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INVOLVEMENT OF CORTICOTROPIN-RELEASING FACTOR AND CORTICOTROPIN-RELEASING FACTOR 2 RECEPTORS IN PATHOGENESIS OF ISCHEMIA/REPERFUSION-INDUCED ENTERITIS IN RATS

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We herein investigated, using a corticotropin-releasing factor (CRF) agonist and antagonists, whether CRF plays a role in the pathogenesis of ischemia/reperfusion-induced small intestinal lesions in rats. Under pentobarbital anesthesia, the superior mesenteric artery was clamped (ischemia) for 75 min, followed by reperfusion with removal of the clamp. After a 24-h reperfusion, the area of hemorrhagic lesions that developed in the small intestine was measured. Urocortin I (CRF receptor 1/2 agonist), astressin (CRF receptor 1/2 antagonist), NBI27914 (CRF receptor 1 antagonist), or astressin 2B (CRF receptor 2 antagonist) was administered i.v. twice: 5 min before ischemia and 6 hours after reperfusion. Ischemia/reperfusion caused hemorrhagic lesions in the small intestine in ampicillin- and aminoguanidine-inhibitable manners, accompanied by enterobacterial invasion and the up-regulation of inducible nitric oxide synthase expression and myeloperoxidase activity. The severity of ischemia/reperfusion-induced lesions was significantly reduced by astressin and astressin 2B, but not by NBI27914, with the suppression of bacterial invasion, myeloperoxidase activity, and inducible nitric oxide synthase expression. In contrast, urocortin I markedly aggravated these lesions, and this response was completely abrogated by the co-administration of astressin 2B, but not NBI27914. The gene expression of CRF, CRF receptor 1, and CRF receptor 2 was observed in the small intestine, and remained unchanged following ischemia/reperfusion. These results suggest that ischemia/reperfusion caused hemorrhagic lesions in the small intestine, the pathogenesis of which involved enterobacteria and inducible nitric oxide synthase/nitric oxide. These lesions were aggravated by urocortin I in an astressin 2B-inhibitable manner, but suppressed by astressin in a CRF receptor 2-dependent manner. Endogenous CRF may be involved in the pathogenesis of ischemia/reperfusion-induced enteritis, possibly *via* the activation of peripheral CRF receptor 2.

Key words: *ischemia/reperfusion, enteritis, corticotropin-releasing factor, urocortin I, corticotropin-releasing factor receptor 1, corticotropin-releasing factor receptor 2, small intestine, enterobacteria*

INTRODUCTION

Corticotropin-releasing factor (CRF), a hypothalamic neuropeptide, has been identified as the principal regulator of the hypothalamus-pituitary-adrenal axis (1, 2), and the CRF family has been expanded by the addition of the following mammalian CRF-related peptides; urocortins I, II, and III (3). CRF and urocortins bind with various affinities to two G-protein-coupled receptors, CRF receptor 1 (CRFR1) and CRF receptor 2 (CRFR2), which are expressed in the brain and peripheral tissues (4-6). Using these CRF ligands, this hormone has been shown to play an important role in the regulation of diverse physiological events, including vascular tone, cardiac function, immune cell activation, feeding behavior, gastrointestinal motility, and mucosal defenses (7-9).

CRF and CRF-related peptides are also involved in inflammatory responses in the intestine; however, their roles remain controversial (10-13). Wlk *et al*. (14) demonstrated that the expression of CRF as well as CRFR1 and CRFR2 in the ileal mucosa was up-regulated after the intraluminal administration of toxin A from *Clostridium difficile* and suggested that peripheral CRF plays a proinflammatory role in toxin A-induced intestinal secretion and inflammation. Kokkotou *et al*. (11) reported that toxin A-induced intestinal inflammation was inhibited in mice lacking CRFR2 and suggested that CRFR2 mediates intestinal inflammatory responses *via* the release of proinflammatory mediators. In contrast, Chatzaki *et al*. (10) found a negative correlation between urocortin levels and mucosal inflammation in gastric biopsies from patients with *Helicobacter pylori* infection, suggesting that urocortin exerts anti-inflammatory effects in the gastric mucosa. We also showed that indomethacin-induced enteropathy in rats was prevented by urocortin I, a CRFR1/2 agonist, and aggravated by astressin, a CRFR1/2 antagonist, with both effects being mediated by the activation of CRFR2, suggesting the involvement of endogenous CRF in intestinal mucosal defenses against non-steroidal anti-inflammatory drugs (NSAIDs) (13). We also demonstrated that the protective effects of urocortin I were functionally associated

with the suppression of intestinal hypermotility caused by indomethacin (13, 15). Since CRF and CRFR2 are both expressed in the gastrointestinal tract as well as in the brain of human and rodents (13, 15-18), urocortin I may exert protective effects against small intestinal lesions through the activation of peripheral CRFR2.

On the other hand, Jonassen *et al*. (19) reported that a CRF peptide reduced apoptotic and necrotic cell death in cardiac myocytes subjected to lethal ischemic-induced stress through the activation of protein kinase A/C-dependent signaling pathways downstream of CRFR2. In contrast, Stevens *et al*. (20) investigated a potential role for CRF in the exacerbation of cerebral injury after ischemic stress and found that cerebral injury after focal ischemia was reduced in CRFR1-deficient mice. However, the influence of CRF on the inflammation and damage induced in the small intestine by ischemia/reperfusion (I/R) and the CRFR subtype involved in these responses remain unclear.

In the present study, we examined the effects of a CRF agonist and antagonists on the intestinal ulcerogenic response induced by I/R in the rat and investigated the roles CRF plays in the pathogenesis of these lesions, including the CRFR subtype involved in the action of this peptide.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200 – 260 g; Nippon Charles River, Shizuoka, Japan) were acclimated to standard laboratory conditions (12:12-h light-dark cycle, temperature of 22 ± 1°C). Experiments were performed using four to six non-fasted animals per group in a conscious state, unless otherwise specified.

All experimental procedures involving animals were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Induction of small intestinal lesions by ischemia/reperfusion

Under pentobarbital anesthesia, the superior mesenteric artery (SMA) was clamped for 75 min, followed by reperfusion with removal of the clamp (*Fig. 1A*). Animals were fed normally thereafter. Animals were killed 3, 6 and 24 hours after reperfusion, the small intestine was excised, tissue walls were fixed with 2% formalin for 10 min, and an opening was made along the mesenteric attachment. The area of macroscopically visible damage (mm^2) was measured under a dissecting microscope with square grids (×10), summed per tissue, and used as a lesion score. In order to highlight hemorrhagic lesions, 1% Evans blue solution was administered i.v. in a volume of 1 ml/animal 30 min before sacrifice. The individual measuring the lesions was blinded to the treatment given to the animals. Urocortin I (a CRFR1/2 agonist: 3 – 30 μg/kg), astressin (a CRFR 1/2 antagonist: 3 – 10 μg/kg), NBI27914 (a selective CRFR1 antagonist: 10 mg/kg), or astressin 2B (a selective CRFR2 antagonist: 60 μg/kg) was administered i.v. twice: 5 min before ischemia and 6 hours after reperfusion (*Fig. 1B*). In some cases, animals were treated with urocortin I (30 μg/kg) i.v. twice: 5 min before ischemia and 6 hours after reperfusion, and NBI27914 (10 mg/kg) or astressin 2B (60 μg/kg) was given 5 min before each administration of urocortin I. In addition, ampicillin (an antibiotic: 800 mg/kg) was administered p.o. twice: 24 h and 30 min before ischemia, while aminoguanidine (a relatively selective inducible nitric oxide synthase (iNOS) inhibitor: 20 mg/kg) was given s.c. twice: 30 min before ischemia and 6 h after reperfusion. The doses of these drugs were selected to exert their pharmacological effects according to previous studies (8, 13, 15, 21). Sham animals were subjected to laparotomy without I/R.

