Estrogen altered facial mechanical pain threshold and trigeminal $P2X_3$ receptor expression

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Abstract

OBJECTIVES: $P2X_3$ receptors are expressed in trigeminal ganglia (TG) and participate in the transduction of facial pain. However, the mechanisms underlying P2X receptor-mediated nociception at different estrogen levels has not been examined.
METHODS: In this study, female rats were randomly divided into sham-operated (sham), ovariectomized (OVX), and estrogen-treated groups. In each group, the facial mechanical pain threshold was tested and the TG were harvested for a real-time PCR analysis of $P2X_3$ receptor mRNA and western blot analysis of protein level.
RESULTS: In OVX rats we found that the mechanical pain threshold was significantly decreased compared with that in sham rats. Estrogen replacement reversed the decrease. The expression of $P2X_3$ mRNA level in TG from OVX rats was significantly increased, consistent with the enhanced $P2X_3$ receptor in protein level. Estrogen replacement could decrease the expression of $P2X_3$ receptor in both mRNA and protein level.
CONCLUSION: These results indicate that estrogen might modulate the transduction of facial pain by inhibiting $P2X_3$ receptor in TG.

INTRODUCTION

Epidemiologic study has shown that pain threshold was lower in woman compared with that in men. Women tend to exhibit higher perceptual responses to the painful stimulus so as to need more analgesics (Chesterton et al. 2003; Frot et al. 2004; Sarlani et al. 2003). Sexual hormone, especially, estrogen may play a role in affecting the pain perception (LaCroix-Fralish et al. 2005). However, conflicting results have been reported regarding the role of gonadal steroid hormones. Several studies indicate that pain threshold is lowest during oesturs and metestrus of oestrous cycle, with pain threshold reducing when the animals are ovariectomized and estrogen replacement can increase thresholds to cutaneous stimuli (Holdcroft et al. 2000). Elevated estrogen levels can also decrease the percentage of escape responses to vaginal canal stimulation (Bradshaw et al. 1999) or ureteral calculosis (Giamberardino et al. 1997). Furthermore, elevated estrogen and progesterone, as occurring during pregnancy, is antinociceptive (Gintzler & Bohan 1990; Dawson-Basoa & Gintzler 1998). It is well known that estrogen receptors are widely distributed throughout the central and peripheral nervous systems (Papka et al. 1997; Taleghany et al. 1999). Therefore, estrogen may potentially par-

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ticipate in the regulation of nociceptive process in the nervous system.

ATP and its receptors play an important role in transduction of noxious stimuli (Burnstock 2007). In sensory neurons, ATP acts by activating ATP-gated ion channels, namely P2X receptors (Burnstock 2007). Among these receptors, homomeric $P2X_3$ receptor and heteromeric $P2X_{2/3}$ receptor play a major role in peripheral nociception (Donnelly-Roberts et al. 2008). The trigeminal neurons have a psedo-unipolar morphology dividing the axon into a peripheral and central branch. The peripheral sensory fibres innervate the large cerebral and meningeal blood vessels (O'Connor & Kooy 1986), whereas the central fibres projects to the trigeminal nucleus caudalis (TNC) in the brain stem, which transmits nociceptive information to higher brain centres and sensory cortex (Lazarov 2002). $P2X_3$ receptor is distributed in the small and medium neurons of trigeminal ganglion (TG) that mediate responses to noxious stimuli. Eriksson has found that the $P2X_3$ receptor is transiently upregulated in the trigeminal ganglion after peripheral nerve injury (Eriksson et al. 1998). These results indicate that $P2X_3$ play an important role in the signal transduction of TG. However, the changes in the oro-facial pain behaviour at different concentration of estrogen and the related alteration of $P2X_3$ receptors in TG have not been determined.

In the present study, we performed the experiments on female SD rats in different estrogen level by ovariectomy and estrogen replacement. We observed changes in the mechanical pain threshold and the expression of $P2X_3$ mRNA and proteins on TG in three groups. The results demonstrate that estrogen regulate the sensory transduction probably by modulating $P2X_3$ receptor in TG.

