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17β-Estradiol Aggravates Temporomandibular Joint Inflammation Through the NF-κB Pathway in Ovariectomized Rats

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Objective. **Women of childbearing age are more likely than men to experience temporomandibular disorders, with pain as the main symptom. Temporomandibular joint (TMJ) inflammation is believed to be a major reason for TMJ pain. Whether sex hormones are involved in the sexual dimorphism of TMJ inflammation and pain remains to be elucidated. The aim of this study was to examine whether estrogen affects TMJ inflammation and pain via the NF-κB pathway.**

Methods. **Female rats were divided into 6 groups: the control group, the sham-ovariectomized group, and 4 groups of ovariectomized rats treated with 17β-estradiol at a dosage of 0 μg/day, 20 μg/day, 80 μg/day, and 200 μg/day, respectively, for 10 days and then injected intraarticularly with Freund's complete adjuvant to induce TMJ inflammation. The behavior-related and histologic effects of 17β-estradiol were evaluated 24 hours after the induction of TMJ inflammation. NF-κB activity in the synovial membrane was examined by electrophoretic mobility shift assay. The expression of the NF-κB target genes tumor necrosis factor α, interleukin-1β (IL-1β), IL-6, cyclooxygenase 2, and inducible nitric oxide synthase in the synovial membrane was examined by real-time polymerase chain reaction.**

Results. **Treatment with estradiol potentiated TMJ inflammation in a dose-dependent manner and also exacerbated the inflammation-induced decrease in food intake. Correspondingly, estradiol potentiated the DNA-binding activity of NF-κB and the transcription of its target genes in the synovial membrane. Furthermore, the estrogen receptor antagonist ICI 182780 or the NF-κB inhibitor pyrrolidine dithiocarbamate partially blocked the effects of estradiol on TMJ inflammation and pain and the NF-κB pathway.**

Conclusion. **These results suggest that estradiol aggravates TMJ inflammation through the NF-κB pathway, leading to the induction of proinflammatory cytokines.**

Temporomandibular disorders (TMDs) are an assorted set of clinical conditions characterized by pain in the temporomandibular joint (TMJ) and/or masticatory muscles. Joint inflammation is thought to be a major cause of pain in patients with TMDs (1–4). The prevalence, severity, and duration of pain in TMDs are greater in women than in men; such pain primarily affects women of childbearing age (5). Estrogen replacement therapy in postmenopausal women is associated with the highest prevalence of TMDs (6). In women with TMDs, serum estradiol levels during the luteal phase are higher than those in healthy control subjects (7), and synovial fluid estradiol levels in patients with TMDs are also higher than those in healthy control subjects (8). These observations suggest that sex hormones, particularly estrogen, may be involved in TMD-associated pain.

Estrogen is involved in joint inflammation. The sex hormone 17β-estradiol enhances the secretion of matrix metalloproteinases in synoviocytes (9). It also enhances interleukin-1β (IL-1β)–induced secretion of IL-6 from synoviocytes in rheumatoid arthritis (10).

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Moreover, the expression and concentration of IL-1β, IL-6, and IL-8 in mouse TMJ cartilage cells increase with increasing doses of 17β-estradiol (11). However, the mechanism underlying estrogen-induced cytokine expression in inflamed joints remains largely unknown.

NF-κB plays a pivotal role in regulating inflammation-associated genes (12,13). Various stimuli, including proinflammatory cytokines, lead to the nuclear translocation of NF-κB and subsequently to up-regulated transcription of IL-1, tumor necrosis factor α (TNFα), IL-6, IL-8, cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS) (13). Ongoing NF-κB activity is implicated in several diseases, including arthritis, asthma, and inflammatory bowel disease (14). Although estrogen can repress the transcriptional activity of NF-κB in several cell lines, such as HeLa, HEp-G2, and MCF-7 (15), estrogen can also enhance the transcriptional activity of NF-κB in endothelial cells, cardiocytes, and T cells (16–18). Whether estrogen enhances or represses NF-κB in the inflamed TMJ remains unknown.

In this study, we hypothesized that estrogen could be involved in TMJ inflammation by modulating the production of cytokines in the synovial membrane. Thus, we explored whether estrogen could aggravate TMJ inflammation and pain and whether the effects of estrogen were facilitated by potentiating the NF-κB pathway in the synovial membrane of the inflamed TMJs of ovariectomized rats.

MATERIALS AND METHODS

Animals. The experimental protocols were approved by the Animal Use and Care Committee of Peking University and were consistent with the Ethical Guidelines of the International Association for the Study of Pain. Adult female Sprague-Dawley rats (180–200 gm) were used. To examine the effects of estrogen on TMJ inflammation, rats were randomly divided into 6 groups (n = 6 rats in each group), as follows: the control group, the sham-ovariectomized group, and 4 groups of ovariectomized rats treated with 0 μg, 20 μg, 80 μg, and 200 μg of 17β-estradiol, respectively.

Estradiol administration and induction of TMJ inflammation. After being anesthetized with 1% pentobarbital sodium administered intraperitoneally, rats were bilaterally ovariectomized or sham-operated (control and sham-ovariectomized groups) and allowed to recover for 1 week. The 17β-estradiol (Huameihuli Biochemical) was dissolved in ethanol and diluted to 10% in saline immediately before administration. The 4 groups of ovariectomized rats were treated with 17β-estradiol, administered by subcutaneous abdominal injection daily in the morning, at doses of 0 μg, 20 μg, 80 μg, and 200 μg per rat, respectively, at a volume of 200 μl for 10 days. The control and sham groups received subcutaneous abdominal injections of the same amount of vehicle. On the tenth day of estradiol treatment, rats in the sham-ovariectomized and ovariectomized groups received 50-μl injections of Freund's complete adjuvant (CFA; Sigma) (1:1 oil:saline emulsion) into each of the TMJs to induce bilateral TMJ inflammation for 24 hours. Rats in the control group received 50-μl injections of saline into each of the TMJs.

