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Bradykinin Upregulated The Expression of Cyclooxygenase-2 in The Submucosal Plexus of Enteric Nervous System of Guinea Pig*

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Abstract **Objective** Bradykinin and bradykinin B2 receptors (B2R) play important roles in the enteric nervous system. Bradykinin is usually involved in inflammation and neuroprotection, dependent on the bradykinin-induced formation of prostaglandins (PGs). Cyclooxygenase-1 (COX1) and cyclooxygenase-2 (COX2) catalyze the conversion of arachidonic acid to PGs. This study aimed to investigate the effect and the signaling mechanism of bradykinin stimulation on the release of prostaglandin E2 (pGE2) and the expression of COX2 in the enteric nervous system of guinea pigs. **Methods** Immunofluorescence was used to detecting the colocalization of COX2 with neural markers Anti-Hu and chAT in the primary cultured ileal submucosal plexus of guinea pigs. PCR and Western blot were used to detecting the effect of bradykinin evoking COX2 expression. Bradykinin B1 receptor (B1R) antagonist Leu-8 and B2R antagonist HOE-140 were preincubated before bradykinin stimulation. COX2 antagonist NS398 and COX1 antagonist FR12207 were used to observing the effect of bradykinin-induced pEG2 release. **Results** The results showed that COX2 was co-localized with neural markers Anti-Hu and chAT on ileal submucosal plexuses. Bradykinin induced COX2 expression was blocked by the B2R antagonist. The release of pGE2 by bradykinin stimulation in ileal submucosal plexuses was significantly decreased when incubating with the COX2 antagonist. **Conclusion** COX2 expression evoked by B2R signaling as an excitatory neurotransmitter in bradykinin stimulated pGE2 secretion, which provides a reasonable explanation for the role of bradykinin in intestinal inflammatory diseases.

Key words bradykinin, COX2, pGE2, B2R, enteric nervous system
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Bradykinin and kallidin are kinins, a family of endogenous peptides discovered in several pathophysiological events. Kinins are a group of structurally related 9-11 receptor agonists (Hyp3) -bradykinin showed effects comparable to bradykinin amino acid peptides that are produced by kallikrein-mediated enzymatic cleavage of kininogen at the site of tissue injury and inflammation[1-2]. Kinins are formed in plasma and tissues *via* the kallikrein-kinin system in response to infection, tissue trauma, or

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inflammatory alterations, such as an increase in vascular permeability, edema formation, and pain[3-4]. Among kinins, bradykinin is widely distributed not only in the periphery but also in the nervous system including the peripheral nervous system, brain, and enteric nervous system (ENS)[5]. Bradykinin receptors are cell surface, G-protein-coupled receptors of the seven-transmembrane family. Based on their pharmacological properties, the bradykinin receptor type 1 (B1R) and type 2 (B2R) are identified[6-7].

The previous studies showed bradykinin B2R was expressed in a majority of the ganglion cells in the submucosal plexuses in the guinea pig small intestine, suggesting that bradykinin might act in the ENS as a paracrine mediator to alter neural control of secretory and motility functions at the organ level[8-9]. Exposing neurons in the guinea pig small intestinal submucosal plexus to bradykinin *in vitro* evoked slowly activating depolarization of the membrane potential and enhanced excitability characterized by increased firing frequency during intraneuronal injection of depolarizing current pulses in both AH- and S-type neurons and the appearance of anodal break excitation at the offset of hyperpolarizing current pulses in AH neurons[10]. The results from electrophysiological recording with intracellular microelectrodes suggested that bradykinin acts on bradykinin B2R on submucosal neurons to stimulate the formation of prostaglandins[11]. Once formed and released, the prostaglandins (pGs) act to elevate the excitability of the same ganglion cells from which they are released and to diffuse and excite neighboring ganglion cells[12-13].

Cyclooxygenase (COX) is the rate-limiting enzyme that catalyzes the formation of prostanoids from arachidonic acid released from membrane phospholipids by phospholipase A2. There are two COX isoforms: COX1 and COX2[14]. COX1 is constitutively expressed for basal level as well as for immediate prostaglandin synthesis upon stimulation, particularly at high arachidonic acid concentrations[15]. COX2 is induced by cytokines or growth factors and thus contributes to the inflammatory states[16]. Bradykinin has previously been reported to induce COX2 expression in various cell types[17-18]. The pro-inflammatory mediator bradykinin stimulated COX2 expression and subsequently prostaglandin E2 (pGE2) synthesis in dermal fibroblasts[13, 19-20].

