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REGULAR ARTICLE

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Estradiol protects cultured articular chondrocytes from oxygen-radical-induced damage

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Abstract Osteoarthritis (OA) is aggravated in menopausal women possibly because of changed serum estrogen levels. Estradiol has been postulated to affect oxidative stress induced by reactive oxygen species (ROS) in articular chondrocytes. We generated ROS in cultured bovine articular chondrocytes by incubating them with combined Fe_2SO_4, vitamin C, and hydrogen peroxide. The release of thiobarbituric-acid-reactive substances (TBARS, lipid peroxidation) and lactate dehydrogenase (LDH, membrane damage) was measured photometrically. Various estradiol doses and vitamin E, serving as control with an established anti-oxidative capacity, were applied either upon each exchange of medium and during radical production (strategy 1) or only during radical production (strategy 2). In chondrocytes incubated according to strategy 1, the production of TBARS and LDH release were significantly suppressed by 10^{-10}–10^{-4} M estradiol or by vitamin E. Under strategy 2, the production of TBARS was significantly suppressed at estradiol concentrations higher than 10^{-6} M, whereas LDH release was inhibited at concentrations of 10^{-6}–10^{-4} M. Vitamin E showed no significant effects. As repeated application of estradiol and vitamin E produced the best results, estradiol, like vitamin E, was speculated to accumulate in the plasma membrane and to decrease membrane fluidity resulting in protection against lipid peroxidation (non-genomic effect). Thus, in contrast to the neuroprotective effect of 17β-estradiol in supraphysiological doses reported recently, the anti-oxidative potential of estradiol appears to protect articular chondrocytes from ROS-induced damage when the hormone is given repeatedly in a physiological range. Decreased estradiol levels may therefore contribute to menopausal OA in the long term.

Keywords Articular cartilage · Reactive oxygen species · Anti-oxidants · Menopause · 17β-Estradiol · Bovine

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Introduction

Clinical, pathological, and epidemiological data have shown that women more often suffer from osteoarthrosis (OA) during and after the menopause than before (Spector and Champion 1989; Spector et al. 1991, 1997; Wluka et al. 2000). Population studies have indicated the extremely sexual-dependent prevalence of OA (Lawrence 1977; Felson 1990; Nevitt et al. 1996). The apparent prominence of peri- and post-menopausal women presenting polyarticular symptoms has fuelled speculations that the onset of OA and the menopausal lack of estrogens are related. Data from the research groups of Spector (Spector and Champion 1989; Spector et al. 1991, 1997) and Wluka (Wluka et al. 2000) suggest that menopausal women given estrogen replacement therapy have a lower incidence of OA. In addition, experiments with ovarectomized macacs receiving estrogen replacement therapy have revealed a diminished expression of OA (Ham et al. 2002). Although numerous groups have studied serum and urinary sex hormone concentrations in women suffering from OA, the way in which estrogens are involved in the pathogenesis of this disease remains unclear (Rogers and Lansbury 1956; Dequeker 1985; Cutollo et al. 1986; Spector et al. 1987). Several studies have shown that estrogens affect oxidative stress induced by reactive oxygen species (ROS). Supraphysiological doses of 17α- and 17β-estradiol protect cultivated neurons against oxidative stress (Schwenke 1998). In addition, cultured neuronal cells are protected against the neurotoxic effects of superoxide ions and hydrogen peroxide by preincubation with 17β-estradiol (Sawada et al.

1998). In comparison with synoviocytes, articular chondrocytes seem to express smaller amounts of scavengers against oxidative stress, such as superoxide dismutase, glutathione peroxidase, or catalase, and have a lower antioxidative status (Cadenas 1989; Mattey et al. 1993; Kurz et al. 1999, 2002). ROS-induced lipid peroxidation in rat articular chondrocytes can be abolished by the addition of vitamin E (Kurz and Schünke 1997). Here, we have looked for influences of 17β-estradiol on oxygen-radical-induced lipid peroxidation and membrane destruction in female bovine articular chondrocytes.