Measurement of food intake and head withdrawal threshold. Food intake and the head withdrawal threshold were measured as described in detail in our previous study (19).

Hormonal determination. Blood was obtained from rats in the experimental groups 24 hours after induction of TMJ inflammation and also from intact female rats at different estrous stages, as determined by the cell types in the vaginal smear according to a previous study (20) (additional information is available from the corresponding author). The plasma levels of 17β-estradiol were measured by radioimmunoassay using an Access immunoassay system (Beckman Coulter).

Tissue preparations. The synovial membrane of the TMJ is composed of a synovial lining layer and a connective sublining layer (21). The TMJ synovial membrane was bilaterally harvested from 3 rats per group for RNA and nuclear extractions. Whole TMJs were removed from the other 3 rats in each group and fixed in 4% paraformaldehyde in 0.1*M* phosphate buffered saline (PBS) and demineralized in 15% EDTA. The specimens were dehydrated in graded alcohols and xylene, embedded in paraffin, and sagittally sectioned at 5 μm. The sections were stained with hematoxylin and eosin.

Histopathologic evaluation and scoring of TMJ inflammation. TMJ inflammation was histopathologically evaluated and scored by 2 senior oral pathologists who were blinded to information regarding the animal groups. The scoring of TMJ inflammation was performed according to the following histopathologic criteria, which were adapted and modified from previous studies (22–25), as follows: 1) proliferation or erosion of the synovial lining, scored on a scale of 0–3, where 0 = ≤3 layers of cells, 1 = 4–6 layers, 2 = ≥7 layers, and 3 = erosion of the synovial layers or disorganized and broken synovial layers; 2) dilated vasculatures and tissue edema, scored on a scale of 0–3, where 0 = absent, 1 = less than one-third of the synovial membrane length involved, 2 = one-third to two-thirds of the synovial membrane length involved, and 3 = more than two-thirds of the synovial membrane length involved; 3) fibrin-like exudate in the articular space, scored on a scale of 0–2, where 0 = absent, 1 = few and scattered, and 2 = marked and cord-like; and 4) infiltration of mononuclear cells, scored on a scale of 0–4, where 0 = absent, 1 = mild infiltration in the sublining layer, 2 = moderate infiltration in the sublining layer and articular space, 3 = severe infiltration in the articular space, and 4 = marked cellular infiltration full of the articular space. Higher scores represent more severe inflammation. For any group, the total scores were averaged by the number of examined joints and are presented as the mean ± SEM.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from the TMJ synovial membrane were prepared using a Nuclear-Cytosol Extraction Kit (Applygen Technologies). The extracts were kept at −80°C until used. Single-stranded oligonucleotides containing the NF-κB–binding motif 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (3′ biotin–labeled and unlabeled) and the complementary strand were synthe-

sized. The binding reaction was performed using the LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's recommendations. The binding samples were separated on a 6% nondenaturing polyacrylamide gel, and membrane-bound probes were detected and visualized after exposure to x-ray film (26).

Real-time quantitative polymerase chain reaction (PCR). Total RNA was isolated from the TMJ synovial membrane, using TRIzol reagent (Invitrogen). Reverse transcription and real-time PCR were performed as described in detail previously (19). The primers synthesized according to the sequences in the previous studies were as follows: for rat β-actin, sense 5′-TGACAGGATGCAGAAGGAGA-3′, antisense 5′-TAGAGCCACCAATCCACACA-3′ (27); for rat IL-1β, sense 5′-CACCTCTCAAGCAGAGCACAG-3′, antisense 5′-GGGTTCCATGGTGAAGTCAAC-3′ (28); for rat IL-6, sense 5′-CCAAGACCATCCAACTCATCTTG-3′, antisense 5′-CACAGTGAGGAATGTCCACAAAC-3′ (28); for rat TNFα, sense 5′-CCAGGTTCTCTTCAAGGGACAA-3′, antisense 5′-CTCCTGGTATGAAATGGCAAATC-3′ (28); and for rat COX-2, sense 5′-TACAAGCAGTGGCAAAGGCC-3′, antisense 5′-CAGTATTGAGGAGAACAGATGGG-3′ (29). Primers for rat iNOS designed with Primer Premier version 5.0 software were synthesized as follows: sense 5′-GAGTGAGGAGCAGGTTGAGG-3′, antisense 5′-CCAAGGTGTTGCCCTTTTT-3′. The efficiency of the newly designed primers was confirmed by sequencing the conventional PCR products.

Immunohistofluorescence. Antigens were retrieved by boiling the TMJ sections in 10 m*M* citrate buffer (pH 6.0) for 10 minutes. Sections were blocked with 10% goat serum for 30 minutes at room temperature and incubated with anti–NF-κB p65 polyclonal antibody (1:100) (sc-109; Santa Cruz Biotechnology) overnight at 4°C. After extensive washing with PBS, the sections were incubated with fluorescein isothiocyanate–conjugated goat anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch) for 30 minutes at room temperature and washed with PBS. The sections were counterstained with 5 μg/ml 4′,6-diamidino-2-phenylindole for 1 minute, washed 3 times with PBS, and covered with fluorescence mounting medium coverslips. Confocal microscopic images were acquired using a Zeiss laser scanning microscope (LSM 510), and the images were processed using LSM 5 Release 4.2 software.

