 4A and B) showed that the mRNA for urocortin and CRF_1-receptors were increased in femoral arteries from the TAO group relative to the levels found in the normal and sham operated rat femoral arteries. We also showed that mRNA for the $CRF_{1\alpha}$-receptor was increased in the model rats (Figure 4A and B). The levels of mRNA for the CRF_2-receptor were not significantly altered in samples from the TAO group.

Subsequently, we also measured protein levels of CRF_1- and CRF_2-receptors in rat femoral arteries by Western blot analysis. As shown in Figure 4C and D, compared with sham operated group, the expression of CRF_1-receptor protein in the TAO model group was significantly increased ($P < 0.05$), while the expression of CRF_2-receptor protein remained constant.

Effects of exogenous urocortin and $CRF_{1/2}$ receptor antagonists on TAO model rats

In order to ascertain whether urocortin contributed to the TAO model, exogenous urocortin and $CRF_{1/2}$ receptor antagonists were added to rats treated with sodium laurate. As shown in Figure 5A, adding exogenous urocortin for 12 days to the TAO model rats exacerbated the gross effects of the ischaemia, with three rats displaying obvious signs of gangrene and three with loss or mummification of paws. These signs of intensified ischaemia were markedly decreased in the groups given the CRF_1-receptor antagonist, NBI-27914 (Figure 5B) or the non-selective CRF-receptor antagonist, astressin (Figure 5D) along with the exogenous urocortin. However, the CRF_2-receptor antagonist, antisauvagine-30 (Figure 5C), did not modify the gross effects of exogenous urocortin.

To quantify the gross effects of ischaemia in each group, these effects were graded into five levels, as described in the *Methods* and shown in Table 2. Rats from each group were graded on the 12th day and the group grades compared, using the Kruskal–Wallis *H* test. Compared with the values from the sham operated group, those from the TAO model and TAO + urocortin groups were significantly higher, that is, more intense signs of ischaemia. In agreement with the gross observations, the grades in the groups given NBI-27914 or astressin were lower and not different from those in the sham operated group. No such attenuation of ischaemic signs was present in the group given antisauvagine-30 and no statistical significance was found between normal and sham operated groups (Table 2). These data indicate that antagonism of CRF_1-receptors, but not of CRF_2-receptors, could alleviate the gross signs of the vasculitis.

In further analysis of the effects of exogenous urocortin and $CRF_{1/2}$ receptor antagonists in the TAO model rats, blood cell counts, blood coagulation and blood rheology of each group were investigated. Blood platelet count was markedly elevated

