Estrogen and isoflavone attenuate stress-induced gastric mucosal injury by inhibiting decreases in gastric tissue levels of CGRP in ovariectomized rats

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Shimozawa N, Okajima K, Harada N. Estrogen and isoflavone attenuate stress-induced gastric mucosal injury by inhibiting decreases in gastric tissue levels of CGRP in ovariectomized rats. Am J Physiol Gastrointest Liver Physiol 292: G615–G619, 2007. First published October 5, 2006; doi:10.1152/ajpgi.00117.2006.—We have previously reported that CGRP plays a critical role in the reduction of stress-induced gastric mucosal injury by increasing gastric prostacyclin (PGI2) levels in rats. Estrogen has been shown to increase the production of CGRP in sensory neurons. Isoflavone has estrogen-like effects and is referred to as a phytoestrogen. Thus, we hypothesized that estrogen and isoflavone might inhibit ovariectomy (OVX)-induced decreases in gastric tissue levels of CGRP, thereby attenuating gastric mucosal injury. We examined these possibilities in the present study. The administration of estradiol and isoflavone for 4 wk completely reversed OVX-induced decreases in CGRP mRNA levels of dorsal root ganglion neurons (DRGs) in rats. OVX-induced decreases in gastric tissue levels of CGRP and 6-keto-PGF1α, a stable metabolite of PGI2, in rats were reversed by estradiol and isoflavone. Water-immersion restraint stress (WIR)-induced increases in gastric tissue levels of CGRP in ovariectomized rats. This inhibition was completely reversed by estradiol and was partially, but significantly, reversed by isoflavone. WIR-induced gastric mucosal injury was exacerbated by OVX, which was reversed by estradiol and isoflavone. In vitro experiments using DRGs isolated from rats demonstrated that neither estradiol nor isoflavone enhanced CGRP release from DRGs, but the former enhanced it in the presence of anandamide, an endogenous agonist for vanilloid receptor-1. These observations suggest that estrogen and isoflavone might inhibit OVX-induced decreases in CGRP levels in DRGs by promoting transcription, thereby contributing to the attenuation of stress-induced gastric mucosal injury in OVX rats.

phytoestrogen; prostacyclin; sensory neurons

ALTHOUGH ESTROGEN HAS BEEN SHOWN to have antiulcer activity (1), the detailed mechanism(s) underlying the therapeutic effect remains obscure. Isoflavone has weak estrogenic activity and is thus referred to as a phytoestrogen (17). Isoflavone has been reported to be effective in the treatment of osteoporosis in healthy postmenopausal women (17) as well as osteoporosis, climacteric syndrome, and cardiovascular disorders in ovariectomized (OVX) animals as an alternative to estrogen (7, 20, 21, 22). However, the mechanism(s) by which isoflavone shows such therapeutic effects is not fully understood. We (5) have previously reported that the activation of capsaicin-sensitive sensory neurons reduces stress-induced gastric mucosal injury by reducing gastric inflammatory responses. In that report, we demonstrated that CGRP released from sensory nerve endings increased the endothelial production of prostacyclin (PGI2), thereby inhibiting neutrophil activation in the stomach of rats subjected to water-immersion restraint stress (WIR). Since estrogen enhances nerve growth factor (NGF)-mediated CGRP production in sensory neurons (3, 9, 10), we hypothesized that estrogen and isoflavone might increase CGRP production, thereby contributing to the attenuation of stress-induced gastric mucosal injury in OVX rats. We examined these possibilities in rats subjected to WIR.

MATERIALS AND METHODS

Animals. Age-matched female specific pathogen-free Wistar rats (220–240 g) were obtained from Nihon SLC (Hamamatsu, Japan). Reagents. Fujiiflavone P40 (a product of isoflavone from soybeans) was supplied by Fujicco (Kobe, Japan) (3). Anandamide and estradiol were purchased from Sigma (St. Louis, MO). Pelanin depot, an estradiol valerate, was purchased from Fujicco (Kobe, Japan) (3). Anandamide and estradiol were injected intramuscularly at a volume of 0.2 ml/100 g body wt once per week for 4 wk starting 7 days postoperation. At 35 days postoperation, animals were used for each experiment as previously described (5).