Application of estrogen receptor antagonist and NF-κB inhibitor. The estrogen receptor–specific antagonist ICI 182780 was dissolved in 10% ethanol. A total of 15 intact female rats were divided into 3 equal groups (i.e., control, inflammation without ICI treatment, and inflammation with ICI treatment) and intraperitoneally injected twice with vehicle or ICI 182780 (500 μg in 100 μl) 24 hours before and immediately before bilateral injections of CFA into the TMJs. The application and dose of ICI 182780 were modified from those used in previous studies (30,31). Food intake was measured 4 hours before (baseline) and 20 hours after induction of TMJ inflammation, whereas the head withdrawal threshold was measured 2 hours before (baseline) and 22 hours after induction of TMJ inflammation. The activation of NF-κB and the expression of its downstream genes in the synovial membrane were examined 24 hours after TMJ inflammation was induced.

To examine whether ICI 182780 could also affect TMJ inflammation in the estradiol-treated rats, an additional 9 female rats were ovariectomized and treated with 200 μg of 17β-estradiol daily for 10 days and then were divided into 3 equal groups, as described above. Determination of the head withdrawal threshold and food intake and histopathologic scoring of TMJ inflammation were also performed.

The NF-κB–specific inhibitor pyrrolidine dithiocarbamate (PDTC; Sigma) was dissolved in dimethyl sulfoxide at a concentration of 1%. A total of 15 female rats were ovariectomized and treated with 200 μg of 17β-estradiol daily for 10 days and then were divided into 3 equal groups (i.e., vehicle, 10 mg PDTC, and 30 mg PDTC). The rats were intraperitoneally injected twice with vehicle or PDTC at doses of 10 mg/kg or 30 mg/kg body weight 24 hours before and immediately after the induction of TMJ inflammation. Food intake was measured 4 hours before (baseline) and 20 hours after the induction of TMJ inflammation, whereas the head withdrawal threshold was measured 2 hours before (baseline) and 22 hours after the induction of TMJ inflammation. Expression of the NF-κB downstream genes in the synovial membrane was examined 24 hours after the induction of TMJ inflammation. For comparison, expression of these genes in the control and sham groups was also examined in the same running batch of real-time PCRs; the expression of these genes in the control group served as the baseline.

Statistical analysis. Statistical analysis was performed with SPSS version 11.5 for Windows. All data are presented as the mean ± SEM. Differences between groups were analyzed by two-way analysis of variance. *P* values less than 0.05 were considered significant.

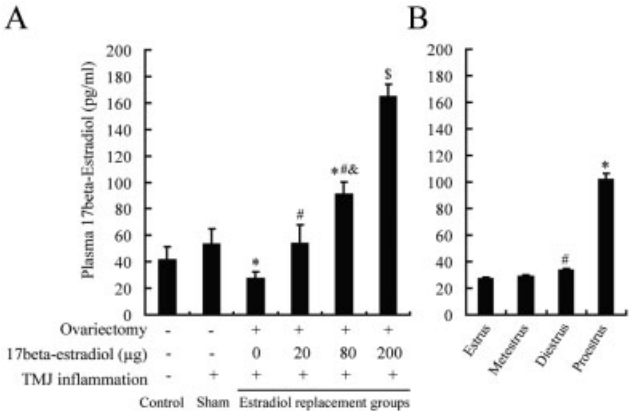

Figure 1. Confirmation of the effectiveness of ovariectomy and estradiol replacement in female rats. **A,** Plasma levels of 17β-estradiol in the experimental groups. Among ovariectomized rats, plasma levels of estradiol were lowest in the group that received no estradiol and increased in a dose-dependent manner in the groups that received estradiol. $*$ = $P < 0.05$ versus control and sham; # = $P < 0.05$ versus 0 μg; & = $P < 0.05$ versus 20 μg; \$ = $P < 0.05$ versus all other groups, by two-way analysis of variance (ANOVA). **B,** Plasma levels of 17β-estradiol in intact female rats during different estrous stages. # = $P < 0.05$ versus estrus; $*$ = $P < 0.05$ versus all other groups, by two-way ANOVA. Bars show the mean ± SEM results for 4–6 rats per group in **A** and for 3–4 rats per group in **B**. TMJ = temporomandibular joint.

