PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BRIEF REPORTS •

Cyclooxygenase 2, pS2, inducible nitric oxide synthase and transforming growth factor alpha in gastric adaptation to stress

Shi-Nan Nie, Hai-Chen Sun, Xue-Hao Wu, Xiao-Ming Qian

Shi-Nan Nie, Hai-Chen Sun, Xue-Hao Wu, Xiao-Ming Qian, Emergency Department, Nanjing General Hospital of Nanjing PLA Command Area/Clinical School of Medical college of Nanjing University, Nanjing 210002, Jiangsu Province, China
Correspondence to: Shi-Nan Nie, M.D., Emergency Department, Nanjing General Hospital of Nanjing PLA Command Area, 305 Eastern Zhongshan Road, Nanjing 210002, Jiangsu Province, China. shnnie630504@.sohu.com
Telephone: +86-25-80860143
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Abstract

AIM: To determine the role of mucosal gene expression of cyclooxygenase 2 (COX-2), pS_2 (belongs to trefoil peptides), inducible nitric oxide synthase (iNOS) and transforming growth factor alpha (TGFα) in gastric adaptation to water immersion and restraint stress (WRS) in rats.

METHODS: Wistar rats were exposed to single or repeated WRS for 4 h every other day for up to 6 d. Gastric mucosal blood flow (GMBF) was measured by laser Doppler flowmeter-3. The extent of gastric mucosal lesions were evaluated grossly and histologically and expressions of COX-2, pS2, iNOS and TGFα were determined by reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot.

RESULTS: The damage to the surface of gastric epithelium with focal areas of deep haemorrhagic necrosis was induced by repeated WRS.The adaptative cytoprotection against stress was developed with activation of cell proliferation in the neck regions of gastric glands. The ulcer index (UI) in groups II, III and IV was markedly reduced as compared with group I (I: 47.23±1.20; IV: 10.39±1.18, $P<0.01$). GMBF significantly decreased after first exposure to WRS with an adaptive increasement of GMBF in experimental groups after repetitive challenges with WRS. After the 4th WRS, the value of GMBF almost restored to normal level (I: 321.87±8.85; IV: 455.95±11.81, $P<0.01$). First WRS significantly decreased the expression of pS2 and significantly increased the expressions of COX-2, iNOS and TGFα. After repeated WRS, pS2 and TGFα expressions gradually increased (pS2: I: 0.37±0.02; IV: 0.77±0.01; TGFα: I: 0.86±0.01; IV: 0.93±0.03, $P<0.05$) with a decrease in the expressions of COX-2 and iNOS (COX-2: I: 0.45±0.02; IV: 0.22±0.01; iNOS: I: 0.93±0.01; IV: 0.56±0.01, $P<0.01$). Expressions of pS2, COX-2, iNOS and TGFα showed regular changes with a good relationship among them.

CONCLUSION: Gastric adaptation to WRS injury involves enhanced cell proliferation,increased expression of pS2 and TGFα, and reduced expression of COX-2 and iNOS. These changes play an important role in adaptation of gastric mucosa after repeated WRS.

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INTRODUCTION

Gastric mucosa can enhance resistance to injury after exposure to repeated insults of noxious agents,such as aspirin, alcohol, stress or *H pylori*-related gastrotoxins. This phenomenon is called gastric adaptation[1]. It has been postulated that gastric adaptation involves enhancement of gastric blood flow and increased mucosal cell proliferation mediated by some growth factors,such as epidermal growth factor (EGF) or TGFα. The fact that the adaptation to stress is associated with increased cell proliferation let us to hypothesize that this process might be mediated by pS2, COX-2, iNOS and TGFα.