WIR-induced gastric mucosal lesion formation in rats. The care and handling of the animals were in accordance with National Institutes of Health guidelines. All experimental procedures described below were approved by the Kumamoto University Animal Care and Use Committee. Before each experiment, rats were deprived of food but not water for 24 h. Rats were then placed in a restraint cage and immersed up to a level of water to the xiphoid process at 22°C as previously described (18). After the indicated times of stress, rats were anesthetized by an injection of pentobarbital sodium (50 mg/kg ip) and exsanguinated via the abdominal aorta. Their stomachs were removed, filled with 10 ml of 1% formalin, and immersed in 1% formalin for 24 h. Stomachs then were cut along the greater curvature and examined for mucosal lesions. Because most gastric mucosal lesions were linear and almost always <2 mm wide, the total length

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(in mm) of each linear hemorrhagic erosion was measured as the ulcer index (UI; in mm) by an independent observer blinded to the previous treatment as previously described (15).

**Determination of gastric tissue levels of CGRP.** Gastric tissue levels of CGRP were determined in rats before and after WIR by a modification of the methods described previously (4). Briefly, stomachs were weighed and then homogenized in 3 ml of 2 N acetic acid. Homogenates were bathed in 90°C water for 20 min and then centrifuged at 4,500 g for 10 min (4°C). CGRP was extracted from the supernatant using reverse-phase C18 columns (Amersham, Little Chalfont, UK). Columns were prepared by washing with 5 ml methanol followed by 10 ml water before being used. The supernatant was applied onto the column followed by a wash with 20 ml of 0.1% trifluoroacetic acid. CGRP was eluted with 3 ml of 60% acetonitrile in 0.1% trifluoroacetic acid, and the solvent was evaporated under a stream of nitrogen gas. The concentration of CGRP was asayed by using a specific enzyme immunoassay kit (SPI-BIO, Massy, France). The sensitivity of the CGRP assay was 10 pg/ml. The antiserum was cross-reacted 100% of rodent α- and β-CGRP according to the manufacturer’s data sheet.

Results are expressed as micrograms of CGRP per gram of tissue.

**Determination of gastric tissue levels of 6-keto-PGF1α.** Gastric tissue levels of 6-keto-PGF1α were determined in rats before and after WIR according to methods described previously (14). Briefly, stomachs were weighed and then homogenized in 3 ml of 0.1 M PBS (pH 7.4) at 5°C. Homogenates were centrifuged at 2,000 g for 10 min to remove the tiny amounts of solid tissue debris. The supernatant was then acidified with 1 N HCl. 6-keto-PGF1α was extracted from the supernatant using columns packed with ethyl-bonded silica gel (ethyl C2, Amersham). Columns were prepared by a wash with 2 ml methanol followed by 2 ml water. The acidified supernatant was applied onto the column followed by a sequentially wash with 5 ml of 10% ethanol and 5 ml of hexane. 6-keto-PGF1α was eluted with 5 ml methyl formate, and the solvent was evaporated under a stream of nitrogen gas. The evaporated supernatant was reconstituted with the buffer equipped in specific enzyme immunoassay kits for the determination of the concentration of 6-keto-PGF1α (Amersham). The cross-reactivities of the assays for 6-keto-PGF1α with PGE2, PGF2α, thromboxane B2, and arachidonic acid were 2.8%, 1.4%, 0.03%, and 0.01%, respectively, according to the manufacturer’s data sheet.

Results are expressed as micrograms of 6-keto-PGF1α per gram of tissue.

**Determination of estrous cycle phases of rats.** The estrous cycle is characterized as proestrus, estrus, metestrus, and diestrus, which were determined according to the cell types observed in a vaginal smear of animals characterized as proestrus, estrus, metestrus, and diestrus, which were identified as previously described (6). The estrous cycle was determined daily at 7–9 AM by modifications of the methods described previously (11). Experiments were performed only after at least one complete estrous cycle had been documented. Five animals in each estrous cycle were subjected to determination of gastric tissue levels of CGRP and 6-keto-PGF1α without WIR. Five animals in each estrous cycle were also subjected to determination of gastric tissue levels of CGRP and 6-keto-PGF1α at 30 min after WIR.

