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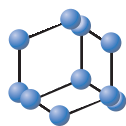
Influence of Adrenalectomy on Protective Effects of Urocortin I, a Corticotropin-Releasing Factor, Against Indomethacin-Induced Enteropathy in Rats

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Abstract: We examined the influence of adrenalectomy on NSAID-induced small intestinal damage in rats and investigated the possible involvement of adrenal glucocorticoids in the protective effects of urocortin I, a corticotropin-releasing factor (CRF) agonist. Male SD rats without fasting were administered indomethacin s.c. and killed 24 h later in order to examine the hemorrhagic lesions that developed in the small intestine. Urocortin I (20 µg/kg) was given i.v. 10 min before the administration of indomethacin. Bilateral adrenalectomy was performed a week before the experiment. Indomethacin (10 mg/kg) caused multiple hemorrhagic lesions in the small intestine, which were accompanied by a decrease in mucus secretion and increases in intestinal motility, enterobacterial invasion, and iNOS expression. Adrenalectomy markedly increased the ulcerogenic and motility responses caused by indomethacin, with further enhancements in bacterial invasion and iNOS expression; severe lesions occurred at 3 mg/kg, a dose that did not induce any damage in sham-operated rats. This worsening effect was also observed by the pretreatment with mifepristone (a glucocorticoid receptor antagonist). Urocortin I prevented indomethacin-induced enteropathy, and this effect was completely abrogated by the pretreatment with astressin 2B, a CRF2 receptor antagonist, but was not significantly affected by either adrenalectomy or the mifepristone pretreatment. These results suggested that adrenalectomy aggravated the intestinal ulcerogenic response to indomethacin, the intestinal hypermotility response may be a key element in the mechanism for this aggravation, and endogenous glucocorticoids played a role in intestinal mucosal defense against indomethacin-induced enteropathy, but did not account for the protective effects of urocortin I, which were mediated by the activation of peripheral CRF2 receptors.

Koji Takeuchi

INTRODUCTION

Corticotropin-releasing factor (CRF), a hypothalamic neuropeptide, is known as the principal regulator of the hypothalamus-pituitary-adrenal (HPA) axis by triggering the release of adrenocorticotropic hormone from the anterior pituitary gland [1, 2]. The CRF family has recently been expanded by the addition of the following mammalian CRF-related peptides; urocortin I, II, and III [3-6]. Using these CRF ligands, this hormone has been shown to play an important role in the regulation of various physiological events, such as cardiovascular function, food intake, visceral pain, and gut motor function as well as mucosal defense [7-11].

CRF and CRF-related peptides are also involved in inflammatory responses in the intestine; however, their roles are not without controversy [12-16]. Kokkotou *et al*. [13] reported that *Clostridium difficile* toxin A-induced intestinal inflammation was inhibited in mice lacking CRF2 receptor (CRF2R) and suggested that CFR/CRF2R mediates intestinal inflammatory responses through the release of pro-inflammatory mediators. We also showed that urocortin I aggravated the intestinal ulcerogenic response to ischemia/reperfusion in a CRF2R-dependent manner [15]. In contrast, a peripheral injection of CRF and urocortin II reduced intestinal inflammation and motility in the mouse terminal ileum [14]. Kubo *et al*. [16] previously reported that indomethacin-induced enteropathy was prevented by urocortin I and worsened by astressin, a nonselective CRFR antagonist, suggesting the involvement of CRF in intestinal mucosal defense against nonsteroidal anti-inflammatory drugs (NSAIDs). Furthermore, the protective effects of

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urocortin I were shown to be mediated by the activation of CRF2R, but not CRF1R, and functionally associated with the suppression of intestinal hypermotility caused by indomethacin [16]. Since CRF and CRF2R were both found to be expressed in the small intestine as well as in the brain [16, 17], urocortin I may exhibit protective effects against NSAID-induced enteropathy *via* the activation of peripheral CRF2R. However, since CRF is known to activate the HPA axis [2] and dexamethasone inhibits indomethacin-induced enteropathy [15], the protective effects of urocortin I may, at least in part, be mediated by endogenous glucocorticoids released from the adrenal gland through activation of the HPA axis.

In the present study, we examined the influences of bilateral adrenalectomy and mifepristone, a glucocorticoid receptor antagonist, on indomethacin-induced enteropathy in rats in order to investigate the possible involvement of adrenal glucocorticoids in the protective effects of urocortin I.

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±1ºC). Experiments were performed using four to eight 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 the Kyoto Pharmaceutical University.

Induction of Small Intestinal Damage by Indomethacin

Animals were administered indomethacin (3 and 10 mg/kg) s.c., sacrificed 24 h later under deep ether anesthesia, and the small intestines were examined for hemorrhagic lesions under a dissecting microscope according to the routine method described in previous papers [16, 18, 19]. Urocortin I (an CRF1R and CRF2R agonist: 2-20 µg/kg) was administered i.v. 10 min before the administration of indomethacin. NBI-27914 (a selective CRF1R antagonist: 200 µg/kg) or astressin 2B (a selective CRF2R antagonist: 60 µg/kg) was administered i.v. 10 min before the administration of urocortin I. Mifepristone (1-10 mg/kg), a glucocorticoid receptor antagonist, was also administered p.o. twice 30 min before and 6 h after the administration of indomethacin, with or without urocortin I (20 µg/kg). In some animals, dexamethasone (1 mg/kg) was administered s.c. once daily for 7 days after adrenalectomy. The doses of NBI-27914, astressin 2B, and mifepristone were selected to antagonize the pharmacological actions of CRF or glucocorticoids mediated by CRF1R, CRF2R, or the glucocorticoid receptor, respectively [15, 16, 20, 21]. A sham operation or bilateral adrenalectomy was performed one week before the experiment, according to a previously described method [20].

Determination of Enterobacterial Counts

Animals were sacrificed under deep diethyl ether anesthesia 6 h after the administration of indomethacin (10 mg/kg, s.c.), and the small intestines were removed. Enterobacteria were enumerated according to a modified version [16, 18] of the method originally described by Deitch *et al.* [22]. Urocortin I (20 µg/kg) was administered i.v. 10 min before the administration of indomethacin. A sham operation or bilateral adrenalectomy was performed one week before the experiment.

Determination of Myeloperoxidase (MPO) Activity

Animals were sacrificed under deep diethyl ether anesthesia 24 h after the administration of indomethacin (10 mg/kg, s.c.), the small intestines were removed, and MPO activity was measured according to a modified version [16, 19] of the method originally described by Krawisz *et al.* [23]. Mifepristone (10 mg/kg) was administered p.o. twice 30 min before and 6 h after the administration of indomethacin. Urocortin I (20 µg/kg) was administered i.v. 10 min before indomethacin treatment. A sham operation or bilateral adrenalectomy was performed one week before the experiment.

Determination of iNOS mRNA Expression by a Reverse Transcriptional Polymerase Chain Reaction (RT-PCR)

Animals were sacrificed under deep diethyl ether anesthesia 6 h after the administration of indomethacin (3 and 10 mg/kg, s.c.), and the expression of mRNA for iNOS in the small intestinal mucosa was determined by a RT-PCR. Extraction and reverse-transcription of total RNA, and RT-PCR amplification were performed according to previous papers [16, 22, 24]. The sequences of the sense and antisense primers for the rat iNOS, and GAPDH, and each product size are shown in Table (**1**). In some cases, urocortin I (20 µg/kg) was given i.v. 10 min before the administration of indomethacin, while mifepristone (10 mg/kg) was given p.o. 30 min before indomethacin. A sham operation or bilateral adrenalectomy was performed one week before the experiment.