Materials and methods

Cell culture

Articular chondrocytes were aseptically isolated from the intertarsal joints of 2–year-old to 7-year-old cows. On average, the cartilage of 30 animals, which had been killed at a local slaughterhouse, was needed for one culture experiment. The harvested cartilage slices were washed three times in Hanks' buffered salt solution (HBSS, Seromed L2035) containing 100 U/ml penicillin/streptomycin (Seromed A2210) and 2.5 μg/ml amphotericin b (Seromed A2610). Slices of cartilage were incubated in 0.5% pronase (Boehringer 1459643)/HBSS and 0.7% hyaluronidase (Sigma H-3506)/HBSS for 60 min at 37°C, washed, and transferred for 6 h into a 2 mg/ml collagenase solution (Sigma C-1889) in Hams F12 (Biochrom FG0815) containing 10% fetal calf serum (FCS, Seromed S0115). After centrifugation (400*g*, 10 min), the cell pellet was resuspended in 10 ml medium containing 10% FCS, 100 IU/ml penicillin/streptomycin, 2.5 μg/ml amphotericin b, and 50 μg/ml ascorbic acid and filtered through a 20 μm-nylon mesh. Vital cells in the final cell suspension were counted by the trypan blue exclusion method with a hemocytometer and seeded at a density of 100,000 chondrocytes/cm^2 on uncoated wells (Costar). For culture in monolayers, chondrocytes were seeded at a density of 400,000 cells/ml medium/well in 12-well culture plates and incubated for 4 days with medium containing serum (see above) at 37°C and 5% CO_2. Medium was changed every third day. At day 5, cells were washed with HBSS and cultured in serum-free medium (Hams F12, 100 IU/ml penicillin/streptomycin, 2.5 μg/ml amphotericin b, 50 μg/ml ascorbic acid) for at least 2 days. At day 7, medium was changed to HBSS with the application of Fe^{2+}, vitamin C, and hydrogen peroxide for radical generation (see below), and incubation was performed for 6 or 18 h.

Radical generation

ROS were generated with Fe_2SO_4 (Merck 103965), vitamin C (Sigma A-8960), and hydrogen peroxide (Roth 8070.1) dissolved in HBSS. The final concentration of Fe_2SO_4 was 3 μM, whereas those of vitamin C and of hydrogen peroxide were 100 μg/ml and 880 μM, respectively.

Incubation with estradiol and vitamin E

Various doses of 17β-estradiol (Sigma E-8875) were applied when chondrocytes were seeded, viz., every time when the medium with or without serum was changed and during radical generation (altogether four times, strategy 1) or during radical generation only (only once, strategy 2). For this purpose, a 10^{-3} M stock solution of estradiol in 100% ethanol was prepared and dissolved stepwise (10^{-11}–10^{-4} M). Controls were stimulated with equivalent amounts of 1% ethanol. Alternatively, cells were incubated with 50 μM vitamin E (Sigma T-1157), a dose reported to decrease Fe^{2+}-induced and ascorbic-acid-induced lipid peroxidation in rat articular chondrocytes to a basal level (Kurz and Schünke 1997), according to the two culture strategies.

Lipid peroxidation

Supernatants were centrifuged (1,500*g*, 5 min), and the cell layers were used for the quantification of DNA. Lipid peroxidation was estimated by measuring the formation of thiobarbituric-acid-reactive substances (TBARS), i.e., malondialdehyde (MDA), in the culture supernatants. The assay was performed as described by Villarca et al. (1989) and Buege and Aust (1978) with some modifications: 400 μl sample was mixed with 800 μl thiobarbituric acid (Sigma T-5500) solution (0.375%, containing 20% trichloracetic acid, 0.25 M HCl) and incubated in a water bath at 100°C for 15 min. Thereafter, the samples were cooled and centrifuged at 1,500*g* for 10 min. The absorbance of the supernatant was measured in a spectrophotometer at 533 nm by using MDA (Sigma T-1642) as the standard.

Toxicity assay

The supernatants were centrifuged (1,000*g*, 5 min). The toxicity of ROS was determined by measuring the lactate dehydrogenase (LDH) activity in the samples with a cytotoxicity detection kit (Boehringer 1 644 793). Each supernatant (100 μl) was pipetted into 100 μl reagent freshly prepared according to the manufacturer's instructions (250 μl solution 1 and 11.25 ml solution 2) in a 96-well culture plate. Incubation was performed for 30 min in darkness at room temperature. The reaction product was measured at 492 nm in a photometer. In addition, for measurement of LDH, a 100% control and a low control (basic release) were needed. The 100% control was stimulated with Triton X-100 (Sigma X-100), a reagent that damages the plasma membrane resulting in a total release of LDH. An unstimulated sample was used as the low control. The percentage concentration of LDH was measured according to the formula: [(sample−low control) × 100]/[100% control−low control].