The present work aimed to investigate how the signals of bradykinin as an excitatory neurotransmitter on secretomotor neurons at the cellular neurophysiological level are transduced to the physiology of intestinal secretion at the level of the integrated system. As an inflammatory signaling molecule, pGE2 is released by bradykinin through B2R signaling activation and COX2 expression. Therefore, this study provides a theoretical reference for the clinical treatment of neurological diseases.

1 Materials and methods

1.1 Animal and tissue preparation

Adult male guinea pigs (Albino-Hartley, 300−600 g) were sacrificed by stunning followed immediately exsanguination from the cervical vessels according to procedures reviewed and approved by the Weifang Medical University Laboratory Animal Care and Use Committee and Weifang Second People's Hospital Laboratory Animal Care and Use Committee. A 10-cm piece of ileum was pinned flat with the mucosal side up to Sylgard 184 encapsulating resin (Dow Corning, Midland, MI, USA) in a dissection dish containing ice-cold Krebs solution. Fine forceps were used to remove the mucosa and expose the submucosal plexus. Submucosal plexuses preparations were prepared by carefully peeling away the mucosal layer with microdissection.

1.2 Immunohistochemistry

Whole-mounts of the submucosal plexus were incubated in 10% normal horse serum in PBS for 1 h at room temperature (RT) before exposure to the primary antisera diluted in hyper-tonic PBS containing 10% normal horse serum, 0.3% Triton X-100, and 0.1% sodium azide. The preparations were placed in humidified chambers and processed for indirect double immunofluorescence staining by incubation for 18 h at RT. The primary antibodies used were rabbit anti-COX2 (1∶500) (Abcam), mouse anti-HuC/HuD neuronal protein (1∶200) (Invitrogen), goat anti-choline acetyltransferase (ChAT) (1∶100) (Chemicon International), sheep anti-vasoactive intestinal peptide (VIP) (1∶100) (Peninsula Laboratories), sheep anti-B2R (B2R) (1∶100) (Chemicon International). The preparations were incubated for 24 h in the primary antibody at RT followed by washes with PBS and then incubated in FITC-labeled donkey anti-rabbit secondary IgG at RT for 1 h. Then they were incubated with Cy3-labeled

donkey anti-mouse secondary IgG, donkey anti-sheep secondary IgG, respectively, for another 1 h at RT. All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The preparations were cover-slipped in Vectorshield (Vector Laboratories, Burlingame, CA, USA). Fluorescence labeling was examined under a Nikon Eclipse 90i fluorescence microscope (Fryer Company, Cincinnati, OH). The specificity of the COX2 antibodies was tested by pre-absorbing the antibodies with corresponding blocking peptides provided by the manufacturers, negative controls consisted of omission of either the primary or the secondary antibodies. Double labeling of COX2 with other specific neurochemical markers was used to identify the cell types that express COX2.

1.3 Tissue culture

For these studies, all solutions were treated with 0.1% DEPC water and autoclaved. The guinea pig ileum submucosal plexuses preparations were prepared by carefully peeling away the mucosal layer with microdissection. The submucosal plexuses preparations were incubated in 2 ml Dulbecco's modified Eagle's medium (DMEM) with antibiotic antimycotic solution (final concentration contain 100 units/ml penicillin G, 100 mg/L streptomycin sulfate, and 0.25 mg/L amphotericin B), glucose (12 mmol/L) in a humidified 37°C, 5% CO_2 incubator before treated by bradykinin or other agents. In the time-dependent examination, 2 pieces of submucosal plexuses were incubated with bradykinin (100 μmol/L) for 0, 2, 4, 6, 8, 24 h before detecting the COX2 expression. To detect whether different concentrations of bradykinin affect the COX2 expression, 2 pieces of plexuses were incubated in bradykinin at the concentration of 0, 1 nmol/L, 10 nmol/L, 100 nmol/L, and 1 μmol/L respectively for 4 h.

To investigate the signal transduction of COX2 expression, the preparations of the submucosal plexuses of the guinea pig small intestine induced with bradykinin, B1R agonist bradykinin 1-8 (1 mmol/L), or the B2R agonist Hyp3-bradykinin (1 mmol/L) for 4 h. Other preparations were pre-incubated with the B1R antagonist Leu-8 (1 mmol/L) or B2R antagonist HOE-140 (1 mmol/L) 30 min followed by incubation with bradykinin (100 nmol/L) for 4 h. The tissues were harvested and stored at −80ºC for Western blot and RT-PCR.