It is now well established that trefoil peptides have cytoprotective functions in protecting the gastrointestinal tract against ongoing damage from agents as diverse as ethanol, non-steroidal anti-inflammatory drugs and restraint stress[2]. The mechanism for this action is unclear. The pronounced and protracted increase in trefoil peptide expression in the inflamed and ulcerated stomach, intestine and colon implicates peptides in reparative processes of the injury gut. Studies reported that pS2 took part in the protracted phase of glandular renewal (marked by proliferation, differentiation and migration) [3]. TGFα, a 50 amino acid polypeptide produced in normal gastric mucosa, promotes cell proliferation, inhibits gastric acid secretion and exhibits gastroprotective activity against acute damage induced by topical irritants or stress[4]. In addition, an increased TGFα mRNA expression has been detected during healing of chronic experimental ulcers and acute damage of gastric mucosa in rats, suggesting its important role in gastric mucosal repair. Previous studies showed that COX-2 was an inducible enzyme. Recently, studies have suggested that COX-2 is a constitutive enzyme expressed in gastrointestinal tract also, even plays a more important role than COX-1 for mucosal integrity[5]. Gut epithelial COX-2 is rapidly induced by inflammatory stimuli, interleukin and TGFα. Suppression of COX-2 could result in exacerbation of inflammation-associated colonic injury, and impair the healing of gastric ulcer. It is generally accepted that nitric oxide (NO) plays an important role in gastric ulcer healing. NO production is highly increased by iNOS.

The aim of the present study was to determine the role of the expression of pS2, COX-2, iNOS and TGFα, and the relationship among them in gastric adaptation to WRS in rats.

MATERIALS AND METHODS

Induction of gastric adaptation to WRS

Thirty male Wistar rats, weighing 210-250 g and fasted for 24 h with free access to water, were used. The animals were deprived of water 1 h before the experiment and devided into: normal control group ($n = 6$) and experimental control group ($n = 24$). After fasted for 24 h, the rats of normal control group were lightly anesthetized with ether and tied up on the rat board, the abdomen was opened, the stomach was exposed and GMBF

was measured in the oxyntic gland area, and then gastric mucosa was sampled. The rats of experimental control group were devided into four subgroups (n = 6 in each group) and exposed to repeated WRS[3]. The rats of group I were lightly anesthetized with ether, tied up on the rat board and exposed to WRS for 4 h by placing in the water at 20-23 ℃ to the rat's xyphoid level at 10:00 am on the 1st d. Then the rats were anesthetized with pentobarbital (30 mg/kg ip), GMBF was measured and gastric mucosa was sampled. The rats of group II were treated similarly except that after WRS they were removed from the water, placed at room temprature, and refed with food and water until 10:00 am on the next day, at which time they were starved again for 24 h, and WRS was repeated. The rats of groups III and IV were exposed to the 3rd or 4th WRS as described above.

Measurement of GMBF

GMBF was measured by using laser Doppler flowmetry (LDF-3 flowmeter, Nankai University, Tianjin, China). In brief, the rats were anesthetized with pentobarbital (30 mg/kg ip), the abdomen was opened, the stomach was exposed and transected, and the gastric contents were gently evacuated to the exterior through the cut made in the stomach. Then, an optical probe was placed gently 0.5 mm above and perpendicular to the mucosal surface in the oxyntic gland area to monitor GMBF displayed in mV (value of Doppler signal voltage) on the digital panel of the flowmeter. After GMBF was stable, four points were selected for measurement (one point for 1 min) and the average value was calculated and expressed as *U/mV*.

Appreciation of UI

Mucosal lesions were evaluated by the score systems reported by Nie *et al*.[3]. Briefly, after the measurement of GMBF, the stomach was dissected out and opened along the greater curvature, then examined with a 10× magnifier for the presence of erosions and scored as follows: 1 point for small round hemorrhagic erosions; 2 points when the length of hemorrhagic erosions was less than 1 mm; 3 points when the length was 1-2 mm; 4 points when the length was 2-3 mm; 5 points when the length was longer than 4 mm; and the score value multiplied 2 when the width of erosions was larger than 1 mm.

Detection of mRNA in pS2,COX-2, iNOS and TGFα by RT-PCR

The stomachs were removed from rats with intact gastric mucosa and from those exposed to a single stress or repeated stresses. Mucosal specimens (about 100 mg) were scraped off using a slide glass and immediately snap frozen in liquid nitrogen and stored at -80 ℃ until analysis. Total RNA was isolated from mucosal samples using a guanidium isothiocyanate/phenol chloroform single step extraction kit from Stratagene (Gibco BRL, USA). Following precipitation, the RNA was resuspended in RNAse-free buffer and the concentration was estimated by absorbance at 260 nm wavelength. Furthermore, the quality of each RNA sample was determined by running the agarose fomaldehyde electrophoresis. RNA samples were stored at -80 ℃ until analysis.