Quantification of DNA

For measurement of the DNA content, 500 µl phosphate-buffered saline (PBS) was pipetted into each well, and the cells were dissolved mechanically with a rubber policeman. A volume of 500 µl PBS was used to catch the cells remaining on the bottom of the wells. DNA was measured fluorometrically after intercalation with ethidium bromide (Karsten and Wollenberger 1977). Samples (containing 1 ml PBS with cells) and blanks (containing only 1 ml PBS) were treated for 15 s with an ultrasonic beam. Subsequently, 500 µl RNAse solution (Sigma R-5220, 5 KU/ml) and 500 µl pronase solution (Boehringer 1459643, 0.7 U/ml) were added, the mixture was incubated for 30 min at 37°C, and 500 µl ethidium bromide (Merck 1.11608, 25 µg/ml) was added. After a 30-min incubation, the samples were measured with a fluorometer (Hitachi F2000; λ_{ex}=365 nm; λ_{em}=590 nm). The DNA content was computed by using a standard curve.

Statistics

All biochemical data are presented as mean values ± SEM. The significance of differences between stimulated samples and non-stimulated controls was calculated by a two-tailed Student's *t*-test.

Results

Cell morphology after radical attack combined with estradiol or without estradiol

Articular chondrocytes were cultured according to strategy 1 with application of estradiol at every change of culture medium and during the generation of oxygen radicals. ROS were generated with 3 µM Fe^{2+}, 100 µg/ml vitamin C, and 880 µM hydrogen peroxide. Chondrocytes incubated with ROS but without estradiol (representing the control) had an unhealthy appearance and were characterized by the production of vesicles possibly belonging to damaged cellular and organelle membranes (Fig. 1a). In some regions of the monolayer, the cells had vanished following disintegration. By contrast, chondrocytes incubated with 10^{-9} M estradiol had grown to confluence, had a polygonal shape, and exhibited no vesicles, even in the presence of ROS (Fig. 1b). Compared with the cells incubated without estradiol, they showed a better morphology despite oxygen radical attack for 18 h. No clear morphological differences between chondrocytes cultured with or without estradiol and finally exposed to ROS were observed when the hormone (10^{-9} M estradiol) was applied only during the production of oxygen radicals (strategy 2; data not shown).

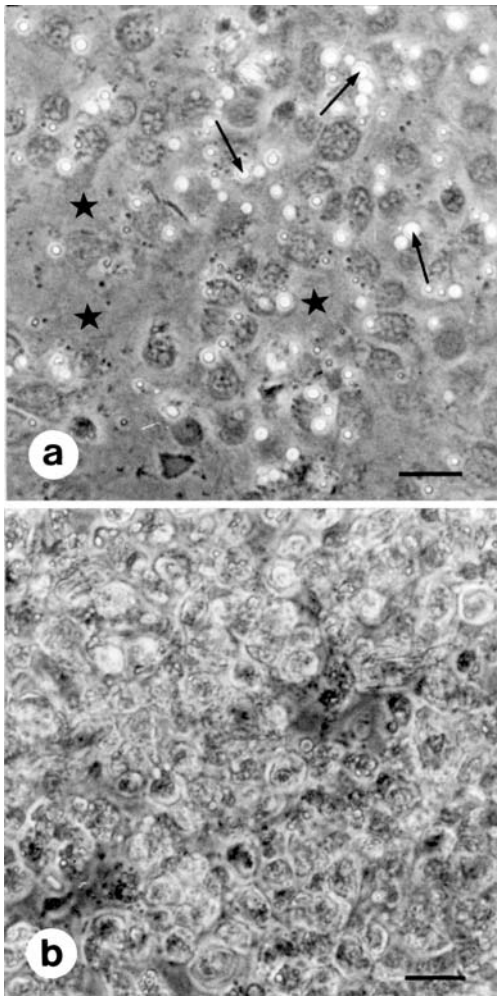

Fig. 1 Morphology of articular chondrocytes cultured in monolayers for 7 days without (**a**) and with (**b**) repeated estradiol incubation (strategy 1) and attacked by the generation of oxygen radicals for at least 18 h by the end of culture. **a** Chondrocytes without estradiol incubation were characterized by the production of vesicles of various sizes (*arrows*) assumed to represent damaged plasma membranes and cell organelles. In some areas (*stars*), chondrocytes had disintegrated. **b** Chondrocytes cultured with repeatedly replenished estradiol at a concentration of 10^{-9} M showed a healthy morphology without production of vesicles, indicating that 17β-estradiol protects articular chondrocytes against ROS-induced lipid peroxidation and plasma membrane damage. *Bars* 20 µm