1.4 Real−time PCR

Submucosal plexuses preparations, within its matrix of connective tissue, were dissected free and homogenized in Trizol (Invitrogen. Carlsbad, CA 92008, USA) with 100 mg of tissue per 1 ml Trizol. Total RNA was extracted according to the manufacturer's protocol and dissolved in diethylpyrocarbonate (DEPC)-treated water. The extracted RNA was treated with DNase and stored at −70ºC until use. RT-PCR was carried out with the SuperScript III Reverse Transcriptase (Invitrogen) in a 20 μl tube containing 2 μg of total RNA, 2 μl (10 mmol/L) dNTP mixture, 5 units RNase inhibitor (Invitrogen), 600 μmol/L random primer (Roche). Reverse transcription was performed according to the manufacturer's protocol. For PCR, 1 μg of the reverse-transcribed cDNA was placed in a tube that contained 2 μl of 2 mmol/L dNTP, 1XPCR buffer, 1 μl dithiothreitol (0.2 mmol/L), 200 μmol/L sense and antisense primers (Integrated DNA Technologies) mix in a final volume of 50 μl. The final products were run on 1% agarose gels. The primers used in the experiments included COX2, 5'-TGGTGGACTG-GAATCTTGAA, 3'-CTCTCAGTTGCTCCTGGTCA, and β-actin, 5'-AGTGTGACGTT-GACATCCGT, 3'-TGATCTTCATTGTGCTGGGT.

1.5 Western blot

Proteins (100 μg/well) of guinea pig submucosal plexuses preparations treated by bradykinin for different periods or different concentrations of bradykinin (Sigma) were prepared and separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes and the membranes were blocked with 5% non-fat dry milk. After blocking, the membranes were incubated with a polyclonal anti-COX2 antibody (1∶2 000, Cayman Chemicals, Ann Arbor, MI). The membranes were washed and then incubated with HRP-conjugated Goat anti-Rabbit IgG (1∶2 000), and then washed again. Pierce ECL Western blot substrate was prepared according to the manufacturers' instructions and added to the membranes for 1 min. The membranes were exposed to film for the 5 min.

1.6 Chemicals

Bradykinin acetate was purchased from Tocris (Ellisville, MO). Stock solutions were prepared in Krebs solution or deionized H_2O. Pharmacological agents were applied by either adding to the Krebs'

bathing solution.

1.7 Statistical analyses

Data were presented as $\bar{x}\pm s$. The student's *t*-test was used for statistical analysis of the significance of differences in the means with $P<0.05$ accepted as significant.

2 Results

2.1 COX2 protein localization in the guinea pig enteric nervous system

Under basal conditions, we observed COX2 immunostaining in the submucosal plexus of guinea pig (Figure 1a,b). COX2-IR was found in (69.7 ± 2.0)% of anti-Hu-IR; (90.2 ± 1.3)% of chAT neurons expressed COX2-IR (Table 1). The results suggested that COX2 is expressed abundantly in guinea pig submucosal plexuses in the enteric nervous system. The results also showed the colocalization of COX2 and B2R in the submucosal plexuses in ENS of the guinea pig ileum (Figure 1c).

Table 1 Distribution of COX2 to chemical codes in the guinea pig submucosal plexus

Chemical codes	[Cox2/chemical codes (cell numbers containing two colors)]/%
Anti-Hu	69.66 ± 2.45
chAT	90.21 ± 1.64