In some cases, the intestinal mucosa was examined under a light microscope following I/R. Animals were euthanized 24 hours after reperfusion, and the small intestine was excised. Tissue samples were then immersed in 10% neutralized formalin, embedded in paraffin, sectioned at a thickenss of 5 μm, and stained with hematoxylin and eosin (H & E).

Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) activity was measured according to a modified method of Krawisz *et al*. (22). Animals were euthanized under deep ether anesthesia 24 h after reperfusion, and the small intestine was removed. After rinsing with cold saline, the whole intestine was weighed and homogenized in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HTAB, pH 6.0; Sigma). Homogenized samples were subjected to freeze-thawing three times and centrifuged at 300 × g at 4°C for 10 min. MPO activity was measured by adding 5 μl of the supernatant to 95 μl of 10 mM phosphate buffer (pH 6.0) and 50 μl of 1.5 M *o*-dianisidine HCl (Sigma) containing 0.0005% w/v hydrogen peroxide. Changes in absorbance at 450 nm in each sample were recorded on a microplate reader (VERSAmax, Molecular Device, Sunnyvale, CA). Sample protein content was estimated by a spectrophotometric assay (protein assay kit; Pierce, Rockford, IL), and MPO activity was obtained from the slope of the reaction curve, according to the following equation: specific activity (μmol H_2O_2/min/mg protein) = (OD/min)/OD/μmol H_2O_2 × mg protein). Urocortin I (30 μg/kg), NBI27914 (10 mg/kg), or astressin 2B (60 μg/kg) was given i.v. twice: 5 min before ischemia and 6 hours after reperfusion. In some cases, NBI27914 or astressin 2B was given i.v. twice 5 min before each administration of urocortin I.

Measurement of enterobacterial counts

Enterobacteria were enumerated according to a modified version (20) of the method originally described by Deitch *et al*. (23). Animals were killed under deep ether anesthesia 6 hours after reperfusion following ischemia for 75 min, and the small intestine was removed. After each intestine was rinsed with sterile saline, the mucosa was scraped with glass slides, weighed, and homogenized in 1 ml of sterile phosphate-buffered saline (PBS) per 100 mg of wet tissue. Aliquots of the homogenate were placed on blood agar and Gifuco anaerobic medium agar (Nissui, Tokyo, Japan). Blood agar plates were incubated at 37°C for 24 h under aerobic conditions (BBL Gas Pack Pouch Anaerobic System; BD Biosciences, San Jose, CA). The number of enterobacteria was counted on plates containing 10 to 300 colony-forming units (CFU), and data were expressed as log CFU per gram of tissue. Ampicillin (800 mg/kg) was administered p.o. twice: 24 h and 30 min before ischemia, while NBI27914 (10 mg/kg) or astressin 2B (60 μg/kg) was administered i.v. 5 min before ischemia.

Measurement of inducible nitric oxide synthase, corticotropin-releasing factor, and corticotropin-releasing factor receptor 1/2 mRNA expression by RT-PCR

The expression of mRNAs for inducible nitric oxide synthase (iNOS), CRF, CRFR1, and CRFR2 in the rat small intestine was examined by RT-PCR. Under deep diethyl ether anesthesia, animals were killed 6 hours after reperfusion following ischemia for 75 min, and the small intestine was removed, frozen in acetone/dry ice, and stored at –80°C prior to use. The brain and small intestine in normal rats were also

examined for the expression of CRF, CRFR1, and CRFR2 for comparison. Total RNA was extracted from tissue samples using Sepasol RNA I (Nacalai Tesque, Kyoto, Japan), and reverse transcribed with a first-strand cDNA synthesis kit (ReverTra Ace alpha, TOYOBO, Osaka, Japan). The sequences of the sense and antisense primers for rat iNOS, CRF, CRFR1, CRFR2, and GAPDH, and each product size are shown in *Table 1*. An aliquot of the RT reaction product served as a template in 32 cycles with 0.5 min of denaturation at 95°C and 1 min of extension at 68°C using the Advantage 2 polymerase mixture (CLONTECH) on a thermal cycler (TAKARA TP-240). A portion of the PCR mixture was electrophoresed on a 1.8% agarose gel in TAE buffer (40 mM Tris, 2 mM EDTA, and 20 mM acetic acid; pH: 8.1), and the gel was then stained with ethidium bromide and photographed. In some cases, ampicillin (800 mg/kg) was administered p.o. twice: 24 h and 30 min before ischemia, while NBI27914 (10 mg/kg) or astressin 2B (60 µg/kg) was administered i.v. 5 min before ischemia.

Analysis of the inducible nitric oxide protein by Western blotting

The protein expression of iNOS was examined in the small intestine 10 h after reperfusion following ischemia for 75 min. Under deep diethyl ether anesthesia, animals were killed 10 h after reperfusion following ischemia for 75 min, and the small intestine was removed, rinsed with cold saline, frozen in acetone/dry ice, and stored at –80°C prior to use. Tissues were homogenized with a Teflon glass homogenizer (Iwaki Glass Co., Chiba, Japan) in lysis buffer containing 50 mM Tris, pH 7.4, 150 mM sodium chloride, 1% Triton X-100, and a protease inhibitor tablet (Roche, Switzerland). Homogenates were then centrifuged at 20,000 × *g* at 4°C for 30 min. The sample protein content of the supernatant was measured by a spectrophotometric assay (BCA Protein Assay Kit, Pierce, IL, USA). A protein extract equivalent to 30 mg of protein was electrophoresed on a 10% polyacrylamide gel, and transferred to a nitrocellulose transfer membranes (Protran, Schleicher & Schuell, NH, USA). The membrane was incubated in 5% BSA at room temperature for 2 hours. After blocking non-specific binding sites, the membrane was incubated at 4°C overnight in Can-Get Signal Immunoreaction enhancer solution 1 (TOYOBO, Japan) or T-PBS containing the following antibody (diluted 1:1000; Santa Cruz Biotechnology, CA, USA): a goat polyclonal antibody against iNOS (sc-651, Santa Cruz Biotechnology). After washing with Tween-PBS (T-PBS), the membrane was incubated in Can-Get Signal Immunoreaction enhancer solution 2 (TOYOBO) containing donkey-anti-goat IgG-HRP (SC-2030, Santa Cruz Biotechnology) at room temperature for 2 hours. After washing with T-PBS, the expression of iNOS was detected by the enhanced chemiluminescence (ECL) method with a VersaDoc5000 (BIO-RAD). In some cases, ampicillin (800 mg/kg) was administered p.o. twice: 24 h and 30 min before ischemia, while NBI27914 (10 mg/kg) or astressin 2B (60 µg/kg) was administered i.v. 5 min before ischemia.