Lipid peroxidation

Articular chondrocytes were cultured according to strategy 1 with an application of estradiol at every change of culture medium and during the generation of oxygen radicals (Fig. 2a). The ROS-induced release of TBARS without estradiol incubation (representing the control) was 0.51±0.21 µM TBARS/µg DNA. After incubation with 10^{-4} M estradiol, the generation of TBARS was decreased to 0.03±0.02 µM TBARS/µg DNA. The values of the DNA measured were: 1.93 µg/well per square centimeter in the control, 1.69 µg/well per square centimeter in vitamin E, and 1.00–1.83 µg/well per square centimeter

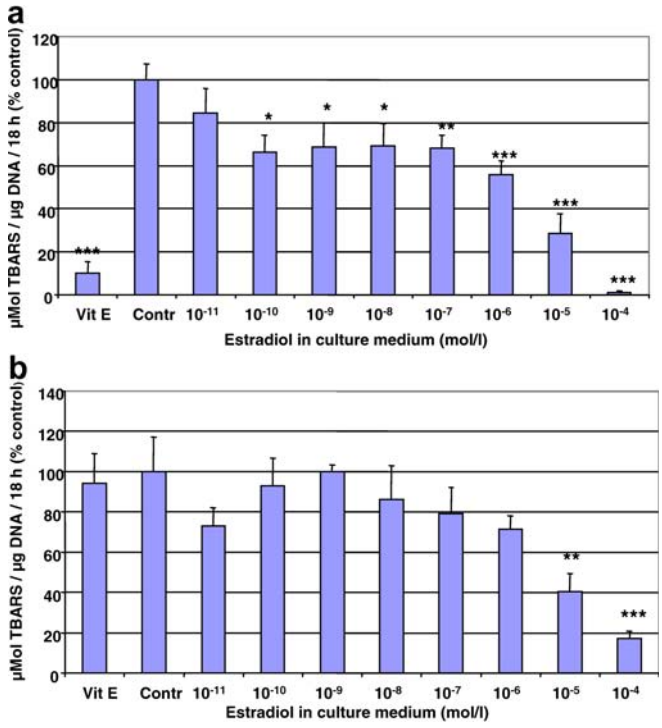

Fig. 2 Influence of estradiol and vitamin E (*Vit E*) on the release of TBARS in cultured female bovine articular chondrocytes under oxygen-radical-induced lipid peroxidation. TBARS was measured photometrically after radical generation for 18 h in the supernatant of the cells. **a** Application of estradiol or vitamin E at every change of medium and during generation of oxygen radicals. **b** Application of estradiol or vitamin E only during the generation of oxygen radicals. Data were normalized to control values (*Contr*), which were set to 100%. Mean values ± SEM, n=10 (**a**), n=6 (**b**). Significance between chondrocytes cultured with estradiol or vitamin E and the control chondrocytes: ***P<0.001, **P<0.01, *P<0.05

in the samples incubated with 10^{-11}–10^{-5} M estradiol. The DNA content decreased significantly (P<0.05) to 0.92 µg/well per square centimeter only after incubation with 10^{-4} M estradiol. In comparison with the control, which was adjusted to 100%, the release of TBARS was significantly depressed by estradiol doses of 10^{-10}–10^{-4} M (Fig. 2a). A comparable effect was seen after the application of 50 µM (5×10^{-5} M) vitamin E according to strategy 1, instead of estradiol. The anti-oxidative effect of vitamin E was approximately in the range of 10^{-5}–10^{-4} M (10–100 µM) estradiol. Articular chondrocytes were cultured according to strategy 2 with estradiol application only during the generation of oxygen radicals (Fig. 2b). The values for DNA measured were: 1.07 µg/well per square centimeter in the control, 0.89 µg/well per square centimeter in vitamin E, and 0.80–1.12 µg/well per square centimeter in the samples incubated with 10^{-11}–10^{-5} M estradiol. The DNA content decreased significantly (P<0.05) to 0.67 µg/well per square centimeter only after incubation with 10^{-4} M estradiol. In comparison with the control, the release of TBARS was significantly depressed by estradiol doses of 10^{-5}–10^{-4} M (Fig. 2b). The application of vitamin E according to strategy 2 had no significant influence on the release of TBARS.

