EXTENDED REPORT

Oestrogens inhibit interleukin 1 β -mediated nitric oxide synthase expression in articular chondrocytes through nuclear factor- κ B impairment

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Objectives: To investigate the presence and functionality of oestrogen receptor α (ER α) in interleukin (IL)1 β -treated rabbit articular chondrocytes in culture, and to determine the mechanisms of 17 β oestradiol (E2) effects on IL1 β -induced inducible nitric oxide synthase (iNOS) expression.

Methods: The presence and functionality of ER α were investigated by immunocytochemistry and transient expression of an E2-responsive reporter construct. iNOS expression and production were determined by transient expression of a chimeric iNOS promoter-luciferase construct and protein immunoblotting. Nitric oxide (NO) production was determined by the Griess reaction. DNA-binding activities of nuclear factor- κ B (NF- κ B) and activated protein 1 were determined by electrophoretic mobility shift assay (EMSA)-ELISA assays. Nuclear translocation of p65 was studied by immunocytochemistry.

Results: ER α was identified in the nucleus of chondrocytes. ER α efficiently transactivated a transiently expressed E2-responsive construct. On IL1 β treatment, ER α partially diffused from its nuclear localisation into the cytoplasm and its transactivation ability was impaired. Nevertheless, E2, tamoxifen and raloxifene efficiently inhibited IL1 β -induced NO production (–34%, –31% and –36%, respectively). E2 decreased IL1 β -induced iNOS protein expression (–40%). Transient expression of an iNOS promoter construct strongly suggested that iNOS expression was inhibited at the transcriptional level, and EMSA-ELISA assays showed that E2 reduced (–60%) the IL1 β -induced p65 DNA-binding capacity. Finally, the p65 nuclear translocation induced by IL1 β was also strongly decreased by E2.

Conclusions: Our data support a reciprocal antagonism between oestrogens and IL1 β , ultimately resulting in the decrease of cytokine-dependent NO production through transcriptional inhibition of iNOS expression. This effect was associated with selective inhibition of p65 DNA binding and nuclear translocation.

Epidemiological studies are consistent with a role for post-menopausal oestrogen deficiency in increasing the risk for osteoarthritis.^{1–3} The increase in cartilage turnover, as observed in menopausal women,⁴ is counteracted by oestrogen replacement therapy (ERT) or selective oestrogen-receptor modulators (SERM).^{4–5}

In animal models, ovariectomy induces osteoarthritis-like pathological changes. These effects are partly inhibited by oestrogens or SERM.^{3–7} Oestrogens enhance synthesis of proteoglycans in cartilage and increase production of insulin-like growth factor system components in synovial fluid and chondrocytes.^{8–10}

The molecular mechanisms by which oestrogens influence cartilage homeostasis are not fully understood. Oestrogen receptor α (ER α) has been found in chondrocytes from humans and other species,³ but its presence and functionality in cartilage cells stimulated by interleukin 1 β (IL1 β) have never been specifically considered. Chondrocytes challenged with IL1 β produce effectors of cartilage degradation and inflammation, including matrix metalloproteases and inducible nitric oxide synthase (iNOS).¹¹ Induction of iNOS leads to the production of nitric oxide (NO), supposed to play a major role in the degradation of cartilage extracellular matrix.^{12–13} Recent in vitro studies suggest that 17 β -oestradiol (E2) reduces IL1 β -induced phosphatidyl glycerol degradation in chondrocytes, presumably by a decrease in the expression and activity of matrix metalloproteases,¹⁴ and protects chondrocytes from oxygen radical-induced damage.¹⁵ In addition, E2 has been shown to modulate iNOS expression in different cell types.^{16–17}

The aim of our work was to investigate ER α functionality in IL1 β -treated articular chondrocytes and to identify the effects of oestrogen on IL1 β -induced iNOS gene expression.

MATERIALS AND METHODS

Cell culture

Articular chondrocytes were obtained from 2-month-old female rabbits (CPA, Orleans, France) and cultured in conditions avoiding cell dedifferentiation as described previously.^{14–18} Chondrocytes were incubated for 20 h in the presence of 2.5 ng/ml IL1 β (PeproTech, New Jersey, USA), alone or in combination with E2 (Sigma-Aldrich, Lyon, France), or the classic SERM compounds 4-OH-tamoxifen (Sigma-Aldrich, Steinheim, Germany) or raloxifene (Lilly, Indiana, USA) at various concentrations. The culture medium was used for nitrite accumulation assay, and the iNOS protein was characterised in cell lysates by western blot. Shorter incubations were performed for immunocytochemistry or nuclear factor- κ B (NF- κ B) and activated protein 1 (Ap-1) factor analysis.