Preparation of drugs

The drugs used were pentobarbital (Nacalai Tesque, Kyoto, Japan), Evans blue, ampicillin, aminoguanidine, urocortin I, astressin, NBI27914, and astressin 2B (Sigma Chemicals, St. Louis, Mo.). Urocortin I was dissolved in 1% acetic acid while astressin, NBI27914, and astressin 2B were dissolved in a trace amount of dimethyl sulfoxide (DMSO), and were further diluted with saline to the desired concentrations (the final concentration of DMSO was less than 1%). All drugs were prepared immediately before use and administered p.o. or s.c. in a volume of 0.5 ml/100 g body weight or i.v. in a volume of 0.1 ml/100 g body weight. Control or sham animals received saline as the vehicle.

Statistical analysis

Data are presented as the means ± S.E. of four to six rats per group. Statistical analyses were performed using a two-tailed unpaired *t*-test and Dunnett's multiple comparison test, and values of $P < 0.05$ were considered to be significant.

RESULTS

Development of small intestinal lesions after ischemia/reperfusion

Ischemia induced by clamping of the SMA for 75 min did not produce any discernible damage in the small intestine, but caused gross lesions on the anti-mesenteric side of the small intestine after reperfusion; the severity of damage increased with time, with the

Table 1. Sequences of sense and antisense primers for rat iNOS, CRF, CRFR1, CRFR2, and GAPDH and their product sizes.

Gene	Sequences	PCR product
iNOS		602 bp
Sense	5'-ACAACAGGAACCTACCAGCTCA-3'	
Antisense	5'-GATGTTGTAGCGCTGTGTGTCA-3'	
CRF		392 bp
Sense	5'-TGATCCGCATGGGTGAAGAATACTTCCTC-3'	
Antisense	5'-CCCGATAATCTCCATCAGTTTCCTGTTGCTG-3'	
CRFR1		248 bp
Sense	5'-CGGTTCACAGTCTTGGTGAAAG-3'	
Antisense	5'-TCCTGCCACCGGCGCCACCTCT-3'	
CRFR2		269 bp
Sense	5'-GGGCATCACCTACATGCTCT-3'	
Antisense	5'-GTCTGCTTGATGCTGTGGAA-3'	
GAPDH		310 bp
Sense	5'-GAACGGGAAGCTCACTGGCATGGC-3'	
Antisense	5'-TGAGGTCCACCACCCTGTTGCTG-3'	

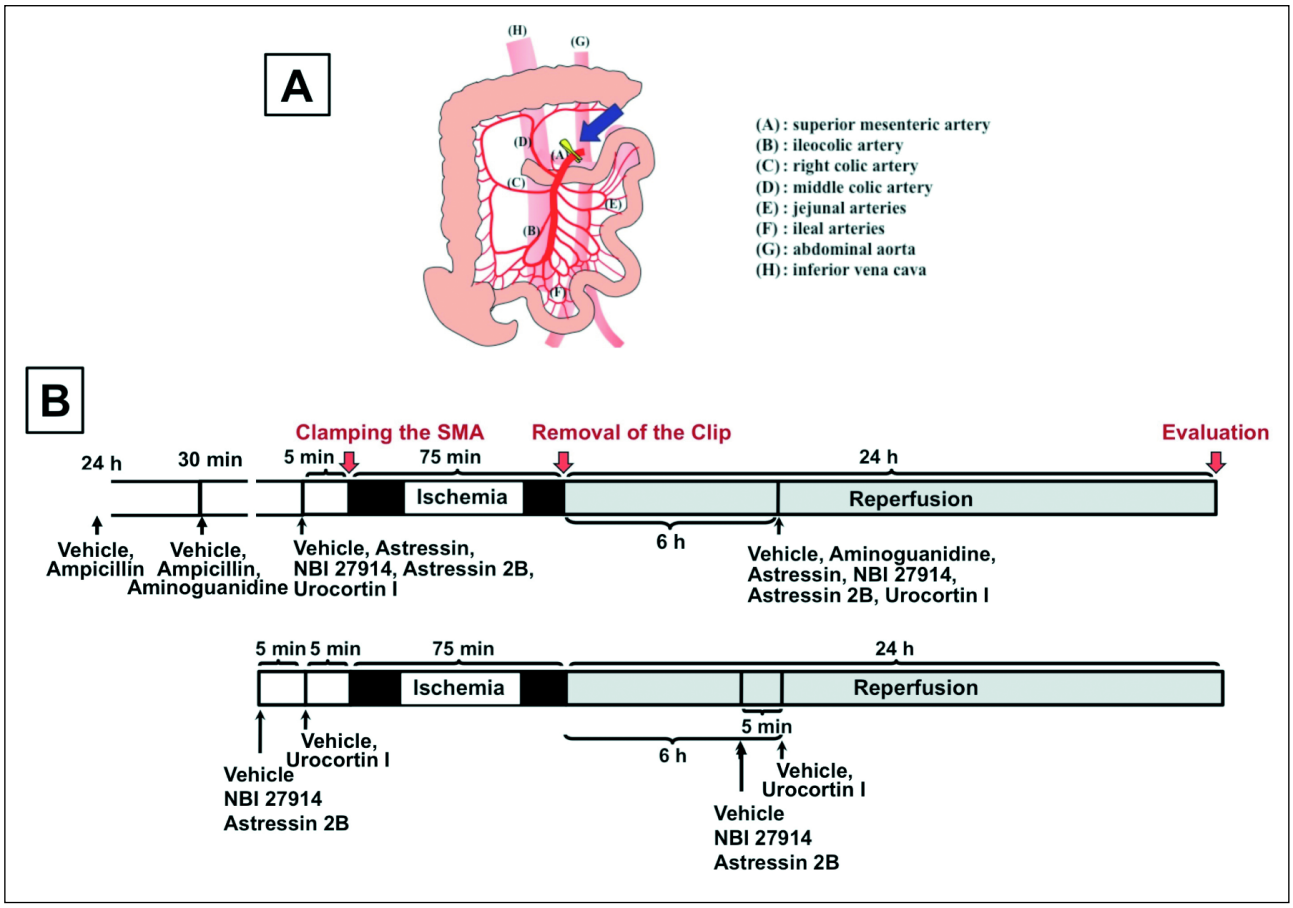

Fig. 1. The operation for ischemia/reperfusion (I/R) in the superior mesenteric artery (SMA)(A) and the time schedule for drug treatments (B). Under pentobarbital anesthesia, the SMA as indicated by the blue arrow, was clamped for 75 min, followed by reperfusion with removal of the clamp. After the operation, animals were fed normally and killed 24 h after reperfusion in order to examine the small intestinal mucosa. Ampicillin was administered p.o. twice: 24 h and 30 min before ischemia, while aminoguanidine was given s.c. twice: 30 min before ischemia and 6 h after reperfusion. Other drugs were given i.v. twice: 5 min before ischemia and 6 h after reperfusion. In some cases, astressin 2B or NBI27914 was given i.v. 5 min before each administration of urocortin I. Control or sham animals received saline as the vehicle.

lesion score 24 h after reperfusion being 140.6 ± 16.3 mm^2 (*Figs. 2* and *3A*). The development of these lesions was significantly prevented by the prior administration of ampicillin (800 mg/kg, p.o.), the antibiotic, and aminoguanidine (20 mg/kg, s.c.), the selective iNOS inhibitor, with the inhibition of lesions being 80.7% and 64.7%, respectively. Histologically, surface epithelial cells were completely sloughed off, and damage was deeper in the mucosa with severe inflammation being evident (*Figs. 3B* and *3C*).