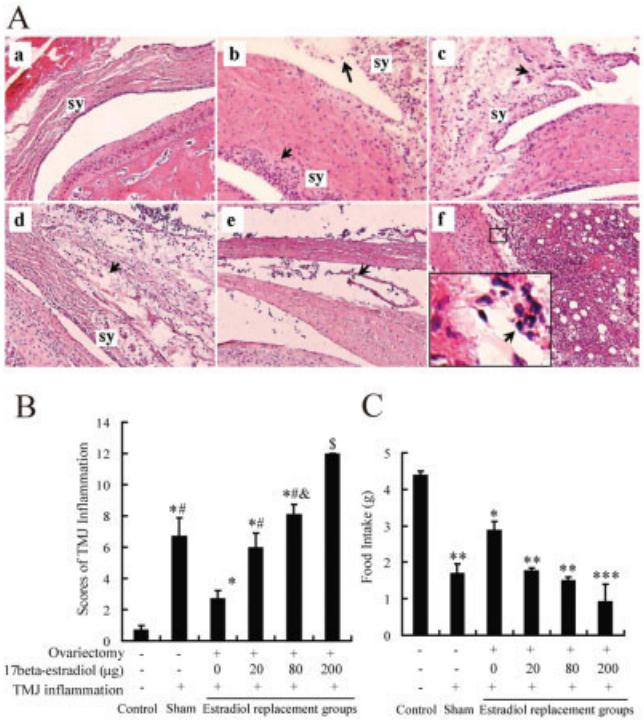

Figure 2. Evaluation of Freund's complete adjuvant–induced TMJ inflammation and pain. **A,** Representative photomicrographs of the TMJ regions in female rats, obtained 24 hours after the induction of inflammation. **a,** Synovial membrane (**sy**) of a control TMJ injected with saline. **b,** Proliferation of synovial cells (**arrowhead**) and erosion of the synovial lining (**arrow**) in the TMJ of a sham-ovariectomized rat. **c,** Dilated vasculature (**arrowhead**) in the TMJ of a rat in the 0-μg group. **d,** Tissue edema (**arrowhead**) in the TMJ of a rat treated with 20 μg of estradiol. **e,** Fibrin-like exudate in the superior joint space (**arrowhead**) in the TMJ of a rat treated with 80 μg of estradiol. **f,** Several infiltrated mononuclear cells (**arrowhead**) in the lower joint space in the TMJ of a rat treated with 200 μg of estradiol. Large boxed area shows a higher-magnification view of the small boxed area. Original magnification × 200. **B,** Estradiol-potentiated TMJ inflammation. Scores for TMJ inflammation increased with increasing doses of estradiol. $* = P < 0.05$ versus control; $\# = P < 0.05$ versus 0 μg; & $= P < 0.05$ versus 20 μg; \$ $= P < 0.05$ versus all other groups, by two-way ANOVA. **C,** Effect of estradiol-enhanced TMJ pain on food intake. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ versus control, by two-way ANOVA. Bars show the mean ± SEM results for 4 rats per group in **B** and for 6 rats per group in **C**. See Figure 1 for other definitions.

RESULTS

Effect of increasing doses of estradiol on plasma levels of estradiol. Plasma levels of estradiol in the ovariectomized groups receiving increasing doses of estradiol increased dose dependently ($P < 0.05$), with levels in the groups receiving 0 μg and 200 μg being, as expected, the lowest (mean ± SEM 27.10 ± 5.12 pg/ml) and the highest (164.73 ± 9.02 pg/ml), respectively (Figure 1A). Plasma levels of estradiol in the control

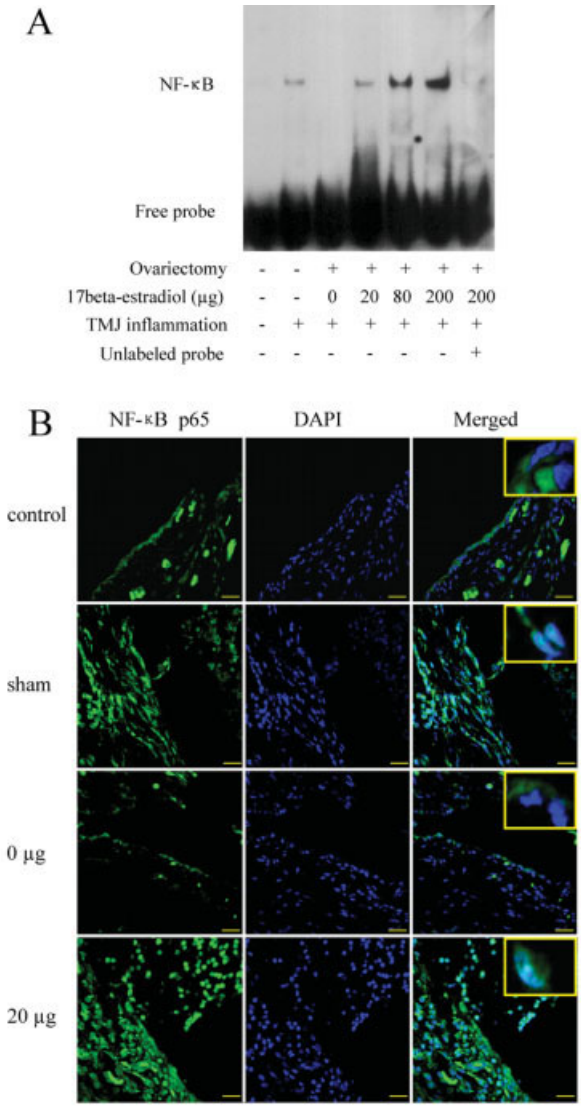

Figure 3. Estradiol-potentiated activation of NF-κB in the synovial membrane of inflamed temporomandibular joints (TMJs). **A,** Potentiation of the DNA-binding activity of NF-κB in the synovial membrane by increasing doses of estradiol. Activation of NF-κB was examined with electrophoretic mobility shift assay 24 hours after the induction of TMJ inflammation. Unlabeled probes completely blocked formation of the DNA-binding complexes. **B,** Translocation of NF-κB p65 in the synovial membrane. The subcellular translocation of p65 was examined using confocal microscopy 24 hours after the induction of TMJ inflammation. Whereas the fluorescence signal of NF-κB p65 (green) was located in the cytoplasm of the cells of the synovial membrane and the connective sublining layer of the control group, it was mainly located in the nuclei of the synovial cells in the sham-ovariectomized and 20-μg groups after induction of inflammation. In contrast, the fluorescence signal of p65 was still located mainly in the cytoplasm and barely translocated into the nuclei of the synovial cells of the 0-μg group after induction of inflammation. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). The green staining of NF-κB p65 changed into watery blue after merging with the blue staining of the nuclei. Boxed areas show higher-magnification views. Bars = 20 μm.

group (mean ± SEM 41 ± 4.06 pg/ml) and the sham-operated group (53 ± 12.12 pg/ml) were higher than that in the group treated with 0 μg ($P < 0.05$), comparable with that in the group treated with 20 μg (53.9 ± 13.94 pg/ml; $P > 0.05$), and lower than that in the groups treated with 80 μg (90.8 ± 6.85 pg/ml) and 200 μg ($P < 0.05$).

