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Estrogen regulates DNA synthesis in human gingival epithelial cells displaying strong estrogen receptor β immunoreactivity

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Background and Objective: Estrogen acts via estrogen receptor (ER) α and β. The expression pattern of ERs and their importance in gingival tissues are not fully understood. In this study, we investigate gingival ER expression and effects of estrogen on gingival epithelial cell proliferation.

Material and Methods: Gingival biopsies were obtained from both healthy and diseased sites in three male and three female subjects. Expression of ERα and β was determined by immunohistochemistry. Effects of 17β-estradiol (E_2) on cell proliferation, monitored by measuring DNA synthesis, were studied in cultured human gingival epithelial HGEPp.05 cells.

Results: Estrogen receptor β, but not ERα, immunoreactivity was demonstrated in nuclei of epithelial cells in all layers of the gingival epithelium, but also in cells of the lamina propria. No differences were observed between male and female subjects. The same pattern, i.e. high ERβ expression but no ERα expression, was observed in both healthy and diseased sites within each individual. No differences in the intensity of the ERβ immunoreactive signal and the number of ERβ-positive nuclei were observed between healthy and diseased gingiva. Treatment with a physiological concentration of E_2 (10 nM) had no effect on DNA synthesis in ERβ- and ERα-expressing HGEPp.05 cells. In contrast, E_2 at high concentrations (500 nM and 10 μM) reduced DNA synthesis by 60–70%.

Conclusion: Human gingival epithelial cells display strong ERβ but low ERα immunoreactivity both *in vivo* and in culture. Estrogen attenuates gingival epithelial cell DNA synthesis at high but not low concentrations, suggesting a concentration-dependent mechanism.

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Two estrogen receptors (ERs) have been identified and named ERα and ERβ (1–3). Estrogen receptor α and ERβ are transcription factors which, in the presence of ligand, bind to unique sequences of DNA to regulate gene activity. These two ER subtypes are expressed in various organs and tissues and show a distinct and specific expression pattern (4). Cultured human periodontal ligament cells have been reported to express ERα mRNA,

but the signal was very weak (5). In another study, human periodontal ligament cells and gingival fibroblasts were shown to lack ERα mRNA expression (6). Cultured human periodontal ligament cells express ERβ protein, as demonstrated by immunocytochemistry, while no ERα immunoreactivity was detected (7,8). Human buccal mucosa has been shown to express ERα mRNA but not ERα protein (9). Epithelial cells within healthy human gingiva have been reported to express ERβ but not ERα protein (10). Taken together, these reports suggest that cells within oral tissues express predominantly ERβ protein in physiological conditions.

The functional importance of oral tissue ER is not fully understood. Data have been presented showing that estrogen stimulates the production of periodontal ligament cell mineralized nodules, suggesting that estrogen promotes periodontal ligament cell bone formation (11). Estrogen has also been reported to reduce *Escherichia coli* lipopolysaccharide-induced periodontal ligament cell cytokine expression and to regulate (both upregulate and downregulate) *E. coli* lipopolysaccharide S-stimulated periodontal ligament cell chemokine expression, suggesting that estrogen is involved in the regulation of periodontal inflammation via these mechanisms (12,13). Both Norderyd *et al.* (14) and Reinhardt *et al.* (15) have reported a higher frequency of gingival bleeding in postmenopausal women with low serum estrogen levels compared with those having high levels of estrogen, suggesting that estrogen exerts a protective effect by reducing gingival inflammation. Plasma estrogen levels rise dramatically in pregnancy owing to massive production of estrogens by the growing placenta (16). In pregnant women gingivitis often develops, suggesting a proinflammatory estrogen-induced effect (17). Estrogen thus seems to execute both anti- and proinflammatory effects on gingival tissues, but the mechanisms have not been identified.

Välimaa *et al.* (10) reported that healthy human gingiva expresses ERβ but not ERα, as studied by immunohistochemistry. The expression pattern of ER subtypes in diseased gingival tissue is not known. Furthermore, no data have been presented on the functional importance of gingival epithelial cell ERs. The objectives of the present study were to determine ER subtype expression in gingival biopsies from both healthy and diseased sites and also to address the physiological importance of gingival ERs by investigating the effects of estrogen on gingival epithelial cell proliferation.

