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Impact of Oestradiol and Progesterone on Antioxidant Activity in Normal Human Breast Epithelial Cells in Culture

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The risk of developing breast cancer increases after long term use of oestrogen and progestagen, and carcinogenesis in the breast is partly due to oxidative damage to DNA bases. Therefore, we studied the effects of 17 β-oestradiol and progesterone on the antioxidative status and the vulnerability to oxidative stress exhibited by normal human breast epithelial cells in culture. After exposure to hydrogen peroxide, cells grown with oestradiol alone or with both oestradiol and progesterone showed significantly decreased viability compared to cells grown in medium without added hormones. There was, however, no difference in hydrogen peroxide degradation rate between controls and hormone treated cultures. When desferrioxamine was added, the viability increased and the hydrogen peroxide degradation rate decreased. The levels of several antioxidants were altered in cells grown in the presence of oestradiol and progesterone: the concentrations of glutathione reductase and catalase decreased significantly while the levels of glutathione peroxidase and reduced glutathione did not change. The alterations in enzyme activity and cell vulnerability were more pronounced in cultures treated with a combination of oestradiol and progesterone.

We conclude that the redox balance in the cultured normal human breast epithelial cells was altered by treatment with oestradiol and progesterone, and that this change led to the increased death of cells subsequently exposed to hydrogen peroxide. This effect may have implications for sex hormone dependent diseases of the breast.

Keywords: Oestradiol, progesterone, glutathione peroxidase, catalase, glutathione reductase, oxidative stress

INTRODUCTION

The risk of developing breast cancer is linked to exposure to both endogenous and exogenous sex steroids. Several epidemiological studies have shown that early menarche and late menopause, as well as long term hormone-replacement therapy increase the risk of breast cancer.[1–3]

There is evidence that oxidative damage to cells is involved in the development of different forms of cancer, including breast cancer.[4,5] Oxidative

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damage to DNA bases induced by hydroxyl radicals may be a significant source of mutations which could lead to cancer.[6] An increased concentration of the hydroxyl-radical-modified DNA base lesion 8-hydroxyguanine has been found in breast cancer.[7,8] Alteration of the antioxidant enzyme activity in cancerous breast tissue compared to adjacent normal breast tissue has also been reported.[9]

The role of oestrogen in pathological conditions is a somewhat contradictory issue. It has been shown that oestrogens, both natural and synthetic, may act as inhibitors of lipid peroxidation and thereby protect against cardiovascular diseases.[10,11] On the other hand, it has also been observed that oestrogens cause oxidative damage to DNA, which in turn leads to the development of cancer.[12,13] This suggests the ability of oestrogens to modulate the cellular redox balance may vary. For example, the action of oestrogens could be different at physiological and supraphysiological levels. Moreover, during oestrogen metabolism, the redox potential of the hormones could change, or the action of oestrogens could be different in the intra- and extracellular compartments. Oestrogens could also have different effects in different cell systems. We have previously shown in breast tissue *in vivo*, that the extracellular levels of the antioxidant glutathione (GSH) increase late in the menstrual cycle when the levels of oestrogen and progesterone are high.[14] Others have demonstrated that the intracellular levels of GSH in rat hepatocytes decreased during oestrogen treatment.[15]

The aim of the present study was to determine whether oestrogen and progesterone influence the intracellular levels of some important antioxidants, such as GSH, glutathione peroxidase (GSHPx), glutathione reductase (GSHRed) and catalase, in normal human breast epithelial cells in culture. We also considered the effects that exposure to hydrogen peroxide had on the survival of untreated cells and cells exposed to sex hormones.