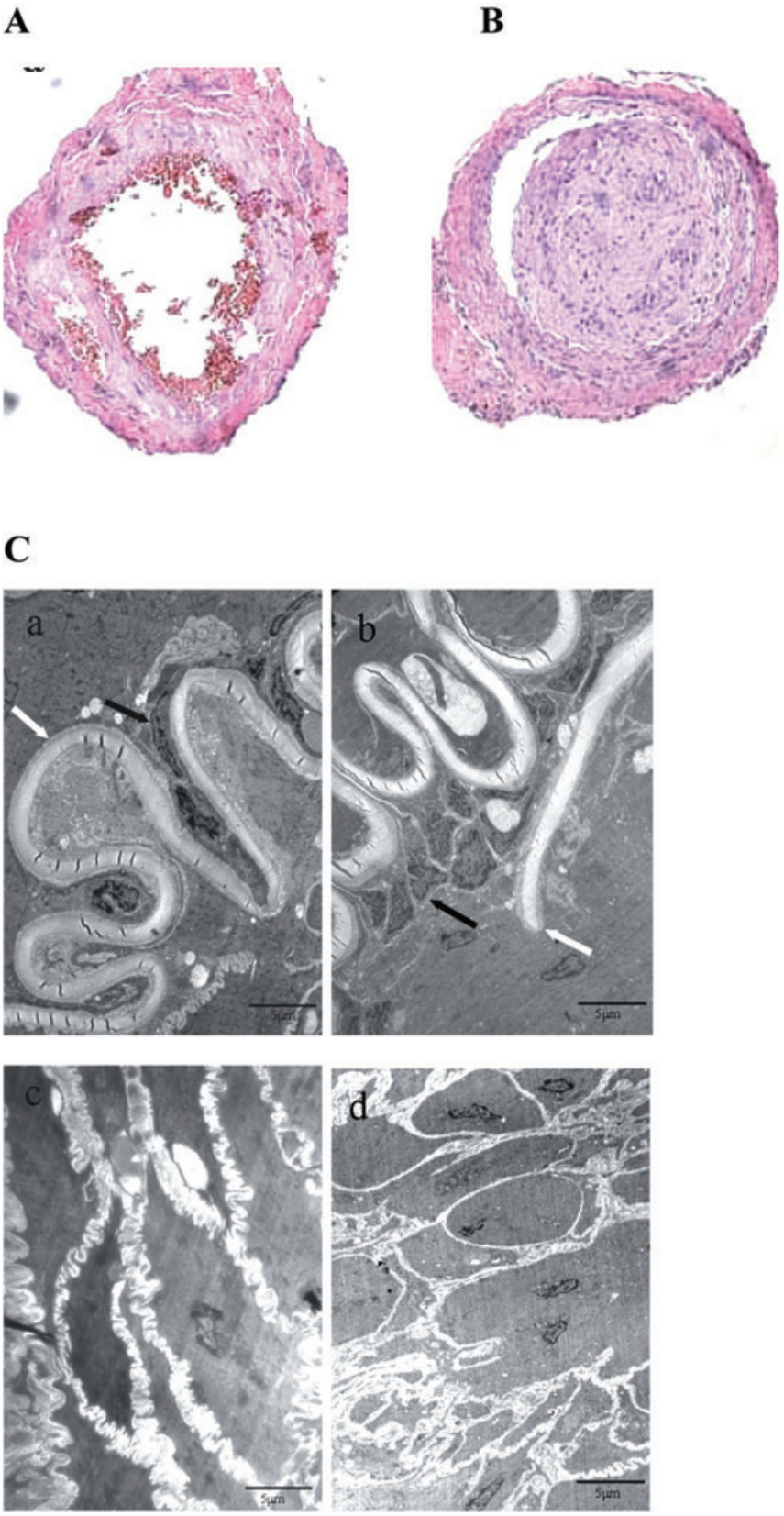

Figure 2 Histological examination of femoral arteries in normal (A) and thromboangiitis obliterans (TAO) model (B) rats (haematoxylin and eosin staining, magnification: 100×). In (C), ultrastructure of femoral arteries in normal (a and c) and TAO model (b and d) rats. a and b, artery intima; c and d, medial membrane. Black arrows show endothelial cells and white arrows show inner elastic layer. In picture b, black arrow indicates endothelial cell proliferation, and the white arrow indicates discontinuity of the internal elastic lamina (transmission electron microscopy; magnification: 4000×).

($P < 0.05$) in TAO model group (Table 3A). As expected, blood coagulation parameters (Table 3B) in the TAO model rats were very different from those in the sham operated group: prothrombin time, thrombin time and activated partial thromboplastin time were all significantly shortened while fibrinogen and D-dimer values were increased. Compared with the TAO model group, adding exogenous urocortin further shortened prothrombin time, thrombin time, activated partial thromboplastin time and increased blood platelet count, fibrinogen and D-dimer levels. Treatment with NBI-27914 and astressin reversed those parameters, to almost normal levels. However, it seems that urocortin had no influence on other parameters of rat blood cells and on blood rheology (Table 3C). Compared with the TAO model or urocortin groups, treatment with antisauvagine-30 had no significant effect on any of the parameters measured.

TXA_2, a potent inducer of platelet aggregation and microvascular contraction (Silver *et al*., 1973; 1974), was measured as its metabolite, TXB_2. As shown in Figure 6, plasma TXB_2 was markedly raised in the TAO model group, over the sham or normal values ($P < 0.05$), and was further elevated on addition of exogenous urocortin. Treatment with the CRF receptor antagonists, NBI-27914 or astressin but not antisauvagine-30, dramatically reduced plasma TXB_2 levels, relative to the TAO model group ($P < 0.05$).

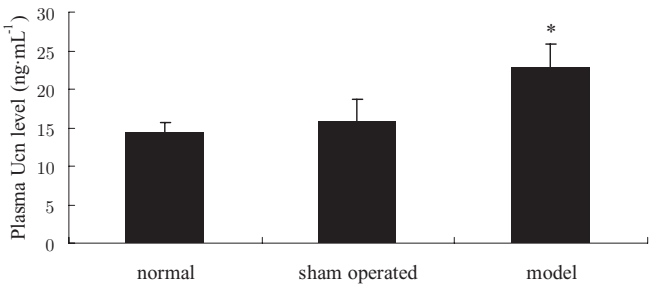

Figure 3 Plasma urocortin (Ucn) levels in normal, sham operated and thromboangiitis obliterans model groups. Data are presented as means ± SEM ($n = 8$). *$P < 0.05$, versus sham operated group.

Effects of urocortin and $CRF_{1/2}$ receptor antagonists on expression of COX-2 and ICAM-1

COX-2 and ICAM-1 are two important components of many inflammatory diseases (Dubois *et al*., 1998; Vane *et al*., 1998; Witkowska, 2005). Clinical reports indicate that ICAM-1 expression is elevated in TAO patients (Halacheva *et al*., 2002; Joras *et al*., 2006) and treatment with COX-2 inhibitors can relieve the disease condition (Nizankowski *et al*., 1985; Fiessinger and Schäfer, 1990). In the present study, RT-PCR