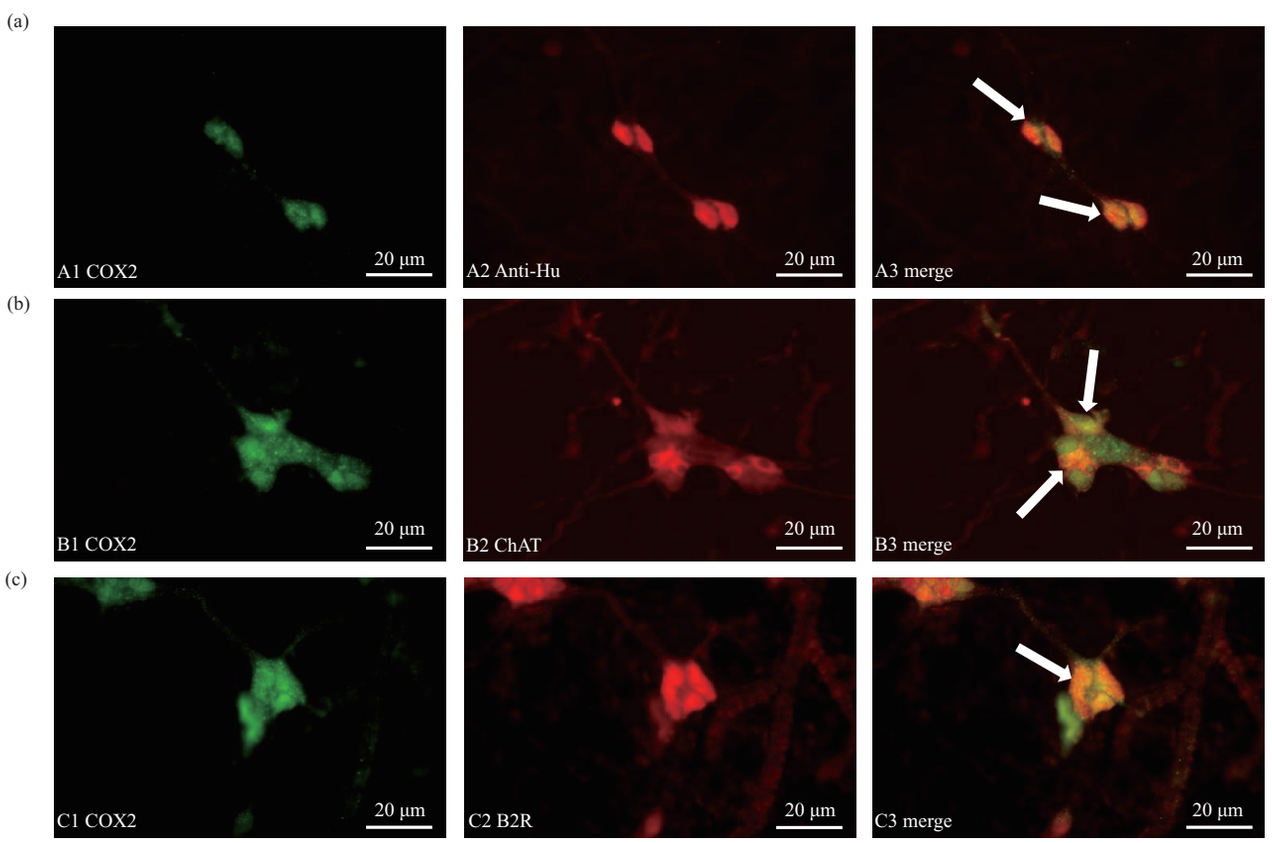

Fig. 1 Immunohistochemical demonstration of COX2 in the ENS

Neurochemical coding of COX2-IR in the submucosal plexus. COX2 was labeled with fluorescein isothiocyanate (FITC, green). The neuronal markers HuC/D (Hu), ChAT, and B2R were labeled with indocarbocyanine (Cy3, red). COX2-IR was colocalized with Hu (a), ChAT (b), and B2R (c). Arrows indicate neurons that are positive for COX2 as well as neurochemical markers.

2.2 COX2 mRNA and protein expression in submucosal plexuses induced by bradykinin treatment

A concentration-response curve for bradykinin was performed with concentrations from 1 nmol/L to 1 μmol/L bradykinin examined by semi-quantitative RT-PCR and Western blot (Figure 2). Incubation of submucosal plexus with bradykinin (1 nmol/L−

1 μmol/L) for 4 h increased COX2 mRNA expression in a concentration-dependent manner, reaching the highest at 10^{-7} mol/L (Figure 2a). The expression level was (3.56±0.82) fold increased when incubated with bradykinin at 100 nmol/L compared to the control, while (1.68±0.45) folds, (2.45±0.25) folds, and (2.16±0.15) folds increased at 1 nmol/L, 10 nmol/L, and 1 μmol/L bradykinin concentration compared to the control, respectively. Figure 2b showed a similar concentration-dependent change of COX2 mRNA expression induced by bradykinin detected by the qRT-PCR. The change of COX2 protein level induced by bradykinin exhibited similar results as the mRNA expression (Figure 2c, d).