In intact female rats, the plasma levels of estradiol during estrus, metestrus, diestrus, and proestrus were 27.35 ± 1.12 pg/ml (n = 4), 29.6 ± 1.08 pg/ml (n = 3), 34 ± 0.9 pg/ml (n = 3), and 102.47 ± 7.03 pg/ml (n = 3), respectively. The level during diestrus was higher than that during estrus, and the level during proestrus was highest ($P < 0.05$) (Figure 1B).

Estradiol-aggravated CFA-induced TMJ inflammation. Twenty-four hours after the injection of CFA into the TMJs, chromodacryorrhea and intense redness and swelling over the TMJ region were observed in all CFA-injected rats but not in the control group. Histopathologic examination showed the features of synovitis, including proliferation of synovial cells, the presence of a fibrin-like exudate in the superior joint space, and dilated vasculatures and infiltrated mononuclear cells under the synovial membrane in the CFA-injected TMJs; no such features were observed in a control TMJ (Figure 2A). The scores for TMJ inflammation significantly increased in the CFA-injected groups compared with those in the control group ($P < 0.05$). Moreover, the scores for the ovariectomized groups treated with estradiol were further potentiated by estradiol in a dose-dependent manner. However, scores in the group receiving 0 μg did not increase as much as those in the sham-operated group ($P < 0.05$) (Figure 2B).

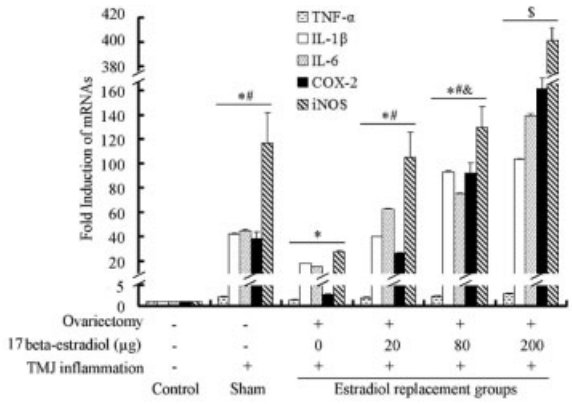

Figure 4. Estradiol-potentiated transcription of NF-κB target genes in the synovial membrane of inflamed temporomandibular joints (TMJs). Transcription of tumor necrosis factor α (TNFα) interleukin-1β (IL-1β), IL-6, cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS) in the synovial membrane was examined by real-time polymerase chain reaction 24 hours after the induction of TMJ inflammation. The mRNA expression of these genes was significantly induced in the groups with inflammation and was further potentiated by estradiol in a dose-dependent manner. Note that the expression of these genes in the 0-μg group did not increase as much as that in the sham-ovariectomized group. Bars show the mean ± SEM results for 3 rats per group. ∗ = $P < 0.05$ versus control; # = $P < 0.05$ versus 0 μg; & = $P < 0.05$ versus 20 μg and sham (except iNOS in the sham group); $ = $P < 0.05$ versus all other groups, by two-way analysis of variance.

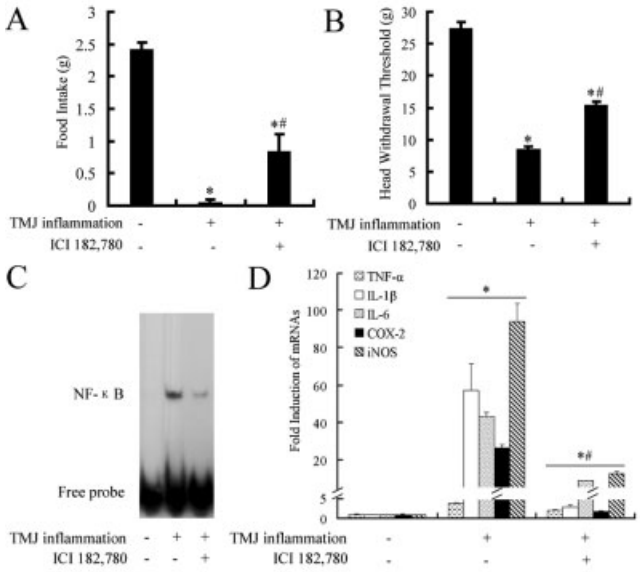

Figure 5. Attenuation of mechanical allodynia and inflammation in the TMJs of female rats by blocking of estrogen receptors. **A** and **B,** Blocking of the estrogen receptor partially reversed the TMJ inflammation–induced decrease in food intake (**A**) and the head withdrawal threshold (**B**). **C,** Blocking of the estrogen receptor attenuated the TMJ inflammation–induced DNA-binding activity of NF-κB in the synovial membrane. Activation of NF-κB was examined by electrophoretic mobility shift assay, 24 hours after the induction of TMJ inflammation. **D,** Blocking of the estrogen receptor attenuated the transcription of NF-κB target genes in the synovial membrane. Inflammation-induced up-regulation of TNFα, IL-1β, IL-6, COX-2, and iNOS mRNA was partially blocked in the group with inflammation that was pretreated with ICI 182780. Bars show the mean ± SEM results for 5 rats per group in **A** and **B** and for 3 rats per group in **D**. ∗ = $P < 0.05$ versus control; # = $P < 0.05$ versus group with inflammation that did not receive treatment with ICI 182780, by two-way analysis of variance. See Figure 4 for definitions.