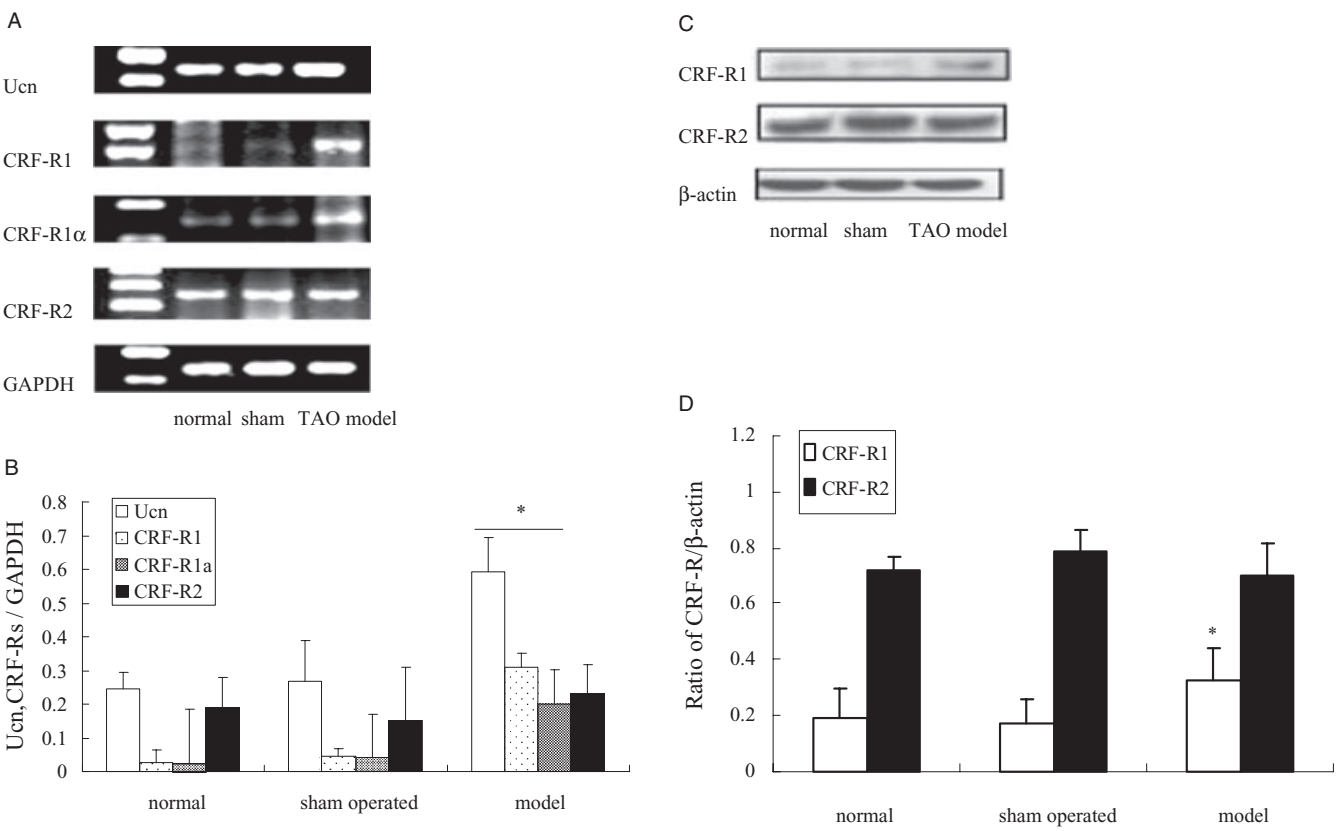

Figure 4 Expression of mRNA and Western blot analysis for urocortin and corticotrophin-releasing factor $(CRF)_{1/2}$ receptors in femoral arteries from normal, sham operated and thromboangiitis obliterans (TAO) model rats. In (A), a representative record of the expression of mRNA is shown (Ucn, urocortin; CRF-R1, CRF_1-receptor; CRF-R1α, $CRF_{1\alpha}$-receptor; CRF-R2, CRF_2-receptor) with GAPDH as an internal control. Summary data are shown in (B); data are presented as means ± SEM ($n = 8$). *$P < 0.05$, versus sham operated group. In (C), a representative Western blot for CRF_1-receptors and CRF_2-receptors is shown (β-actin as control) with summary data in (D); data presented as means ± SEM ($n = 8$). *$P < 0.05$, versus sham operated group.

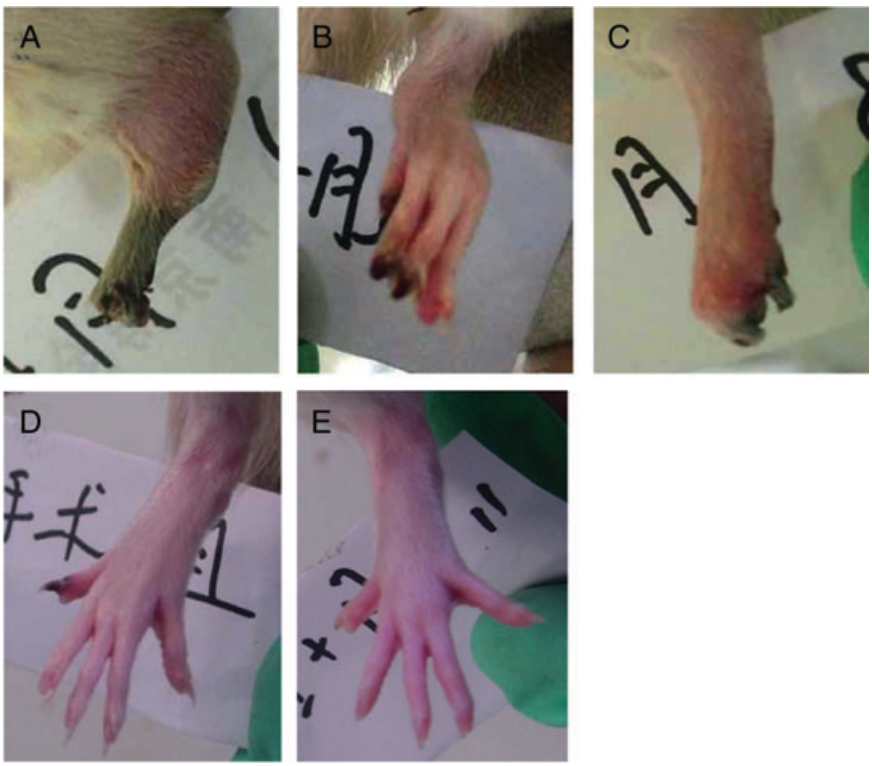

Figure 5 Gross appearance of rat paws after urocortin and corticotrophin-releasing factor type 1 and 2 receptor antagonists (12th day). A, urocortin group; B, urocortin + NBI-27914 group; C, urocortin + anti-sauvagine-30 group; D, urocortin + astressin group; E, sham operated group.