Material and methods

Subjects and biopsies

Punch biopsies (4 mm in diameter) were obtained from both healthy and diseased gingival tissues from six individuals (three male and three female subjects). The biopsies included tissue from the marginal gingival epithelium, i.e. gingiva in direct connection to the gingival margin. Healthy gingival tissue was selected by clinical periodontal status, i.e. probing pocket depth <4 mm and no bleeding on probing. The teeth adjacent to the site where healthy gingiva was collected did not suffer from periodontitis. The criteria probing pocket depth >5 mm and bleeding on probing identified diseased sites. The patients included in the study were suffering from generalized severe chronic periodontitis. They were referred to the periodontal clinic for treatment, which included flap surgery at teeth not responding with pocket closure (<6 mm) after initial cause-related therapy and scaling. They were over 40 years of age and they were nonsmokers (see Table 1). We excluded premenopausal women because of the fluctuations in plasma estrogen levels occurring with phases of the menstrual cycle (16), by having >50 years of age as an inclusion criterion for female subjects. The women included in the study were not on hormone replacement therapy. The biopsies were fixed in 4% paraformaldehyde (in phosphate-buffered saline) and then kept in 70% ethanol until further processing. The experiments were undertaken with written consent of each subject and in accordance with the World Medical Association Declaration of Helsinki and with the approval of the Regional Ethical Committee at Lund, Sweden.

Immunohistochemistry

The fixed biopsies were embedded in paraffin and cut into 4 μm sections. The sections were dewaxed, rehydrated with descending concentrations of ethanol and rinsed in distilled water. Antigen retrieval was performed by microwaving for 15 min in citrate buffer (pH 6.0). Sections were stained with either a polyclonal ERα antibody (NeoMarkers code Ab-16; Thermo Scientific, Waltham, MA, USA) raised in

Table 1. Age, sex and medication of the six patients (three male and three female subjects) included in the present study

Identification	Sex	Age (years)	Diseases and medication
Patient 1	Male	64	High blood pressure, Trombyl®; Pfizer AB, Sollentuna, Sweden (anti-thrombotic)
Patient 2	Female	79	None
Patient 3	Male	44	High blood pressure, Enalapril®; Sandoz Novartis Sverige AB, Täby, Sweden (angiotensin-converting enzyme inhibitor)
Patient 4	Female	51	Abnormal thyroid gland function, Levaxin®; Nycomed AB, Stockholm, Sweden (synthetic thyroid hormones), Metoprolol®; Hexal AB, Helsingborg, Sweden (β-adrenergic blocker) and Simvastatin®; Merck NM AB, Stockholm, Sweden (cholesterol synthesis inhibitor)
Patient 5	Male	57	None
Patient 6	Female	56	None

rabbits at a dilution of 1:100 or an ERβ antibody raised in chickens (ERβ 503; kindly provided by the Professor Jan-Åke Gustafsson's laboratory at the Karolinska Institute, Stockholm, Sweden) at a dilution of 1:500. In preliminary experiments, different dilutions of both primary antibodies were tested, and 1:100 for the ERα antibody and 1:500 for the ERβ antibody resulted in high-intensity fluorescence with low background staining. The ERβ 503 antibody has been extensively characterized in different cell systems before use in immunohistochemistry (18,19). After preabsorption of the ERβ 503 antibody with ERβ protein (protein in 50-fold excess), no ERβ immunoreactivitiy was observed in rat aorta and rat prostate, tissues known to be ERβ positive. In preliminary experiments, we confirmed the nuclear staining of gingival epithelial cells observed with the ERβ 503 antibody by using a commercially available rabbit polyclonal ERβ antibody (Affinity Bioreagents code PA1-311; Thermo Scientific). Data on ERβ immunoreactivity presented in Figs 1–4 and in Table 2 are on the chicken ERβ 503 antibody. The site of antigen–antibody reaction was visualized with fluorescein isothiocyanate-conjugated secondary anti-rabbit or anti-chicken antibodies (both from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Mouse (C57BL/6 strain) uterus was included as a positive control for both ERα and ERβ antibodies. The animal experiments were approved by the Animal Ethics Committee at Lund University, Lund, Sweden. It is well known that uterus expresses ERα as well as ERβ, as shown in both humans and rodents, making uterus from these species a most suitable positive control (19–21). For negative controls, the primary ERα and ERβ antibodies were omitted.