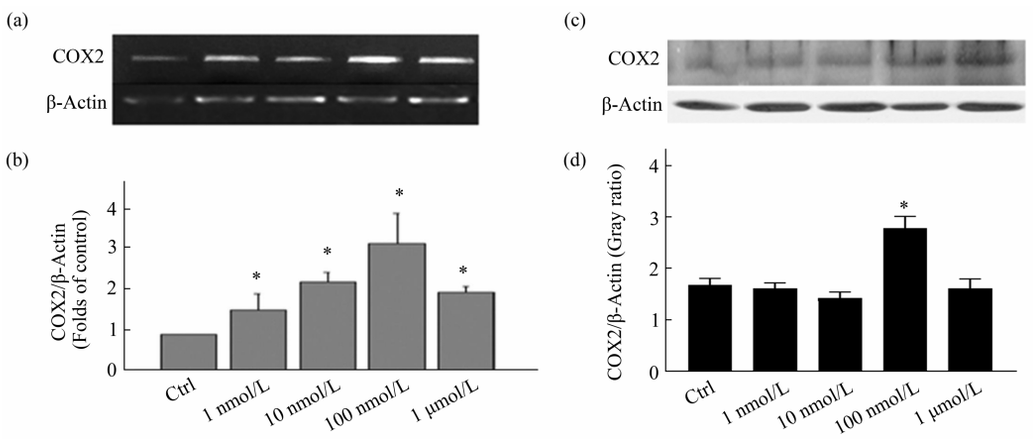

Fig. 2 Concentration dependence of bradykinin induced COX2 mRNA expression and protein expression in the submucosal plexuses incubated with various concentrations of bradykinin for 4 h

(a) Semi-quantitative RT-PCR detected a pattern of concentration-response of bradykinin (1 nmol/L−1 μmol/L) induced COX2 mRNA expression for 4 h in submucosal plexuses. (b) Statistical analysis of (a). (c) Western blot showing concentration-dependent COX2 protein induction by bradykinin (100 nmol/L) in the submucosal plexuses. β-actin was used as an internal control. (d) Statistical analysis of (c). Bars represent $\bar{x}\pm s$ for 3 experiments. * $P < 0.05$ *vs*. control.

2.3 The submucosal plexuses incubated with bradykinin stimulated the expression of COX2 in a time−dependent manner

Based on the above study, we used 100 nmol/L of bradykinin treatment for the following experiments. The time course of bradykinin induced COX2 mRNA expression and protein level in the submucosal plexuses of the guinea pig small intestine was detected by RT-PCR, and Western blot, respectively. COX2 mRNA expression by bradykinin (100 nmol/L) treatment in the submucosal plexus was detected at 0, 2, 4, 6, 8, and 24 h. The highest COX2 mRNA expression was detected at 4 h of incubation with bradykinin with (5.64±1.91) folds of increase compared to the control sample using β-actin as an internal reference. COX2 mRNA expression was increased to (1.40±0.33) folds, (4.76±0.51) folds, (4.90±1.24) folds, and (2.82±1.54) folds at 2, 6, 8, and 24 h, respectively (Figure 3a, b).

Western blot analysis showed that bradykinin induced COX2 protein expression patterns were similar to the mRNA expression (Figure 3c, d).

2.4 Bradykinin induced COX2 expression is mediated through B2R but not B1R in the enteric nervous system

COX2 mRNA expression and protein level were determined with semi-quantitative RT-PCR (Figure 4a, b) and Western blot (Figure 4c,d). Bradykinin induced COX2 mRNA (Figure 4a,b) and protein (Figure 4c,d) expression in the submucosal plexus was unaffected by the B1 receptor antagonist Leu-8 but was suppressed by the B2 receptor antagonist HOE-140. B1 receptor agonist bradykinin 1-8 did not increase COX2 expression, but B2 receptor agonist Hyp3-bradykinin stimulated COX2 expression. The results indicated that bradykinin induced COX2 expression is mediated through B2R but not B1R in the enteric nervous system.