MATERIALS AND METHODS

Chemicals

Insulin, hydrocortisone, isoproterenol, ethanolamine, phosphoryl ethanolamine, epidermal growth factor, transferrin, t-butyl hydroperoxide and n-octyl sodium sulphate were purchased from Sigma (St. Louis, MO, USA). Bovine pituitary extract was from GIBCO BRL (Paisley, Scotland, UK), and 17β-oestradiol and progesterone were from Apoteksbolaget (Stockholm, Sweden). NADH, NADPH, GSH and oxidised glutathione (GSSG) came from Boehringer Mannheim (Mannheim, Germany). Desferrioxamine B from Ciba (Basel, Switzerland). Other chemicals were obtained from standard sources.

Cell Culture

Normal human breast epithelial cells (AG1134) were purchased from the Coriell Institute for Medical Research (Camden, NJ, USA). The cells were cultured from breast tissue obtained from a 28 year old woman undergoing reduction mammoplasty and the cells were well characterised as normal human mammary epithelial cells.[16,17] They were cultured in MCBD 170 medium (GIBCO BRL, Paisley, Scotland, UK) supplemented with insulin (5 µg/ml), hydrocortisone (0.5 µg/ml), isoproterenol (10^{-5} mol/L), ethanolamine (10^{-5} mol/L), phosphoryl ethanolamine (10^{-4} mol/L), epidermal growth factor (5 ng/ml), bovine pituitary extract (70 µg/ml) and transferrin (5 µg/ml). Culture was performed at 37°C in humidified air with 5% CO_2. The cells were subcultured and seeded into 35-mm dishes or 12 or 24-well plates (Costar, Cambridge, NJ, USA) using 0.05% trypsin supplemented with 0.02% ethylenediaminetetra-acetate (EDTA). Different concentrations of 17 β-oestradiol and progesterone were added in growth medium 24 h after subcultivation; the cultures were treated for seven days before experiments. The medium in all cultures was changed every day.

Enzyme Assays

Cells seeded at a cell density of 20,000 cells/cm^2 in 35-mm dishes, were washed twice in ice-cold phosphate buffered-saline (PBS) and then subjected to three cycles of freezing and thawing to rupture cell membranes. Enzyme activities were determined as described elsewhere; GSHPx,[18] GSHRed[19] and catalase[20] were analysed on an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) and then related to protein content.[21] When measuring GSHPx activity, t-butyl-hydroperoxide was used as a substrate to allow determination of both Se-dependent and Se-independent activity. The GSHPx and GSHRed activities were quantitated as oxidation of NADPH at 340 nm (37°C) and expressed as nmol NADPH oxidised/min/mg protein using $\varepsilon = 6.22\,mM^{-1}\,cm^{-1}$. Catalase activity (37°C) was monitored at 240 nm and expressed as μmol/L H_2O_2 consumed/min/mg protein; $\varepsilon = 56\,mM^{-1}\,cm^{-1}$.

GSH Assay

Dishes seeded with 20,000 cells/cm^2 were rapidly rinsed with cold PBS and 300 μl of 0.5 mol/L perchloric acid supplemented with 1 mmol/L EDTA was added. The samples were collected in tubes on ice and centrifuged at $300 \times g$ for 10 min. The supernatant was analysed for GSH by using a high performance liquid chromatography (HPLC) instrument equipped with a Kromasil 100-5C18 reverse phase column, 250 × 4.6 mm (Hichrom, Reading, UK). The mobile phase, which was supplied by a Consta-Metric III pump, consisted of 0.1 M NaH_2PO_4, 0.1 mmol/L EDTA, 0.2 mmol/L n-octyl sodium sulphate (pH 2.5) and 5% methanol. The flow rate was 0.5 ml/min, the volume of the injection loop was 20 μl, and the retention time for GSH was 9 min. GSH was detected using a BAS LC-4B Amperometric Detector (Bioanalytical Systems Inc., West Lafayette, IN, USA) equipped with an Au electrode at +0.6 V. GSH concentrations were calculated from standard curves obtained daily before the analysis.[22] The intra-assay variation was 1.2 ± 0.4% and the detection limit was 1×10^{-12} mol GSH.