The ERα and ERβ immunoreactivities in healthy and diseased gingival tissue were compared by scoring the nuclear immunoreactive signal according to the following four criteria: +++, intense staining, all or almost all nuclei positive; ++, intermediate staining, many positive nuclei; +, weak staining, few positive nuclei: and 0, no staining. The immunostaining was scored by three independent observers in a blinded fashion, and a summarized verdict was reached. The three observers obtained identical or almost identical results. For each biopsy and staining, at least three sections were analysed.

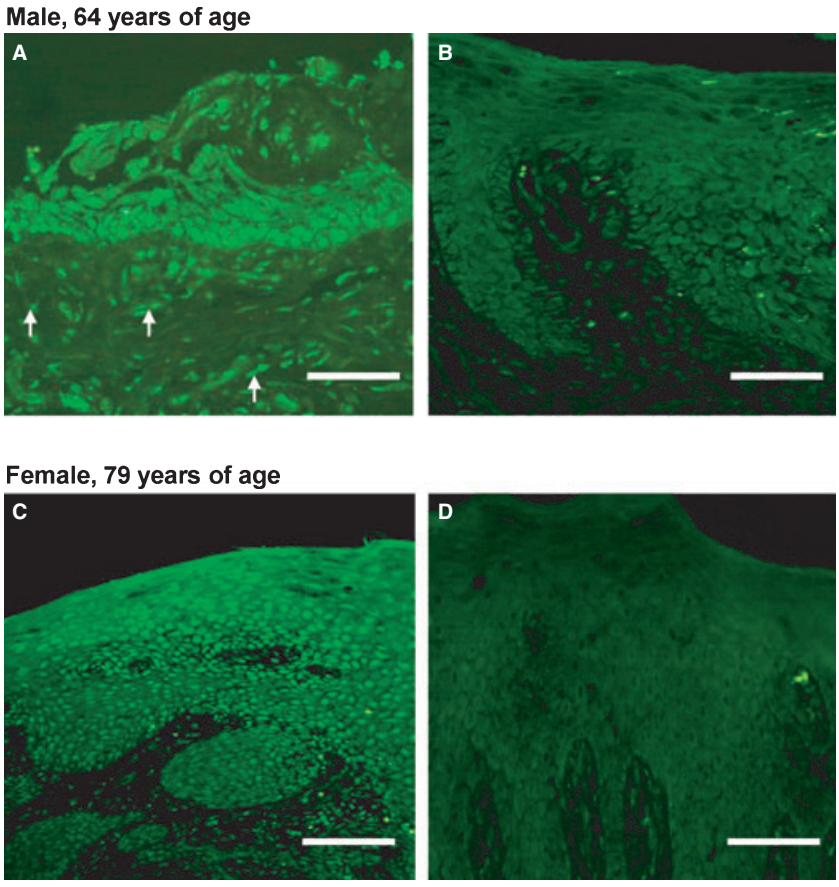

Fig. 1. Nuclear estrogen receptor (ER) β but not nuclear ERα immunoreactivity is shown in epithelial cells of healthy gingival tissues from a male subject, 64 years of age (A and B), and a female subject, 79 years of age (C and D). The figure shows staining with ERβ antibody (A and C) and staining with ERα antibody (B and D). Not only epithelial cells but also the cells of the underlying connective tissue, i.e. the lamina propria, express ERβ (arrows). No nuclear ERα immunoreactivity is observed in cells within the lamina propria. For each biopsy, three sections were analysed. Identical results were observed in biopsies obtained from three male and three female subjects. Scale bars represent 50 μm.

Cells and cell culture

Human primary gingival epithelial HGEPp.05 cells were purchased from CellnTec (CellnTec Advanced Cell Systems AG, Bern, Switzerland) and cultured in CnT-24 cell culture medium (CellnTec) supplemented with antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) and fetal calf serum (10%). After reaching confluence, cells were trypsinized (0.25% trypsin) and reseeded at a density of 100,000 cells/mL. The cells were cultured in air containing 5% CO_2 and used at passages 2–4, when they had reached about 80% confluence. For ERα and ERβ immunocytochemistry, cells were seeded on glass coverslips and fixed in a mixture of 2% formaldehyde and 0.2% picric acid in phosphate buffer (pH 7.2). The same antibodies and dilutions as used for tissue sections (please see previous subsection) were applied also for the HGEPp.05 cells. Before experiments, standard cell culture medium was replaced with serum-free and phenol red-free medium to obtain standardized conditions with quiescent cells and to remove the estrogen-like activity of phenol red.