**Isolation of dorsal root ganglion neurons in rats for mRNA.** Dorsal root ganglion neurons (DRGs) were isolated from control animals, OVX animals, OVX animals with estradiol replacement, and OVX animals with isoflavone administration. Under pentobarbital sodium (50 mg/kg ip) anesthesia, thoracic and lumbar DRGs were immediately dissected from both sides of the spinal cord of Wistar rats as described previously (8) and rapidly frozen in liquid nitrogen and stored at −80°C until the subsequent analysis of CGRP mRNA.

**Quantitative mRNA analysis.** RNA was extracted from DRGs of rats using TRIzol (Life Technologies, Gaithersburg, MD) reagent according to manufacturer’s instructions as described previously (12). This procedure yielded 1–1.5 mg total RNA/mg DRG tissue. RNA samples were diluted to a final concentration of 0.5 mg/ml in RNase-free water and stored at −80°C until use. Synthesis of cDNA was performed with 1 mg total RNA by using TaqMan Reverse Transcription Reagents (Applied Biosystems, Branchburgh, NJ). The 20-μl RT reaction consisted of 2 μl Taqman RT buffer, 25 mM magnesium chloride, 500 mM deoxy-NTPs mixture, 2.5 mM random hexamers, 0.4 U/ml RNase inhibitor, and 1.25 U/ml MultiScribe reverse transcriptase. Thermal cycling parameters for RT were a 10-min hold at 25°C, 30-min hold at 48°C, and 5-min hold at 95°C. Real-time PCR amplification and determination were done using the ABI PRISM 7700 Sequence Detection System, TaqMan Universal PCR Master Mix, and commercially available predesigned, gene-specific primers and FAM-labeled probe sets for quantitative gene expression (TaqMan Gene Expression Assays, Rodent CGRP, Rodent GADPH, Applied Biosystems). All probes used in these experiments spanned an exon-intron boundary. CGRP and GADPH mRNA were quantified by parallel estimation. The thermal cycler conditions were a 2-min hold at 95°C and 10-min hold at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

**Isolation and culture of DRGs.** Under pentobarbital sodium (50 mg/kg ip) anesthesia, thoracic and lumbar DRGs were immediately dissected from both sides of the spinal cord of Wistar rats as previously described (6) and placed in ice-cold sterile calcium- and magnesium-free Dulbecco’s PBS (GIBCO, Grand Island, NY). Ganglia were chopped and incubated at 37°C for 15 min in Dulbecco’s PBS containing 20 U/ml papain (Worthington Biochemical, Lakewood, NJ). The tissue was then incubated at 37°C for 15 min in Dulbecco’s PBS containing 4 mg/ml collagenase type II (Worthington Biochemical). The tissue was incubated for a further 30 min in Dulbecco’s PBS containing 2,000 U/ml dispase I (Godo Shusei, Tokyo, Japan) at 37°C. Individual cells were then dissociated by trituration through a fire-polished Pasteur pipette. After centrifugation at 250 g for 5 min, the resultant pellet was washed twice in serum-free Ham’s F-12 medium (Glycclone, Logan, UT). Cells were plated on 60-mm polystyrene dishes precoated with Vitrogen (Cohesion Technologies, Palo Alto, CA) in Ham’s F-12 medium containing 10% supplemented calf serum, 2 mmol/l glutamine, and 50 mg/ml mouse 2.5S NGF (Upstate Biotecnology, Lake Placid, NY). After 24 h, the culture medium was removed and replaced every 2 days.

**Determination of CGRP release from cultured DRGs.** CGRP release from cultured DRGs was determined as previously described (6). The medium was aspirated gently and washed with serum-free Ham’s F-12 medium after 5 days in culture with DRGs. Cells were incubated with estradiol (10 or 50 nM), isoflavone (1 or 10 μM), or anandamide, an endogenous activator of sensory neurons (13) (1, 5, or 10 μM), and estradiol (50 nM) or isoflavone (10 μM) in combination with anandamide (5 μM) for 60 min in Ham’s F-12 medium containing 1% supplemented calf serum with NGF (100 ng/ml). After incubation, supernatants were sampled and stored at −20°C for CGRP measurements. CGRP levels were determined using a commercial rat CGRP enzyme immunoassay kit (SPI-BIO).