Cell Viability

Human breast epithelial cells were seeded at a cell density of 15,000 and 20,000/cm^2 and exposed to different concentrations of hydrogen peroxide in HBSS supplemented with 10 mmol/L 4-(-2-hydroxylethyl)-1-piperazine-ethanesulphonic acid (HEPES), pH 7.4. Before the H_2O_2 treatment some of the cells were treated with 1 mmol/L desferrioxamine B for three hours. After treatment with hydrogen peroxide, the cultures were rinsed and incubated for 24 h in fresh medium without added hormones, and viability was measured in the cell layer and in the surrounding medium and reported as percentage of total lactate dehydrogenase (LDH) activity in the cells.[23]

Immunostaining

Cells grown on 22-mm coverslips were fixed in 4% paraformaldehyde. The cells were subsequently washed in PBS containing 0.1% sapfonine and 5% foetal bovine serum and incubated for 20 h at 4°C with polyclonal mouse antihuman oestrogen receptor antibodies (Novocastra, Newcastle, UK) or mouse antihuman progesterone receptor antibodies (Dakopatts, Älvsjö, Sweden). Thereafter FITC-conjugated antimouse IgG antibodies (Vector Lab., Burlingame, CA, USA) were added, and the specimens were incubated for 30 min at room temperature. The cells were washed in PBS containing sapfonine and then in distilled water, mounted in gelvatol and examined in a Nikon microscope. The control cells, which were treated in the same manner but not exposed the specific oestrogen and progesterone receptor exhibited a weak green autofluorescence.

Degradation of Hydrogen Peroxide

Human breast epithelial cells were grown in 35 mm dishes and exposed to H_2O_2 in HBSS (2 ml/35 mm dish) at 37°C. In some experiments 1 mmol/l desferrioxamine was added 3 h before H_2O_2 exposure. Aliquots (50 µl) were taken from the cell supernatant and the H_2O_2 concentration was assayed using *p*-hydroxyphenylacetic acid (pHPA) as a probe. In the presence of horse radish peroxidase, H_2O_2 oxidises pHPA to its fluorescent dimer. Fluorescence intensity was measured at $\lambda ex = 315\,nm/\lambda em = 410\,nm$ using a RF-540 fluorescence spectrophotometer (Shimadzu).[24,25]

Statistical Analysis

Data are presented as mean ± SD. Groups were compared using ANOVA, (Statview 4.5) and Fisher's post-hoc test; $p < 0.05$ was considered statistically significant.

RESULTS

The cultures used in our study were in passage 22–24 in all experiments. The cell line has an estimated lifespan of about 70 passages[16] and we used early passages to avoid variations due to long term culturing. The hormone treatment did not alter the growth rate of the cells as measured by cell count and protein content. Oestrogen or progesterone receptors were not detected in the cells, i.e. cultures stained for the receptors did not differ from controls incubated without the primary antibody.

Exposing cells to hydrogen peroxide caused a time- and concentration-dependent loss of viability. A concentration of hydrogen peroxide less than 250 µmol/L had no effect on cell viability while 1 mmol/L had a time-dependent effect (Figure 1). Treatment with 1 mmol/L H_2O_2 for two hours resulted in 50% loss of viability in cells seeded at a density of 15,000 cells/cm^2 while 1.5 mmol/L gave the same result in cells seeded at a density of 20,000 cells/cm^2 and this regime was used in the experiments. Viability after exposure to hydrogen peroxide was significantly lower ($p = 0.0001$) in cultures pre-treated with oestradiol (10^{-8} mol/L) or a combination of oestradiol (10^{-8} mol/L) and progesterone (10^{-8} mol/L) than in non pre-treated cultures (Figure 2). The negative effect on viability was not augmented by raising the concentrations of both oestradiol and progesterone to 10^{-7} mol/L or 10^{-5} mol/L.