Transient expression and luciferase assay

Chondrocytes were cultured in Ham's F12 medium containing 10% fetal calf serum-supplement, trypsinised and plated in six-well plates. At 70% confluence, cells were passed in serum-free

Abbreviations: Ap-1, activated protein 1; ER α , oestrogen receptor α ; ERE, oestrogen responsive element; ERT, oestrogen replacement therapy; iNOS, inducible nitric oxide synthase; NF- κ B, nuclear factor κ B; SERM, selective oestrogen receptor modulators

conditions (0.3% bovine serum albumin) and transfected either with 1 µg of the 3×ERE–luciferase construct (3×ERE–Luc) or 1 µg of the murine iNOS promoter–luciferase construct in the presence of 200 ng of a β-galactosidase vector (pSV-βGal, Promega, Paris, France).^{19, 20} Data were expressed as luciferase activity normalised to the β-galactosidase activity and protein concentration.

Immunocytochemistry

Chondrocytes were grown as primary cultures on 2 cm² glass slides (Nunc, Strasbourg, France), treated with 10 nM E2 or IL1β (2.5 ng/ml) for 60 min, alone or in combination. Immunocytochemistry was performed as described previously.²¹ The anti-ER 1D5 monoclonal antibody (1/40 dilution) was purchased from Dako (Dakocytomation, Trappes, France). We used an anti-p65 goat polyclonal antibody (Santa Cruz, Tebu-Bio SA, France) and an Alexa 488-coupled second antibody to mouse IgGs (Invitrogen SARL, Cergy Pontoise, France). About 100–200 cells were counted per condition. Cells shown in the figures are representative of the major population observed.

Western blotting

Cells treated with IL1β alone or combined with different E2 concentrations for 20 h were washed twice with cold Gey's balanced solution and scrapped off the flask in cold lysis buffer containing Complete protease inhibitors (Roche, Meylan, France). Protein extracts and western blotting were performed as described previously.²¹

Nitrites assay

NO production was measured as the amount of nitrites released into the culture medium, determined on centrifuged medium by the Griess reaction assay.²²

Ap-1 and NF-κB electrophoretic mobility shift assay – ELISA assays

The DNA binding activities of NF-κB or Ap-1 present in crude nuclear protein extracts from chondrocytes were determined with the TransAM Ap-1 and NF-κB family ELISA (Active Motif, Rixensart, Belgium). Subconfluent chondrocytes were treated with 10 nM E2 for 1 h. IL1β was then added at 2.5 ng/ml for either 15 or 60 min of incubation. In all, 5 µg of cellular protein was used for the assay. The NF-κB proteins were detected with mouse anti-P65, anti-P50 or anti-Rel-B antibodies, followed by a peroxidase-conjugated goat secondary antibody. Ap-1 proteins were similarly detected with mouse anti-cFos, anti-FosB, anti-Fra1, anti-Fra2, anti-c-Jun, anti-JunB or anti-JunD antibodies and peroxidase-conjugated goat secondary antibody. For colorimetric detection, microwells were read at 450 nm with an MRX II microplate reader (Dynatech Laboratories, Guyancourt, France).

Data analysis

Each experiment was conducted at least three times. The mean (standard deviation) of each variable was calculated. Analysis of variance was performed on all of the experiments. When an F value was found to be significant, the analysis of variance was followed by multiple comparisons with the Tukey test. p Values <0.05 were considered significant.

RESULTS

The oestrogen receptor is present and functional in rabbit chondrocytes

Immunocytofluorescence analysis showed ERα to be nuclear in chondrocytes in basal conditions (fig 1A, panel 1). This nuclear staining was slightly enhanced in cells receiving E2 treatment (fig 1A, panel 2). IL1β treatment strongly decreased ERα

nuclear staining and elicited a diffused cytonuclear localisation (fig 1A, panel 3). This was counteracted partly by 10 nM E2 (fig 1A, panel 4).

Chondrocytes transfected with the 3×ERE–Luciferase construct reporter construct were treated with increasing amounts of for 24 h, alone or in combination with 2.5 ng/ml IL1β. Oestradiol elicited a dose-dependent transactivation of the reporter gene with a 4.2-fold increase at 10 nM E2. Cotreatment with IL1β strongly impaired (–55%) the effect of 10 nmol oestradiol, which still remained significant (fig 1B).