Single-stranded complementary DNA (cDNA) was generated from 5 μg of total cellular RNA using StrataScript™ reverse transcripase (Gibco BRL, USA) and oligo (dT) primers (Gibco BRL, USA). Briefly, 5 μg of total RNA was used as the template to synthesize complementary DNA with 2.5 units of Maloney murine leukemia virus reverse transcriptase in 5 μL of buffer containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 5 mmol/L $MgCl_2$,1 mmol/L of each deoxyribonucleaside triphospate, 2.5 mmol/L of oligo (dt) primers and 1.4 U/μL RNase blocker. Reverse-transcription was performed at room temperature for 20 min, then at 37 ℃ for 15 min, at 90 ℃ for 5 min and at 5 ℃ for min. The resulting cDNA was used as a template for subsequent PCR.

A 124-base pair (bp) fragment of pS2 was amplified from single-stranded DNA by PCR using two oligonucletide primers to pS2 sequence: sense primer, 5'-CCATGGAGCACAAGGTGA CCTG-3' and antisense primer, 5'-GGGAAGCCACAATTTAT TCT-3'. A 230-base pair (bp) fragment of COX-2 was amplified from single-stranded DNA by PCR using two oligonucletide primers to COX-2 sequence: sense primer, 5'-GCCACCTCTGCG ATGCTCTT-3' and antisense primer, 5'-GTGTTTGGGGTGGGC TTCAG-3'. A 576-base pair (bp) fragment of iNOS was amplified from single-stranded DNA by PCR using two oligonucletide primers to iNOS sequence: sense primer, 5'-GTGTTCCACCAGG AGATGTTG-3' and antisense primer, 5'-CTCCTGCCCACTGA GTTCGTC-3'. A 246-base pair (bp) fragment of TGFα was amplified from single-stranded DNA by PCR using two oligonucletide primers to TGFα sequence: sense primer, 5'-TCTGGGTACGTGGGTGTTCG-3'and antisense primer, 5'-AGAGTGGCAGCAGGCAGTCC-3'. Concomitantly, amplification of the 521 bp fragment of rat β-actin was performed on the same RNA samples to assess RNA integrity using two oligonucletide primers to β-actin sequence: sense primer, 5'-TGGGACGATATGGAGAAGAT-3' and antisense primer, 5'-ATTGCCGATAGTGATGACCT-3'. The nucleotide sequences of the primers for pS2, COX-2, iNOS and TGFα were based on the published cDNA sequences encoding pS2, COX-2, iNOS and TGFα[3,6,7]. The primers were synthesized by Bo-Ya Biotechnical Co. Ltd, Shanghai, China.

Reaction mixture for PCR contained cDNA template (2 μL), 50 pmoL of each primer, and 2.5 U of Termus aquaticus DNA (Promega) in 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L dNTPs in a volume of 50 μL. RT blanks (no RNA included) were incubated in each analysis. The mixture was overlaid with 25 μL of mineral oil to prevent evaporation. Amplification was performed using a DNA thermal cycler for 35 cycles, each cycle consisting of denaturation for 2 min at 94 ℃, annealing for 45 s at 55 ℃ (pS2), 52 ℃ (COX-2 , iNOS) and 60 ℃ (TGFα), and extension for 1 min at 72 ℃. The final cycle included an extension for 5 min at 72 ℃ to ensure a full extension of the product. The number of amplification cycles was previously determined to keep amplification in the linear to avoid the "plateau effect" associated with increased number of PCR cycles.Eight microliters of each PCR product was electrophoresed on 16 g/L agarose gel stained with ethidium bromide, and then visualized under *uv* light. Location of predicted PCR products was confirmed by using DNA digest phix 174/Hae III as a stained size marker. The gel was then photographed under *uv* transillumination. In addition to size analysis by agarose gel electrophoresis, specificity of the primer pairs for pS2, COX-2, iNOS and TGFα was assessed by sequencing PCR products. For quantification, we determined the intensity of PCR products on the negative film of gel photographs according to Morrissey *et al*.[6]. Expression of the products was quantified using a video image analysis system (Tanon GIS-1000, Tanon Technical Co, Ltd, Shanghai, China). An index of mRNA expression was determined in each sample according to Konturek *et al*.[8].