The hydrogen peroxide degradation rate did not differ between the hormone treated cells compared with controls (Figure 3). The concentration

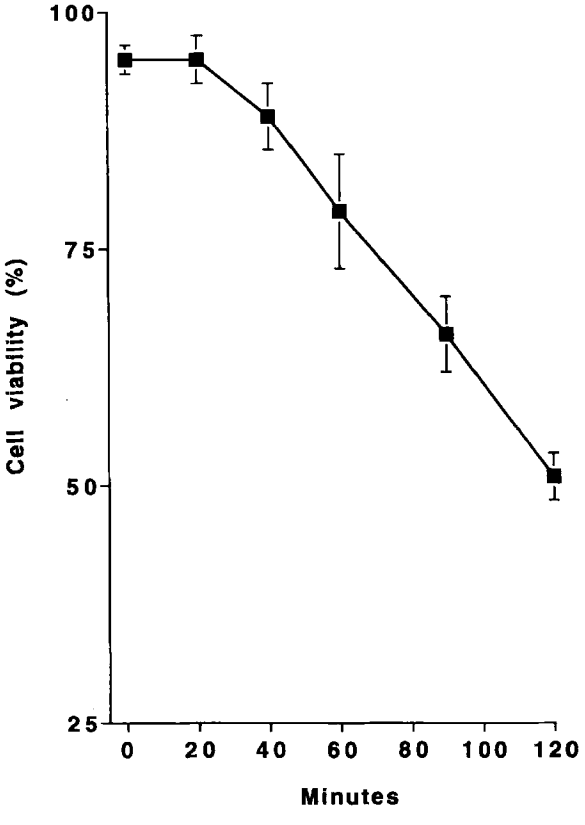

FIGURE 1 Time dependent loss of viability in cultured normal breast epithelial cells (15,000 cells/cm^2) after exposure to 1 mmol/L H_2O_2 for 0–120 min. Results are expressed as percentage of LDH activity remaining in the cells. Values are mean ± SD, $n = 2$–6.

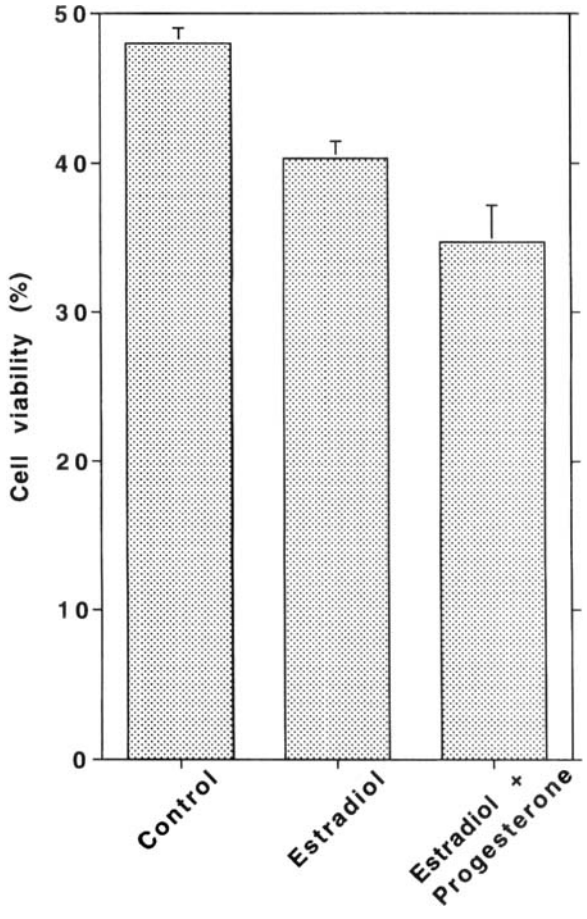

FIGURE 2 The effects of seven-day pre-treatment with 17β-oestradiol (10^{-8} mol/L) and progesterone (10^{-8} mol/L), on the viability of cultured normal breast epithelial cells (15,000 cells/cm^2) exposed to 1 mmol/L H_2O_2 for two hours. Results are expressed as percentage of LDH activity remaining in the cells, and the values represent means ± SD ($n = 3$); $p = 0.0001$ (ANOVA, Statview). Fisher's post-hoc test: control vs oestradiol $p = 0.0015$; control vs oestradiol + progesterone $p < 0.0001$; oestradiol vs oestradiol + progesterone $p = 0.0065$. The LDH activity in cells pre-treated with hormones but not exposed to H_2O_2 were 94–96%.