Table 2 Grades of disease on the 12th day after sodium laurate injection

	Grade of disease					*Significance*
	0	1	2	3	4	
Normal	8	0	0	0	0	
Sham operated	8	0	0	0	0	
TAO model	2	0	0	4	2	**
Ucn	1	1	0	3	3	**
Ucn + NBI-27914	5	0	0	2	1	†
Ucn + antisauvagine-30	2	0	2	1	3	**
Ucn + astressin	5	1	1	1	0	†#

On the 12th day after sodium laurate injection, rats from each group were graded according to the gross appearance of the affected paw (see *Methods*). Kruskal–Wallis *H* test was carried out to compare the disease condition among groups.
**$P < 0.01$, versus sham operated group; #$P < 0.05$, versus TAO model group; †$P < 0.05$, versus Ucn group.
TAO, thromboangiitis obliterans; TAO model, single injection of sodium laurate only; Ucn, TAO model + daily urocortin for 12 days; Ucn + NBI 27914 or + antisauvagine-3 or + astressin, Ucn + daily injections of each antagonist for 12 days.

analysis (Figure 7) showed that mRNA for COX-2 and ICAM-1 extracted from femoral arteries were increased in the TAO model and urocortin groups, compared with the sham operated group ($P < 0.05$) and that treatment with NBI-27914 or astressin but not antisauvagine-30 attenuated the increase due to urocortin group ($P < 0.05$). Western blot assays to determine protein levels of COX-2 and ICAM-1 (Figure 8) gave results largely in accordance with the RT-PCR data. Again, both NBI-27914 and astressin but not antisauvagine-30 blocked the effect of exogenous urocortin ($P < 0.05$) on expression of COX-2 and ICAM-1.

We also measured plasma PGE_2, the main metabolite of COX-2 and sICAM-1 in the experimental groups of rats. As shown in Figure 9, in accordance with the expression of COX-2 and ICAM-1 in rat femoral arteries, plasma PGE_2 and sICAM-1 were enhanced in the TAO model group, compared with the sham operated group ($P < 0.05$) and were further increased on addition of exogenous urocortin ($P < 0.05$ vs. TAO model group). As observed earlier, the CRF-receptor antagonists NBI-27914 and astressin but not antisauvagine-30 abolished these effects of urocortin ($P < 0.05$).

Discussion

Urocortin is a peptide of the CRF family. In our model of TAO in rats, urocortin potentiated the effects of the peripheral vasculitis and ischaemia, which could be attributed to exacerbation of the hypercoagulable state of the blood. This exacerbation was mediated by CRF_1-receptors, probably the $CRF_{1\alpha}$-receptor subtype.

Urocortin was originally identified in the CNS in 1995 (Vaughan *et al.*, 1995) and more recently, it was found to be widely expressed in the periphery (Fekete and Zorrilla, 2007). Except for its protective roles in some diseases (Oki and Sasano, 2004), urocortin has been found to play a pro-inflammatory role in some inflammatory diseases, such as

Table 3A Effects of urocortin and $CRF_{1/2}$ receptor antagonists on rat blood cell counts

	Blood platelet count (10^8 mL^{-1})	*Red blood cell count (10^9 mL^{-1})*	*Haemoglobin ($g \cdot L^{-1}$)*	*Leucocyte count (10^6 mL^{-1})*	*Neutrophil (10^6 mL^{-1})*
Normal	7.12 ± 0.31	6.51 ± 0.31	131.25 ± 6.17	4.93 ± 0.21	1.08 ± 0.20
Sham operated	7.35 ± 0.51	6.21 ± 0.49	128.22 ± 4.73	5.01 ± 0.40	1.16 ± 0.11
TAO model	9.30 ± 0.49*	6.11 ± 0.63	129.98 ± 7.44	5.11 ± 0.61	1.19 ± 0.28
Ucn	11.71 ± 0.72*#	6.23 ± 0.14	130.05 ± 5.10	5.08 ± 0.58	1.09 ± 0.09
Ucn + NBI-27914	7.87 ± 0.62#†	6.83 ± 0.72	135.12 ± 9.39	4.95 ± 0.13	1.13 ± 0.19
Ucn + antisauvagine-30	11.07 ± 0.82	6.61 ± 0.42	132.26 ± 8.17	5.00 ± 0.46	1.14 ± 0.24
Ucn + astressin	7.61 ± 0.71#†	6.47 ± 0.55	128.78 ± 6.85	4.97 ± 0.71	1.15 ± 0.17

Values are presented as means ± SEM ($n = 8$). *$P < 0.05$, versus sham operated group; #$P < 0.05$, versus model group; †$P < 0.05$, versus urocortin group. $CRF_{1/2}$, corticotrophin-releasing factor type 1 and 2 receptors; TAO, thromboangiitis obliterans; TAO model, single injection of sodium laurate only; Ucn, TAO model + daily urocortin for 12 days; Ucn + NBI 27914 or + antisauvagine-3 or + astressin, Ucn + daily injections of each antagonist for 12 days.