Estradiol-exacerbated TMJ inflammation and pain. Food intake is associated with TMJ inflammation and pain, with lower intake associated with more severe pain (19). As shown in Figure 2C, food intake significantly decreased in all of the CFA-treated groups compared with the control group ($P < 0.05$). Although the difference was not statistically significant, food intake by

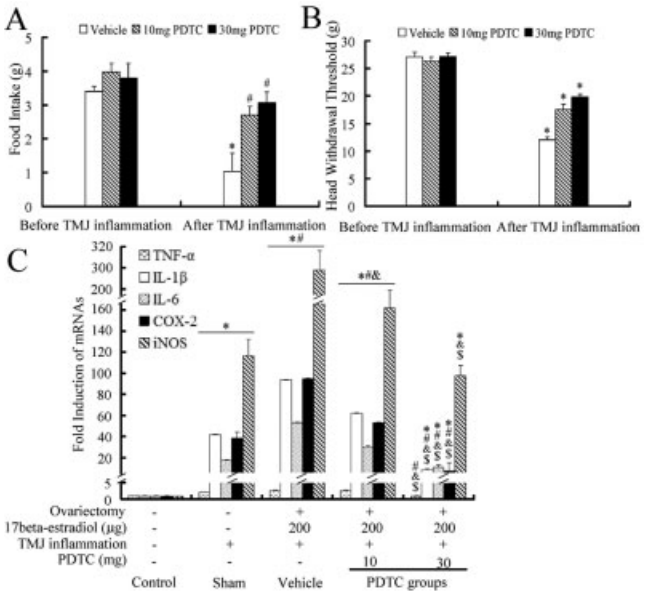

Figure 6. Attenuation of TMJ mechanical allodynia and estradiol-potentiated transcription of NF-κB target genes by blocking of NF-κB. Ovariectomized rats treated with 200 μg of estradiol per day for 10 days were pretreated with pyrrolidine dithiocarbamate (PDTC; 10 mg or 30 mg) or vehicle 24 hours before and immediately before induction of TMJ inflammation. **A** and **B,** Blocking of NF-κB almost completely reversed and partially reversed the TMJ inflammation–induced decrease in food intake and the head withdrawal threshold, respectively. $* = P < 0.05$ versus all other groups both before and after induction of TMJ inflammation; $\# = P < 0.05$ versus all groups before induction of TMJ inflammation and versus vehicle after induction of TMJ inflammation, by two-way analysis of variance (ANOVA). **C,** Blocking of NF-κB attenuated the estradiol-potentiated transcription of NF-κB target genes in the synovial membrane. The estradiol-potentiated induction of NF-κB target genes was partially and dose-dependently reversed in the groups pretreated with PDTC. $* = P < 0.05$ versus control; $\# = P < 0.05$ versus sham; $\& = P < 0.05$ versus vehicle; $\$ = P < 0.05$ versus 10 mg PDTC, by two-way ANOVA. Bars show the mean ± SEM results for 5 mice per group in **A** and **B** and for 3 mice per group in **C**. See Figure 4 for other definitions.

rats in the 0-μg group seemed higher than that by rats in the other CFA-treated groups, whereas food intake by rats in the ovariectomized groups showed a trend toward decreasing as the doses of estradiol increased.

Estradiol-potentiated activation of NF-κB in the synovial membrane of inflamed TMJs. To evaluate whether the estradiol-induced aggravation of TMJ inflammation is related to NF-κB, the DNA-binding activity of NF-κB in the synovial membrane of the TMJ was examined by EMSA. As shown in Figure 3A, the DNA-binding activity of NF-κB was significantly enhanced in the synovial membrane in the CFA-treated groups compared with the control group but not in the 0-μg group. Moreover, the DNA-binding activity of NF-κB was dose-dependently activated in the ovariectomized groups receiving increasing doses of estradiol. The unlabeled probes completely blocked formation of the DNA-binding complexes, indicating that the binding complexes were specific for the NF-κB binding sites.

To further confirm the activation of NF-κB in the synovial membrane of inflamed TMJs, we also examined the subcellular translocation of p65, using confocal microscopy. Before the induction of inflammation, the fluorescence signal of p65 was located in the cytoplasm of cells in the synovial membrane (including the connective sublining layer). After the induction of TMJ inflammation, the fluorescence signal of p65 was mainly located in the nuclei of synovial cells in the sham and 20-μg groups. In contrast, the fluorescence signal of p65 was still mainly located in the cytoplasm, barely translocated into the nuclei of synovial cells in the 0-μg group (Figure 3B).

Estradiol-potentiated transcription of NF-κB target genes in the synovial membrane of inflamed TMJs. As shown in Figure 4, the expression of TNFα, IL-1β, IL-6, COX-2, and iNOS messenger RNA (mRNA) was significantly induced in the groups with inflammation compared with the control group ($P < 0.05$). Corresponding to the activation of NF-κB, expression of these genes in the ovariectomized groups was further potentiated by estradiol in a dose-dependent manner ($P < 0.05$). However, the expression of these genes in the 0-μg group did not increase as much as that in the sham-operated group.