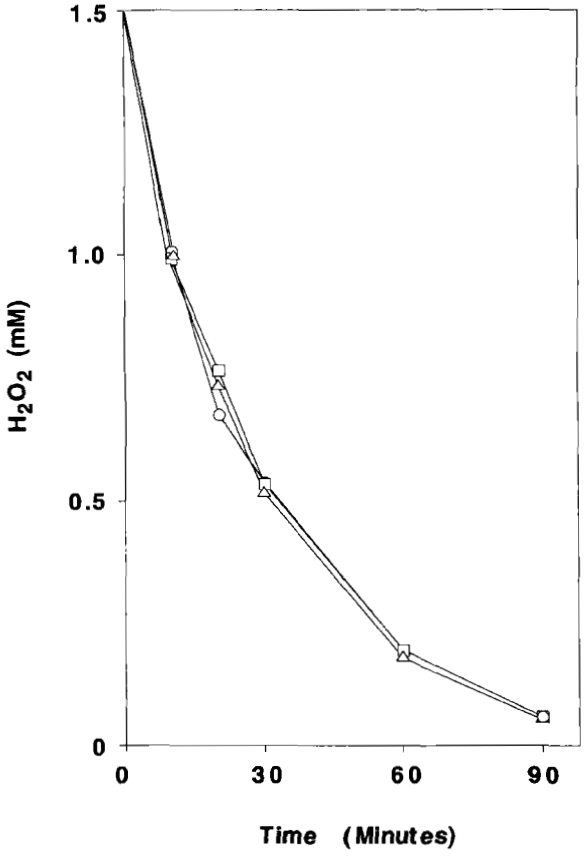

FIGURE 3 Degradation of H_2O_2 in normal human breast epithelial cells (20,000 cells/cm^2) cultured in media only and cells pre-treated with 17β-oestradiol (10^{-8} mol/L) or 17β-oestradiol (10^{-8} mol/L) and progesterone (10^{-8} mol/L) in combination for seven days. There were no differences between the groups. Values are mean, the SD varied between 0.006–0.03 ($n = 4$).

of GSH was 27.8 ± 3 nmol/mg protein in both untreated and harmone-treated cultures. The activities of catalase and GSHRed decreased significantly ($p = 0.0007$) in cells exposed to oestradiol (10^{-8} mol/L) and progesterone (10^{-8} mol/L), whereas the same treatment led to a slight, but not significant, increase in GSHPx activity ($p = 0.095$; Figure 4).

The viability increased in cells pre-treated with desferrioxamine for three hours before hydrogen peroxide exposure ($p < 0.05$; Figure 5). The desferrioxamine treatment also decreased the hydrogen peroxide degradation rate, regardless of hormone treatment (Figure 6).

DISCUSSION

This study showed that sex hormones altered the intracellular antioxidant capacity and increased