Ischemia alone did not produce macroscopically visible damage; however, gross damage was induced after reperfusion, expanded with time, and became hemorrhagic from 6 h later (*Fig. 3*). Therefore, in subsequent experiments, we examined the effects of the CRFR agonist and antagonists on I/R-induced enteritis 24 hours after reperfusion.

Effects of the CRFR agonist and antagonists on small intestinal lesions induced by ischemia/reperfusion

Ischemia induced by clamping the SMA for 75 min followed by reperfusion caused hemorrhagic lesions in the small intestine, with the lesion score observed 24 h after reperfusion being 136.3 ± 12.1 mm^2. The severity of these lesions was dose-dependently reduced by the pretreatment with astressin (3 – 30 µg/kg, i.v.), a non-selective CRFR antagonist, and a significant effect was observed at 30 µg/kg, with the inhibition of lesions being 71.1% (*Fig. 4*). The protective effects of astressin against I/R-induced intestinal lesions was mimicked by the administration of astressin 2B (60 µg/kg, i.v.), the selective CRFR2 antagonist, but not by NBI27914 (1000 µg/kg, i.v.), the selective CRFR1 antagonist; the inhibition of lesions by astressin 2B was 72.4%, which was equivalent to that obtained by astressin at 30 µg/kg. In contrast, the severity of I/R-induced intestinal lesions was dose-dependently aggravated by the pretreatment with urocortin I (3 – 30 µg/kg, i.v.), the non-selective CRFR agonist, and the effects observed were significant at 10 and 30 µg/kg, with the lesion scores being 160.1 ± 26.1 mm^2 and 228.4 ± 18.1 mm^2, respectively (*Fig. 5*). The aggravating effects of urocortin I (30 µg/kg) were significantly abrogated by the co-treatment with astressin 2B (60 µg/kg, i.v.), but not by NBI27914 (10 mg/kg, i.v.).

Effects of the CRFR agonist and antagonists on changes in myeloperoxidase activity in the small intestinal mucosa induced by ischemia reperfusion

MPO activity in the intestinal mucosa of sham-operated rats was 0.038 ± 0.014 µmol H_2O_2/min/mg protein and significantly increased after I/R, reaching 0.124 ± 0.008 µmol H_2O_2/min/mg protein 24 hours after reperfusion (*Fig. 6*). The increase observed

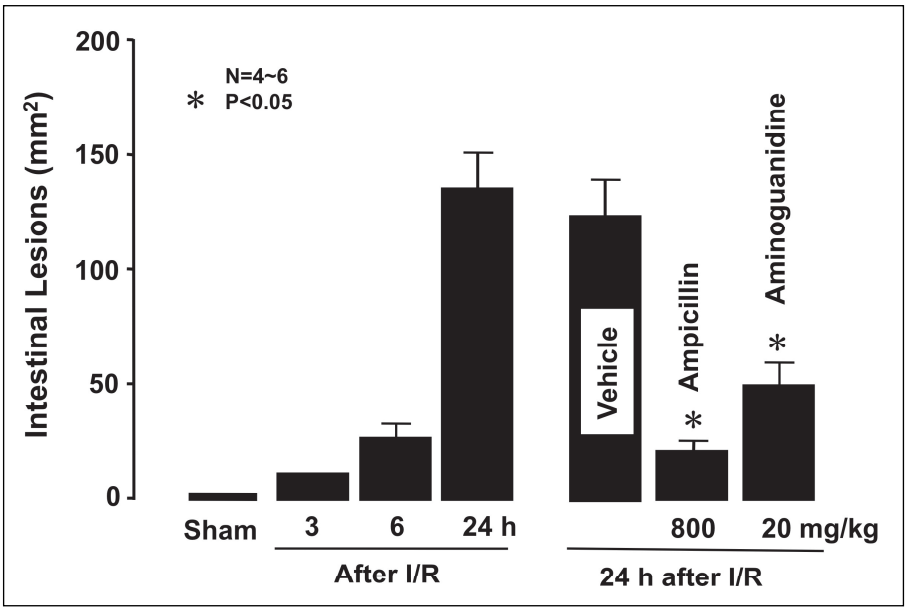

Fig. 2. Time-course development of small intestinal lesions following I/R with or without a pretreatment with ampicillin or aminoguanidine. Under pentobarbital anesthesia, the SMA was clamped for 75 min, followed by reperfusion. Animals were then fed normally and killed 3, 6, and 24 h after reperfusion. Ampicillin (800 mg/kg) was administered p.o. twice: 24 h and 30 min before ischemia, while aminoguanidine (20 mg/kg) was given s.c. twice: 30 min before ischemia and 6 h after reperfusion. Data are presented as the means ± S.E. of 4 – 6 rats. *Significant difference from the vehicle at $P < 0.05$.

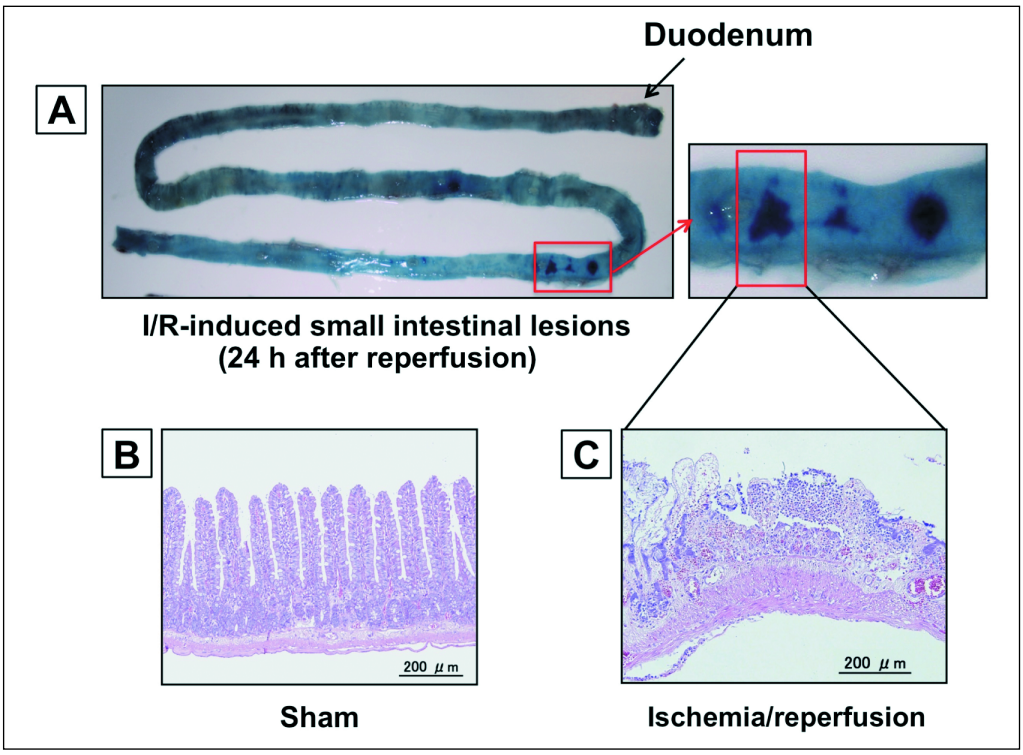

Fig. 3. Gross appearance of I/R-induced small intestinal lesions in a rat 24h after reperfusion (A); the left panel shows a higher magnification of the lesions indicated by a red arrow. Histological observations of the small intestinal mucosa;
(B): a sham-operated rat;
(C): a rat subjected to I/R.
The damage in *Fig. 3C* corresponded to that surrounded by the red line in *Fig. 3A*.

in MPO activity following I/R was significantly suppressed by the prior treatment with astressin 2B (60 μg/kg, i.v.), the CRFR2 antagonist, but not by the CRFR1 antagonist NBI27914 (10 mg/kg, i.v.), with inhibition by the former being 41.9%. On the other hand, the increase in MPO activity following I/R was further enhanced by the prior treatment with urocortin I (30 μg/kg, i.v.), the non-selective CRFR agonist, with activity reaching 0.162 ± 0.014 μmol H_2O_2/min/mg protein, which was 130.6% of the values obtained in control rats. The enhancing effects of urocortin I on increases in MPO activity following I/R were completely attenuated by the co-administration of astressin 2B, but not NBI27914. Ampicillin (800 mg/kg, p.o.) and aminoguanidine (20 mg/kg, s.c.) both significantly suppressed increases in MPO activity following I/R (data not shown).