Table 3B Effects of urocortin and $CRF_{1/2}$ receptor antagonists on rat blood coagulation

	Prothrombin time (s)	*Thrombin time (s)*	*Activated partial thromboplastin time(s)*	*Fibrinogen level ($g \cdot L^{-1}$)*	*D-dimer ($ng \cdot mL^{-1}$)*
Normal	37.93 ± 1.61	25.27 ± 2.12	38.91 ± 3.10	2.98 ± 0.11	0.60 ± 0.02
Sham operated	38.20 ± 0.98	24.00 ± 1.91	35.90 ± 2.74	3.12 ± 0.20	0.57 ± 0.06
TAO model	26.50 ± 1.23*	10.33 ± 1.69*	20.03 ± 2.18*	5.94 ± 0.47*	1.34 ± 0.10*
Ucn	20.20 ± 1,50*#	8.07 ± 1.78*#	15.11 ± 3.53*#	6.90 ± 0.35*#	1.66 ± 0.19*#
Ucn + NBI-27914	35.38 ± 0.72#†	22.65 ± 3.12#†	36.80 ± 4.61#†	3.53 ± 0.29#†	0.78 ± 0.09#†
Ucn + antisauvagine-30	21.01 ± 1.11	11.20 ± 2.19	18.31 ± 3.50	6.28 ± 0.61	1.58 ± 0.21
Ucn + astressin	36.83 ± 1.31#†	23.79 ± 1.70#†	38.77 ± 5.07#†	3.30 ± 0.42#†	0.59 ± 0.13#†

Values are presented as means ± SEM ($n = 8$). *$P < 0.05$, versus sham operated group; #$P < 0.05$, versus model group; †$P < 0.05$, versus urocortin group. $CRF_{1/2}$, corticotrophin-releasing factor type 1 and 2 receptors; TAO, thromboangiitis obliterans; TAO model, single injection of sodium laurate only; Ucn, TAO model + daily urocortin for 12 days; Ucn + NBI 27914 or + antisauvagine-3 or + astressin, Ucn + daily injections of each antagonist for 12 days

Table 3C Effects of urocortin and $CRF_{1/2}$ receptor antagonists on rat blood rheology

	Erythrocyte sedimentation rate ($mm \cdot h^{-1}$)	*Haematocrit (%)*	*Blood viscosity*		*Plasma viscosity (mPa s)*
			200 S^{-1} (mPa s)	*5 S^{-1} (mPa s)*	
Normal	1.08 ± 0.23	40.88 ± 1.34	5.68 ± 0.03	11.34 ± 1.39	1.39 ± 0.03
Sham operated	1.15 ± 0.09	40.03 ± 0.31	5.77 ± 0.28	12.01 ± 2.80	1.34 ± 0.05
TAO model	1.11 ± 0.05	39.60 ± 0.18	5.81 ± 0.41	11.73 ± 0.47	1.36 ± 0.02
Ucn	1.09 ± 0.17	39.21 ± 0.99	5.69 ± 0.18	11.58 ± 1.19	1.39 ± 0.04
Ucn + NBI-27914	1.13 ± 0.20	39.67 ± 2.12	5.71 ± 0.33	11.21 ± 1.30	1.34 ± 0.04
Ucn + antisauvagine-30	1.16 ± 0.09	40.30 ± 2.08	5.61 ± 0.01	11.94 ± 1.71	1.31 ± 0.01
Ucn + astressin	1.01 ± 0.17	39.63 ± 1.62	5.73 ± 0.17	11.84 ± 1.55	1.33 ± 0.02

$CRF_{1/2}$, corticotrophin-releasing factor type 1 and 2 receptors; TAO, thromboangiitis obliterans; TAO model, single injection of sodium laurate only; Ucn, TAO model + daily urocortin for 12 days; Ucn + NBI 27914 or + antisauvagine-3 or + astressin, Ucn + daily injections of each antagonist for 12 days Values are presented as means ± SEM ($n = 8$).

ulcerative colitis (Saruta *et al.*, 2004) and rheumatoid arthritis (Kohno *et al.*, 2001; Uzuki *et al.*, 2001). Our previous study demonstrated that CRF accelerated progression of atherosclerosis in LDLr–/– mice via CRF_1-receptors (Wu *et al.*, 2009). However, its function in peripheral vasculitis and a potential role in modulating TAO progression are not well analysed.