**Statistical analysis.** Data are expressed as means ± SD. Results were compared using ANOVA followed by Scheffé’s post hoc test. A level of P < 0.05 was considered statistically significant.

**RESULTS**

Effects of estradiol replacement and administration of isoflavone on OVX-induced decreases in cellular levels of CGRP mRNA in DRGs isolated from rats. Cellular levels of CGRP mRNA in DRGs isolated from OVX female rats were significantly lower than those of intact female rats (Fig. 1). These OVX-induced decreases in CGRP mRNA levels in DRGs were completely reversed by estradiol replacement and isoflavone administration (Fig. 1).

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Effects of OVX, estradiol replacement, and isoflavone administration on gastric tissue levels of CGRP and 6-keto-PGF\(_{1\alpha}\) in rats with and without WIR.

In rats without WIR, gastric tissue levels of CGRP and 6-keto-PGF\(_{1\alpha}\) were significantly lower in OVX animals than in intact female animals, OVX animals with estradiol replacement, and OVX animals administered isoflavone (Fig. 2A).

Gastric tissue levels of CGRP and 6-keto-PGF\(_{1\alpha}\) were significantly lower in OVX animals than in intact female animals, OVX animals with estradiol replacement, and OVX animals administered isoflavone (Fig. 2A).

Gastric tissue levels of CGRP and 6-keto-PGF\(_{1\alpha}\) were significantly lower in OVX animals than in intact female animals, OVX animals with estradiol replacement, and OVX animals administered isoflavone (Fig. 2A).

Although gastric tissue levels of both CGRP and 6-keto-PGF\(_{1\alpha}\) were significantly increased at 30 min after WIR compared with those in animals without WIR, these increased rapidly to those in animals without WIR at 1 h after WIR and remained unchanged until 8 h after WIR (Fig. 3).

Fig. 1. Effects of ovariectomy (OVX), estradiol (EST) replacement, and administration of isoflavone (IF) on cellular levels of CGRP mRNA in dorsal root ganglion neurons (DRGs) isolated from female rats. Cellular levels of CGRP mRNA were determined by quantitative RT-PCR from total RNA of isolated DRGs from control female rats (n = 6), OVX female rats (n = 5), OVX + EST female rats (n = 5), and OVX + IF female rats (n = 5). EST replacement and administration of IF were performed as described in MATERIALS AND METHODS. Values are expressed as means ± SD. *P < 0.01 vs. control; †P < 0.01 vs. OVX female rats.

Fig. 2. Effects of OVX, EST replacement, and administration of IF on gastric tissue levels of CGRP and 6-keto-PGF\(_{1\alpha}\) in female rats without water-immersion restraint stress (WIR) and those subjected to WIR. Gastric tissue levels of CGRP and 6-keto-PGF\(_{1\alpha}\) were assessed in control (n = 12), OVX (n = 12), OVX + EST (n = 12), and OVX + IF (n = 12) female rats. OVX was performed 5 wk before WIR. EST replacement and administration of IF were performed as described in MATERIALS AND METHODS. Values are expressed as means ± SD. *P < 0.05 vs. WIR(-); †P < 0.05 vs. female + OVX [WIR(-)]; ‡P < 0.05 vs. female + OVX [WIR(+)]; §P < 0.05 vs. female + OVX + IF [WIR(-)].

Fig. 3. Changes in cellular levels of CGRP mRNA in DRGs isolated from female rats subjected to WIR. Changes in cellular levels of CGRP mRNA were determined by quantitative RT-PCR from total RNA of isolated DRGs from female rats subjected to WIR. Values are expressed as means ± SD from 6 animal experiments. *P < 0.01 vs. Pre.

Fig. 4. Changes in the ulcer index (UI) in female rats subjected to WIR. The UI was calculated as described in MATERIALS AND METHODS. Values are expressed as means ± SD from 6 animal experiments. ND, not detectable. *P < 0.01 vs. Pre.
Effects of estradiol or isoflavone and/or anandamide on CGRP release from cultured DRGs isolated from female rats in vitro. Neither estradiol nor isoflavone increased CGRP release from DRGs at concentrations of 50 nM and 10 μM, respectively, in the absence of anandamide (Fig. 6). Anandamide increased CGRP release from cultured DRGs in a concentration-dependent manner (Fig. 6). Although isoflavone at a concentration of 10 μM did not enhance CGRP release in the presence of 5 μM anandamide, estradiol at a concentration of 50 nM significantly enhanced it (Fig. 6).