Western blot analysis of pS2, COX-2, iNOS and TGFα proteins

Gastric tissues for the analysis of protein expressions of pS2, COX-2, iNOS and TGFα were homogenized in a proteinase inhibitor buffer containing 50 mmol/L Tris HCl (pH 7.5), 150 mmol/L NaCl, 5 g/L β-cholate sodium, 1 g/L SDS, 2 mmol/L EDTA, 10 mL/L Triton X-100, 100 g/L glycerol, 1 mmol/L PMSF and aprotinin, and then centrifuged at 10 000 *g* for 15 min at 4 ℃. The supernatant was collected and the protein content was determined with the dye-binding (Bio-Rad) method. Thirty micrograms of total protein was loaded onto SDS-polyacrylamide

gel and blotted onto hybrid C membranes (Amersham Life Science, Little Chalfont, Buckinghamshire, England) by electrophoresis. Pre-stained rainbow recombinant protein molecular mass markers (Amersham International plc, Little Chalfont, Buckinghamshire, England) were used for molecular mass determinations. Membranes were blocked with blocking buffer containing 50 g/L fat free milk powder, 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl and 1 mL/L Tween 20 for 1 h at room temperature. The blots were incubated overnight at 4 ℃ with 1:1 000 dilution of polycolonal antibodies against pS2 and TGFα (Stress-Gen, Victoria, Canada), monoclonal antibody against iNOS (Transduction Lab, Lexington, Kentucky, USA), polyclonal antibody against COX-2 (Santa Cruz Biotechnology INC, Santa Cruz, California, USA). After washed in washing buffer for 30 min, the membranes were treated with HRP conjugated secondary antibody (1:4 000 dilution) (Bio-Rad) for 1 h at room temperature, followed by another 30 min of washing. The ECL Western blotting system (Amersham Life Sciences) was used in accordance with the manufacturer's instructions for chemiluminescence of proteins, and the blots were then exposed to photographic films.

Statistical analysis

Results were expressed as mean±SD. Statistical comparisons were made by Student's *t* test. Linear correlation analysis was used to analyse the relationship between two variants. *P* values less than 0.05 were considered statistically significant.

RESULTS

Damage to the surface of gastric epithelium with focal areas of deep haemorrhagic necrosis was induced by repeated WRS. The adaptative cytoprotection against stress was developed with activation of cell proliferation in the neck regions of gastric glands. The UI in groups II, III and IV was markedly reduced as compared with group I (I: 47.23±1.20; IV: 10.39±1.18, $P<0.01$). GMBF significantly decreased after the first exposure to WRS with a adaptive increasement of GMBF in experimental groups after repetitive challenges with WRS. After the 4th WRS, the value of GMBF almost restored to normal level (I: 321.87±8.85; IV: 455.95±11.81, $P<0.01$). The first WRS significantly decreased the expression of pS2 and significantly increased the expression of COX-2, iNOS and TGFα. After repeated WRS, pS2 and TGFα expressions gradually increased (pS2: I: 0.37±0.02; IV: 0.77±0.01; TGFα: I: 0.86±0.01; IV: 0.93±0.03, $P<0.05$) with a decrease in the expression of COX-2 and iNOS (COX-2: I: 0.45±0.02; IV: 0.22±0.01; iNOS: I: 0.93±0.01; IV: 0.56±0.01, $P<0.01$). The expressions of pS2, COX-2, iNOS and TGFα showed regular changes with a good relationship among them (decrease of COX-2 and iNOS was accompanied by an increased expression of pS2 and TGFα after 4 consecutive WRS). (Figure 1,Table 1).