Determination of Mucus Secretion

Animals were sacrificed under deep diethyl ether anesthesia 3 h after the administration of indomethacin (10 mg/kg, s.c.), and the small intestine was removed. The amount of mucus secreted in the small intestine was determined using periodic acid-Schiff (PAS) staining according to a previously described method [19, 24].

Table 1.   Sequences of sense and antisense primers for rat iNOS and GAPDH.

Gene	Sequences	PCR Product
INOS Sense Antisense	5'-CGGTTCACAGTCTTGGTGAAAG-3' 5'-CAGGTGTTCCCCAGGTAGGTAG-3'	780 bp
GAPDH Sense Antisense	5'-GAACGGGAAGCTCACTGGCATGGC-3' 5'-TGAGGTCCACCACCCTGTTGCTG-3'	311 bp

Urocortin I (20 µg/kg) was administered i.v. 10 min before the indomethacin treatment. A sham operation or bilateral adrenalectomy was performed one week before the experiment.

Measurement of Small Intestinal Motility

Animals with or without adrenalectomy, fasted for 18 h, were anesthetized with urethane (1.25 g/kg, i.p.), and the trachea was cannulated to facilitate respiration. Intestinal motility was measured according to a modified version [19, 25] of the method originally described by Calignano *et al.* [26]. Indomethacin (3 or 10 mg/kg) was administered s.c. after basal intestinal motility had stabilized, and motility was measured for 3 h thereafter. Urocortin I (20 µg/kg) was administered i.v. 2 h after the indomethacin treatment. A sham operation or bilateral adrenalectomy was performed one week before the experiment.

Preparation of Drugs

The drugs used were indomethacin, urocortin I, astressin-2B, mifepristone, dexamethasone, Evans blue (Sigma Chemicals, St. Louis, Mo.), and urethane (Tokyo Kasei, Tokyo, Japan). Indomethacin was suspended in saline with a drop of Tween 80 (Wako, Osaka, Japan). NBI-27914, astressin 2B, and mifepristone were dissolved in dimethyl sulfoxide (DMSO), while urocortin I was dissolved in 1% acetic acid, and they were further diluted with saline to the desired concentrations. All drugs were prepared immediately before use and administered s.c., p.o., or i.v. in a volume of 0.5 ml/100 g body weight or 0.1 ml/100 g body weight, respectively. Control animals received saline as the vehicle.

Statistics

Data are presented as the means±SE of four to eight 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 significant.

RESULTS

Effects of Urocortin I on Indomethacin-Induced Small Intestinal Lesions in Normal Rats

Indomethacin (10 mg/kg) administered s.c. to normally fed rats produced multiple hemorrhagic lesions in the small intestine within 24 h, mainly in the jejunum and ileum, with the lesion score being 267.3±40.8 mm². When the animals were pretreated with urocortin I (2-20 µg/kg, i.v.), a CRF1R and CRF2R agonist, the severity of these lesions was reduced in a dose-dependent manner, and the effect was significant at 10 µg/kg or greater, with the degree of inhibition being 53.6% at 10 µg/kg and 64.3% at 20 µg/kg, respectively Fig. (**1A**). The inhibitory effect of urocortin I was significantly abrogated by the prior administration of astressin 2B (60 µg/kg, i.v.), a selective CRF2R antagonist, with the lesion score being 325.4±56.5 mm², which was approximately 4-fold greater than that of the group treated with indomethacin plus urocortin I Fig. (**1B**). However, the selective CRF1R antagonist NBI-27914 (200 µg/kg, i.v.) had no influence on the protective effects of urocortin I on these intestinal lesions, with the lesion score being 66.8±17.6 mm².
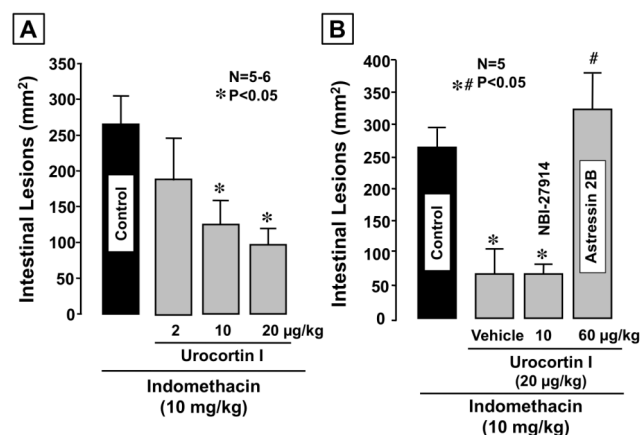

Fig. (1). Effects of Urocortin I on indomethacin-induced small intestinal lesions in rats in the absence **(A)** or presence **(B)** of CRFR antagonists. Animals were administered indomethacin (10 mg/kg, s.c.) and killed 24 h later. Urocortin I (2, 10, and 20 µg/kg) was given i.v. 10 min before the administration of indomethacin. NBI-27914 (a CRF1R antagonist; 200 µg/kg) or astressin 2B (a CRF2R antagonist; 60 µg/kg) was given i.v. 10 min before the administration of urocortin I. Data are presented as the means±SE of 5-6 rats. Significant difference at P<0.05; ∗ from control; # from vehicle.

Effects of Adrenalectomy and Mifepristone on the Intestinal Ulcerogenic Response to Indomethacin

Indomethacin administered s.c. at 3 mg/kg did not damage the small intestine, but caused multiple hemorrhagic lesions 24 h after the administration of 10 mg/kg, the with the lesion score being 236.7±21.6 mm². This intestinal ulcerogenic response to indomethacin was markedly worsened by bilateral adrenalectomy; indomethacin produced severe damage even at 3 mg/kg, and the lesion score at 10 mg/kg was 446.2±44.3 mm² Fig. (**2A** and **3A**). The worsening effect of adrenaletomy was reversed by the repeated administration of dexamethasone (1 mg/kg, s.c.). On the other hand, the intestinal ulcereogenic response to indomethacin (10 mg/kg, s.c.) was significantly suppressed by the prior administration of urocortin I (20 µg/kg, i.v.) in both sham-operated and adrenalectomized rats, with the degree of inhibition being 49.8% and 50.7%, respectively Fig. (**2B**).