Membrane damage

Articular chondrocytes were cultured according to strategy 1 with estradiol application at every change of culture medium and during the generation of oxygen radicals (Fig. 3a). The ROS-induced release of LDH (cytotoxicity) without estradiol incubation (representing the control) was 61%. Cytotoxicity dropped to a maximum of 7% after incubation with 10^{-4} M estradiol. In comparison with the control, cytotoxicity was significantly depressed by estradiol doses of 10^{-10}–10^{-4} M (Fig. 3a). A significant suppression of LDH release was also seen after application of 50 µM (5×10^{-5} M) vitamin E according to strategy 1. Articular chondrocytes were cultured according to strategy 2 with estradiol application only during the generation of oxygen radicals (Fig. 3b). The release of LDH (cytotoxicity) in the control was 79%. Cytotoxicity dropped to a maximum of 28% after incubation with 10^{-4} M estradiol. In comparison with the control, cytotoxicity was significantly depressed by estradiol doses of 10^{-6}–10^{-4} M (Fig. 3b). A significant effect of vitamin E was lacking.

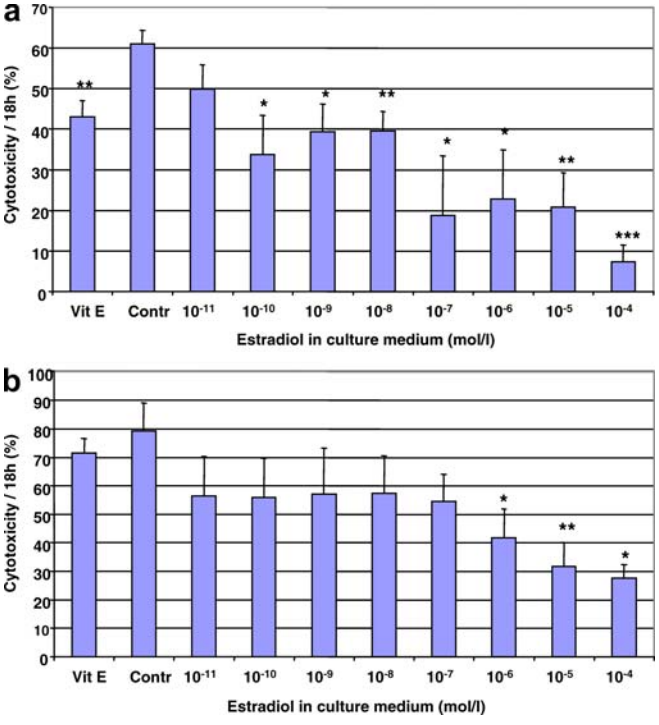

Fig. 3 Influence of estradiol and vitamin E (*Vit E*)on the release of lactate dehydrogenase (LDH, cytotoxicity) in cultured female bovine articular chondrocytes under oxygen-radical-induced membrane damage. LDH was measured photometrically after radical generation for 18 h in the supernatant of the cells. **a** Application of estradiol or vitamin E at every change of medium and during generation of oxygen radicals. **b** Application of estradiol or vitamin E only during generation of oxygen radicals. LDH release was presented as percentage cytotoxicity. Mean values ± SEM, n=5 (**a**), n=4 (**b**). Significance between chondrocytes cultured with estradiol or vitamin E and the control chondrocytes: ***P<0.001, **P<0.01, *P<0.05