Effects of CRFR antagonists on enterobacterial invasion in the small intestinal mucosa following ischemia reperfusion

Aerobic and anaerobic bacterial counts in the intestinal mucosa of sham-operated rats were 6.80 ± 0.14 log CFU/g tissue and 6.81 ± 0.12 log CFU/g tissue, respectively. Following I/R, bacterial counts under aerobic and anaerobic conditions were markedly increased 12 h after reperfusion, with values reaching 7.82 ± 0.14 log CFU/g tissue and 8.30 ± 0.12 log CFU/g tissue, respectively (*Fig. 7*). The pretreatment of animals with ampicillin (800 mg/kg, p.o.) completely prevented bacterial invasion in the mucosa following I/R. The prior administration of astressin 2B (60 μg/kg, i.v.) also significantly suppressed enhancements in bacterial invasion in the intestinal mucosa

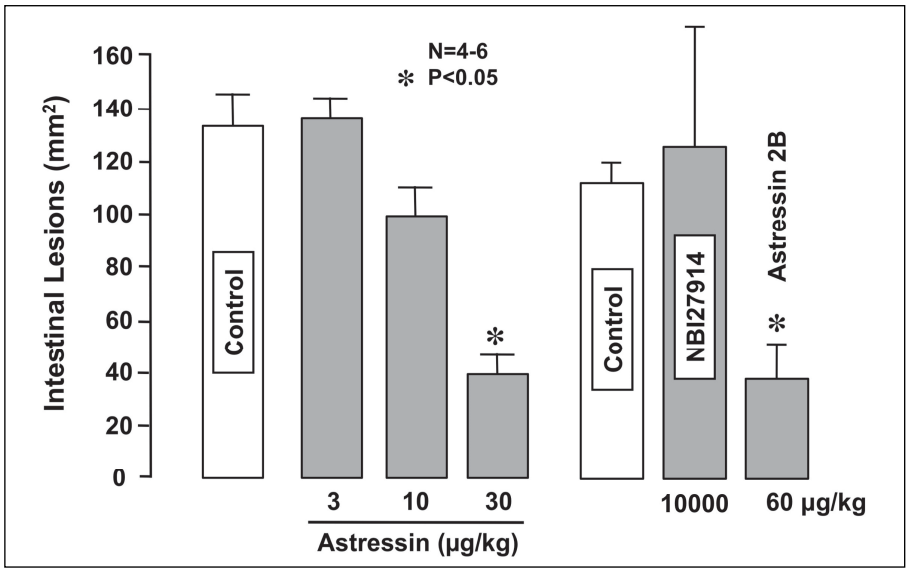

Fig. 4. Effects of astressin (a non-selective CRF antagonist), NBI27914 (a selective CRFR1 antagonist), or astressin 2B (a selective CRFR2 antagonist) on I/R-induced small intestinal lesions in rats. Under pentobarbital anesthesia, the SMA was clamped for 75 min, followed by reperfusion. Animals were then fed normally and killed 24 h after reperfusion. Astressin (3 – 30 µg/kg), NBI27914 (10 mg/kg), or astressin 2B (60 µg/kg) was given i.v. twice: 5 min before ischemia and 6 h after reperfusion. Data are presented as the means ± S.E. of 4 – 6 rats. *Significant difference from the control at $P < 0.05$.

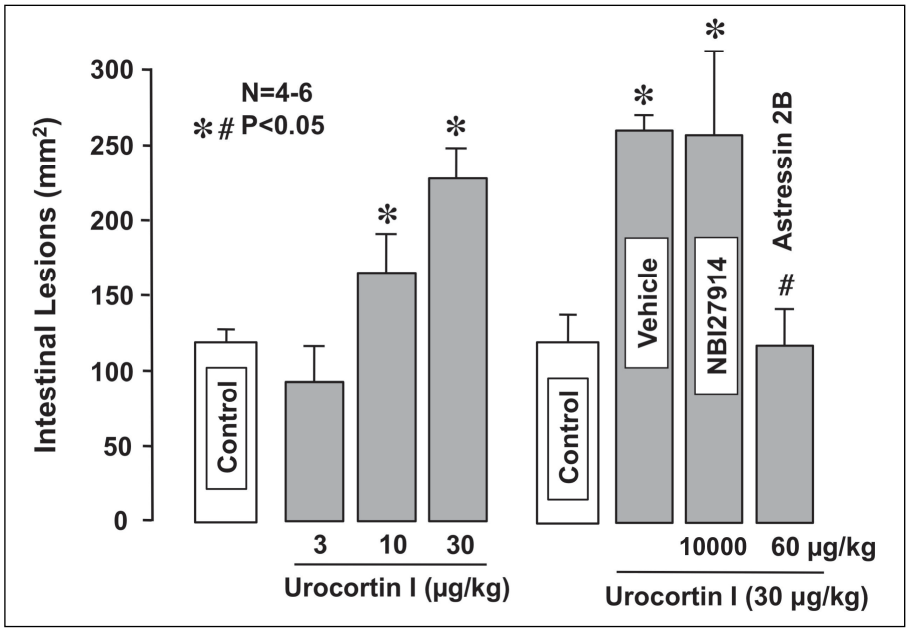

Fig. 5. Effects of urocortin I on I/R-induced small intestinal lesions in rats with or without a pretreatment with NBI27914 or astressin 2B. Under pentobarbital anesthesia, the SMA was clamped for 75 min, followed by reperfusion. Animals were then fed normally and killed 24 h after reperfusion. Urocortin I (3 – 30 µg/kg) was given i.v. twice; 5 min before ischemia and 6 h after reperfusion, while NBI27914 (10 mg/kg) or astressin 2B (60 µg/kg) was given i.v. 5 min before each administration of urocortin I (30 µg/kg). Data are presented as the means ± S.E. of 4 – 6 rats. Significant difference at $P < 0.05$; *from the control, #from the vehicle.