Oestradiol inhibits IL1β-induced NO production

Chondrocytes were incubated with increasing concentrations of E2 with or without IL1β to investigate effects of E2 on NO production. No nitrite accumulation was detected in the culture medium from basal conditions, or in the presence of E2 or SERM alone (fig 2A). IL1β treatment induced nitrite accumulation, which decreased (–34%, *p*<0.01) on cotreatment with 10 nM E2 (fig 2B). The SERM compounds 4-OH-tamoxifen and raloxifene also showed an oestrogen agonistic effect on IL1β-induced NO production (fig 2B,C), with a significant inhibitory effect at 1 nmol/l (–31% and –36%, respectively; *p*<0.01).

Figure 1 Presence and functionality of oestrogen receptor α (ERα) in interleukin (IL)1β-treated chondrocytes. (A) ERα was detected in chondrocytes in presence or absence of 10 nM 17 β-oestradiol (E2) combined with 2.5 ng/ml IL1 β. Untreated chondrocytes showed a nuclear localisation for ERα (panel 1), which was slightly enhanced in E2 treated cells (panel 2). IL1β treatment decreased the ERα nuclear staining (panel 3) and this was counteracted by 10 nM E2 (panel 4). A total of 100 cells/coverslip were counted for a semiquantitative analysis of the cellular localisation of oestrogen receptor. Cells shown are representative of the major population in each condition. (B) Chondrocytes were transiently transfected with the pERE3-luciferase (Luc) vector and a β-galactosidase vector and further treated with effectors for 24 h, alone or in combination. Luciferase activity was calculated as fold-induction in treated cells compared with the activity in untreated cells. Luciferase activity were normalised with β-galactosidase activities. ***p*<0.01.

Figure 2 Effects of oestrogens on interleukin (IL)1 β -induced nitrite accumulation. Chondrocytes were treated for 24 h with the indicated concentrations of oestradiol; (A), 4-OH-tamoxifen (B) or raloxifene (C) with or without IL1 β (2.5 ng/ml). Nitrite accumulation in culture medium was determined by the Griess reaction. ** $p < 0.01$.

Estradiol counteracts IL1 β -mediated iNOS protein expression and promoter transactivation

As observed by western blotting (fig 3A), naive chondrocytes did not express iNOS (lane 1) whereas IL1 β -treated chondrocytes displayed a robust induction (lane 2). Addition of E2 reduced iNOS expression in a dose-dependent manner (lanes 3–5), with a significant (-40% ; $p < 0.01$) decrease at 10 nmol/l (lane 5).

We next investigated inhibitory effects of E2 on IL1 β -induced iNOS transcription. Chondrocytes were transiently transfected with an iNOS promoter-luciferase construct and incubated for 24 h with increasing E2 concentrations alone or in combination with IL1 β . E2 alone was unable to induce luciferase activity whereas IL1 β increased it sevenfold (fig 3B). This induction was inhibited (-41%) when cells were cotreated with IL1 β and 10 nM E2 ($p < 0.01$). These data suggest that E2 inhibits IL1 β -induced iNOS expression at the transcriptional level.

Oestradiol impairs the NF- κ B pathway in chondrocytes

We investigated a possible effect of E2 against IL1 β -induced signalling pathways by analysing E2-mediated modulation of NF- κ B and Ap-1 DNA binding activities. Chondrocytes were challenged or not with 10 nM E2 treatment in a preliminary 60 min period. Cells were then challenged or not with IL1 β (2.5 ng/ml) for another 15 min. Protein extracts from the various conditions were assayed for NF- κ B and Ap-1 binding activities.

E2 alone had no effect on the NF- κ B binding capacity (fig 4A). By contrast, IL1 β strongly induced p65 DNA binding.

Figure 3 Effects of 17 β -oestradiol (E2) on inducible nitric oxide synthase (iNOS) protein expression and gene promoter transactivation. (A) Western blot analysis of iNOS expression. Chondrocytes were treated with interleukin (IL)1 β (2.5 ng/ml) alone or with different E2 concentrations for 20 h (1, no treatment; 2, IL1 β alone; 3, IL1 β with E2 0.1 nM E2; 4, IL1 β with E2 1 nM E2; 5, IL1 β with E2 10 nM E2). The electrophoresed proteins were blotted on a nylon membrane and revealed with an anti-iNOS antibody. Blots were revealed by chemoluminescence. (B) Chondrocytes were transiently transfected with a luciferase (Luc) reporter construct bearing a 1749 bp fragment of the iNOS murine gene promoter and a β -galactosidase expression vector and further treated with effectors for 24 h, alone or in combination. Luciferase activity was calculated as in fig 1B. ** $p < 0.01$. Ap-1, activated protein 1; NF- κ B, nuclear factor- κ B.