Discussion

ROS seem to be involved in the pathogenesis of OA (Tiku et al. 1999), a disease showing aggravation in menopausal women. The onset of OA in menopausal women is thought to be related to a changed estrogen metabolism (Spector and Champion 1989). Physiological levels of serum estradiol concentration range from 10^{-11} M to 10^{-9} M in the menstrual cycle and from 10^{-8} M to 10^{-7} M during pregnancy. The following values have been reported for the menstrual cycle (Strecker and Lauritzen 1989): 25–75 pg/ml ($2.5–7.5\times10^{-11}$ M) in the early follicle phase, 300–600 pg/ml ($3–6\times10^{-10}$ M) immediately before ovulation, and 150–200 pg/ml ($1.5–2.0\times10^{-10}$ M) in the luteal phase. During late pregnancy, the serum estradiol concentration reaches values of approximately 20 ng/ml (2×10^{-8} M; Kuhl 2001). In menopause, the serum estradiol level decreases to 5–15 pg/ml ($0.5–1.5\times10^{-11}$ M; Dudenhausen et al. 2003). Estrogens have an anti-oxidant activity because of the phenol structure of 17β-estradiol and its metabolites (Liehr and Roy 1998). Here, we have investigated whether estradiol has an influence on ROS-induced lipid peroxidation and membrane damage in cultured female bovine articular chondrocytes. In addition, the influence of estradiol has been compared with that of vitamin E.

Several studies have shown that ROS are involved in the degradation of the extracellular matrix of articular cartilage (Mazetti et al. 2001) and the suppression of proteoglycan synthesis (Bates et al. 1984; Schalwijk et al. 1985; Tiku et al. 1999). Superoxide radicals, generated enzymatically by the action of xanthine oxidase on hypoxanthine, significantly reduce proteoglycan synthesis of cultured bovine articular cartilage as demonstrated by ^{35}S-sulphate incorporation (Bates et al. 1984). Additionally, Bates et al. (1984) have observed an inhibition of protein synthesis. Suppressed proteoglycan synthesis has also been found in murine patellar cartilage after the application of hydrogen peroxide (Schalwijk et al. 1985). Autoradiography has shown an inhibition of $^{35}SO_4^{2-}$ uptake by the chondrocytes of the patella. In cultured rabbit articular chondrocytes, treatment with lipopolysaccharides leads to the release of ^{35}S-labeled aggrecan, whereas treatment with catalase significantly prevents this release (Tiku et al. 1999). Tiku et al. (1999) suggest that the matrix degradation is attributable to chondrocyte-derived hydrogen peroxide resulting from stimulation with lipopolysaccharides. Thus, ROS might be involved in the degradation of cartilage, and anti-oxidative substances might prevent this type of tissue destruction.

In our culture model of bovine articular chondrocytes, ROS were generated by the use of Fe_2SO_4, vitamin C, and hydrogen peroxide. In previous studies, a strong dose-dependent induction of TBARS was observed by the combination of Fe^{2+} and ascorbic acid in homogenates and supernatants of rat articular chondrocytes cultured in monolayers (Kurz and Schünke 1997). In addition, after exposure to 0.1–10 mM hydrogen peroxide, rat articular cells showed plasma membrane swelling and swelling of subcellular organelles within a few hours of injury (Kurz et al. 1999). The reaction of Fe^{2+} and hydrogen peroxide results in the formation of Fe^{3+}, a hydroxyl radical and a hydroxide anion (Fenton reaction). For radical generation, we changed the medium to HBSS. Thus, radical generation was assured not to be impaired by the medium contents. The application of estradiol at every change of medium and during the generation of ROS resulted in a significant suppression of the release of TBARS and LDH at doses of 10^{-10}–10^{-4} M. When estradiol was applied only during the generation of ROS, the release of TBARS was significantly suppressed at concentrations higher than 10^{-6} M, whereas the release of LDH was inhibited at doses of 10^{-6}–10^{-4} M.

The oxidation of low-density lipoprotein (LDL) is an important factor in the development of atherosclerosis. Estradiol, because of its anti-oxidant capacity, has been shown to induce a dose-dependent decrease in MDA (a TBARS) concentration as a marker of LDL oxidation (Arteaga et al. 2003). Oxidative stress is also important in the process of dopaminergic neuronal degeneration in Parkinson's disease. Cultured rat mesencephalic dopaminergic neurons are protected from glutamate-induced or hydrogen-peroxide-induced oxidative damage by preincubation with 100 μM (10^{-4} M) 17β-estradiol (Sawada et al. 1998). However, the simultaneous administration of 17β-estradiol and glutamate does not produce any significant effects. These results are comparable with our experiments showing that estradiol application only during ROS generation is not as effective in suppressing the release of TBARS (lipid oxidation), and that supraphysiological doses of 10–100 μM (10^{-5}–10^{-4} M) estradiol are needed for significant anti-oxidative effects.