To further confirm the aggravating influence of adrenalectomy on the intestinal ulcerogenic response to indomethacin, we also examined the effects of mifepristione, a glucocorticoid receptor antagonist, on indomethacin-induced enteropathy. The pretreatment of animals with mifepristone (1-10 mg/kg, p.o.) dose-dependently worsened the severity of small intestinal lesions in response to indomethacin (10 mg/kg, s.c.), and this worsening effect was significant at 3 mg/kg or greater, with the lesion score at 10 mg/kg being 350.3±58.2 mm², which was approximately 1.7-fold greater than that of control rats Figs. (**3B** and **4A**). The intestinal ulcerogenic response to indomethacin was significantly suppressed by the prior administration of urocortin I (20 µg/kg, i.v.) in normal and mifepristone-pretreated rats, with the degree of inhibition being 57.7% and 49.9%, respectively Fig. (**4B**).
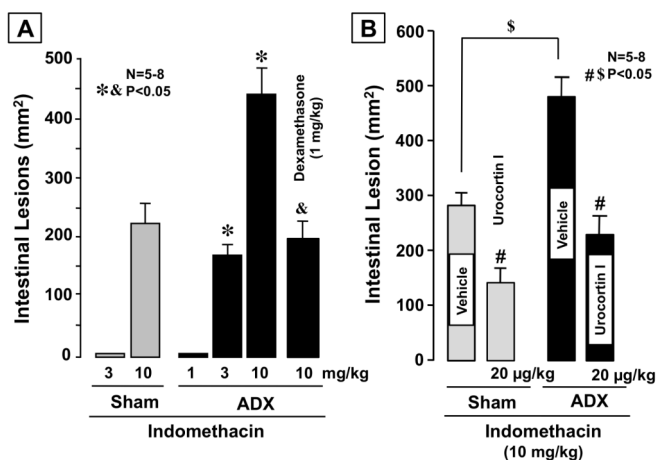
Fig. (2). Influence of adrenalectomy on the intestinal ulcerogenic response induced by indomethacin in rats **(A)** and effects of urocortin I on indomethacin-induced intestinal lesions in sham-operated (Sham) or adrenalectomized (ADX) rats **(B)**. A sham operation or bilateral adrenalectomy was performed one week before the experiment. Animals were administered indomethacin (1-10 mg/kg) s.c. and killed 24 h later. In some animals, dexamethasone (1 mg/kg) was given s.c. once daily for 7 days after adrenalectomy. Urocortin I (20 μg/kg) was given i.v. 10 min before the administration of indomethacin (10 mg/kg). Data are presented as the means±SE of 5-8 rats. Significant difference at P<0.05; **A:** ∗ from Sham at the corresponding dose; & from ADX (10 mg/kg) alone; **B:** # from vehicle in Sham or ADX; $ from vehicle in Sham.
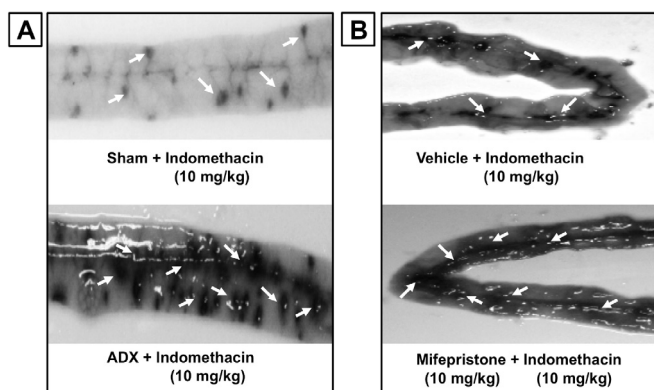

Fig. (3). Gross appearance of indomethacin-induced small intestinal lesions in sham-operated (Sham) or adrenalectomized (ADX) rats **(A)** and vehicle or mifepristone-treated rats **(B)**. Animals were administered indomethacin (10 mg/kg) s.c. and killed 24 h later. A sham operation or bilateral adrenalectomy was performed one week before the experiment. Mifepristone (10 mg/kg) was given twice 30 min before and 6 h after the administration of indomethacin. Note that the intestinal ulcerogenic response to indomethacin was markedly aggravated in rats subjected to bilateral adrenalectomy or the mifepristone pretreatment.

Effects of Urocortin I on Changes in MPO Activity Induced by Indomethcin in Sham-Operated and Adrenalectomized Rats

MPO activity in the intestinal mucosa of sham-operated rats was 0.038±0.008 μmol H_2O_2/min/mg protein and

significantly increased in response to indomethacin (10 mg/kg, s.c.), reaching 0.164±0.007 μmol H_2O_2/min/mg protein after 24 h Fig. **(5)**. This elevation in MPO activity was almost completely suppressed by the prior administration of urocortin I (20 μg/kg, i.v.), with the degree of inhibition being 69.3%. On the other hand, the increase observed in MPO activity after the administration of indomethacin was markedly enhanced in adrenalectomized rats, with the value reaching 0.680±0.114 μmol H_2O_2/min/mg protein 24 h later. The prior administration of urocortin I significantly suppressed the increase in MPO activity in adrenalectomized rats, with the degree of inhibition being 63.6%. The increase in MPO responses to indomethacin was also observed in rats pretreated with mifepristone (10 mg/kg, p.o.), and urocortin I similarly decreased the increase in MPO activity in both normal and mifepristone-pretreated rats (data not shown).
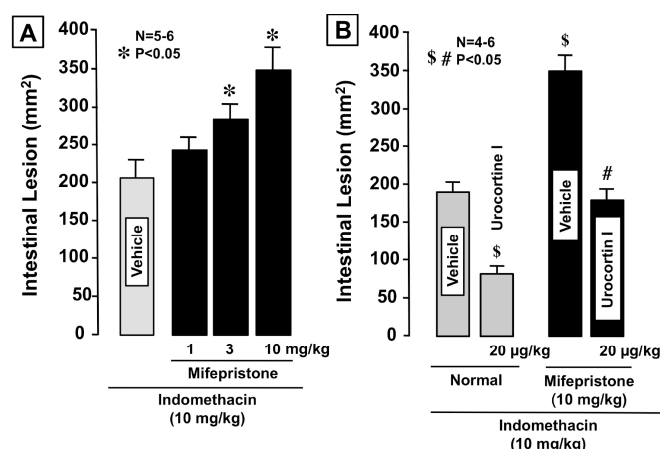

Fig. (4). Influence of mifepristone on the intestinal ulcerogenic response induced by indomethacin in rats **(A)** and effects of urocortin I on indomethacin-induced intestinal lesions in normal or mifepristone-pretreated rats **(B)**. Animals were administered indomethacin (10 mg/kg) s.c. and killed 24 h later. Mifepristone (10 mg/kg) was given p.o. twice 30 min before and 6 h after the administration of indomethacin. Urocortin I (20 μg/kg) was given i.v. 10 min before the administration of indomethacin. Data are presented as the means±SE of 5-6 rats (A) and 4-6 rats (B), respectively. Significant difference at P<0.05; **A:** ∗ from vehicle; **B:** $ from vehicle in normal; # from vehicle in Mifepristone.