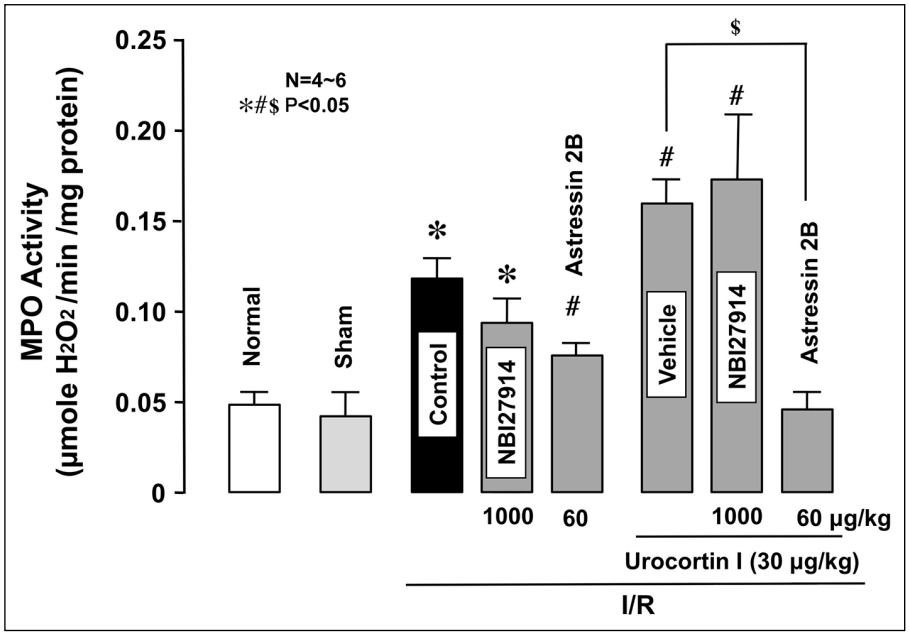

Fig. 6. Effects of urocortin I, NBI27914, and astressin 2B on changes in MPO activity in the small intestinal mucosa in response to I/R in rats. Under pentobarbital anesthesia, the SMA was clamped for 75 min, followed by reperfusion. Animals were then fed normally and killed 24 h after reperfusion. Urocortin I (30 µg/kg), NBI27914 (10 mg/kg), or astressin 2B (60 µg/kg) was given i.v. twice: 5 min before ischemia and 6 h after reperfusion. In some cases, animals were pretreated i.v. with NBI27914 or astressin 2B 5 min before each administration of urocortin I. Data are presented as the means ± S.E. of 4 – 6 rats. Significant difference at $P < 0.05$; *from the sham, #from the control, and $from the vehicle.

following I/R, while that of NBI27194 (10 mg/kg, i.v.) did not; the number of bacteria in the astressin 2B-treated group was similar to that in sham-operated animals without I/R.

Effects of CRFR antagonists on the up-regulation of inducible nitric oxide synthase expression in the small intestinal mucosa by ischemia/reperfusion

The expression of iNOS mRNA was negligible in the small intestinal mucosa of normal rats. The sham operation without I/R did not induce the expression of iNOS in the small intestine. However, the expression of iNOS was markedly up-regulated in the small intestine 6 h after reperfusion following ischemia for 75 min (*Fig. 8A*). This up-regulated expression of iNOS in the small intestine was potently suppressed by the prior administration of ampicillin (800 mg/kg, p.o.). The pretreatment with astressin 2B (60 μg/kg, i.v.), the CRFR2 antagonist, also suppressed the up-regulated expression of iNOS, while that of NBI27914 (10 mg/kg, i.v.), the CRFR1 antagonist, did not.

The up-regulated expression of iNOS in the small intestine following I/R was also confirmed at the protein level by Western blotting; however, this was not observed in the small intestine of normal or sham-operated animals (*Fig. 8B*). The up-regulated expression of the iNOS protein following I/R was also prevented by the pretreatment with ampicillin as well as astressin 2B, but not by NBI27914.

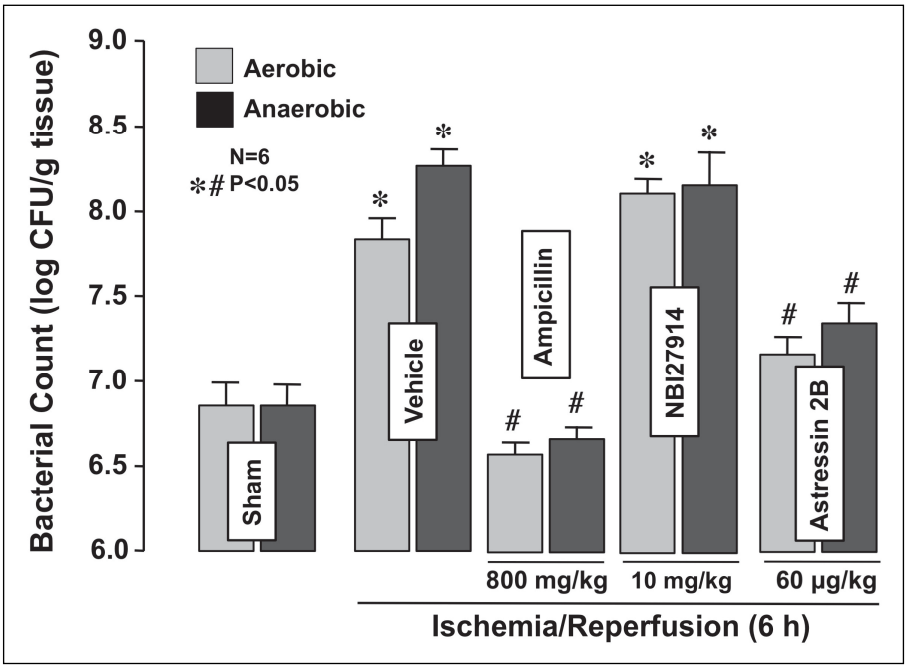

Fig. 7. Effects of ampicillin and CRFR antagonists on enterobacterial invasion in the intestinal mucosa induced by I/R in rats. Under pentobarbital anesthesia, the SMA was clamped for 75 min, followed by reperfusion. Animals were then fed normally and killed 6 h after reperfusion in order to measure the enterobacterial count. Ampicillin (800 mg/kg) was administered p.o. twice: 24 h and 30 min before ischemia, while NBI27914 (10 mg/kg) or astressin 2B (60 μg/kg) was administered i.v. 5 min before ischemia. Data are presented as the means ± S.E. of 6 rats. Significant difference at $P < 0.05$; *from the sham, #from the vehicle.