MATERIALS AND METHODS

Surgical preparation

Adult Sprague-Dawley female rats (200–220 g) were obtained from Shanghai Super – B&K laboratory animal Corp. Ltd. 30 rats were randomly divided into three groups: a sham, an ovariectomized (OVX) and an estrogen replacement group. Each group has 10 rats. The rats of sham group were anesthesized with 3% isoflurane and the abdominal skin was shaved and wiped with an alcohol pad. Using sterile equipment, a small incision was made on the side of the abdomen below the ribs, then the intestinal tract was agitated. After that, the muscle and the skin were closed with sutureclips. After the operation, the rats were received Sodium Chloride (0.4 ml/day) for 6 weeks. Both ovaries were isolated and removed in the OVX group followed by daily subcutaneous injection of Sodium Chloride (0.4 ml/day) for 6 weeks. The rats of estrogen replacement group had bilateral ovariectomy followed by daily subcutaneous injection of 30 μg/kg of 17-estradiol in saline for 6 weeks.

BEHAVIOR STUDY

All rats were placed under a plexiglass dome and allowed to acclimatise for at least 30 min before beginning behavioral experiments. Mechanical pain threshold was assessed with a set of von Frey filaments, which power were 1 (0.0115 g), 2 (0.0229 g), 3 (0.0358 g), 4 (0.052 g), 5 (0.1 g), 6 (0.494 g), 7 (0.634 g), 8 (1.19 g), 9 (2.05 g), 10 (4.01 g), 11 (6 g), 12 (8.08 g), 13(10.8 g), 14 (12.2 g) and 15 (14.9 g) respectively. The ipsilateral and contralateral facial whisker pads were tested. If backward movement or bending or head withdrawal were observed after probing with a filament, the rat was considered responsive to that filament. The minimal force required to elicit a reproducible escape behavior on each side of ten times of application of the von Frey filaments (Takeda et al. 2000). The response threshold was defined as the mean value of twenty trials.

Real-time reverse transcription-PCR

RNA extraction and RT-PCR

Total RNA was extracted using a RNeasy Mini Kit (QIAGEN; Clifton Hill, Australia). RNA purity was determined using a method of ultraviolet spectrophotometry at a wavelength of 260–280 nm. Two μg of total RNA was reversely transcribed to complementary DNA in a 20 μl reaction mixture containing 1× reverse transcriptase buffer (15 mM $MgCl_2$, 375 mM KCl, 50 mM DTT, 250 mM Tris-HCl, pH 8.3), 10 mM dNTP, 20 U RNase inhibitor, 200 U M-MLV reverse transcriptase, and 50 ng of oligo(deoxythymidine)$_{15}$ primer. Reaction time was at least 1 h at 42 °C. The cDNA was stored at −20 °C until real-time polymerase chain reaction (RT-PCR). All reagents, with the exception of the RNeasy Mini Kit, were from Promega Corp. (Madison, WI).

Real-time RT-PCR

Quantitative RT-PCR amplification was performed with SYBRGreen (Applied Biosystems; Scoresby, Australia) using Roto-gene RG3000 (Australia) in a 20 μl reaction mixture. The solution consisted of 1.0 μl diluted RT-PCR product, 0.25 μM of each of the paired primers, and 10 μl real-time PCR SYBR Green Master Mix (QIAGEN, Clifton Hill, Australia). RNA levels were measured with specific primers designed, for $P2X_3$ was: TGGCGTTCTGGGTATTAAGATCGG (forward); CAGTGGCCTGGTCACTGGCGA (reverse) against the sequences downloaded from

Genbank (accession no. X90651, 708–731 and 1126–1147). The PCR condition was 95 °C for 2 min, followed by 40 cycles of 95 °C, 20 s; 65 °C, 25 s; 72 °C, 25 s for $P2X_3$ and was 95 °C for 2 min, followed by 40 cycles of 95 °C, 20 s; 63 °C, 25 s; 72 °C. RT-PCR for the housekeeping gene β-actin was performed for each sample. The primer for amplification of β-actin was: ATGGTGGGTATGGGTCAGAAGG (forward); TGGCTGGGGTGTTGAAGGTC (reverse). The PCR

condition was: 95 °C for 2 min, followed by 40 cycles of 95 °C, 20 s; 58 °C, 25 s; 72 °C, 25 s. The absolute mRNA level of target gene in each sample was calculated using a standard curve and then by the ratio to β-actin in each sample. The specificity of the primers was verified by examining the melting curve as well as sequencing of the QT-RT-PCR products. The melting curve of QT-RT-PCR showed a single sharp peak for $P2X_3$ and β-actin PCR products. The lengths of PCR products of $P2X_3$ and β-actin mRNA were 440 bp and 265 bp, respectively.