Effects of Urocortin I on Enterobacterial Invasion Caused by Indomethacin in Sham-Operated and Adrenalectomized Rats

Aerobic and anaerobic bacterial counts in the intestinal mucosa of sham-operated rats were 5.40±0.42 log CFU/g tissue and 5.82±0.35 log CFU/g tissue, respectively. Following the administration of indomethacin (10 mg/kg, s.c.), bacterial counts under both aerobic and anaerobic conditions were markedly increased and reached 7.52±0.42 log CFU/g tissue and 7.67±0.34 log CFU/g tissue, respectively Table **(2)**. The pretreatment of animals with urocortin I (20 μg/kg, i.v.) significantly prevented bacterial invasion in the mucosa following the administration of indomethacin to sham-operated rats. On the other hand,
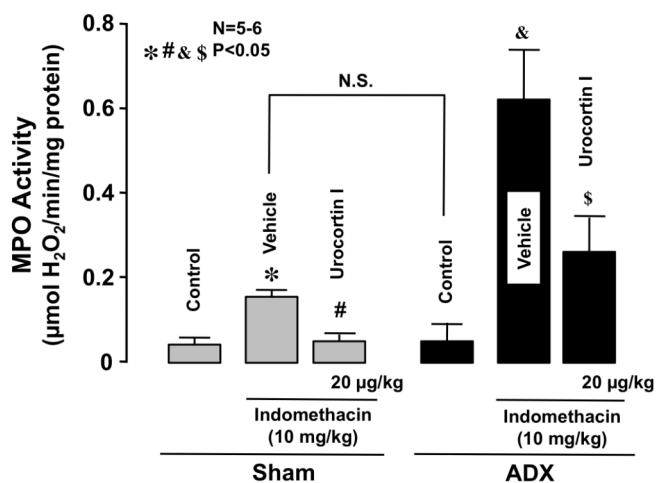
Fig. (5). Effects of indomethacin on MPO activity in the small intestine of sham- operated (Sham) or adrenalectomized (ADX) rats with or without the urocortin I pretreatment. A sham-operation or bilateral adrenalectomy was performed one week before the experiment. Animals were administered indomethacin (10 mg/kg) s.c. and killed 24 h later. Urocortin I (20 µg/kg) was given i.v. 10 min before the administration of indomethacin. Data are presented as the means±SE of 5-6 rats. Significant difference at P<0.05: ∗ from control in sham; # from vehicle in sham; & from control in ADX; $ from vehicle in ADX.

bacterial counts in the small intestine of adrenalectomized rats were not significantly different from those in sham-operated rats, and increased markedly in response to indomethacin. Furthermore, enhanced bacterial invasion in adrenalectomized rats following the indomethacin treatment was significantly suppressed by the pretreatment of these animals with urocortin I, with the degree of inhibition being equivalent to that observed in sham-operated rats.

Effects of Urocortin I on Mucosal Expression of iNOS mRNA Following the Indomethacin Treatment Under Various Conditions

The expression of iNOS mRNA was hardly detected in the intestinal mucosa of normal or sham-operated rats. In a sham-operated rat, this expression was markedly up-regulated in the intestinal mucosa 6 h after the administration of indomethacin (10 mg/kg, s.c.), and this response was suppressed by the prior administration of urocortin I (20 µg/kg, i.v.) Fig. (**6A**). Indomethacin also clearly up-regulated the expression of iNOS mRNA in an adrenalectomized rat, and this up-regulation was suppressed by the pretreatment with urocortin I. The up-regulated expression of iNOS following the indomethacin treatment (10 mg/kg, s.c.) was not markedly affected by adrenalectomy. The same results were obtained in rats pretreated with mifepristone; indomethacin up-regulated the expression of iNOS 6 h after its administration, and this expression was markedly suppressed by the prior administration of urocortin I Fig. (**6B**). On the other hand, a low dose of indomethacin (3 mg/kg, s.c.) up-regulated the expression of iNOS in the intestinal mucosa of adrenalectomized rats, whereas this treatment did not up-regulate the expression of iNOS in sham-operated rats Fig. (**7**).

Effects of Urocortin I on the Intestinal Hypermotility Response to Indomethacin in Sham-Operated and Adrenalectomized Rats

Indomethacin (10 mg/kg, s.c.) produced a marked increase in small intestinal motility from approximately 20 min after its administration to sham-operated rats Fig. (**8A**). An i.v. injection of urocortin I (20 µg/kg) suppressed intestinal hypermotility in response to indomethacin, resulting in fluctuations at baseline, which were difficult to characterize as contraction spikes Fig. (**8B**). As shown in Fig. (**8C**), indomethacin markedly enhanced intestinal

Table 2.   Effects of urocortin I on enterobacterial invasion in the intestinal mucosa induced by indomethacin in sham-operated or adrenalectomized rats.

Treatment	N	Enterobacterial Count (Log CFU/g Tissue)	
		Aerobic	**Anaerobic**
Sham-Operated Rats (Sham)			
Vehicle	5	5.40 ± 0.42	5.82 ± 0.35
Indomethacin (10 mg/kg)	6	7.52 ± 0.42 [a]	7.67 ± 0.34 [a]
Indomethacin + Urocortin I (20 µg/kg)	6	6.09 ± 0.15 [b]	7.04 ± 0.13 [b]
Adrenalectomized Rats (ADX)			
Vehicle	6	5.71 ± 0.83	6.48 ± 0.62
Indomethacin (10 mg/kg)	6	8.48 ± 0.21 [c]	8.84 ± 0.23 [c]
Indomethacin + Urocortin I (20 µg/kg)	6	6.64 ± 0.41 [d]	7.42 ± 0.05 [d]

Animals were administered saline or indomethacin (10 mg/kg, s.c.) and killed 6 h later. Urocortin I (20 µg/kg, i.v.) was given 10 min before the administration of indomethacin. A sham operation (Sham) or bilateral adrenalectomy (ADX) was performed one week before the experiment. Data are presented as the mean±SE of 5-6 rats. Significant difference at P<0.05; a) from vehicle in Sham, b) from indomethacin in Sham, c) from vehicle in ADX, d) from indomethacin in ADX.
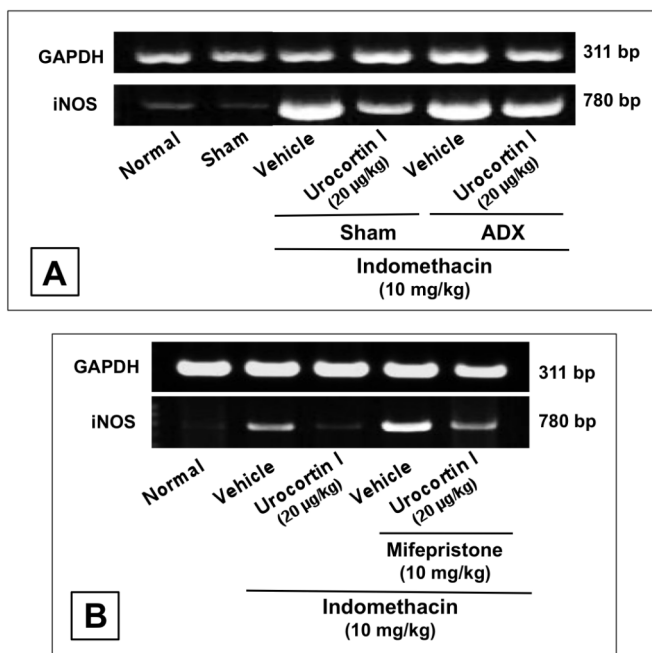
Fig. (6). Effects of urocortin I on the expression of iNOS mRNA in the small intestine of adrenalectomized rats **(A)** or mifepristone-treated rats **(B)**. A sham operation (Sham) or bilateral adrenalectomy (ADX) was performed one week before the experiment. Animals were administered indomethacin (10 mg/kg) s.c. and killed 6 h later to examine the expression of iNOS mRNA by RT-PCR. Mifepristine (10 mg/kg) was administered p.o. 30 min before the administration of indomethacin. Urocortin I (20 μg/kg) was given i.v. 10 min before indomethacin. Note that indomethacin up-regulated the expression of iNOS in the small intestine of sham-operated, adrenalectomized, and mifepristine-treated rats, whereas these responses all appeared to be mitigated by the prior administration of urocortin I.
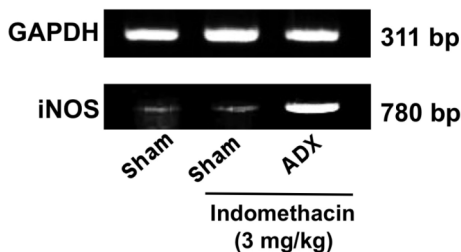