**Discussion**

We (5) have previously demonstrated that the activation of sensory neurons reduces stress-induced gastric mucosal injury by the release of CGRP. Gastric tissue levels of CGRP were significantly increased at 30 min after WIR compared with those in animals without WIR, and these were decreased rapidly to those in animals without WIR at 1 h after WIR and remained unchanged until 8 h after WIR in rats (5). We (16) also recently reported that CGRP is colocalized with vanilloid receptor (VR)-1 in stomachs of mice at 30 min after WIR, suggesting that increases in gastric tissue levels of CGRP at 30 min after WIR might be mainly explained by increases in CGRP levels in sensory neurons. Cellular levels of CGRP mRNA in DRGs isolated from female rats were also significantly increased at 30 min after WIR, as shown in the present study. These observations suggest that WIR-induced increases in gastric tissue levels of CGRP in rats might be mainly due to increased transcription in sensory neurons. However, the mechanism(s) by which transcription in CGRP synthesis is increased in rats subjected to WIR is not fully understood. This point should be clarified in further studies.

In the present study, both estradiol replacement and isoflavone administration significantly reversed OVX-induced decreases in gastric CGRP levels to normal levels in rats by increasing transcription in DRGs. Estrogen has been shown to increase CGRP levels in sensory neurons by enhancing NGF-mediated production of CGRP (3, 9, 10), suggesting that estradiol replacement in OVX rats might enhance NGF-mediated production of CGRP. The mechanism(s) by which isoflavone increases CGRP levels in sensory neurons of OVX rats is...
not clear at present. Since isoflavone has biological activities similar to those of estrogen (1), it is possible that isoflavone increases CGRP production via enhancement of NGF-mediated production of CGRP in sensory neurons. This possibility should be examined using DRGs isolated from OVX rats in further studies in the near future. We (5) have previously reported that CGRP plays a critical role in the reduction of stress-induced gastric mucosal injury by increasing gastric PGI2 levels in rats. OVX significantly decreased gastric tissue levels of CGRP and 6-keto-PGF1α, and these decreases were completely reversed by estradiol replacement and isoflavone administration. Since CGRP increases gastric tissue levels of PGI2 in rats (5), OVX-induced decreases in gastric tissue levels of 6-keto-PGF1α might be due to the decrease in CGRP levels. Estradiol replacement completely reversed OVX-induced decreases in gastric tissue levels of CGRP and 6-keto-PGF1α at 30 min after WIR. Isoflavone administration partially, but significantly, reversed these decreases. However, both estradiol replacement and isoflavone administration significantly reversed OVX-exacerbated gastric mucosal injury, as shown in the present study. We (5) have previously demonstrated that capsaicin reduces WIR-induced gastric mucosal injury by increasing the release of CGRP in rats. Since estrogen has been shown to release CGRP from sensory neurons (3), it is possible that estrogen attenuates OVX-induced gastric mucosal injury both by increasing CGRP production and promoting the release of CGRP from sensory neurons. Consistent with this notion, estrogen alone did not increase CGRP release from DRGs but enhanced CGRP release in the presence of anandamide, an endogenous agonist for VR-1 (13). In contrast, isoflavone did not increase CGRP release from DRGs even in the presence of anandamide, suggesting that isoflavone might reverse the OVX-induced exacerbation of gastric mucosal injury not by increasing CGRP release but mainly by increasing CGRP levels in DRGs of OVX rats. This may explain why estrogen reversed the OVX-induced complete inhibition of WIR-induced increases in gastric tissue levels of CGRP and 6-keto-PGF1α but isoflavone reversed them only partially.

Taken together, the observations in the present study demonstrated that the administration of isoflavone as well as estrogen significantly inhibited OVX-induced decreases in CGRP levels in DRGs, thereby attenuating stress-induced gastric mucosal injury by maintaining gastric tissue levels of PGI2. These observations also raised the possibility that isoflavone might become an alternative to estrogen replacement for the prevention of gastric mucosal injury in elderly women after menopause.

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