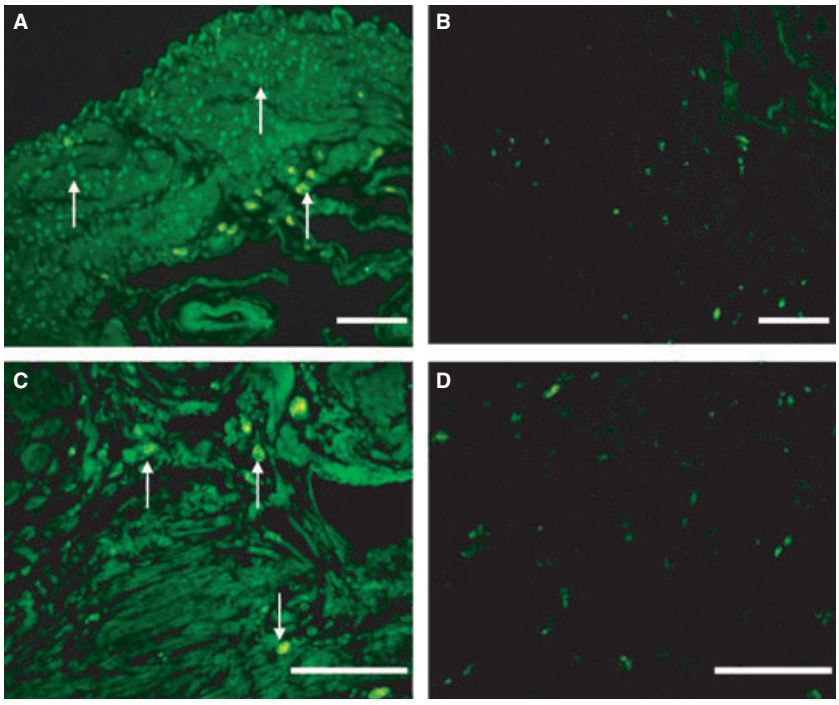

Fig. 2. Mouse uterus, used as a positive control, shows nuclear immunoreactivity for ERβ (A) and ERα (C) in myometrial smooth muscle cells and in the stroma cells of the endometrium. Arrows represent examples of ERβ- and ERα-positive nuclei. For each tissue specimen, at least three sections were analysed. No immunoreactivity was observed after omission of ERβ (B) and ERα antibodies (D). Scale bars represent 50 μm.

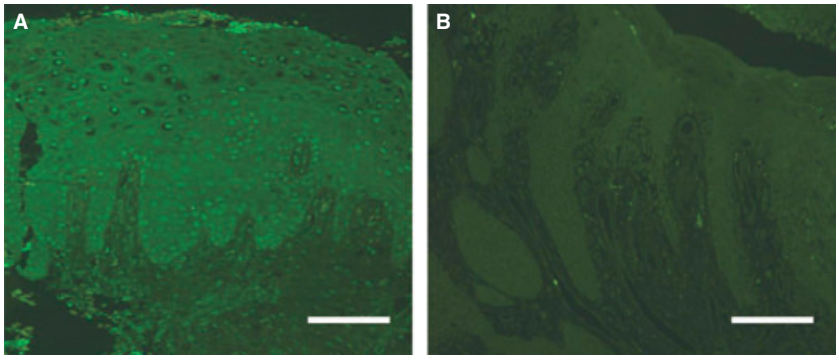

Fig. 3. Nuclear ERβ (A) but not nuclear ERα immunoreactivity (B) is observed in epithelial cells of diseased gingival tissue from a female subject, 79 years of age. Not only epithelial cells but also the cells of the underlying connective tissue, lamina propria, express ERβ (A). No nuclear ERα immunoreactivity is observed in cells within the lamina propria (B). The ERα and ERβ expression pattern in healthy gingival tissue obtained from this subject is shown in Fig. 1C,D. Identical results were observed in biopsies obtained from three male and three female subjects. For each biopsy, three sections were analysed. Scale bars represent 50 μm.

Measurements of DNA synthesis were performed with 5% dextran-coated charcoal-stripped fetal calf serum as a growth promoter representing submaximal growth stimulation. Stripped fetal calf serum was used to remove steroids from the serum. The 17β-estradiol (E_2) was purchased from Sigma (Sigma Chemicals, St Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Control cultures received DMSO as vehicle.