Fig. (7). The up-regulation of iNOS expression induced by indomethacin at 3 mg/kg in adrenalectomized rats. A sham operation (Sham) or bilateral adrenalectomy (ADX) was performed one week before the experiment. Animals were administered indomethacin (3 mg/kg) s.c. and killed 6 h later to examine the expression of iNOS mRNA by RT-PCR.

motility in adrenalectomized rats even at 3 mg/kg, a dose that did not produce any damage in sham-operated rats [see Fig. (**2A**)]. This hypermotility response to a low dose of indomethacin (3 mg/kg, s.c.) in adrenalectomized rats was effectively suppressed by urocortin I Fig. (**8D**).
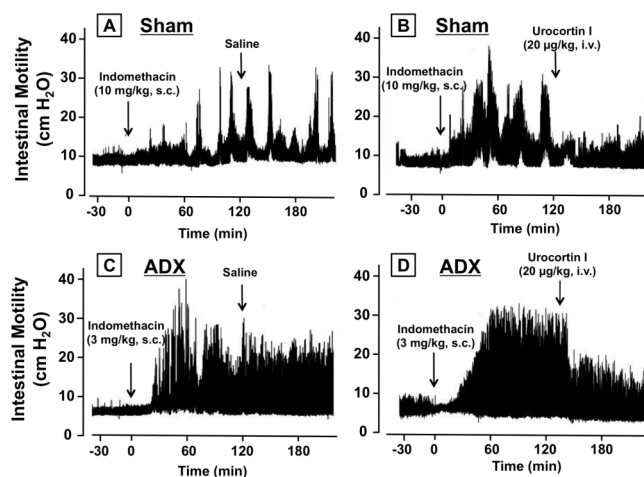

Fig. (8). Representative recordings showing the effects of urocortin I on intestinal hypermotility induced by indomethacin in sham-operated or adrenalectomized rats. A sham operation (Sham) or bilateral adrenalectomy (ADX) was performed one week before the experiment. After basal motility had stabilized, animals were given indomethacin (3 or 10 mg/kg) s.c. and motility was measured for 4 h thereafter. Urocortin I (20 μg/kg) was given i.v. 2 h after the administration of indomethacin. Figures show: **(A)** indomethacin (10 mg/kg) + saline in Sham; **(B)** indomethacin (10 mg/kg) + urocortin I in Sham; **(C)** indomethacin (3 mg/kg) + saline in ADX; **(D)** indomethacin (3 mg/kg) + urocortin I in ADX. Note that indomethacin, even at 3 mg/kg, induced intestinal hypermotility in adrenalectomized rats, and urocortin I appeared to suppress the intestinal hypermotility response to indomethacin in both sham-operated and adrenalectomized rats.

Effects of Urocortin I on Changes in PAS-positive Substances Induced by Indomethacin in Sham-Operated and Adrenalectomized Rats

In the intestinal mucosa of sham-operated rats, PAS-positive materials were observed in surface epithelial cells and the lamina propria along the intestinal gland Fig. (**9A**). In sham-operated rats, indomethacin (10 mg/kg, s.c.) markedly reduced PAS-positive materials in the intestinal mucosa, particularly in surface epithelial cells, reaching approximately 1/3 of that in the vehicle-treated group Table (**3**) and Fig. (**9B**). The decrease in PAS-positive materials by indomethacin was significantly restored by the pretreatment with urocortin I (20 μg/kg, i.v.) Fig. (**9C**). Bilateral adrenalectomy by itself led to slightly less PAS-positive materials than that in sham-operated rats Table (**2**) and Fig. (**9D**). Indomethacin markedly decreased PAS-positive materials in the intestinal mucosa of adrenalectomized rats, and this response was significantly reverted by the prior administration of urocortin I Figs. (**9E** and **9F**).

DISCUSSION

NSAID-induced enteropathy has recently been attracting attention due to the development of capsule endoscopy and double-balloon endoscopy [27-29]. These techniques have enabled the identification of previously undetectable lesions in the human small intestine and demonstrated that such
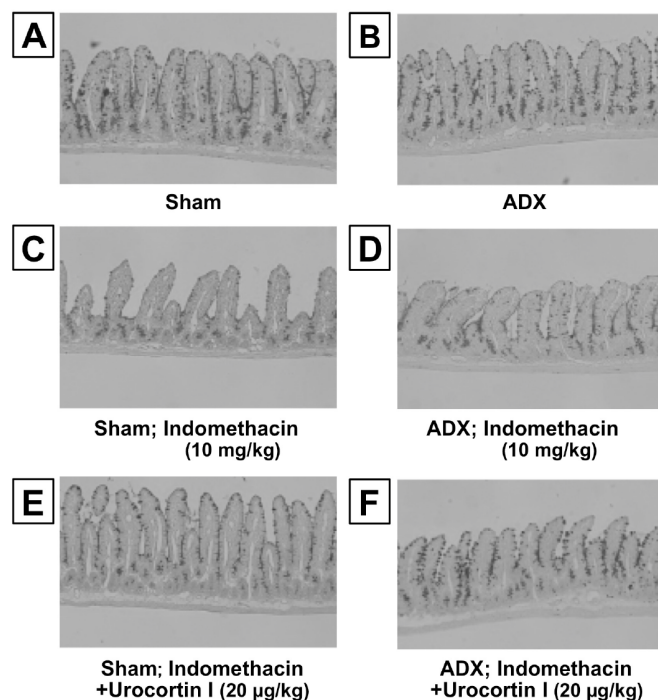
Fig. (9). Histology of the small intestinal mucosa showing changes in PAS staining in sham-operated or adrenalectomized rats with or without the urocortin I pretreatment. A sham operation (Sham) or bilateral adrenalectomy (ADX) was performed one week before the experiment. Animals were administered indomethacin (10 mg/kg) s.c. and killed 3 h later. Urocortin I (20 μg/kg) was given i.v. 10 min before the administration of indomethacin. Figures show; **A**: saline in Sham; **B**: saline in ADX; **C**: indomethacin in Sham; **D**: indomethacin in ADX; **E**: indomethacin+urocortin I in Sham: urocortin I; **F**: indomethacin+urocortin I in ADX. Note that the staining of PAS-positive materials was decreased by indomethacin in sham-operated and adrenalectomied rats, and these changes appeared to be restored by the pretreatment with urocortin I.

Table 3. Effects of urocortin I on changes in PAS-positive substances induced in the small intestine by indomethacin in sham-operated or adrenalectomized rats.