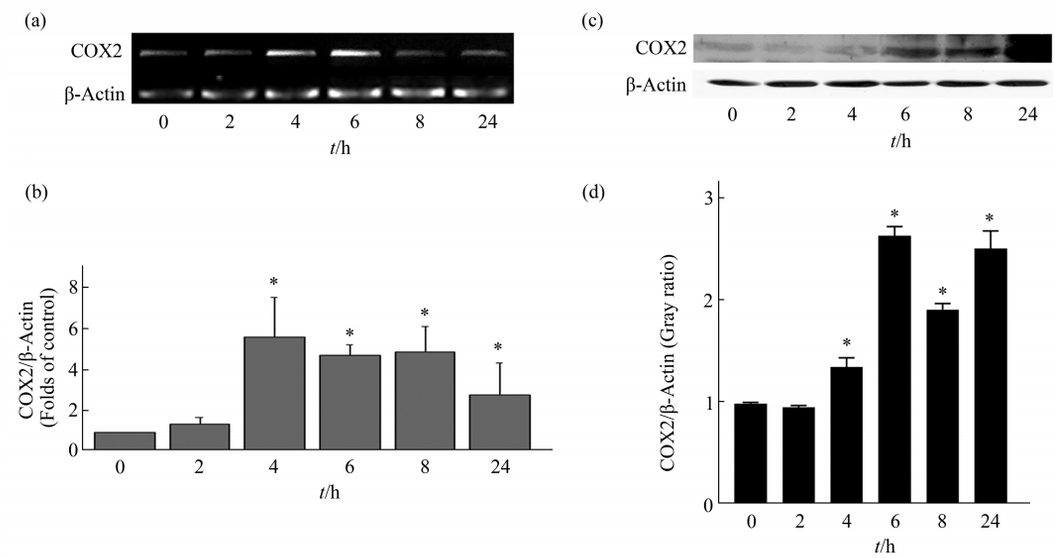

Fig. 3　Time course of bradykinin induced COX2 mRNA expression in the submucosal plexuses of the guinea pig small intestine

(a) Semi-quantitative RT-PCR found a time-dependent pattern of COX2 mRNA expression induced by bradykinin (100 nmol/L) in the submucosal plexuses at 0, 2, 4, 6, 8, and 24 h (n=3). (b) Statistical analysis of (a). (c) Western blot showing time-dependent COX2 protein induction by bradykinin (100 nmol/L) in the submucosal plexuses. β-Actin was used as an internal control. (d) Statistical analysis of (c). Bars represent $\bar{x}\pm s$ for 3 experiments. * $P < 0.05$ *vs*. control.

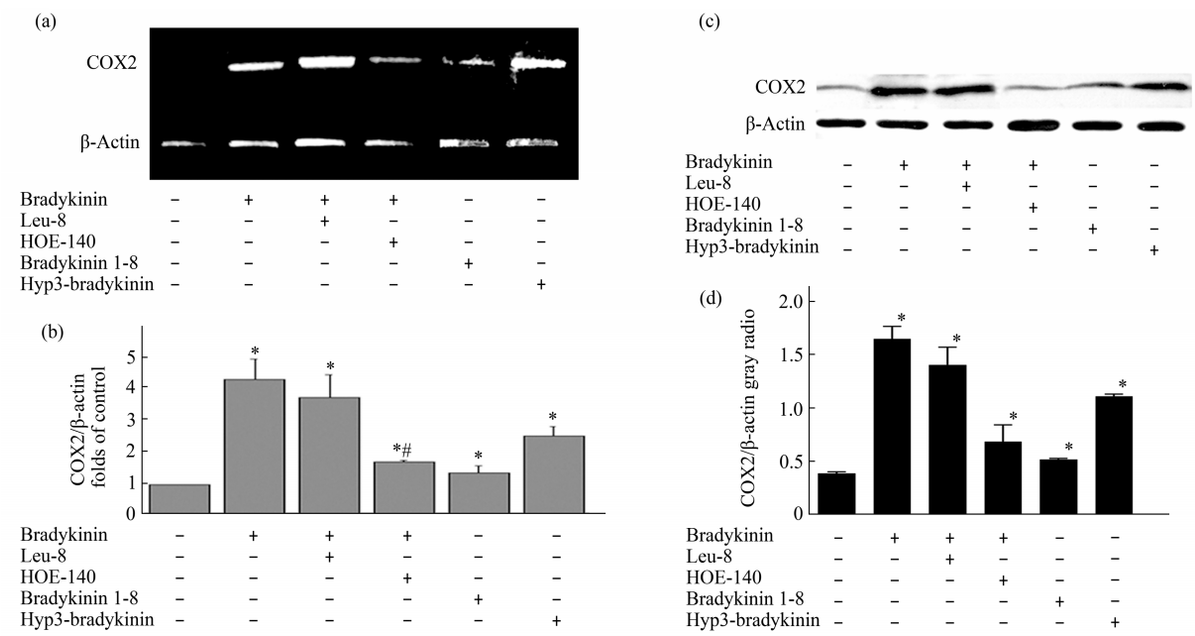

Fig. 4　Bradykinin−induced COX2 expression is mediated through B2 but not B1 bradykinin receptors in the enteric nervous system