Measurement of DNA synthesis

The DNA synthesis was determined by measuring the incorporation of methyl-[^{3}H]-thymidine (PerkinElmer Inc., Boston, MA, USA) into newly synthesized DNA as described by Jönsson *et al.* (22). The radiolabelled thymidine (5 μCi) was present during the last hour of the 24 h incubation with E_2. Radioactivity was measured in a Beckman liquid scintillation counter (Beckman LS6500; Beckman Instruments Inc., Fullerton, CA, USA). The radioactivity was expressed as disintegrations per minute and normalized to total protein concentration determined by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

Statistics

Values are presented as means ± SEM. Statistical significance was calculated using ANOVA and Student's two-tailed *t*-test for unpaired comparisons with Bonferroni correction for *post hoc* analysis as appropriate. Values of $p < 0.05$ were regarded as denoting statistical significance.

Results

Expression of ERα and ERβ in healthy gingiva

In biopsies collected from healthy sites, nuclear ERβ but not ERα immunoreactivity was observed in all layers of the gingival epithelium (Fig. 1). Nuclear ERβ but not ERα immunoreactivity was observed also in cells residing in the underlying connective tissue, i.e in the lamina propria (Fig. 1). Almost all of the cell nuclei within all layers of the epithelium and the lamina propria were ERβ positive. In the lamina propria, both round cells with small nuclei, probably representing lymphocytes, and cells with large elongated nuclei, probably representing fibroblasts, were ERβ positive. Identical results were observed in biopsies collected from three male and three female subjects (Fig. 1). In Table 1 age, sex, diseases and medication of the patients included in this study are presented. In mouse uterus, serving as positive control,

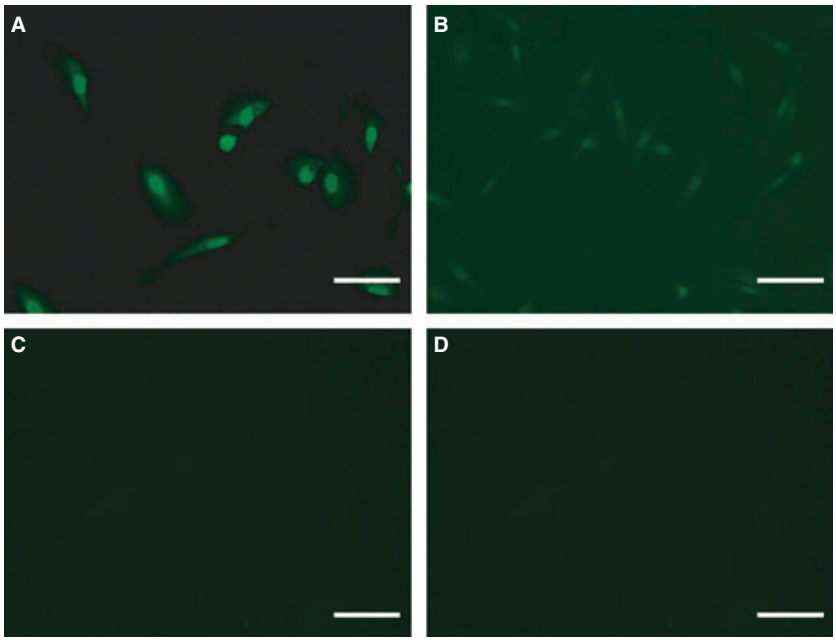

Fig. 4. Cultured human gingival epithelial HGEPp.05 cells express strong ERβ (A) but weak ERα immunoreactivity (B). Both ERβ and ERα immunoreactivities were almost exclusively localized to nuclei. No immunoreactivity was observed after omission of ERβ (C) and ERα antibodies (D). Determination of ERα and ERβ immunoreactivity in HGEPp.05 cells was repeated in cells at passages 2–4, showing identical results. At least two glass coverslips were analysed at each passage for each staining. Scale bars represent 10 μm.

