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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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 (PGL2) 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 in 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 and 6-keto-PGF1α, were inhibited 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.

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. Fujiisovone 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 Mochida Pharmaceutical (Tokyo, Japan). All other reagents were of analytic grade.

OVX and administration of isoflavone and estradiol. Rats were subjected to bilateral OVX or sham operated under pentobarbital sodium anesthesia (50 mg/kg ip) as described previously (19). On the day of the operation, rats were divided into four groups. The first group (the sham group) was given a sham operation and treated with vehicle (sesame oil). The second group (the OVX group) was subjected to OVX and treated with vehicle. The third group (the OVX + estradiol group) was subjected to OVX and treated with estradiol valerate (1.0 mg/kg). These three groups received AIN-93M pellets (isoflavone-free foods, Clea Japan, Osaka, Japan). The fourth group (the OVX + isoflavone group) was subjected to OVX and received AIN-93M pellets containing 0.5% Fujiisovone P40 [0.22% (wt/wt) isoflavone] and treated with vehicle (2, 17). Both the vehicle 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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The estrous cycle is characterized as proestrus, estrus, metestrus, and diestrus, which were determined daily at 7–9 AM by modifications of the methods described previously (1). Briefly, stools from each estrous cycle were subjected to determination of characterizations of the assays for 6-keto-PGF\(_{1\alpha}\) and \(\alpha\)-GCAP according to the manufacturer’s data sheet. Results are expressed as micrograms of CGRP per gram of tissue.

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 0.1% trichloroacetic acid. CGRP was eluted with 3 ml of 60% acetonitrile in 0.1% trichloroacetic acid, and the solution was evaporated under a stream of nitrogen gas. The concentration of CGRP was assayed by using a specific enzyme immunoassay kit (SPI-BIO, Massy, France). The sensitivity of the CGRP assay was 10 pg/ml. The antiserum cross-reacted 100% of rodent \(\alpha\)- and \(\beta\)-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-PGF\(_{1\alpha}\). Gastric tissue levels of 6-keto-PGF\(_{1\alpha}\) 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 4,500 g for 10 min to remove the tiny amounts of solid tissue debris. The supernatant was then acidified with 1 N HCl. 6-keto-PGF\(_{1\alpha}\) 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-PGF\(_{1\alpha}\) 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-PGF\(_{1\alpha}\) (Amersham). The cross-reactivities of the assays for 6-keto-PGF\(_{1\alpha}\), PGE\(_2\), PGB\(_3\), thromboxane B\(_2\), 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-PGF\(_{1\alpha}\) per gram of tissue.

I. Isolation of dorsal root ganglion neurons in rats for mRNA. Dorsal root ganglion neurons (DRGs) were isolated from control animals, O VX animals, O VX animals with estradiol replacement, and O VX 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 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 (HyClone, Logan, UT). Cells were plated on 60-mm polylysine 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.

II. 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 \(\mu\)M), or anandamide, an endogenous activator of sensory neurons (13) (1, 5, or 10 \(\mu\)M), and estradiol (50 nM). Cells were incubated with anandamide (5 \(\mu\)M) 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 \(-20^\circ\)C for CGRP measurements. CGRP levels were determined using a commercial rat CGRP enzyme immunoassay kit (SPI-BIO).

III. 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).
Effects of OVX, estradiol (EST) replacement, and administration of isoflavone (IF) on gastric tissue levels of CGRP and 6-keto-PGF$_1$α in rats with and without WIR.

In rats without WIR, gastric tissue levels of CGRP and 6-keto-PGF$_1$α were significantly lower in OVX animals than in intact female animals, OVX animals with estradiol replacement, and OVX animals administered IF (Fig. 2A).

Gastric tissue levels of CGRP and 6-keto-PGF$_1$α in animals without WIR [WIR(−)] and at 30 min after WIR [WIR(+)1] 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(+)].

**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. control; †P < 0.01 vs. OVX female rats.

**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.
Fig. 5. Effects of O VX, EST replacement, and administration of IF on UI in female rats subjected to W IR. The UI at 4 h after W IR was assessed in control (n = 6), O VX (n = 6), O VX + EST (n = 6), and O VX + IF (n = 6) female rats. O VX was performed 5 wk before W IR. EST replacement and administration of IF were performed as described in MATERIALS AND METHODS. Values are expressed as means ± SD from 5 experiments. *P < 0.01 vs. control; †P < 0.01 vs. O VX.

dental groups, these levels at 30 min after W IR in O VX animals were significantly lower than those of control animals, O VX rats with estradiol replacement, and O VX rats administered isoflavone (Fig. 2). Although estradiol replacement completely reversed the O VX-induced inhibition of W IR-induced increases in gastric tissue levels of CGRP and 6-keto-PGF1α, isoflavone administration reversed such inhibition partially but significantly (Fig. 2).

Effects of O VX, estradiol replacement, and isoflavone administration on gastric mucosal injury in rats subjected to W IR. The UI began to increase at 1 h after W IR and increased with time, peaking at 4 h after W IR (Fig. 4). The UI at 4 h after W IR was not significantly different among the proestrus, estrus, metestrus, and diestrus phases in intact female animals (data not shown). The UI at 4 h after W IR was significantly higher in O VX animals than in intact females (Fig. 5). The O VX-induced exacerbation of gastric mucosal injury was almost completely reversed in animals with estradiol replacement and in those administered isoflavone (Fig. 5).

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 W IR compared with those in animals without W IR, and these were decreased rapidly to those in animals without W IR at 1 h after W IR and remained unchanged until 8 h after W IR 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 W IR, suggesting that increases in gastric tissue levels of CGRP at 30 min after W IR 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 W IR, as shown in the present study. These observations suggest that W IR-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 W IR is not fully understood. This point should be clarified in further studies.

In the present study, both estradiol replacement and isoflavone administration significantly reversed O VX-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 O VX rats might enhance NGF-mediated production of CGRP. The mechanism(s) by which isoflavone increases CGRP levels in sensory neurons of O VX 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 PGI2 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 decrement and isoflavone administration. Since CGRP increases gastric tissue levels of CGRP and 6-keto-PGF1α, these decreases were completely reversed by estradiol replacement and isoflavone administration. Since CGRP increases gastric tissue levels of CGRP and 6-keto-PGF1α, these decreases were completely reversed by estradiol replacement and isoflavone administration.

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