(a) Semi-quantitative RT-PCR found a time-dependent pattern of COX2 mRNA expression induced by bradykinin (100 nmol/L) with B1R antagonist Leu-8 (1 μmol/L) or the B2R antagonist HOE-140 (1 μmol/L) or B1R agonist bradykinin1-8 (1 μmol/L) or the B2R agonist Hyp3-bradykinin (1 μmol/L) for 4 h. (b) Statistical analysis of (a). (c) Western blot showing expression of COX2 under (a) condition. β-Actin was used as an internal control. (d) Statistical analysis of (c). Bars represent $\bar{x}\pm s$ for 3 experiments. *$P < 0.05$ *vs*. control, $^{\#}P < 0.05$ *vs*. bradykinin.

2.5 Bradykinin induced pGE2 production was mediated by COX2

COX catalyzes the release of arachidonic acid from membrane phospholipids by phospholipase A2 to form pGE2[21]. Bradykinin induced pGE2 releasing was blocked by COX2 inhibitor NS398, but not COX1 inhibitor FR12207 in the submucosal plexuses of the guinea pig small intestine. Preparations of the submucosal and submucosal plexuses were pre-incubated for 30 min with the COX1 inhibitor, FR12207 (1 mmol/L) or the COX2 inhibitor, NS398 (1 mmol/L), followed by incubation with bradykinin (100 nmol/L) for 4 h. pGE2 was analyzed using ELISA kits. The results showed that COX2 inhibitor NS398 significantly decreased the bradykinin evoked releasing of pGE2 (Figure 5a) in the submucosal plexuses. Diagram described a signaling pathway in this study (Figure 5b).

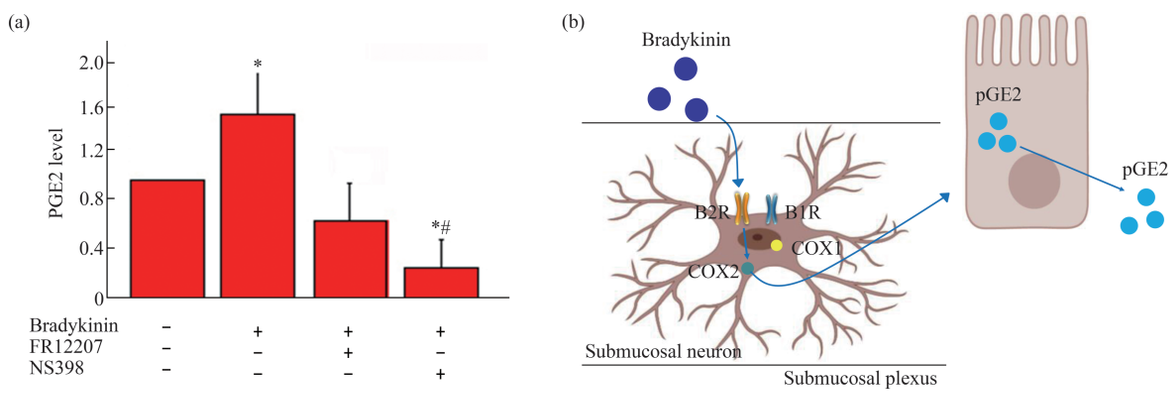

Fig. 5　Inhibition of bradykinin induced pGE2 production by COX1 or COX2 inhibitors in the submucosal plexuses

(a) Preparations of the submucosal plexuses were pre-incubated with the COX1 inhibitor, FR12207 (1 μmol/L) or the COX2 inhibitor, NS398 (1 μmol/L) for 30 min, followed by incubation with bradykinin (100 nmol/L) for 4 h. pGE2 was analyzed using specific ELISA kits. (b) Summary diagram of signaling pathway of this study. Results are expressed as $\bar{x}\pm s$ of 3 independent samples, each assayed in duplicate. $^{*}P < 0.05$ *vs.* control. $^{\#}P < 0.05$ *vs.* bradykinin.