Western Blot

Trigeminal ganglia were harvested and homogenized in cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Deoxycholic acidsodium salt, 0.1% SDS, and a protease inhibitor mixture) using a homogenizer(Ultra Turrax T 18 basic, IKA, German). The homogenate was centrifuged at 12 000 × *g* (10 min, 4 °C) and the supernatant was collected. Total protein concentration was determined by the Bradford method using bovine serum albumin as a standard (Bio-Rad). Proteins were separated using SDS-PAGE on 10% TRIS-HCl gels (Bio-Rad, Hercules, CA) and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked in blocking buffer consisting of 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% Tween 20, and 5% nonfat milk at room temperature for 2 h and then incubated with the goat anti-mouse $P2X_3$ primary antibody(1:1 000, Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (1:1 000, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C.The blots were washed, incubated with HRP-conjugated secondary antibody(1:1 000, Proteintech Group, Proteintech, USA)for 2 h at room temperature, and finally visualized in ECL solution(Santa Cruz Biotechnology, Santa Cruz, CA). For 1 min and exposed onto Kodak film (Rayco company) for 1–30 min. For control of correct gel loading, β-actin quantification was used. To quantify Western blot signals, band density was measured using UMAX PowerLook III(Synpix) and normalized with respect to the control.

Statistics

Data are expressed as mean ± SEM, and n indicates the number of animals in experiments. Differences among three groups were analysed for statistical significance using one-way analysis of variance (ANOVA), $p<0.05$ was taken as the significance level.

RESULTS

Tests of the mechanical pain threshold

Mechanical pain threshold was tested by Von Frey filaments. The results were shown in Figure 1. OVX group presented significantly lower nociceptive threshold than that in sham group ($p<0.01$), with the Von Frey Hair force as 7.90 ± 1.62 g, and 12.75 ± 1.18 g, respectively. Estrogen replacement reversed this decrease, with the threshold as 12.93 ± 0.95 g, which is significantly higher than that in OVX group ($p<0.01$).

Expression of $P2X_3$ receptor mRNA in TG

Figure 2 showed that OVX strongly elevated $P2X_3$ receptor mRNA levels compared with that in sham group (OVX: 1.21 ± 0.27 vs Sham: 0.72 ± 0.29; $p<0.01$). Treatment of estrogen reversed the increase to 0.88 ± 0.08, which was significantly lower than that in OVX group ($p<0.05$).

Expression of $P2X_3$ receptor protein in TG

Then we further examined expression of $P2X_3$ receptor protein in TG (Figure 3). Similar to that in the mRNA level, in OVX rats we found that the expression of $P2X_3$ was significantly increased compared with that in the

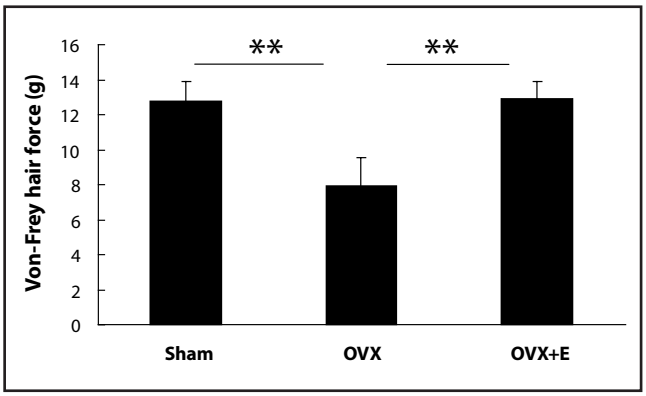

Fig. 1. Changes in the mechanical pain threshold for evoking withdrawl behavior in the whisker pad region in sham, OVX and estrogen-treated (OVX+E) groups. Von Frey Hair force of the OVX group was significantly decreased compared with that of the sham group rats. ($p<0.01$). Estrogen replacement reversed the decrease ($p<0.01$). $^{**}p<0.01$