This increase in p65 DNA-binding capacity was significantly reduced (-60% ; $p < 0.01$) when cells were cotreated with E2. IL1 β elicited a minor increase in p50 and RelB DNA binding activities, unmodified by E2.

Similar experiments performed on Ap-1 proteins showed that IL1 β strongly induced JunB, JunD, c-Fos and Fra1 DNA binding activities (fig 4B), but not FosB, c-Jun or Fra2 (not shown). E2 alone also significantly increased JunB, c-Fos and Fra-1 but not JunD binding. Cotreatment of the cells with IL1 β and E2 had no further effect on JunB, JunD or c-Fos, whereas it elicited a synergistic response on Fra1-binding activity ($p < 0.01$).

Oestradiol inhibits the IL1 β -mediated nuclear translocation of NF- κ B

We finally explored the possibility that E2-mediated inhibition of NF- κ B binding to DNA might be caused by inhibition of NF- κ B nuclear translocation. Figure 5 shows that the p65 protein belonging to the NF- κ B complex was detected in the cytoplasm of untreated chondrocytes (panel 1) or E2-treated cells (panel 2), whereas it appeared nuclear or cytonuclear in cells treated with IL1 β alone for 60 min (80–90% of total cells) (panels 3,4). A short IL1 β treatment (15 min) elicited only a minor shift of p65 towards the nucleus (10–20% of total cells, not shown). The

nuclear translocation of p65 induced by IL1 β was strongly decreased by co-exposure to 10 nM E2 (20–25% of total cells, $p < 0.01$; panels 5,6).

DISCUSSION

Oestrogen receptors (ER α , ER β) have been detected in chondrocytes from humans and monkeys.^{10–23} We focused our investigations on ER α in our model of rabbit chondrocytes as it was shown to be the principal mediator of oestrogens' anti-inflammatory properties, whereas ER β seemed ineffective.¹⁶

ER α was nuclear in chondrocytes, irrespective of the presence of ligand (fig 1, panels 1,2).²⁴ The diffusion of ER α from the nucleus to the cytoplasm provoked by IL1 β (fig 1, panels 1,3) is likely to take part in the antagonism between ER α and this cytokine. The ability of E2 to challenge this delocalisation (panels 3, 4) suggests that the outcome of the crosstalk depends on ligand concentration. We are currently investigating the hypothesis that IL1 β -induced ATP depletion is involved in this process,^{25–26} as the nuclear localisation of steroid receptors is energydependent.²⁷

ER α mediated E2 transactivation of an E2-responsive reporter gene in chondrocytes. This transactivation was partly abolished by cotreatment with IL1 β (fig 1B). Despite the decrease in nuclear presence and general functionality of ER α after IL1 β stimulation, E2 and SERM remained able to counteract IL1 β -induced nitrite production in a dose-dependent manner (fig 2).

Our range of oestrogen concentrations starts from menstrual cycle up to pregnancy values (0.1–10 nmol/l). The beneficial effects of oestradiol were observed at the 10 nmol concentration, which cannot be achieved by ERT. However, our data should be replaced in the context of an in vitro study. Various hormonal compounds show a gap between their active concentrations in vivo and in vitro, the reported differences being as high as 1000-fold in some cases.^{28–29}

We observed an agonistic effect of SERM on chondrocytes. Previous in vivo studies reported that SERM mimic oestrogen effects on articular cartilage.^{5–6} As long term use of ERT has been raising increasing concern about potential adverse effects,³⁰ a SERM-based treatment may seem to be a safer alternative for the

treatment of cartilage degradation. A novel SERM, totally devoid of oestrogenic activity but able to promote oestrogen receptor-mediated NF- κ B inhibition has been designed.³¹ This compound has been used in two models of arthritis,³² and highlights the potential utility of pathway-selective oestrogen receptor ligands. Nevertheless, these in vivo studies did not investigate cartilage degradation or chondrocyte biology.

Oestrogen's effects on iNOS are controversial, as different groups have reported increases or decreases in iNOS activity after E2 treatment. The in vitro inhibitory effect of E2 on nitrite production and iNOS expression has been consistently reported in lipopolysaccharide or IL1 β -stimulated cells.^{16–17–33–34} Conversely, the stimulatory effect of oestradiol on iNOS expression was observed in naive cells.^{35–36}

The inhibitory effect of E2 on nitrites production seems to be caused by a reduction in iNOS protein expression in our chondrocyte model (fig 3A). This concurs with the results from Cake *et al*,³⁷ who showed an increase in cartilage iNOS expression in ovariectomised ewes. A transient expression assay using a fragment of the iNOS promoter showed that E2 down regulated the promoter function (fig 3B). We did not detect any oestrogen-responsive element-like sequence in the DNA fragment used, which suggested an indirect effect of E2 on promoter activity.