DISCUSSION

The cytoprotective functions of pS2, COX-2, iNOS and TGFα in the gastrointestinal tract against ongoing damage may be accomplished in several ways, and there evidence is that thes factors participate in both the early phase of epithelial repair known as restitution (marked by increased cell migration but no proliferation), and in the subsequent protracted phase of glandular renewal (marked by proliferation, differentiation and migration)[9-11].

In this study, when WRS was applied once,it produced numerous gastric mucosal erosions, the adaptative cytoprotection against stress was developed after repeated stresses,and mucosal lesions were reduced markedly after the 2nd, 3rd and

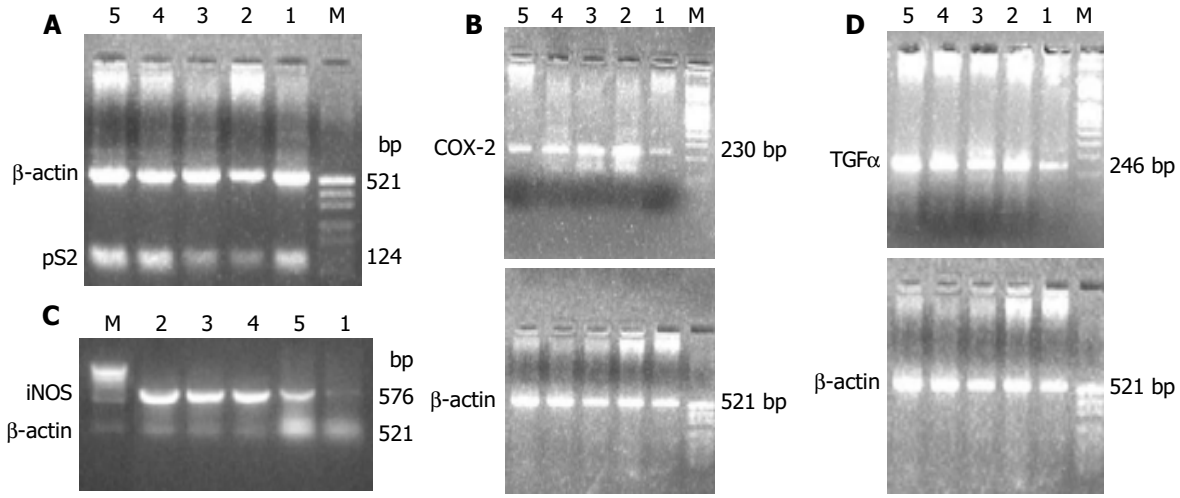

Figure 1 Expression of pS2 mRNA and β-actin (A), COX-2 mRNA and β-actin (B), iNOS mRNA and β-actin (C),TGFα mRNA and β-actin (D) in gastric mucosa of rats after repeated exposure to WRS and in control intact rats. Lane M: PCR size marker; Lane 1: control group; Lane 2-5: groups I-IV.

Table 1 Changes of gene expression of pS2, COX-2, iNOS, TGFα and GMBF, UI in gastric mucosa after repeated exposure to WRS

Group	GMBF (*U*/mV)	UI	iNOS	pS2	TGFα	COX-2
Control	484.01±10.97	0.00	0.16±0.01	0.63±0.01	0.26±0.01	0.10±0.01
Experimental						
I	321.87±8.85^{b}	47.23±1.20	0.93±0.01^{b}	0.37±0.02^{b}	0.86±0.01^{a}	0.45±0.02^{b}
II	418.35±7.94^{bd}	30.54±1.12^{d}	0.78±0.01^{bd}	0.42±0.01^{bd}	0.87±0.02^{ad}	0.38±0.02^{bd}
III	446.09±10.98^{bd}	20.75±1.54^{d}	0.67±0.02^{bd}	0.72±0.02^{bd}	0.88±0.01^{ad}	0.29±0.01^{bd}
IV	455.95±11.81^{bd}	10.39±1.18^{d}	0.56±0.01^{bd}	0.77±0.01^{bd}	0.93±0.03^{bc}	0.22±0.01^{bd}

$^{a}P<0.05$, $^{b}P<0.01$ *vs* experimental control group; $^{c}P<0.05$, $^{d}P<0.01$ *vs* group I.