Treatment	N	PAS-Positive Materials (% from Sham)
Sham-Operated Rats (Sham)		
Vehicle	5	100 ± 18.9
Indomethacin (10 mg/kg)	5	33.8 ± 5.80 [a]
Indomethacin + Urocortin I (20 μg/kg)	5	60.4 ± 13.6 [b]
Adrenalectomized Rats (ADX)		
Vehicle	4	83.4 ± 22.3
Indomethacin (10 mg/kg)	5	23.7 ± 9.10 [c]
Indomethacin + Urocortin I (20 μg/kg)	4	61.2 ± 12.0 [d]

A sham operation (Sham) or bilateral adrenalectomy (ADX) was performed one week before the experiment. Animals were administered indomethacin (10 mg/kg) s.c. and killed 3 h later. Urocortin I (20 μg/kg) was given i.v. 10 min before the administration of indomethacin. Data are expressed as a % of the amount of Vehicle of sham-operated rats and represented the means±SE of 5-6 rats. Significant difference at P<0.05: a) from vehicle in sham; b) from indomethacin in sham; c) from vehicle in ADX; d) from vehicle indomethacin in ADX.

lesions are more common than originally considered. The occurrence of these lesions has been causally related to various functional alterations induced by the inhibition of cyclooxygenase, such as intestinal hypermotility, decreased mucus secretion, and enterobacterial invasion, followed by a series of inflammatory responses, including iNOS expression and neutrophil migration [24, 30-34]. We recently reported that indomethacin-induced enteropathy was prevented by urocortin I, a CRF-related peptide, and aggravated by astressin, a CRFR antagonist, suggesting the involvement of endogenous CRF in intestinal mucosal defense against NSAIDs [16]. However, the protective effects of urocortin I may, at least partly, be accounted for by endogenous glucocorticoids released from the adrenal glands because this peptide may activate the adrenal glands through a peripheral effect and dexamethasone was shown to inhibit indomethacin-iduced intestinal lesions [18].

The results of the present study were consistent with the previous findings in which urocortin I showed protective effects against indomethacin-induced enteropathy, and these effects were mitigated by astressin 2B, a selective CRF2R

antagonist, but not NBI-27914, a selective CRF1R antagonist [16]. Moreover, these intestinal lesions were significantly aggravated by astressin 2B alone, suggesting the role of endogenous CRF/CRF2R in mucosal defense against NSAIDs [16]. Since CRF and CRF2R were both found to be expressed in the rat small intestine [16, 17], urocortin I may act peripherally to prevent intestinal lesions. Indeed, previous studies reported that CRF and CRF-related peptides given peripherally effectively suppressed inflammatory responses in the gastrointestinal mucosa [12, 14]. This protection may be partly attributed to glucocorticoids released from the adrenal glands. This idea was supported by the present results in which indomethacin-induced enteropathy was markedly aggravated by the glucocorticoiud deficiency produced by bilateral adrenalectomy as well as the mifepristone pretreatment, a glucocorticoid receptor antagonist. We previously demonstrated that indomethacin-induced gastric damage was aggravated in adrenalectomized rats, and the dose required to produce lesions was decreased in these rats [20]. The present study also showed that indomethacin provoked severe damage in the small intestine of adrenalectomzied rats, even at 3 mg/kg, a dose that did not damage the intestinal mucosa of sham-operated animals. Filaretova *et al*. [21] also reported that adrenalectomy aggravated indomethacin-induced gastric lesions with a marked decrease in plasma corticosterone levels. This aggravation was prevented by corticosterone replacement, suggesting that a glucocorticoid deficiency is the main reason for the aggravation of indomethacin-induced gastropathy in adrenalectomized rats [20, 35]. These findings suggest that endogenous glucocorticoids derived from the adrenal glands contribute to mucosal defense against NSAID-induced gastrointestinal damage. However, we

demonstrated that urocortin I significantly attenuated the aggravation of intestinal ulcerogenic and MPO responses to indomethacin in rats subjected to bilateral adrenalectomy or the mifepristone pretreatment, and the extent of its effects was almost equivalent to those observed in sham-operated or normal rats. Thus, it is assumed that urocortin I exhibited protective effects against NSAID-induced enteropathy independent of adrenal glucocorticoids.

Intestinal hypercontraction has been identified as an important pathogenic mechanism of NSAID-induced enteropathy [25, 34]. This hypermotility, together with decreased mucus secretion, led to epithelial barrier dysfunction through vigorous mixing with food residue in the lumen. Since the epithelial barrier plays an important part in innate host defense against intestinal pathogens and irritants, it is possible that enhanced intestinal contraction may have accelerated enterobacterial invasion and up-regulated the expression of iNOS in the intestinal mucosa [32, 34]. In the present study, we found that adrenalectomy markedly enhanced the intestinal hypermotility response to indomethacin; this agent even at 3 mg/kg caused the hypermotility in adrenalectomized rats but not normal rats. Although the mechanism by which adrenalectomy increased the sensitivity of the intestinal motility response to indomethacin currently remains unknown, this event appears to be an important mechanism for the aggravation of NSAID-induced enteropathy by adrenalectomy. Similar hypermotility responses were reported in the stomach in association with the aggravation of indomethacin-induced gastric lesions in adrenalectomized rats [19].

Taché *et al.* [10] first reported that CRF and its related peptides, such as urocortin I & II, inhibited the gastric emptying of solid food. The same group also showed that stress-induced gastric stasis was reversed by a central or peripheral pretreatment with non-selective or selective CRF2R antagonists [7-10, 36]. We also reported that urocortin I inhibited intestinal hypermotility caused by indomethacin, and this effect was antagonized by the co-administration of astressin 2B, but not NBI-27914, suggesting the involvement of CRF2R in the inhibitory effects of CRF on small intestinal motility [16]. However, CRF and its related peptides reportedly stimulated colonic motility mainly through the activation of CRF1R [36, 37]. It is assumed that CRF inhibits gastric and small intestinal motility mediated by the activation of CRF2R, but stimulates colonic motility through CRF1R.

As expected, urocortin I significantly suppressed the intestinal hypermotility response to indomethacin in sham-operated and adrenalectomized rats. The present study also showed that indomethacin decreased PAS-positive materials, as an indicator of mucus secretion, in sham-operated and adrenalectomized rats, and these changes were similarly reverted by the urocortin I treatment. We previously reported that urocortin I prevented the mucosal invasion of enterobacteria as well as the up-regulation of iNOS expression, and these effects were abrogated by a pretreatment with astressin 2B, but not NBI-27914 [16]. In the present study, adrenalectomy or the mifepristone pretreatment slightly enhanced the up-regulation of iNOS

expression following the indomethacin treatment; however, these responses were potently suppressed by urocortin I, similar to that observed in sham-operated/normal rats. The administration of indomethacin, even at 3 mg/kg, to adrenalectomized rats caused intestinal hypermotility, up-regulated the expression of iNOS, and provoked damage in the small intestine, supporting a causal relationship between intestinal hypermotility, iNOS expression, and intestinal damage. Thus, it is reasonable to assume that urocortin I, by inhibiting intestinal hypermotility, prevented not only the occurrence of enteropathy, but also the accompanying inflammatory changes such as increases in iNOS expression and MPO activity.

Kokkotou *et al.* [13] reported that the severity of intestinal inflammation in CRF2R-knockout mice was reduced following luminal exposure to *Clostridium difficile* toxin A, and this effect was mimicked by a pretreatment with astressin 2B, suggesting the mediation of inflammatory responses by CRF/CRF2R. We also recently reported that urocortin I aggravated the intestinal ulcerogenic response to ischemia/reperfusion in an astressin 2B-inhibitable manner [15]. CRF may worsen tissue injury *via* CRF2R if motility does not play any role in the pathogenic mechanism. Because intestinal hypermotility plays a critical role in the pathogenesis of indomethacin-induced intestinal lesions, CRF may prevent these lesions by inhibiting the hypermotility response.