Mechanisms of mutual inhibition have been described between oestrogen receptors and NF- κ B³⁸ or Ap-1.³⁹ These cross talks include inhibition of NF- κ B shuttling¹⁶ and DNA binding impairment,⁴⁰ and are thought to be involved in the anti-inflammatory activities of oestrogens.⁴¹ NF- κ B mediates IL1 β effects on iNOS expression in chondrocytes.^{42–44} The regulation of iNOS expression in mammalian cells seems to be predominantly governed by NF- κ B^{45–48} and to a lesser extent by Ap-1.⁴⁹

We investigated the effects of E2 on the ability of NF- κ B and Ap-1 proteins to bind DNA and/or segregate into the nucleus. E2 rapidly (in 15 min) inhibited p65 binding to DNA (fig 4A) and impaired (in 60 min) its nuclear translocation (fig 5). The E2 effect was restricted to p65 as no modification of DNA binding was observed for p50 and RelB. The delay between the inhibition of p65 ability to bind DNA (15 min) and that of its

Figure 4 Effects of oestradiol (E2) on nuclear factor κ B (NF- κ B) and activated protein 1 (Ap-1) activities in chondrocytes. (A) Chondrocytes were treated with 10 nM E2 for 60 min, and then in combination with 2.5 ng/ml interleukin (IL)1 β for another 15 min. After cell lysis, proteins from crude nuclear extract (5 μ g) were assayed for NF- κ B-binding activity by electrophoretic mobility shift assay (EMSA)-ELISA. (B) Chondrocytes were treated with 10 nM E2 for 60 min, and then in combination with 2.5 ng/ml IL1 β for 15 min. Cells were lysed and proteins from crude nuclear extract (5 μ g) were assayed for Ap-1 binding activity by EMSA-ELISA. ** $p < 0.01$. OD, optical density.

Figure 5 Effects of 17 β -oestradiol (E2) on p65 localisation in chondrocytes. (A) Chondrocytes were treated with 10 nM E2 for 60 min, and then 2.5 ng/ml interleukin (IL)1 β was added in combination for another hour. The localisation of p65 was assessed by immunocytochemical analysis with a polyclonal anti-p65 antibody (Santa-Cruz). Panels 1 and 2 show that p65 was essentially cytoplasmic in control and E2 conditions. In the presence of IL1 β , p65 segregated into the nucleus (panels 3, 4). This change in localisation was hampered by E2 (panels 5, 6). (B) Quantification of the E2 effect on p65 intracellular localisation. Cells displaying p65 in cytoplasmic localisation are plotted as a percentage of the total number of counted cells (n=200). **p<0.01.

nuclear transfer (60 min) suggests that DNA binding is the primary target of oestrogen action.^{38 50}

Although NO is known to sustain p65 nuclear translocation,⁴² we do not think that the E2-dependent NO decrease is involved in the modulation of p65 translocation by E2. Indeed, the kinetics of our experiments (15 and 60 min) is much faster than the 1–6 h delay for NG-monomethyl-L-arginine inhibition of the NO effect reported by these authors.

A similar experiment on Ap-1 proteins showed no significant effect of E2 on JunD (fig 4B). E2 or IL1 β alone induced JunB

and c-Fos binding, whereas Fra-1 was induced by both compounds with an additive effect. JunB and c-Fos are E2 target genes, and their increased expression was expectable. *Fra1* activity is involved in the negative regulation of Ap-1-dependent transactivation.⁵¹ *Fra1*-mediated inhibition of Ap-1 also influences endogenous cellular pathways such as the inhibition of proliferation and induction of apoptosis of C6 glioma cells.⁵² This E2-mediated increase in *Fra1* may synergise with the inhibition of p65 to repress iNOS expression in chondrocytes.

CONCLUSION

IL1 β decreased the presence and functionality of ER α in rabbit chondrocytes. Nevertheless, the activation of ER α by E2 or SERM elicited an anti-inflammatory effect in these cells as E2 counteracted IL1 β -induced NO production and iNOS expression. This seemed to occur through inhibition of p65 DNA binding and nuclear translocation.

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