In the present study, rats with a sodium laurate-induced vasculitis were used to model TAO, which is also defined as a vasculitis. This model has previously been used to study TAO (Ashida *et al.*, 1980) and other groups have found that injection of sodium laurate into the rat femoral artery could mimic the signs and symptoms displayed in patients suffering from TAO (Nakata *et al.*, 1976; Nielubowicz *et al.*, 1980). Here, we observed that most of the model rats showed symptoms typical of ischaemia and vasculitis in the hind limb, and that the femoral artery was structurally changed with thrombi or proliferation of endothelial cells. Our results were consistent with previous reports (Nakata *et al.*, 1976; Nielubowicz *et al.*, 1980; Cui Pan Cui *et al.*, 2000). We also found that expression of mRNA and protein for urocortin, CRF_1- and $CRF_{1\alpha}$-receptors (but not for CRF_2-receptors) were elevated in the femoral arteries from our TAO model rats, as was plasma urocortin. The $CRF_{1\alpha}$-receptor, widely expressed throughout the body, is the main mediator of the actions of CRF and urocortin (Teli *et al.*, 2008). Moreover, the $CRF_{1\alpha}$-receptor is the most efficient receptor in terms of stimulation of cAMP production, while the CRF_{1c}- and $CRF_{1\beta}$-receptors have a decreased CRF binding capacity (Pisarchik and Slominski, 2004). Taken together,

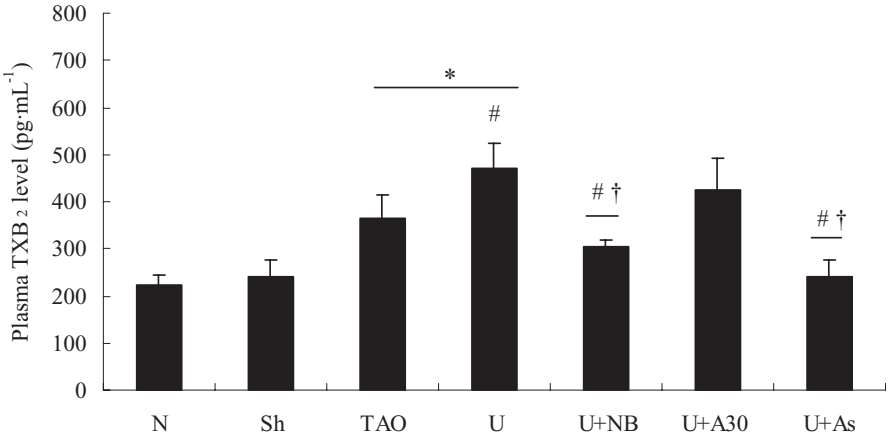

Figure 6 Plasma thromboxane B_2 (TXB_2) levels in experimental groups. TXB_2 levels were higher (than sham) in the thromboangiitis obliterans (TAO) model rats and further increased on addition of exogenous urocortin (Ucn; see *Methods*). These effects of urocortin were prevented by treatment with NBI-27914 or astressin, but not by antisauvagine-30. Values are presented as means ± SEM ($n = 8$). *$P < 0.05$, versus sham operated group; #$P < 0.05$, versus TAO model group; †$P < 0.05$, versus urocortin group. N, normal; Sh, sham; TAO, TAO model; U, urocortin; U + NB, U + NBI-27914; U + A30, urocortin + antisauvagine-30; U + As, urocortin + astressin.

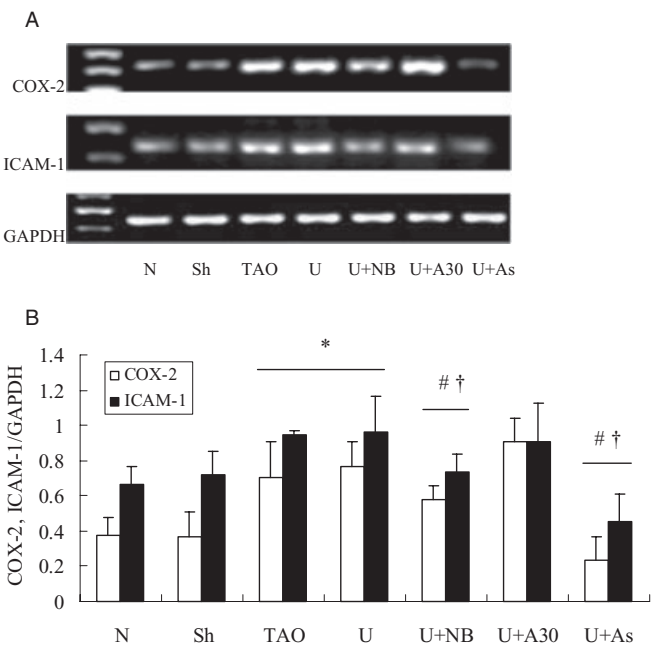

Figure 7 Expression of mRNA for cyclooxygenase (COX)-2 and intercellular adhesion molecule-1 (ICAM-1) in rat femoral arteries from experimental groups. In (A), a representative record of the expression of mRNA is shown, with GAPDH as an internal control. Summary data are shown in (B); data are presented as means ± SEM ($n = 8$), *$P < 0.05$, versus sham operated group; #$P < 0.05$, versus TAO model group; †$P < 0.05$, versus urocortin group. N, normal; Sh, sham; TAO, TAO model; U, urocortin; U + NB, U + NBI-27914; U + A30, urocortin + antisauvagine-30; U + As, urocortin + astressin.