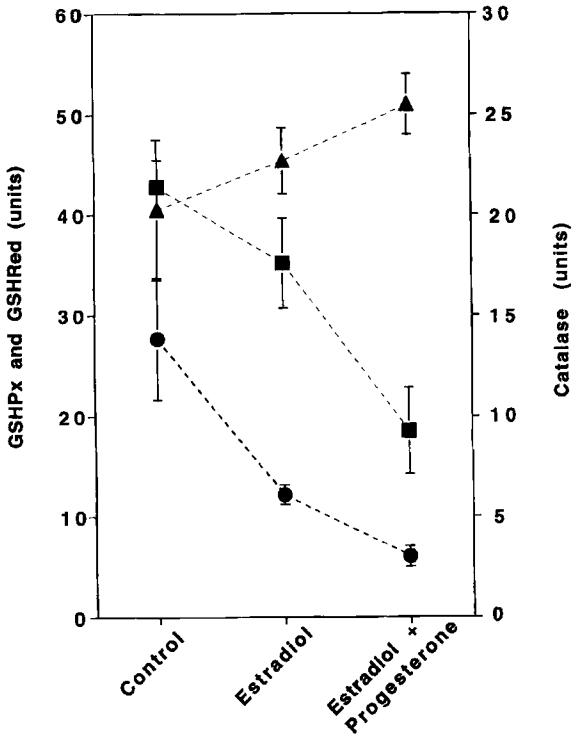

FIGURE 4 The activities of glutathione reductase (GSHRed ■), glutathione peroxidase (GSHPx ▲) and catalase (●) in normal breast epithelial cells cultured with or without 17β-oestradiol (10^{-8} mol/L) and 17β-oestradiol (10^{-8} mol/L) and progesterone (10^{-8} mol/L) in combination for seven days. The activities for GSHRed and GSHPx were determined as decrease of NADPH-absorption and one unit is defined as nmol NADPH oxidised/min/mg protein. Catalase activity was determined as H_2O_2 consumption and expressed as μmol/L H_2O_2 consumed/min/mg protein. Values represent means ± SD ($n = 6$). The activity of catalase and glutathione reductase decreased in cells treated with sex hormones ($p = 0.0007$). The increase in glutathione peroxidase in cells exposed to the hormones was not significant ($p = 0.095$; ANOVA, Statview). Fisher's post-hoc test for catalase: control vs oestradiol $p = 0.0016$; control vs oestradiol + progesterone $p = 0.0003$; oestradiol vs oestradiol + progesterone $p = 0.07$. Fisher's post-hoc test for glutathione reductase: control vs oestradiol $p = 0.055$; control vs oestradiol + progesterone $p = 0.0003$; oestradiol vs oestradiol + progesterone $p = 0.002$.

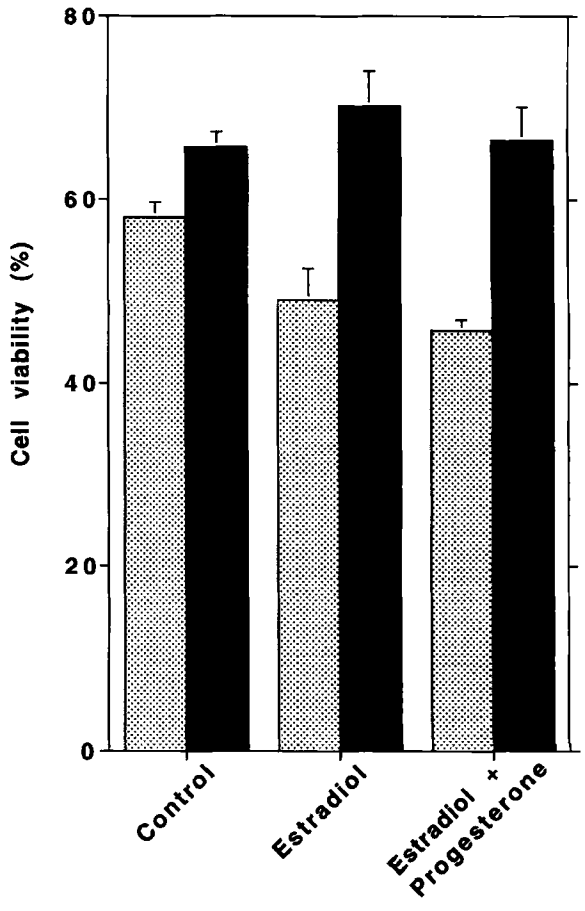

FIGURE 5 The effects of seven-day pre-treatment with 17β-oestradiol (10^{-8} mol/L) and progesterone (10^{-8} mol/L), on the viability of cultured normal breast epithelial cells (20,000 cells/cm^2) exposed to 1.5 mmol/L H_2O_2 for two hours. Results in black bars represents cells pre-treated with desferrioxamine for 3 h before the H_2O_2 exposure. Results are expressed as percentage of LDH activity remaining in the cells, and the values represent means ± SD ($n = 4$) $p < 0.05$ between all pairs.

cell death in cultured normal human breast epithelial cells exposed to hydrogen peroxide.