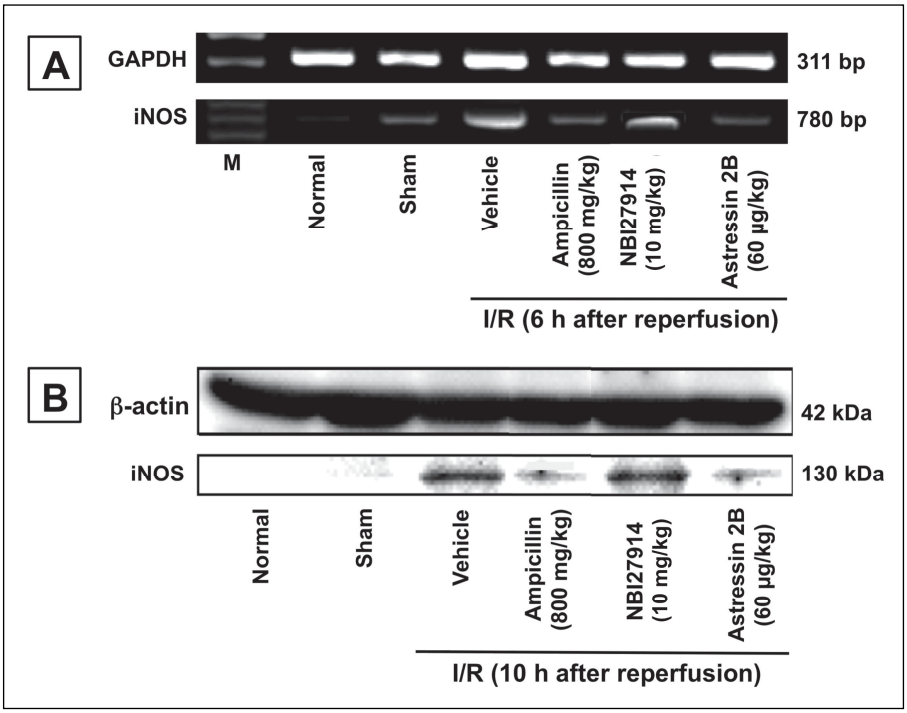

Fig. 8. Effects of ampicillin and CRFR antagonists on iNOS mRNA (A) and protein expression (B) induced in the rat small intestine by I/R. Under pentobarbital anesthesia, the SMA was clamped for 75 min, followed by reperfusion. Animals were then fed normally and killed 6 h or 10 h after reperfusion, respectively, to examine the expression of iNOS mRNA by RT-PCR or the iNOS protein by Western blotting. Ampicillin (800 mg/kg) was administered p.o. twice: 24 h and 30 min before ischemia, while NBI27914 (10 mg/kg) or astressin 2B (60 μg/kg) was administered i.v. 5 min before ischemia. Note that iNOS mRNA and protein expression was up-regulated in the small intestinal mucosa after I/R, and this up-regulated expression was clearly suppressed by the pretreatment with astressin 2B, but not NBI27914. M in *Fig. 8A*: marker.

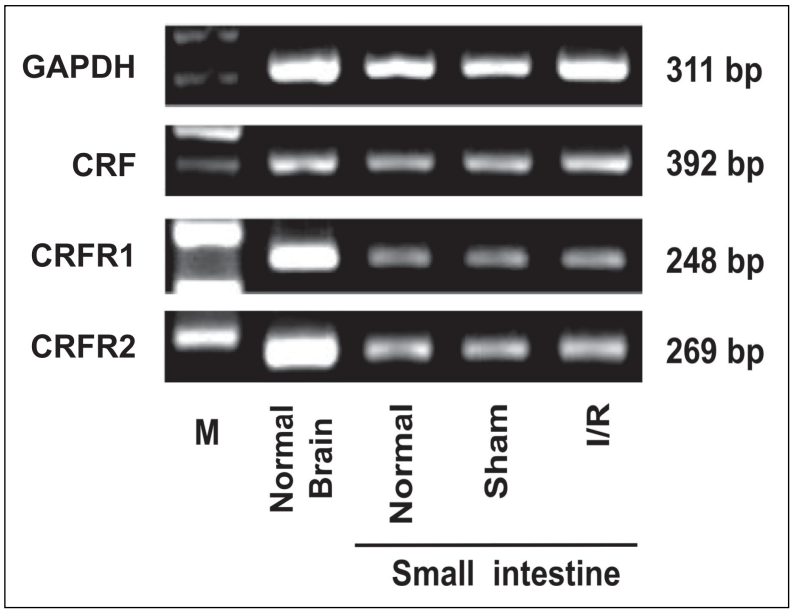

Fig. 9. Gene expression of CRF, CRFR1, and CRFR2 in the brain and small intestine of normal rats, and the small intestine of rats subjected to the sham operation or I/R. Animals were killed 10 h after reperfusion in order to examine the mRNA expression of CRF, CRFR1, CRFR2, and GAPDH. M: marker.

Gene expression of CRFR, CRFR1, and CRFR2 in the small intestine and brain

Since endogenous CRF was found to be involved in the pathogenesis of I/R-induced small intestinal lesions, we examined the expression of CRF and its receptors (CRFR1/2) in the rat small intestine with or without I/R. As shown in *Fig. 9*, CRF and CRFR1/2 mRNAs were all expressed in the normal rat small intestine, and their expression was not affected by either the sham operation or I/R. Furthermore, the gene expression of CRF and its receptors was also observed in the normal rat brain.

DISCUSSION

CRF and CRF-related peptides play important roles in the regulation of diverse physiological events, including gastrointestinal inflammatory responses and mucosal defenses; however, these findings remain controversial (10-14). We recently reported that urocortin I, a non-selective CRFR agonist, prevented indomethacin-induced enteropathy by activating CRFR2 and without the involvement of endogenous glucocorticoids released from the adrenal glands, suggesting the involvement of endogenous CRF in intestinal mucosal defenses against NSAIDs (13, 15). However, it currently remains unknown whether CRF influences the intestinal ulcerogenic response induced by I/R. In the present study, we examined the roles CRF plays in the pathogenesis of I/R-induced intestinal lesions, including the CRFR subtype involved in the actions of this peptide, and found that endogenous CRF is involved in the pathogenesis of I/R-induced enteritis *via* the activation of CRFR2 receptors.

We recently reported a new animal model of ischemic enteritis caused by partial ligation of the SMA; ischemia alone caused superficial lesions, whereas damage increased with time and became hemorrhagic 24 h later (24). We also found that ischemia induced by clamping of the SMA for 75 min caused minimal damage, whereas the severity of damage increased with time after reperfusion and became hemorrhagic from 6 h later. Thus, we examined the effects of a CRFR agonist and antagonists on I/R-induced intestinal lesions 24 h after reperfusion. We previously demonstrated that ischemic enteritis was accompanied by enterobacterial invasion as well as iNOS expression/NO production, and the development of lesions was prevented by a treatment with ampicillin, an antibiotic, and aminiguanidine, an iNOS inhibitor (24). Consistent with these findings, we also observed the involvement of enterobacterial invasion and up-regulated expression of iNOS in the pathogenesis of the present model of I/R. Furthermore, the severity of these lesions was significantly reduced by the prior administration of ampicillin or aminoguanidine.

We initially showed that the severity of I/R-induced enteritis was dose-dependently reduced by the prior administration of astressin, the non-selective CRFR antagonist, and this effect was mimicked by astressin 2B, the selective CRFR2 antagonist, but not by NBI27914, the selective CRFR1 antagonist. These results suggest that endogenous CRF exerts deleterious effects on the intestinal ulcerogenic response induced by I/R *via* the activation of CRFR2 receptors. This was further supported by the severity of these lesions being dose-dependently aggravated by the administration of urocortin I, the non-selective CRF receptor agonist, and significantly abrogated by the co-administration of astressin 2B, but not NBI27914. These results are consistent with previous findings showing that CRF and CRF-related peptides are involved in inflammatory responses in the gastrointestinal tract (10, 11, 12, 17, 25). Larauche *et al.* (17) demonstrated the interrelationship between CRF and CRFR1/2 and stress, advocating the role of CRF signalings in stress-related impact in relation to inflammatory bowel diseases and irritable bowel syndrome. Paschos *et al.* (25) reported that the CRF system is involved in the inflammatory process within the gastrointestinal tract, and the blocking of CRF receptors may theoretically exert beneficial anti-inflammatory effects in colonic tissues. Kokkotou *et al.* (11) showed that *C. difficile* toxin A-induced intestinal inflammation was inhibited in CRFR2-deficient mice. In contrast, La Fleur *et al.* (12) demonstrated that a peripheral injection of CRF and urocortin II reduced intestinal inflammation and motility in the mouse terminal ileum. We also previously reported that urocortin I exerted protective effects against indomethacin-induced enteropathy, and these effects were abrogated by astressin 2B, but not NBI27914 (13, 15). In addition, these lesions were significantly aggravated by astressin 2B alone, suggesting the roles of endogenous CRF and