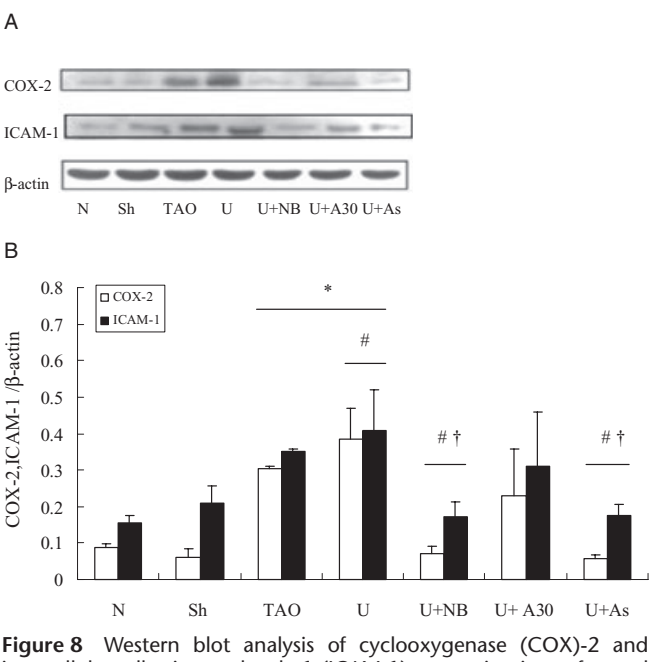

Figure 8 Western blot analysis of cyclooxygenase (COX)-2 and intercellular adhesion molecule-1 (ICAM-1) expression in rat femoral arteries from experimental groups. In (A), a representative blot is shown with β-actin as an internal control. Summary data are shown in (B); data are presented as means ± SEM ($n = 8$). *$P < 0.05$, versus sham operated group; #$P < 0.05$ versus TAO model group; †$P < 0.05$, versus urocortin group. N, normal; Sh, sham; TAO, TAO model; U, urocortin; U + NB, U + NBI-27914; U + A30, urocortin + antisauvagine-30; U + As, urocortin + astressin.

these observations suggest that urocortin does contribute to the signs and symptoms of TAO.

Previous reports have observed a hypercoagulable state of blood in TAO model rats (Shirakura *et al.*, 1994). In the present study, such a hypercoagulable state was observed in our rat model of vasculitis. Plasma TXA_2, a potent inducer of platelet aggregation and microvascular contraction (Silver *et al.*, 1973; 1974), was measured as its stable product, TXB_2, and was greatly elevated in the TAO model rats. This result could be mainly attributed to the augmentation of platelet aggregation, which is the main producer of TXA_2. Previous studies indicated that serum levels of a soluble form of ICAM-1 (sICAM-1) were correlated with possibility of disseminated intravascular coagulation or maximum disseminated intravascular coagulation scores (Gando *et al.*, 2002; Chen *et al.*, 2005). Our data showed that plasma sICAM-1 was

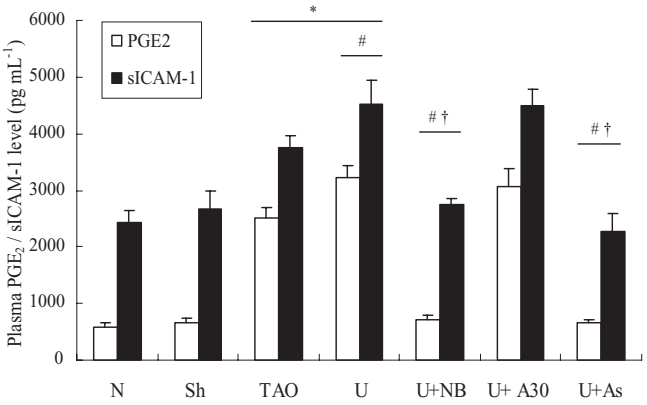

Figure 9 Plasma prostaglandin (PG)E_2 and soluble intercellular adhesion molecule (sICAM)-1 levels from experimental groups. Values are presented as means ± SEM ($n = 8$). *$P < 0.05$, versus sham operated group; #$P < 0.05$. versus TAO model group; †$P < 0.05$, versus urocortin group. N, normal; Sh, sham; TAO, TAO model; U, urocortin; U + NB, U + NBI-27914; U + A30, urocortin + antisauvagine-30; U + As, urocortin + astressin.

increased in the TAO model rats, consistent with clinical findings of increased sICAM-1 in TAO patients (Joras *et al.*, 2006).

To investigate the role of urocortin in TAO, the TAO model rats were treated with exogenous urocortin and $CRF_{1/2}$ receptor antagonists. Adding urocortin significantly enhanced the grossly visible signs of ischaemia and vasculitis and further raised plasma TXB_2 and sICAM-1 levels. Treatment of these rats with the CRF_1-receptor antagonist, NBI-27914 or the non-selective CRF-receptor antagonist, astressin, the gross signs of vasculitis were attenuated; plasma TXB_2 and sICAM-1 were returned to near normal and signs of the hypercoagulable state were decreased. When the rats were treated with the CRF_2-receptor antagonist, antisauvagine-30, no such changes were observed.

From the gross, macroscopic findings (Figure 5 and Table 2), the non-selective CRF-receptor antagonist, astressin, could completely reverse whereas the selective CRF_1-receptor antagonist, NBI-27914, could only partially reverse, the effects of urocortin and this might suggest a contribution from CRF_2-receptors. However, selective blockade of CRF_2-receptors with antisauvagine-30 had no significant effect. Overall, it is likely that urocortin promotes a hypercoagulable state, at least partially, through increasing plasma TXB_2 and sICAM-1 and that this promoting effect was modulated via CRF_1-receptors, rather than CRF_2-receptors.