4th WRS. The expressions of COX-2, iNOS and TGFα were all up-regulated, but that of pS2 was down-regulated after the first stress in rats with WRS-induced ulcers. But the major finding of this study was the gastric adaptation to WRS involving a gradually decrease of overexpression of COX-2 and iNOS, and a gradually increase of expression of pS2 and TGFα, and an increased rate of cell proliferation in the gastric mucosa.

Members of the trefoil peptide family, including pS2, share a common structural feature, which is a motif of six cysteine residues termed a trefoil or a P domain. There is increasing evidence that trefoil peptides are important in maintaining the integrity of gastric mucosae and involved in the repair of ulcerated areas in the gastrointestinal tract[12-16]. This is supported by an observation that an increased expression of trefoil peptides was found in the ulcer-associated cell lineage (UACL), which is a glandular structure adjacent to the ulcerated mucosa[17], and by the findings from *in vitro* studies showing that trefoil peptides exhibited a mitogenic effect on different cell lines[18,19]. It has also been reported that the trefoil peptide family could contribute to gastric mucosal defence and repair by affecting cell proliferation[1,20].

It has been found that NO produced from inflammatory cells by the inducible isoforms of NOS has antimicrobial, antitumor and cytotoxic effects, but an excessive amount may lead to peroxynitrite formation, protein tyrosine nitration, hydroxyl radical production and tissue damage[21]. The present study also demonstrated that overexpression of iNOS on d 1 after the first stress was accompanied with the enlargement of ulcer crater. The expression of iNOS protein was declined when the ulcer began to heal. High expression and enzymatic activity of COX at the late ulcer healing stage were observed in stress. The changes of COX activity might be mainly due to the changes of protein level and activity of COX-2. Since tissue remodeling including reepithelization of gastric mucosa, maturation of granulation tissue, and reconstruction of extracellular matrix mainly occurs at the late ulcer healing stage, COX-2 plays an essential role in these remodeling processes[5,22-31].

There may be interactions among pS2, COX-2, iNOS and TGFα. In the present study, after the 4th WRS, GMBF almost restored to normal level, and during the process of tolerant cytoprotection, GMBF, UI and expressions of pS2, COX-2, iNOS and TGFα showed regular changes and a good relationship among them. A dramatic decrease of COX-2 and iNOS was accompanied with an increase expression of pS2 and TGFα after the 4th stress. This inverse relationship between COX-2, iNOS and pS2, TGFα expressions support the existence of a close interaction among these factors.

Trefoil peptides are a new class of regulatory peptides involving mucosal protection and repair in the gastrointestinal tract. NO and epithelium-associated mucin have important roles in sustaining mucosal integrity in the gastrointestinal tract,and trefoil peptide modulate epithelial NO production via the iNOS pathway[32-35].

Treatment with COX-2 inhibitors and COX-2 antisense oligonucleotides could suppress these responses induced by TGFα, suggesting the involvement of COX-2 in proliferation of gastric mucosal epithelium,and the synergistic stimulation of COX-2 expression by TGFα. Gut epithelial COX-2 could be rapidly induced by inflammatory stimuli, interleukin and TGFα[4,36-40].

We confirmed that WRS-adapted mucosae exhibited an augment action of GMBF, but it is not clear whether COX-2, iNOS and pS2 could directly or indirectly account for the mucosal adaptation, or what is the mechanism of this mucosal hyperemia in the stomach. TGFα has been shown to increase GMBF[41], while trefoil peptides could promote synthesis of TGFα[42-48]. Hence, we believe that hyperaemia observed during the development of adaptation might be mediated by the release of COX-2, iNOS and pS2.

In summary, different expressions of pS2, COX-2, iNOS and TGFα occur during gastric ulceration and healing. COX-2 and iNOS may contribute to tissue inflammation during ulcer formation, while pS2 and TGFα may promote ulcer healing by proliferation, since their expressions are correlated with the protracted phase of glandular renewal of ulcer tissues.

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