Effect of blocking estrogen receptors on TMJ mechanical allodynia and inflammation. We first examined whether the estrogen receptor antagonist ICI 182780 could suppress TMJ inflammation and pain in female rats that had not undergone ovariectomy. As shown in Figures 5A and B, food intake and the head withdrawal threshold significantly decreased in the group with inflammation 20 hours and 22 hours after the induction of TMJ inflammation, respectively, compared with the control group ($P < 0.05$). The decreased food intake and head withdrawal threshold were partially reversed in the group with inflammation that received pretreatment with ICI 182780 compared with the control group and the group with inflammation that did not receive pretreatment with ICI 182780 ($P < 0.05$). The inflammation-induced DNA-binding activity of NF-κB was also partially blocked in the synovial membrane of the group with inflammation that received pretreatment with ICI 182780 compared with that of the control group and the group with inflammation that did not receive pretreatment with ICI 182780 (Figure 5C). Correspondingly, the inflammation-induced transcription of the

NF-κB target genes TNFα, IL-1β, IL-6, COX-2, and iNOS was also partially blocked by pretreatment with ICI 182780 ($P < 0.05$) (Figure 5D).

We then examined whether the estrogen receptor antagonist ICI 182780 could suppress TMJ inflammation and pain in ovariectomized rats treated with estradiol at a dosage of 200 μg/day for 10 days. Similar to the results described above, the inflammation-induced decrease in food intake and the head withdrawal threshold in the group with inflammation was completely and partially reversed, respectively, by pretreatment with ICI 182780 ($P < 0.05$) (data not shown); the inflammation-induced increase in the scores for TMJ inflammation in the group with inflammation was also partially reversed by pretreatment with ICI 182780 ($P < 0.05$) (data not shown).

Effect of blocking NF-κB on TMJ mechanical allodynia and estradiol-potentiated transcription of NF-κB target genes. As shown in Figures 6A and B, among ovariectomized rats treated with 200 μg of estradiol, food intake and the head withdrawal threshold before induction of TMJ inflammation were not different between the groups treated with PDTC (10 mg or 30 mg) and the group treated with vehicle ($P > 0.05$). After the induction of inflammation, both food intake and the head withdrawal threshold significantly decreased in the group that received vehicle compared with the groups pretreated with PDTC ($P < 0.05$). However, the TMJ inflammation–induced decrease in food intake and the head withdrawal threshold was almost completely and partially reversed, respectively, in the groups that received pretreatment with PDTC (10 mg or 30 mg), compared with groups before induction of TMJ inflammation and the group that received vehicle after the induction of TMJ inflammation ($P < 0.05$). Moreover, the estradiol-potentiated induction of TNFα, IL-1β, IL-6, COX-2, and iNOS mRNA expression in the synovial membrane was also partially and dose-dependently blocked in the group pretreated with PDTC ($P < 0.05$) (Figure 6C).

DISCUSSION

In the present study, we observed 2 important findings regarding the involvement of estradiol in TMJ inflammation through the NF-κB pathway. First, TMJ inflammation and synovial NF-κB and its downstream genes were potentiated by estradiol in ovariectomized rats. Second, blocking of the estrogen receptor or NF-κB partially reversed the effects of estradiol on the inflamed TMJ. These results may help our understanding of why the prevalence, severity, and duration of TMD-related pain are greater in women of childbearing age than in men.

Estradiol may exacerbate TMJ pain through the aggravation of synovial inflammation, a possible local mechanism underlying the effects of estradiol on TMJ inflammation and pain. We observed that increasing plasma levels of estradiol were associated with increasingly severe inflammation and pain in TMJs with CFA-induced inflammation. This phenomenon was further supported by our data that estradiol could potentiate the DNA-binding activity of NF-κB and the transcription of the proinflammatory cytokines TNFα, IL-1β, IL-6, COX-2, and iNOS. Blocking estrogen receptors with ICI 182780 or inhibiting NF-κB with PDTC in both nonovariectomized female rats and ovariectomized rats that received estradiol replacement could partially reverse the effects of estradiol on TMJ inflammation and pain. These data suggest that estradiol could potentiate TMJ inflammation, at least partially, through estrogen receptors and the NF-κB pathway. Our data also suggest that estradiol-aggravated TMJ inflammation could be an important reason for the sex differences observed in TMD.

The aggravation of TMJ inflammation and pain by estradiol is consistent with the results observed in a formalin-induced pain model, in which nociceptive responses were lower in estrogen receptor β–knockout female mice than in wild-type female mice (32). Our data on the estradiol-enhanced transcription of NF-κB target genes in the synovial membrane also agree with the report that estradiol induces IL-1α gene expression in rheumatoid synovial cells (33).

The effects of estradiol on the inflamed TMJ partially depended on the NF-κB pathway, suggesting that the estrogen receptor positively cross-talked with NF-κB. Although estradiol suppresses NF-κB in several cell types (15), we observed that estradiol enhanced NF-κB in the synovial cells of inflamed TMJs. Hardly any activation and translocation of NF-κB in the synovial membrane were observed in the inflamed TMJs of rats in the ovariectomized group that received no estradiol replacement; thus, the activation and translocation of NF-κB in the synovial membranes of inflamed TMJs were facilitated unexpectedly, depending on the plasma level of estradiol. Correspondingly, TMJ inflammation and pain were also less severe in this group. Moreover, estradiol-potentiated TMJ inflammation could be partially blocked by the NF-κB–specific inhibitor PDTC. Interestingly, estrogen receptor antagonist ICI 182780 could also partially block the inflammation-induced ac-

tivation of NF-κB and the transcription of its downstream genes, further supporting the idea that the estrogen receptor cross-talked with NF-κB.