The high and not physiological levels of H_2O_2 was used as a model to study the effects on viability after oxidative stress. Due to the high H_2O_2 level the time of exposure could be short with minor risk of cell detachment. Immediately after H_2O_2 exposure no LDH activity was lost from the cell layer. Since the cells were washed and then incubated for 24 h before analysis the H_2O_2 could not affect the LDH activity. Moreover, the cells which might have undergone apoptosis during the H_2O_2 treatment would probably after the 24 h incubation show LDH release due to secondary cell membrane rupture (secondary necrosis).

Hydrocortisone is required to grow breast epithelium cells[16] and of course it could not be

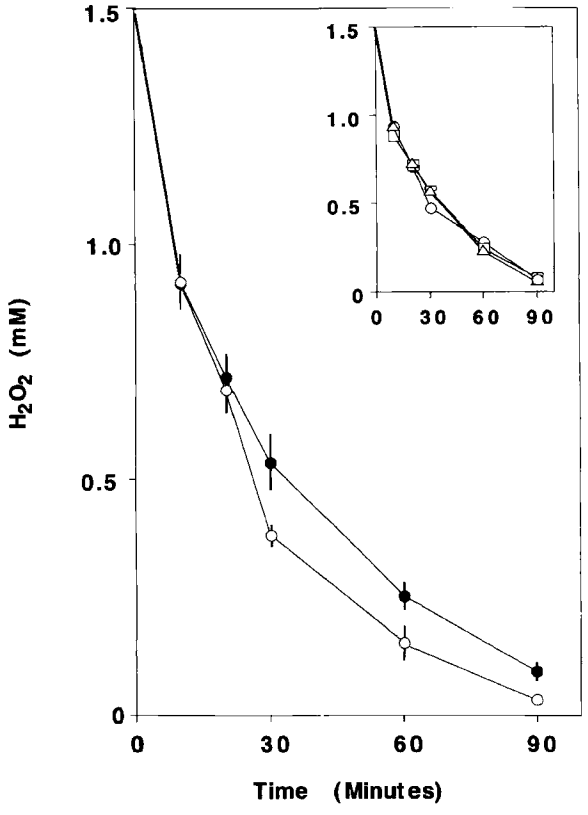

FIGURE 6 Degradation of H_2O_2 in normal human breast epithelial cells (20,000 cells/cm^2) pre-treated with desferrioxamine for 3 h before H_2O_2 exposure (●) and control cells (○). Values are mean ± SD, ($n = 3$) $p < 0.05$ at 30, 60 and 90 min. The inset shows the effects of desferrioxamine in cells cultured in media only and in cells pre-treated with 17β-oestradiol (10^{-8} mol/L) or 17β-oestradiol (10^{-8} mol/L) and progesterone (10^{-8} mol/L) in combination for seven days. There were no differences between the groups ($n = 3$).

ruled out that this could affect the results. However, the hydrocortisone was present in all groups, regardless of sex hormones or not, and should therefore not have any substantial effect on our results.

There was no difference in the rate of H_2O_2 degradation between the groups. In cells treated with sex hormones the catalase levels were decreased. This could lead to an increased generation of hydroxyl radicals and thereby increased cytotoxicity. Hydroxyl radicals could be produced by transition metal ions catalysed H_2O_2 breakdown (Fenton reaction). By inhibition of iron, the most abundant transition metal in the body, by the iron-chelating agent desferrioxamine, iron-catalysed oxidative reactions are prevented.[26] We could also show a cytoprotective effect of desferrioxamine against the H_2O_2 induced cell damage. The desferrioxamine treatment also led to a slight decreased degradation of H_2O_2. This effect was not dependent on sex hormones.