COX-2, the inducible isoform of the rate-limiting enzyme in the biosynthesis of TXA_2 and PGs is well established as an important component of many inflammatory diseases (Dubois *et al.*, 1998; Vane *et al.* and., 1998). It plays a critical role in atherosclerosis and modulates atherosclerotic plaque stability through PG output (Cipollone and Fazia, 2006). In case–control studies, prostacyclin derivatives have shown to be more effective than placebo for the therapy of TAO patients (Nizankowski *et al.*, 1985; Fiessinger and Schäfer, 1990). Another key component of peripheral inflammatory disease, ICAM-1, is regarded as a marker for vasculitis (Witkowska, 2005), in conditions such as atherosclerosis (Witkowska, 2005) and TAO (Halacheva *et al.*, 2002). In patients with TAO, inflammatory cells infiltrate local lesion sites, and ICAM-1 expression in endothelial cells and inflammatory sites was increased greatly (Halacheva *et al.*, 2002; Joras *et al.*, 2006). Our present study found that urocortin increased COX-2 and ICAM-1 expression in femoral arteries via CRF_1-receptors, consistent with previous reports that in rat aortic smooth muscle cells, urocortin induced the expression of COX-2 in a time- and dose-dependent manner (Kageyama *et al.*, 2006). Also, CRF-related peptides (CRF and urocortin) up-regulated COX-2 expression and PG output in cultured human placental trophoblasts via CRF_1-receptors (Gao *et al.*, 2008). Furthermore, CRF was biologically active in cultured keratinocytes and enhanced expression of hCAM and ICAM-1, stimulated by interferon-γ (Quevedo *et al.*, 2001).

Our present study showed that both endogenous and exogenous urocortin were involved in our model of TAO in rats. On the one hand, endogenous urocortin was increased in these TAO model rats. On the other hand, exogenous urocortin application exacerbated the condition of the TAO rats. Furthermore, treatment with NBI-27914 or with astressin, CRF-receptor antagonists, reduced the urocortin-enhanced TXB_2, COX-2 and ICAM-1 expression to a level lower than those in the TAO model group, indicating antagonism of both endogenous and exogenous urocortin.

The pro-inflammatory role of CRF_1-receptors in peripheral inflammatory sites has been demonstrated (Kohno *et al.*, 2001; Saruta *et al.*, 2004). Yokotani *et al.* (2001) found that activation of brain CRF_1-receptors raised brain TXA_2, which is involved in the central adrenomedullary outflow. In the synovium of rheumatoid arthritis patients, urocortin and CRF-receptors were overexpressed and were significantly correlated with the degree of inflammation (Kohno *et al.*, 2001). Furthermore, peripheral administration of CRF-receptor antibody (Karalis *et al.*, 1991) or the specific CRF_1-receptor non-peptide antagonist, antalarmin (Webster *et al.*, 1996), prior to subcutaneous carrageenan injection, significantly suppressed inflammatory exudate volume and cell infiltration. Although there are reports indicating a protective role of urocortin in the cardiovascular system, this effect is mainly mediated via activating CRF_2-receptors (Oki and Sasano, 2004). Recent studies have demonstrated that the CRF_2-receptor is a tonic suppressor of vascularization (Bale *et al.*, 2002). Genetic deletion of CRF_2-receptors resulted in profound postnatal hypervascularization in mice, which was characterized by both an increase in total vessel number and a dramatic increase in vessel diameter. Furthermore, CRF_2-receptor activation can inhibit tumour growth via effects on vascularization and cell proliferation (Hao *et al.*, 2008; Wang *et al.*, 2008). However, in the present study, expression of mRNA or protein of CRF_2-receptors were the same in normal, sham operated and TAO model groups. In addition, the selective CRF_2-receptor antagonist, antisauvagine-30 had no obvious effect on the vasculitis or the hypercoagulable state in TAO rats.

Urocortin induced degranulation of rat lung mast cells by increasing intracellular calcium via CRF_1-receptors (Wu *et al.*, 2008). In the present study, levels of CRF_1-receptors were elevated in the TAO model group. This might represent both the infiltration of immune cells bearing CRF_1-receptors and an effect of inflammatory mediators on CRF_1-receptor

expression. On the one hand, the pro-inflammatory action of CRF peptides is partially the result of their effects on immune cells (such as mast cell, macrophages and lymphocytes) (Bamberger Wald Bamberger *et al*., 1998; Theoharides *et al*., 1998; Agelaki *et al*., 2002), which can synthesize CRF peptides and express their receptors, CRF_1 and CRF_2 (Theoharides *et al*., 1998; Baigent, 2001). On the other hand, inflammatory mediators could induce expression of CRF_1-receptors (Inada *et al*., 2009). Thus any increase of CRF_1-receptors could be due to increased immune cells or to increased inflammatory mediators.

In conclusion, we have shown, in a model of TAO induced by sodium laurate in rats, that urocortin could exacerbate the pathological condition by potentiating the hypercoagulable state and the vasculitis. Blockade of CRF_1-receptors attenuated the signs and symptoms of TAO by normalizing the hypercoagulable state. Up-regulation of COX-2 and ICAM-1 might also contribute to the exacerbation induced by urocortin.

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Conflict of interest

The authors have no conflict of interest.

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