The present results demonstrate that the application of 50 μM (5×10^{-5} M) vitamin E at every change of medium and during the generation of ROS results in a significant suppression of the release of TBARS, comparable with the effect of 10^{-5}–10^{-4} M (10–100 μM) estradiol. However, the suppression of the release of LDH is not as strong as that of TBARS, but is nevertheless significant, comparable with the effect of 10^{-9}–10^{-8} M (1–10 nM) estradiol. Application of vitamin E only during the generation of ROS shows no significant effects. Since Arteaga et al. (2000) have shown that the reduction of MDA (as a TBARS) reaches statistical significance at 50 μM with alpha-tocopherol, the oxidative stress in our culture system might be so potent that a single application of this vitamin has little effect.

Vitamin E is characterized by potent anti-oxidant properties, and its beneficial effects in the prevention of chronic diseases is believed to be associated with oxidative stress (for reviews, see Brigelius-Flohé and Traber 1999; Brigelius-Flohé et al. 2002). According to Jain (1983), the treatment of red blood cells with hydrogen peroxide results in a movement of phosphatidylserine and phosphatidylethanolamine from the inner to the outer bilayer of the plasma membrane and is correlated with the concentration of generated MDA (as a TBARS). Vitamin E abolishes the effect of peroxide treatment on fatty acid peroxidation.

The TBARS-suppressing effect of estradiol might be similar to the respective effect of vitamin E. Since the repeated application of vitamin E or estradiol results in a more effective suppression of TBARS in comparison to a single application of these substances, estradiol and vitamin E probably accumulate during the repeated applications and therefore are more effective.

Indeed, several studies have provided evidence that 17β-estradiol accumulates in plasma membranes conditioned by diffusion into the lipid membrane phase, and that membrane fluidity is decreased as a non-genomic effect of this hormone (Suleimanov et al. 1985; Schwartz et al. 1996; Liang et al. 2001). Ayres et al. (1996) have shown that estradiol is as effective an anti-oxidant as alpha-tocopherol in terms of fatty acid peroxidation but is far more effective than this vitamin in terms of cholesterol peroxidation. In accordance with this concept, 17β-estradiol protects against quinolinic-acid-induced lipid peroxidation in rat brains (Heron and Daya 2000).

Although we favor a plasma membrane-stabilizing effect of estradiol during ROS-induced damage of articular chondrocytes, an additional estradiol influence on the redox status of chondrocytes cannot be excluded. According to Mattson et al. (1997), the generalized neuroprotective action of estrogens in Alzheimer's disease is attributable to estradiol preventing decreases in mitochondrial transmembrane potentials. The cytoprotective effect of estradiol against tert-butyl hydroperoxide-induced toxicity in hepatocytes might be related to the maintenance of the normal redox status of the cell, which partially recovers its intracellular glutathione peroxidase levels (Leal et al. 1998). Furthermore, a study in pre- and postmenopausal women has revealed a positive correlation between estradiol and glutathione peroxidase (Bednarek-Tupikowska et al. 2001).

Peripheral serum estradiol concentrations resulting in physiological reactions of the respective tissues are in the range of 60 pg/ml (6×10^{-11} M) for the vaginal epithelium and 20 pg/ml (2×10^{-11} M) for bone (Dudenhausen 2003). Under conditions whereby the estradiol concentrations showing physiological effects in bone are similar to those in articular cartilage, menopausal estradiol levels of 5–15 pg/ml ($0.5–1.5\times10^{-11}$ M) are perhaps too low to exert anti-oxidative effects in articular cartilage cells of some menopausal women.

Taken together, these data show that estradiol can limit ROS-induced damage in articular chondrocytes. Comparable with the neuroprotection of rat mesencephalic neurons against oxidative stress after preincubation with 100 μM (10^{-4} M) 17β-estradiol (Sawada et al. 1998,) supraphysiological estradiol doses are needed to protect articular chondrocytes against ROS-induced damage when the hormone is applied only during the generation of ROS. However, when estradiol is applied repeatedly (strategy 1) to cultured articular chondrocytes, statistically significant anti-oxidative effects are observed in the physiological range of the hormone (10^{-10}–10^{-9} M). Therefore, 17β-estradiol might be a real candidate for the protection of articular chondrocytes from ROS-induced damage. The decrease in estradiol levels may therefore contribute to menopausal OA in the long term.

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