We performed experiments *in vitro* using physiological concentrations of the naturally occurring steroids 17 β-oestradiol and progesterone. Plasma levels of oestradiol vary between 0.1–1 nmol/L during the menstrual cycle and rise to 50 nmol/L during pregnancy. The progesterone levels vary between 1–60 nmol/L.[27] The levels of oestradiol and progesterone which women are exposed to during a large part of their life was therefore chosen for the experiments. Although oestrogen receptors were not detected by the immunofluorescence method we employed, the cells did respond to oestradiol. There are several possible explanations for this: the level of the receptor may have been below the detection limit; the steroids could have interfered with other unidentified receptors; the effects of the steroids may not have been dependent on receptors.

It is known that oestrogens are metabolised to catechol forms, which in turn are oxidised to quinones and semiquinones,[13] and catechol oestrogen is one of the major metabolites of oestradiol.[28] Moreover, it has been reported that oestrogen quinones/semiquinones may enter a redox cycle that produces reactive oxygen species capable of causing DNA damage,[29,30] and other findings show that the DNA damage induced by oestrogen quinones does not require oestrogen receptors in human breast cancer cell lines.[31] Oestrogens are known to increase the risk of uterine and breast cancer, and the redox cycling of catechol oestrogens resulting in the formation of free radicals could be a part of the carcinogenic effect of these hormones.

In a study of Syrian hamsters, oestrogen treatment induced kidney tumours but had no effect on the liver.[32] In these animals, alterations of the antioxidative defence system in the kidney and liver occurred in reverse during oestrogen treatment, and catalase levels were low in the kidney and high in the liver. It has also been shown that catalase activity is lowered and GSHPx activity is elevated in human breast cancer tissue compared to normal breast tissue.[9]

We have previously shown *in vivo* that the GSH levels in both subcutaneous fat and breast tissue change during the menstrual cycle.[14] This overall change may be due to differences in liver metabolism of sex hormones as the liver has been shown to be the main site of GSH synthesis in humans.[33]

In the present experiments oestradiol alone and in combination with progesterone affected the antioxidant enzymes but not the intracellular GSH levels. The catalase and GSHRed activities decreased in cells treated with oestradiol, and that effect was enhanced after treatment with a combination of oestradiol and progesterone. This suggests that the two hormones act synergistically, not antagonistically, in breast epithelial cells. This is in agreement with epidemiological data, which show that progestagen treatment does not lower the risk of breast cancer as it does the risk of endometrial cancer.[1,2]

We have not analysed whether the low levels of catalase and GSHRed we observed were the result of decreased synthesis of the enzymes or increased turnover. The GSHPx levels increased slightly after combined oestradiol–progesterone treatment, which probably had only a minor effect on H_2O_2 degradation since reduced GSH is needed as an electron donor in this reaction. The GSH turnover was probably decreased as a consequence of the reduced GSHRed activity in the hormone treated cultures. The toxic effect of hydrogen peroxide was greatest in the cells treated with both oestradiol and progesterone, and these cells showed the lowest levels of catalase and GSHRed. In breast tissue, the cellproliferation rate and apoptosis reach their maxima during the luteal phase of the menstrual cycle, when both oestradiol and progesterone levels are high.[34] Proliferation is probably the most vulnerable stage in the cell cycle, with the highest risk of expressing DNA damage, and our findings suggest that it its during that period of vulnerability that the defence system against oxidative damage is least effective.

In summary, oestradiol and progesterone altered the enzymatic antioxidative defence system in cultured normal human breast epithelial cells and thereby increased the vulnerability of the cells to oxidative stress. This may have implications for sex hormone dependent diseases of the breast.

Acknowledgements

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