Considering that NF-κB is a pivotal regulator of the inflammatory response, the cross-talk between the estrogen receptor and NF-κB in the TMJ synovial membrane may be one of the major causes for estrogen-induced aggravation of TMJ inflammation. The enhancement of the NF-κB pathway by estrogen was also observed in other cell types (16–18). Recently, the positive cross-talk between the estrogen receptor and NF-κB was demonstrated to be more prominent than that of reciprocal antagonism in MCF-7 cells (34). Whether estrogen enhances or suppresses NF-κB may be related to different cell types and contexts. To the best of our knowledge, the present study is the first to demonstrate that the proinflammatory effects of estradiol depend on the NF-κB pathway in the synovial membrane of the inflamed TMJ.

The cytokine network plays an important role in estradiol-mediated TMJ inflammation. Our data on the transcription of NF-κB target proinflammatory cytokines robustly potentiated by estradiol provide further details on the proinflammatory effects of estradiol on joint inflammation and pain. TNFα, IL-1β, and IL-6 appear to be the major proinflammatory cytokines involved in TMJ pathology (35). These cytokines can stimulate synoviocyte proliferation and subsequent activation (36); they can also activate NF-κB (13). Therefore, estradiol-induced potentiation of these cytokines in the synovial membrane will certainly result in aggravated TMJ inflammation.

In addition, COX-2 is a key enzyme involved in the synthesis of prostaglandins, among which prostaglandin E_2 can particularly contribute to joint pain (37). Inducible NOS is also involved in the pathologic process of inflammatory pain and is associated with persistent inflammation and synovial membrane destruction in osteoarthritis (38). Our data on estradiol-potentiated transcription of COX-2 and iNOS through NF-κB in the inflamed TMJ reveal another profile of the possible mechanisms underlying the role of estrogen in TMJ pain. Our data on the transcription of COX-2 and iNOS depending on NF-κB in the synovial membrane are also consistent with those of previous studies (39,40).

Measurement of circulating estradiol during the estrous cycle in normal female rats served as the reference with which to confirm that the concentration of circulating estradiol in ovariectomized rats that received estradiol replacement remained mostly within the physiologic level of normal female rats. Even in the ovariectomized group that received 200 μg of replacement estradiol, the plasma level of estradiol was approximately the same as that observed in rats during the late stage of pregnancy (41). It is noteworthy that the level of circulating estradiol in the ovariectomized rats that did not receive estradiol replacement was not negligible; rather, it was comparable with that observed in normal female rats during estrus. The reason for this is that estradiol can also be produced in extragonadal sites, such as adipose tissue, adrenal gland, and brain, and acts locally at these sites as a paracrine signal (42). Therefore, the circulating level in the ovarectomized group that did not receive estradiol replacement may reflect rather than direct this extragonadal synthesis of estradiol and release into the blood (42).

To explore the effects of estrogen on TMJ inflammation, a precise evaluation of TMJ inflammation is important. Previously, TMJ inflammation was commonly evaluated by visually assessing swelling of the TMJ (43), chromodacryorrhea (44), or exudation in the joint cavity (45). We also observed significant swelling of the TMJ and chromodacryorrhea after the induction of TMJ inflammation. However, these 2 indices are not sensitive enough to reflect the different degrees of TMJ inflammation. Therefore, we developed a histopathologic scoring standard to evaluate CFA-induced TMJ inflammation. This scoring standard showed good sensitivity for differentiating the degrees of TMJ inflammation as well as good repeatability and thus could be suggested for evaluating TMJ inflammation in the future.

The mechanism by which estrogen modifies inflammation is exceedingly complex. Our findings on the induction of proinflammation by estradiol in the inflamed TMJ seem to be in contrast with the general antiinflammatory effects of estrogen in the brain, bone, vasculature, and skin (46). However, proinflammation induced by estrogen was also observed in the setting of prostatitis (47), B cell–driven systemic lupus erythematosus (48), and allergic lung inflammation (31). A recent study showed that in synoviocytes, 17β-estradiol is mainly converted to proinflammatory metabolites such as 16α-hydroxylated estradiol (49). This conversion of estradiol into proinflammatory metabolites in synoviocytes may be an important mechanism underlying the proinflammatory effects of estradiol in the inflamed TMJ. As pointed out by Straub (46), the dual proinflammatory and antiinflammatory effects of estrogens in human and experimental inflammation depend on the immune stimulus, the cell types involved during different disease phases, the specific microenvironment, the

timing of estrogen administration in relation to the disease course, the concentration of estrogens, the variability in estrogen receptor expression, and the intracellular conversion of estrogens. The proinflammatory and antiinflammatory effects of estrogen may also be influenced by other sex hormones such as progesterone (31). Therefore, the mechanism underlying the proinflammatory effects of estrogen in the TMJ still need to be fully elucidated.

In conclusion, our study demonstrated that estradiol potentiated TMJ inflammation and pain through activation of the NF-κB pathway to induce the transcription of proinflammatory genes. These results may help us understand why women of childbearing age are clinically predominant among patients with TMDs. These results also imply that the estrogen receptor or the NF-κB pathway may be a target for the treatment of TMJ inflammation and pain.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Gan and Ma had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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