CRFR2 in intestinal mucosal defenses against NSAIDs. These findings are opposite to the results of the present study showing the involvement of endogenous CRF and CRFR2 in the pathogenesis of I/R-induced enteritis. The protective effects of urocortin I against indomethacin-induced enteropathy were previously reported to be functionally associated with the inhibition of the intestinal hypermotility response to indomethacin *via* the activation of CRFR2 (13). Intestinal hypermotility is known to be important in the pathogenesis of NSAID-induced enteropathy, and this event is critical for the occurrence of enterobacterial invasion and subsequent inflammatory events, such as iNOS expression/NO production (26, 27). CRF may worsen tissue injury *via* the activation of CRFR2, if motility does not play any role in the pathogenic mechanism. A previous study reported that the proinflammatory role of CRF was largely mediated by the induction of chemokines, because blocking this receptor reduced the expression of several inflammatory chemokines in toxin A-exposed mice, including keratinocyte chemokines and monocyte chemoattractant protein 1 (11). Moreover, in murine TNBS-induced colitis, the knockout of CRF also reduced inflammation with a decline in the up-regulation of local IL-1β (11, 28). We herein found that urocortin I aggravated intestinal lesions in response to I/R, with an increase in MPO activity, in an astressin 2B-inhibitable manner. CRF may weaken intestinal mucosal defenses during I/R by affecting apoptosis, cell proliferation, and immune cell activation (9, 10, 19).

The present study demonstrated that CRF, generated endogenously or administered exogenously, aggravated the intestinal ulcerogenic response to I/R through the activation of CRFR2. These results are in contrast to those of other studies showing protection by urocortin against I/R in other tissues such as the heart and liver (18, 29-31). Adao *et al*. (30) found that urocortin/CRFR2 exerted favorable effects on the cardiovascular system, including coronary vasodilatation, increased coronary blood flow and conductance, and augmented cardiac contractility and output, and also protected against I/R injury. Walczewska *et al*. (31) indicated that urocortin/CRFR2 is a potential therapeutic target in coronary heart disease, congestive heart failure, and hypertension. Although the reason why CRF exerts different effects on I/R-induced injury in the heart, liver, and small intestine currently remains unknown, these findings may be attributed to different mechanisms of injury in response to I/R depending on the tissue.

The results of the present study demonstrated that enterobacteria and iNOS expression are involved in the pathogenic mechanism of I/R-induced enteritis. The invasion of bacteria into the intestinal mucosa plays a pivotal role in the pathogenesis of various intestinal diseases, including those induced by hemorrhagic shock (32), intestinal obstruction (24, 33), ischemia (34), and NSAIDs (20, 26, 27). Several factors, such as the breakdown of the intestinal barrier, a decrease in the secretion of mucus, and the loss of resistance against bacterial overgrowth in the intestinal tract, are considered to be responsible for bacterial invasion (27, 35-38). In the present model, the enterobacterial count was increased in the ileal mucosa after stenosis of the SMA, and the severity of enteritis was significantly suppressed by the administration of ampicillin. Enterobacteria release lipopolysaccharide, which plays a crucial role in the pathogenic existence of various forms of enteritis induced by ischemia or NSAIDs (26, 27, 39, 40). When patients are diagnosed with ischemic enteritis, an antibiotic is prescribed (41, 42). We found that the increased bacterial count in the mucosa after I/R was significantly attenuated by the pretreatment with astressin 2B, but not NBI27914. These results clearly suggest that enterobacteria play a major role in the pathogenesis of I/R-induced enteritis, and this process is facilitated by endogenous CRF *via* the activation of CRFR2.

On the other hand, bacterial endotoxins up-regulate intestinal iNOS expression, which plays important roles in the genesis of enteritis caused by NSAIDs or intestinal ischemia due to ligation of the SMA (20, 24, 42). Indomethacin up-regulated iNOS/NO production, which preceded the onset of intestinal damage, and aminoguanidine attenuated NSAID-induced enteropathy by suppressing NO production due to iNOS (27, 36, 37). In the present study, the expression of iNOS at the mRNA and protein levels was up-regulated in the small intestine after I/R, and this up-regulated expression was mitigated by the pretreatment with astressin 2B, but not NBI27914. Although we did not measure NO production in the present study, the development of enteritis following I/R was markedly suppressed in the presence of aminoguanidine, suggesting the involvement of iNOS/NO in the pathogenesis of these lesions. We also found that urocortin I significantly aggravated the intestinal ulcerogenic response caused by I/R with an increase in MPO activity, and these effects were abrogated by the co-treatment with astressin 2B, but not NBI27914, again confirming the mediation of the deleterious effects of CRF by the activation of CRFR2.

Consistent with the findings of others (14, 17, 18), we herein confirmed that CRF and CRFR2 were expressed in the small intestine and brain. Yarushkina *et al*. (18) reported the central localization and the peripheral functioning of CRF/CRFR2 in analgesia on somatic pain sensitivity, suggesting that the CRF-induced analgesic effect is mediated by CRFR2 located in the midbrain. We recently showed that the protective effects of urocortin I on NSAID-induced enteropathy were not affected by adrenalectomy or the prior administration of mifepristone (a glucocorticoid antagonist) (15). The results of the present study demonstrated that urocortin I, given peripherally, aggravated I/R-induced enteritis. Thus, endogenous CRF or urocortin I may exert protective or deleterious effects in the small intestine depending on the model of enteritis, both of which are mediated by the activation of peripheral CRFR2 and are independent of adrenal glucocorticoids. In the present study, up-regulated iNOS expression following I/R was suppressed by astressin 2B, a CRFR2 antagonist. Glucocorticoids are known to prevent the up-regulation of iNOS expression by inhibiting the process at the transcription level (20, 42). These results also support protection by a CRFR2 antagonist being independent of endogenous glucocorticoids. Vieira *et al*. (43) reported that glucocorticoids prevented I/R-induced enteritis in mice. Since CRFR1 is involved in the hypothalamus-pituitary-adrenal axis (44), a selective CRFR1 antagonist may suppress corticosterone levels, resulting in the aggravation of I/R-induced enteritis. However, as described above, this peptide modified the intestinal ulcerogenic response to I/R without the involvement of endogenous glucocorticoids released from the adrenal glands. Thus, the CRFR1 antagonist had no effect on I/R-induced enteritis.

In conclusion, I/R in the small intestine caused severe hemorrhagic lesions in the mucosa within 6 h after reperfusion. This model of enteritis was accompanied by enterobacterial invasion and the up-regulated expression of iNOS in the mucosa and prevented by a pretreatment with ampicillin or aminoguanidine, suggesting the involvement of iNOS/NO in the pathogenesis of this model of enteritis. Furthermore, the present study showed for the first time that I/R-induced intestinal lesions were prevented by the administration of astressin and astressin 2B, but not NBI27914, and were aggravated by urocortin I in an astressin 2B-inhibitable manner. Endogenous CRF is assumed to be a pathogenic factor in I/R-induced enteritis, and, thus, CRFR2 antagonists may be useful for maintaining the intestinal mucosa under I/R-induced conditions.

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