In conclusion, the results of the present study confirmed that urocortin I, a CRF-related peptide, afforded a protective influence on the development of indomethacin-induced enteropathy, and this action may be mediated by the activation of peripheral CRF2R and functionally associated with the suppression of intestinal hypermotility caused by indomethacin. The intestinal ulcerogenic response was worsened by adrenalectomy or the pretreatment with mifepristone, suggesting that endogenous glucocorticoids play a role in the maintenance of intestinal mucosal integrity under adverse conditions such as a NSAID treatment. Since urocortin I prevented NSAID-induced enteropathy in glucocorticoid-deficient rats, similar to normal animals, it may have exhibited such protective effects without the involvement of endogenous glucocorticoids released from the adrenal glands. Furthermore, the present study strongly suggested that a glucocorticoid deficiency enhanced the intestinal hypermotility response to indomethacin, an important functional event underlying the aggravation of intestinal damage by adrenalectomy.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES

[1] Vale, W.; Spiess, J.; Rivier, C.; Rivier, J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science,* **1981**, *213*(4514), 1394-1397. [http://dx.doi.org/10.1126/science.6267699] [PMID: 6267699]

[2] Carrasco, G.A.; Van de Kar, L.D. Neuroendocrine pharmacology of stress. *Eur. J. Pharmacol.,* **2003**, *463*(1-3), 235-272. [http://dx.doi.org/10.1016/S0014-2999(03)01285-8] [PMID: 12600714]

[3] Hauger, R.L.; Grigoriadis, D.E.; Dallman, M.F.; Plotsky, P.M.; Vale, W.W.; Dautzenberg, F.M. International Union of Pharmacology. XXXVI. Current status of the nomenclature for receptors for corticotropin-releasing factor and their ligands. *Pharmacol. Rev.,* **2003**, *55*(1), 21-26. [http://dx.doi.org/10.1124/pr.55.1.3] [PMID: 12615952]

[4] Bale, T.L.; Vale, W.W. CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu. Rev. Pharmacol. Toxicol.,* **2004**, *44*, 525-557. [http://dx.doi.org/10.1146/annurev.pharmtox.44.101802.121410] [PMID: 14744257]

[5] Vaughan, J.; Donaldson, C.; Bittencourt, J.; Perrin, M.H.; Lewis, K.; Sutton, S.; Chan, R.; Turnbull, A.V.; Lovejoy, D.; Rivier, C.; Rivier, J.; Sawchenko, P.E.; Vale, W. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature,* **1995**, *378*(6554), 287-292. [http://dx.doi.org/10.1038/378287a0] [PMID: 7477349]

[6] Lewis, K.; Li, C.; Perrin, M.H.; Blount, A.; Kunitake, K.; Donaldson, C.; Vaughan, J.; Reyes, T.M.; Gulyas, J.; Fischer, W.; Bilezikjian, L.; Rivier, J.; Sawchenko, P.E.; Vale, W.W. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc. Natl. Acad. Sci. USA,* **2001**, *98*(13), 7570-7575. [http://dx.doi.org/10.1073/pnas.121165198] [PMID: 11416224]

[7] Taché, Y.; Martinez, V.; Million, M.; Wang, L. Stress and the gastrointestinal tract III. Stress-related alterations of gut motor function: role of brain corticotropin-releasing factor receptors. *Am. J. Physiol. Gastrointest. Liver Physiol.,* **2001**, *280*(2), G173-G177. [PMID: 11208537]

[8] Kihara, N.; Fujimura, M.; Yamamoto, I.; Itoh, E.; Inui, A.; Fujimiya, M. Effects of central and peripheral urocortin on fed and fasted gastroduodenal motor activity in conscious rats. *Am. J. Physiol. Gastrointest. Liver Physiol.,* **2001**, *280*(3), G406-G419. [PMID: 11171623]

[9] Martinez, V.; Wang, L.; Million, M.; Rivier, J.; Taché, Y. Urocortins and the regulation of gastrointestinal motor function and visceral pain. *Peptides,* **2004**, *25*(10), 1733-1744. [http://dx.doi.org/10.1016/j.peptides.2004.05.025] [PMID: 15476940]

[10] Taché, Y.; Bonaz, B. Corticotropin-releasing factor receptors and stress-related alterations of gut motor function. *J. Clin. Invest.,* **2007**, *117*(1), 33-40. [http://dx.doi.org/10.1172/JCI30085] [PMID: 17200704]

[11] Tao, J.; Li, S. Effects of urocortin *via* ion mechanisms or CRF receptors? *Biochem. Biophys. Res. Commun.,* **2005**, *336*(3), 731-736. [http://dx.doi.org/10.1016/j.bbrc.2005.07.078] [PMID: 16061206]

[12] Chatzaki, E.; Charalampopoulos, I.; Leontidis, C.; Mouzas, I.A.; Tzardi, M.; Tsatsanis, C.; Margioris, A.N.; Gravanis, A. Urocortin in human gastric mucosa: relationship to inflammatory activity. *J. Clin. Endocrinol. Metab.,* **2003**, *88*(1), 478-483. [http://dx.doi.org/10.1210/jc.2002-020853] [PMID: 12519893]

[13] Kokkotou, E.; Torres, D.; Moss, A.C.; O'Brien, M.; Grigoriadis, D.E.; Karalis, K.; Pothoulakis, C. Corticotropin-releasing hormone receptor 2-deficient mice have reduced intestinal inflammatory responses. *J. Immunol.,* **2006**, *177*(5), 3355-3361. [http://dx.doi.org/10.4049/jimmunol.177.5.3355] [PMID: 16920976]

[14] la Fleur, S.E.; Wick, E.C.; Idumalla, P.S.; Grady, E.F.; Bhargava, A. Role of peripheral corticotropin-releasing factor and urocortin II in intestinal inflammation and motility in terminal ileum. *Proc. Natl. Acad. Sci. USA,* **2005**, *102*(21), 7647-7652. [http://dx.doi.org/10.1073/pnas.0408531102] [PMID: 15883387]

[15] Abe, N.; Kumano, A.; Kotani, T.; Takeuchi, K. Involvement of corticotropin- releasing factor (CRF)/CRF2 receptors in pathogenesis of ischemia/ reperfusion- induced intestinal lesions in rats. *Gastroenterology,* **2010**, *138* (Abstract), S-74

[16] Kubo, Y.; Kumano, A.; Kamei, K.; Amagase, K.; Abe, N.; Takeuchi, K. Urocortin prevents indomethacin-induced small intestinal lesions in rats through activation of CRF2 receptors. *Dig.*

[17] Chang, J.; Hoy, J.J.; Idumalla, P.S.; Clifton, M.S.; Pecoraro, N.C.; Bhargava, A. Urocortin 2 expression in the rat gastrointestinal tract under basal conditions and in chemical colitis. *Peptides,* **2007**, *28*(7), 1453-1460. [http://dx.doi.org/10.1016/j.peptides.2007.05.008] [PMID: 17586086]

Dis. Sci., **2010**, *55*(6), 1570-1580. [http://dx.doi.org/10.1007/s10620-009-0930-1] [PMID: 19707872]

[18] Tanaka, A.; Kunikata, T.; Mizoguchi, H.; Kato, S.; Takeuchi, K. Dual action of nitric oxide in pathogenesis of indomethacin-induced small intestinal ulceration in rats. *J. Physiol. Pharmacol.,* **1999**, *50*(3), 405-417. [PMID: 10574470]

[19] Takeuchi, K.; Satoh, H. , **2010**.