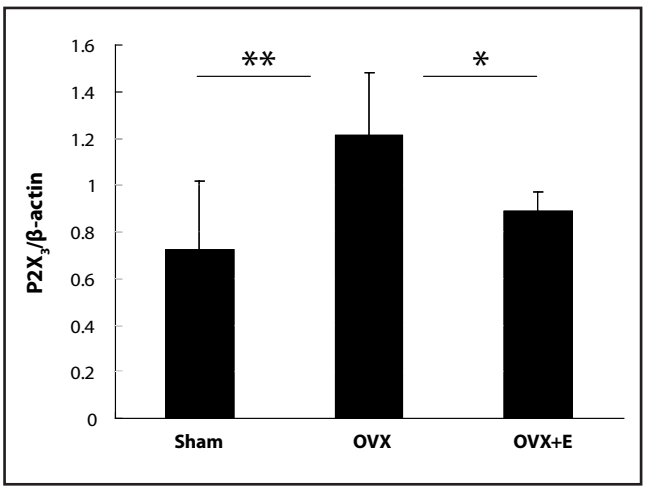

Fig. 2. Expression of $P2X_3$ receptor mRNA in TG from Sham, OVX and OVX+E rats. $P2X_3$ receptor mRNA expression in OVX group was significantly increased compared with that of the sham group rats ($p<0.01$). Estrogen replacement significantly reversed the increase ($p<0.05$). $^{**}p<0.01$; $^{*}p<0.05$.

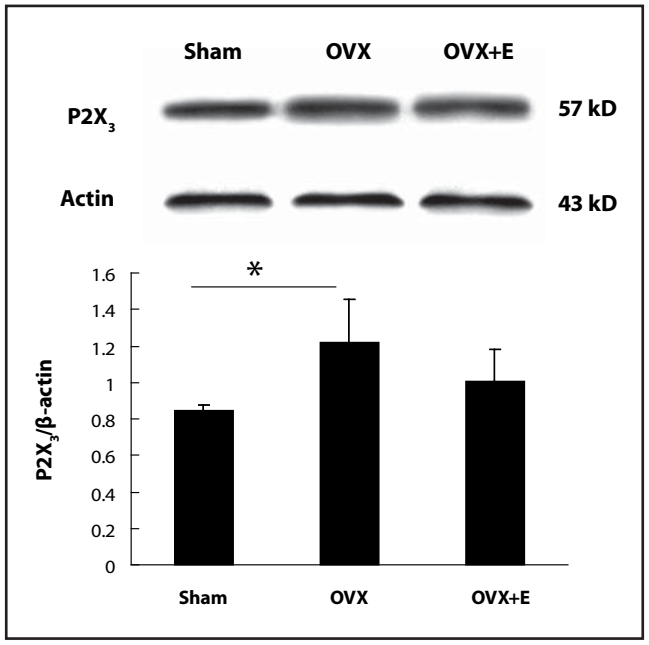

Fig. 3. Expression of $P2X_3$ receptor in TG from Sham, OVX and OVX+E rats. $P2X_3$ receptor expression was significantly increased compared with that of the sham group rats ($p<0.01$), while has no significant difference with that in estrogen replacement rats ($p>0.05$). *$p<0.05$.

sham group ($p<0.05$). Estrogen replacement decreased the expression, but there was no significant difference between the sham group and estrogen replacement group ($p>0.05$).

DISCUSSION

The behavior tests in this study demonstrate that mechanical pain threshold was reduced significantly after ovariectomy, and then reversed after estrogen replacement. These effects were reasonably similar to those described previously in estrogen knock-out rats, which presented an increased nociceptive behavior in the oro-facial model by a formalin injection in the lip and completely normalized by preceding daily estradiol administration (Sylvie et al. 2005). Another researcher found that OVX mice developed a robust mechanical hyperalgesia and allodynia in the abdomen, hindlimbs and proximal tail, this abnormal pain sensitivity is dependent on lack of circulating estrogens and can be reversed by exogenous and continuous administration of 17β-estradiol (Sanoja & Cervero 2005). These results suggest that the plasma estrogen level in rats have a strong effect on the mechanical pain threshold.