3 Discussion

The enteric immune/inflammatory system communicates with the ENS through the release of chemical mediators, including bradykinin, histamine, prostaglandins, and cytokines[22]. Bradykinin belongs to the Kallikrein-kinin system (KKS), which is widely distributed in the ENS and plays an important regulatory role in various pathological processes and physiological functions. Hu *et al.* [23] showed that bradykinin stimulates enteric muscle neurons of guinea pigs. The cellular effects of kallidin and bradykinin are mediated by two receptors B1R, B2R. B2R is constitutively expressed in a variety of tissues and shows a higher affinity to kinins[24]. Our previous results showed that bradykinin stimulates neurogenic chloride secretion in the guinea pig ileum by activating B2R and increasing pGE2 production[25]. COX2, known as PGs-endoperoxide synthase, can be induced by various stimuli in a variety of tissues to promote the biosynthesis of PGs, pGE2 especially, during the inflammatory response of multiple cell types[26-27]. Bradykinin regulates the activity and expression of COX2 through different mechanisms in a variety of cell types including astrocytes[28-29]. This study aimed to reveal the signaling pathway of bradykinin evoking pEG2 releasing in the submucosal plexuses of guinea pig ileum. The current study showed the colocalization of COX2 with B2R and neuron marker Anti-Hu, ChAT in submucosal plexuses of guinea pigs. Incubated with bradykinin, the submucosal plexuses showed an increased COX2 expression evoked by bradykinin in a time- and concentration-dependent manner.

Amann *et al.* [30] showed that pGE2 released rapidly after bradykinin stimulating and kept high for at least 3 h from sections of guinea pig ileum tissue *in vitro*. As the key enzyme in the synthesis of prostaglandins from arachidonic acid, COX was regarded as an inflammation marker and drug target

for inflammatory diseases. The two COX isoforms, COX1 and COX2, are reported expressing in neuronal and glial cells responsible for the production of the pGE2 [31]. In this study, we validated the bradykinin/pGE2 signaling pathway in the submucosal plexuses in the guinea pig ileum. As shown in Figure 5b, bradykinin promoted the expression of COX2 through B2R in the submucosal plexuses in the ileum of guinea pig. This was further supported by the expression of COX2 stimulated by bradykinin was blocked by B2R antagonist HOE-140, but not B1R antagonist Leu-8. The secretion of pGE2 in the intestine by bradykinin evoking was blocked by COX2 antagonists other than COX1 antagonists, which implied COX2 was the key factor in pGEs releasing.

4 Conclusion

In summary, we showed that bradykinin induced COX2 protein expression in the submucosal plexuses, which activated COX2/PGE2 signal pathway through B2R. This study revealed the downstream of B2R signaling in the submucosal plexuses and the relationship of pGE2 releasing the COX2 expression, providing targets in the anti-inflammation drug research in the future.

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缓激肽上调豚鼠肠道黏膜下神经丛环氧合酶2的表达*

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摘要　目的　缓激肽和缓激肽B2受体在肠神经系统中起重要作用。缓激肽通常参与肠道的炎症反应和神经保护，这种作用取决于缓激肽诱导前列腺素的形成。环氧合酶1（COX1）和环氧合酶2（COX2）催化花生四烯酸转化为前列腺素。本研究旨在探讨缓激肽刺激对豚鼠肠神经前列腺素E2（pGE2）释放和COX2表达的影响及信号机制。**方法**　本文通过免疫荧光检测肠神经细胞中COX2与神经细胞标志物Anti-Hu和chAT的表达；采用PCR及蛋白质印迹（Western blot）检测不同条件下缓激肽刺激对COX2表达的影响；使用缓激肽B1受体的选择性拮抗剂Leu-8和B2受体的选择性拮抗剂HOE-140，研究缓激肽影响COX2表达的信号机制；利用COX2选择性拮抗剂NS398和COX1拮抗剂FR12207，观察COX2在缓激肽诱导pEG2释放的作用。**结果**　COX2与神经细胞标志物Anti-Hu和chAT在肠神经细胞上共同表达，缓激肽可通过B2受体诱导肠神经细胞COX2的表达。缓激肽刺激引起的肠神经细胞pGE2的释放与COX2表达升高密切相关。**结论**　缓激肽通过B2R影响肠道黏膜下神经丛COX2的表达，肠道缓激肽刺激引起pGE2释放与肠神经COX2信号激活有关。这为缓激肽在肠道炎症疾病中的作用提供了合理的解释。

关键词　缓激肽，环氧合酶2，缓激肽B2受体，前列腺素E2，肠神经系统
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