[20] Takeuchi, K.; Nishiwaki, H.; Okada, M.; Niida, H.; Okabe, S. Bilateral adrenalectomy worsens gastric mucosal lesions induced by indomethacin in the rat. Role of enhanced gastric motility. *Gastroenterology,* **1989**, *97*(2), 284-293. [http://dx.doi.org/10.1016/0016-5085(89)90063-2] [PMID: 2744352]

[21] Filaretova, L.; Tanaka, A.; Miyazawa, T.; Kato, S.; Takeuchi, K. Mechanisms by which endogenous glucocorticoid protects against indomethacin-induced gastric injury in rats. *Am. J. Physiol. Gastrointest. Liver Physiol.,* **2002**, *283*(5), G1082-G1089. [http://dx.doi.org/10.1152/ajpgi.00189.2002] [PMID: 12381521]

[22] Deitch, E.A.; Ma, L.; Ma, W.J.; Grisham, M.B.; Granger, D.N.; Specian, R.D.; Berg, R.D. Inhibition of endotoxin-induced bacterial translocation in mice. *J. Clin. Invest.,* **1989**, *84*(1), 36-42. [http://dx.doi.org/10.1172/JCI114164] [PMID: 2661590]

[23] Krawisz, J.E.; Sharon, P.; Stenson, W.F. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Assessment of inflammation in rat and hamster models. *Gastroenterology,* **1984**, *87*(6), 1344-1350. [PMID: 6092199]

[24] Hayashi, S.; Kurata, N.; Yamaguchi, A.; Amagase, K.; Takeuch, K. Lubiprostone prevents NSAID-induced small intestinal damage by suppressing the expression of inflammatory mediators *via* EP4 receptors. *J. Pharmacol. Exp. Ther.,* **2014**, *349*, 470-479. [http://dx.doi.org/10.1124/jpet.114.213991] [PMID: 24713141]

[25] Takeuchi, K.; Miyazawa, T.; Tanaka, A.; Kato, S.; Kunikata, T. Pathogenic importance of intestinal hypermotility in NSAID-induced small intestinal damage in rats. *Digestion,* **2002**, *66*(1), 30-41. [http://dx.doi.org/10.1159/000064419] [PMID: 12379813]

[26] Calignano, A.; Whittle, B.J.; Di Rosa, M.; Moncada, S. Involvement of endogenous nitric oxide in the regulation of rat intestinal motility *in vivo. Eur. J. Pharmacol.,* **1992**, *229*(2-3), 273-276. [http://dx.doi.org/10.1016/0014-2999(92)90567-N] [PMID: 1490527]

[27] Goldstein, J.L.; Eisen, G.M.; Lewis, B.; Gralnek, I.M.; Zlotnick, S.; Fort, J.G. Video capsule endoscopy to prospectively assess small bowel injury with celecoxib, naproxen plus omeprazole, and placebo. *Clin. Gastroenterol. Hepatol.,* **2005**, *3*(2), 133-141. [http://dx.doi.org/10.1016/S1542-3565(04)00619-6] [PMID: 15704047]

[28] Maiden, L.; Thjodleifsson, B.; Theodors, A.; Gonzalez, J.; Bjarnason, I. A quantitative analysis of NSAID-induced small bowel pathology by capsule enteroscopy. *Gastroenterology,* **2005**, *128*(5), 1172-1178. [http://dx.doi.org/10.1053/j.gastro.2005.03.020] [PMID: 15887101]

[29] Fujimori, S.; Seo, T.; Gudis, K.; Ehara, A.; Kobayashi, T.; Mitsui, K.; Yonezawa, M.; Tanaka, S.; Tatsuguchi, A.; Sakamoto, C. Prevention of nonsteroidal anti-inflammatory drug-induced small-intestinal injury by prostaglandin: a pilot randomized controlled trial evaluated by capsule endoscopy. *Gastrointest. Endosc.,* **2009**, *69*(7), 1339-1346. [http://dx.doi.org/10.1016/j.gie.2008.08.017] [PMID: 19243767]

[30] Whittle, B.J. Temporal relationship between cyclooxygenase inhibition, as measured by prostacyclin biosynthesis, and the gastrointestinal damage induced by indomethacin in the rat. *Gastroenterology,* **1981**, *80*(1), 94-98. [PMID: 6778761]

[31] Konaka, A.; Kato, S.; Tanaka, A.; Kunikata, T.; Korolkiewicz, R.; Takeuchi, K. Roles of enterobacteria, nitric oxide and neutrophil in pathogenesis of indomethacin-induced small intestinal lesions in rats. *Pharmacol. Res.,* **1999**, *40*(6), 517-524. [http://dx.doi.org/10.1006/phrs.1999.0550] [PMID: 10660951]

[32] Takeuchi, K.; Yokota, A.; Tanaka, A.; Takahira, Y. Factors involved in upregulation of inducible nitric oxide synthase in rat small intestine following administration of nonsteroidal anti-inflammatory drugs. *Dig. Dis. Sci.,* **2006**, *51*(7), 1250-1259. [http://dx.doi.org/10.1007/s10620-006-8045-4] [PMID: 16944022]

[33]   Yamada, T.; Deitch, E.; Specian, R.D.; Perry, M.A.; Sartor, R.B.; Grisham, M.B. Mechanisms of acute and chronic intestinal inflammation induced by indomethacin. *Inflammation,* **1993**, *17*(6), 641-662. [http://dx.doi.org/10.1007/BF00920471] [PMID: 7906675]

[34]   Takeuchi, K.; Satoh, H. NSAID-induced small intestinal damage--roles of various pathogenic factors. *Digestion,* **2015**, *91*(3), 218-232. [http://dx.doi.org/10.1159/000374106] [PMID: 25791157]

[35]   Filaretova, L.; Tanaka, A.; Komoike, Y.; Takeuchi, K. Selective cyclooxygenase-2 inhibitor induces gastric mucosal damage in adrenalectomized rats. *Inflammopharmacology,* **2002**, *10*, 413-422. [http://dx.doi.org/10.1163/156856002321544882]

[36]   Martínez, V.; Wang, L.; Rivier, J.; Grigoriadis, D.; Taché, Y. Central CRF, urocortins and stress increase colonic transit *via* CRF1 receptors while activation of CRF2 receptors delays gastric transit in mice. *J. Physiol.,* **2004**, *556*(Pt 1), 221-234. [http://dx.doi.org/10.1113/jphysiol.2003.059659] [PMID: 14755002]

[37]   Taché, Y.; Martinez, V.; Million, M.; Maillot, C. Role of corticotropin releasing factor receptor subtype 1 in stress-related functional colonic alterations: implications in irritable bowel syndrome. *Eur. J. Surg. Suppl.,* **2002**, *587*(587)(Suppl.), 16-22. [PMID: 16144197]