Table 2. Estrogen receptor (ER) α and ERβ nuclear immunoreactivities in biopsies from healthy and diseased (periodontitis) gingiva obtained from six different patients

Identification	ERα epithelium	ERα lamina propria	ERβ epithelium	ERβ lamina propria
Patient 1, healthy gingiva	0	0	+ + +	+ + +
Patient 1, diseased gingiva	0	0	+ + +	+ + +
Patient 2, healthy gingiva	0	0	+ + +	+ + +
Patient 2, diseased gingiva	0	0	+ + +	+ + +
Patient 3, healthy gingiva	0	0	+ + +	+ + +
Patient 3, diseased gingiva	0	0	+ + +	+ + +
Patient 4, healthy gingiva	0	0	+ + +	+ +
Patient 4, diseased gingiva	0	0	+ + +	+
Patient 5, healthy gingiva	0	0	+ + +	+ + +
Patient 5, diseased gingiva	0	0	+ + +	+ + +
Patient 6, healthy gingiva	0	0	+ + +	+ + +
Patient 6, diseased gingiva	0	0	+ + +	+ + +

Immunoreactivities in biopsies were classified as follows: + + +, intense staining, all or almost all nuclei positive; + +, intermediate staining, many positive nuclei; +, weak staining, few positive nuclei; and 0, no staining. At least three sections were analysed for each biopsy and ER staining. The immunostaining was scored by three independent observers in a blinded fashion. The summarized scores of the three observers are presented. Age, sex and medication for the individual patients are presented in Table 1.

nuclear immunoreactivity for both ERα and ERβ was observed in the smooth muscle cells of the myometrium and in the stromal cells of the endometrium (Fig. 2). No ERα and ERβ immunoreactivity was observed after omission of the respective primary antibody (Fig. 2).

Expression of ERα and ERβ in diseased gingiva

Expression of ERα and ERβ was analysed by immunocytochemistry in healthy (probing pocket depth < 4 mm and no bleeding on probing) and diseased gingival tissues (probing pocket depth > 5 mm and bleeding on probing) obtained from six patients (three male and three female subjects). Age, sex and medication of the six patients included in the present study are presented in Table 1. Gingival biopsies were collected from both healthy and diseased sites within each of the six patients. Nuclear ERβ but not ERα immunoreactivity was demonstrated within both epithelial cells and connective tissue cells of the lamina propria in diseased gingival tissue (Fig. 3). Epithelial cells as well as connective tissue cells in diseased gingival tissues showed high nuclear ERβ but no nuclear ERα immunoreactive signal in all six patients. The nuclear ERα and ERβ immunoreactivity in both the gingival epithelium and the lamina propria were scored according to predetermined criteria as described in the Material and methods. Almost all the cells in both healthy and diseased gingival epithelium and lamina propria were ERβ positive (Table 2). The ERβ expression was identical in healthy and diseased gingiva. No detectable nuclear ERα immunoreactive signal was observed in either healthy or diseased gingival epithelium and lamina propria (Table 2). The gingival ERα and ERβ expression pattern was almost identical in all six patients under study (Table 2).

Expression of ERα and ERβ and effects of E_2 on DNA synthesis in cultured human primary gingival epithelial cells

Cultured human primary gingival epithelial HGEPp.05 cells expressed strong nuclear ERβ and weak nuclear ERα immunoreactivity (Fig. 4). As demonstrated in Fig. 4, the ERβ immunoreactive signal was much stronger than that for ERα. No ERα and ERβ immunoreactivity was observed after omission of the respective primary antibody (Fig. 4). Stimulation with E_2 at 500 nM for 24 h reduced growth-promoter-induced (5% fetal calf serum) DNA synthesis by about 60% (Fig. 5). Treatment with 10 μM E_2 attenuated DNA synthesis by about 70% (Fig. 5). A low physiological concentration of E_2 (10 nM) had no effect on DNA synthesis (Fig. 5).

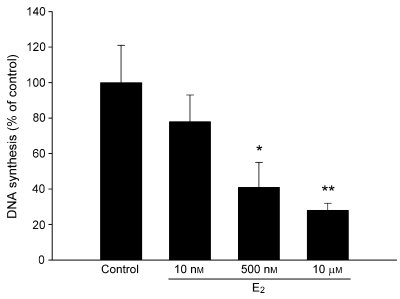

Fig. 5. Effects of stimulation with 17β-estradiol (E_2) for 24 h on DNA synthesis in cultured human gingival epithelial HGEPp.05 cells. DNA synthesis was determined by measuring incorporation of radiolabelled thymidine into newly synthesized DNA during the last hour of the 24 h incubation. Values are presented as means ± SEM of five to 10 observations in each group. $*p < 0.05$ and $**p < 0.01$, compared with control.