ATP is present in virtually all cells and participates in the pain signal transduction (Burnstock 2007). Iontophoretically applied ATP onto normal skin in human induced dose-dependent pain (Hamilton et al. 2000). ATP acts via a family of ligand-gated ion channels, namely P2X receptors and a family of G-protein-coupled P2Y receptors. Seven subtypes of P2X receptors and eight subtypes of P2Y receptors have been cloned (Burnstock 2007). Among these receptors, homomeric $P2X_3$ receptor and heteromeric $P2X_{2/3}$ receptor play a major role in peripheral nociception (Donnelly-Roberts et al. 2008). Intraplantar infection of α,β-me ATP, a $P2X_3$ subunit-contained receptors agonist resulted in spontaneous and short-lasting licking, biting and lifting of the injured hindpaw (Bland-Ward & Humphrey 1997). $P2X_3$-null mice exhibit a marked urinary bladder hyporeflexia and have reduced pain-related behaviour in response to injection of ATP and formalin (Cockeyne et al. 2000). These results suggest that $P2X_3$ is critical in modulating peripheral pain signal transduction and transmission.

In trigeminal ganglion (TG), six subtypes of P2X receptors ($P2X_{1-6}$) have been found to be localized, with $P2X_3$ receptor expressed exclusively in those small and medium neurons that mediate responses to noxious stimuli (Xiang et al. 1998). The trigeminal neurons transmit nociceptive information from peripheral to higher brain centres and sensory cortex (Lazarov 2002). When craniofacial organs were injured, endogenous ATP might be released from injury cells, which results in activation of $P2X_3$ receptors as a result of depolarization of TG nerve endings and up-regulation discharges of sensory neuron. Another research found that significant reversible increases in ATP were observed after infusion of 100 mM KCl or 1 mM capsaicin within guinea pig trigeminal ganglia in vivo (Matsuka et al. 2001). These results suggest that ATP and $P2X_3$ receptor might be the vital members in modulating nociceptive transduction in TG..

In the present study, we found that $P2X_3$ mRNA expression significantly increased after surgical ovariectomy compared with that in the sham group. Estrogen replacement did reverse this effect. The similar results were obtained in the protein level by western blot. In conjunction with the mechanical threshold results, we conclude that estrogen might inhibit facial pain by modulating $P2X_3$ receptors in TG neurons. Our previous study showed that estrodial rapidly inhibited $P2X_3$ mediated inward currents in dorsal root ganglion neurons via estrogen receptors (Ma et al. 2005). Recently we reported that OVX induced over-expression of $P2X_3$ receptors in DRG and the related peripheral mechanical hyperalgesia (Ma et al. 2011). Some evidence has also shown the modulatory effect by estrogen on $P2X_3$ receptor expression (Carley et al. 2002; Papka et al. 2005). The mechanisms underlying the altered $P2X_3$-containing receptors function in the various gonadal hormone levels remain unclear, thus more studies are required to clarify the mechanisms of ovarian hormones effects on the transduction and transmission of peripheral pain.

It has been reported that estradiol treatment was associated with increased activation of the endogenous mu opioid system during exposure to the pain stimulus,

whereas in the low estradiol state, this system appeared to de-activate when women were exposed to the pain stimulus (Smith et al. 2006). A recent study reported that rising levels of estrogen appear to offer some protection against migraine via increasing the capacity for 5-HT synthesis and release which could alter the dilation and constriction of extra- and intra-cranial blood vessel in patients (Macgregor et al. 2006; Hirol & Neumaier 2009). We now provide evidence that estrogen could inhibit the facial mechanical pain behavior and the expression of $P2X_3$ receptors in both mRNAs and protein level. These results suggest that $P2X_3$ receptors appear to be important contributors to altered pain perception with relation to the estrogen modulation, thus they might be regarded as potential targets for the drug treatment of pain.

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