Discussion

Here, we show that ERβ is the predominant estrogen receptor in healthy as well as diseased human gingiva, implying that the effects of estrogen on gingival tissues in both health and disease are mediated via this receptor. Gingival epithelial cells in healthy gingiva have been reported previously to express the ERβ protein (10). Our data confirm the findings reported by Välimaa *et al*. (10), but in addition to their report, we show now that diseased gingiva has a similar ER subtype expression pattern to healthy gingiva, i.e. strong ERβ immunoreactivity but no ERα immunoreactivity. Semiquantitative analysis of ERβ expression in healthy and diseased gingiva within individual subjects showed no difference in ERβ immunoreactive signal between healthy and diseased sites, suggesting that periodontal/gingival inflammation does not affect the expression level of ERβ observed in healthy tissue. Thus, ERβ seems to be responsible for estrogen signalling in both healthy and diseased gingiva. Our data demonstrating high ERβ expression in gingival tissues are in accordance with previous reports showing high ERβ concentrations in other nonreproductive tissues (4).

We report no difference in gingival ER subtype expression between male and female subjects, suggesting that gingival ERβ, which seems to be the dominating gingival ER subtype, plays an important role in male as well as female gingiva. Three of six subjects included in this study took medication because of various diagnoses, e.g. high blood pressure. We have no reason to believe that the patients' medication affected their ER subtype expression pattern, because the pattern of ER subtype expression was similar in all patients whether or not they were on medication and similar in all patients independent of their type of medication. Furthermore, the drugs used by the three patients on medication were not steroids or other possible ER ligands, which might interfere with ER expression level. Thus, we suggest that the patients' medication had no influence on gingival ERα and ERβ expression, although we cannot completely rule out the possibility that the patients' medication and their medical condition affected expression of ERs.

In the present study, we show that cultured human gingival epithelial HGEPp.05 cells express strong immunoreactivity for ERβ but also a weak ERα immunoreactive signal. In gingival biopsies, no immunoreactivity for ERα was detected, suggesting plasticity in gingival epithelial cell ERα expression depending on the milieu where the cells reside, i.e. if they are in culture or localized in their natural *in vivo* context with blood vessels, nerves and extracellular matrix. High concentrations (500 nM and 10 μM) of E_2 reduced DNA synthesis in cultured human gingival epithelial HGEPp.05 cells, but in contrast, a low concentration of E_2 (10 nM) had no effect, suggesting differential effects of estrogen on gingival epithelial cell proliferation depending on concentration. The preovulatory plasma concentration of E_2 is about 2 nM but increases severalfold in pregnancy (16). Thus, the low concentration (10 nM) of E_2 that we used is within the physiological range. In contrast, 500 nM and 10 μM are regarded as high concentrations, probably reflecting a pharmacological rather than a physiological situation (16). In the cultured human skin keratinocyte cell line NCTC 2544 cells, E_2 (10 nM) produces a slight but significant increase in proliferation (23). Estrogen has been reported to accelerate cutaneous wound healing in female mice independent of its anti-inflammatory activities (24,25). Estrogen-induced improvement of cutaneous wound healing in mice is proposed to be mediated via ERβ (25). Thus, previous studies in mice and in a cultured human skin keratinocyte cell line suggest that estrogen promotes skin epithelial cell proliferation. We demonstrate in the present study that estrogen has no effect on proliferation of cultured human gingival epithelial cells at a physiologically relevant concentration, but attenuates proliferation at a pharmacological dose. Estrogen seems thus to have different effects on skin and gingival epithelial cell proliferation. These differences might reflect differences in ER subtype expression pattern between these two epithelial cell types.

Redundant, nonredundant and sometimes opposite effects have been reported for ERα and ERβ (4). Differential cellular expression of ER subtypes makes it possible for estrogen to have selective effects in different cell types (4). Our data suggest that estrogen, acting via ERβ, regulates gingival epithelial cell proliferation, causing anti-proliferation at high but not low concentrations. Gingival epithelial ERβ may, however, also mediate estrogenic effects other than proliferation, e.g. on the metabolic system. Estrogen has been shown to regulate enzymes involved in oxidative phosphorylation in different tissues and cells, such as blood vessels and periodontal ligament cells (26,27), suggesting that estrogen regulates the cellular production of ATP, thereby influencing many cellular processes. Phytoestrogens, such as the isoflavonoid genistein, have been shown to activate ERβ with high specificity (28), implying that this type of ERβ ligand also influences gingival epithelial estrogenic signalling.

In summary, we conclude that ERβ seems to be the predominant ER subtype in human gingival tissue and that a pharmacological concentration of estrogen attenuates gingival epithelial cell proliferation.

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