Proliferation of ovarian theca-interstitial cells is modulated by antioxidants and oxidative stress

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BACKGROUND: Maintenance of ovarian homeostasis requires precise regulation of proliferation of thecal-interstitial (T-I) cells. Recent evidence indicates that oxidative stress and antioxidants modulate proliferation of various tissues under both physiological and pathological conditions. This study evaluated the effects of oxidative stress and antioxidants on T-I proliferation.

METHODS: Rat T-I cells were cultured in serum-free medium and proliferation was assessed by determination of DNA synthesis using the thymidine incorporation assay, by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and by direct counting of steroidogenically active and steroidogenically inactive cells.

RESULTS: Antioxidants and reactive oxygen scavengers induced a dose-dependent decrease of T-I proliferation. Vitamin E succinate was inhibitory at 10–100 \(\mu\)mol/l, ebselen was inhibitory at 0.3–30 \(\mu\)mol/l, and superoxide dismutase was inhibitory at 300–1000 IU/ml. In contrast, oxidative stress resulted in a biphasic effect. Modest oxidative stress induced by 1 mmol/l hypoxanthine and xanthine oxidase (3–30 \(\mu\)U/ml) stimulated proliferation of T-I cells, while greater oxidative stress induced by xanthine oxidase (1 \(\mu\)U/ml) profoundly inhibited proliferation. Direct cell counting demonstrated comparable effects on steroidogenically active and inactive cells.

CONCLUSIONS: Reactive oxygen species may play a role in the regulation of growth of ovarian mesenchyme. Under pathological conditions, such as those encountered in polycystic ovary syndrome, excessive oxidative stress and depletion of antioxidants may contribute to ovarian mesenchymal hyperplasia.

Key words: antioxidants/oxidative stress/proliferation/theca

Introduction

Appropriate ovarian development and homeostasis require precise mechanisms regulating proliferation of thecal-interstitial (T-I) cells. Our previous studies have demonstrated that insulin and tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) stimulate T-I proliferation (Duleba et al., 1997, 1999; Spaczynski et al., 1999). A plausible clinical corollary of these findings may be represented by conditions such as polycystic ovary syndrome (PCOS). This condition is characterized by thecal and stromal hyperplasia (Hughesdon, 1982), insulin resistance, compensatory hyperinsulinaemia (Burghen et al., 1980; Dunai et al., 1987) and increased levels of TNF-\(\alpha\) (Naz et al., 1995; Gonzalez et al., 1999). Recently, it has been shown that PCOS is associated with excessive oxidative stress (Sabuncu et al., 2001). Since several in vivo and in vitro experimental models demonstrate that insulin and TNF-\(\alpha\) induced oxidative stress (Adamson and Billings, 1992; Krieger-Brauer and Kather, 1992; McDonagh et al., 1992; Rifici et al., 1994), we hypothesized that the effects of insulin and TNF-\(\alpha\) on proliferation may be, at least in part, mediated by increased oxidative stress. The concept that reactive oxygen species (ROS) may modulate proliferation stems from growing evidence that oxidants and antioxidants are involved in regulation of gene expression under physiological and pathological conditions. High concentrations of ROS induce oxidative damage and are cytotoxic. However, it is now well established that, at moderate concentrations, ROS play a role in signal transduction processes involving growth and protection from apoptosis (Clement and Pervaiz, 1999; Kamata and Hirata, 1999; Kunsch and Medford, 1999). ROS induce proliferation of various cell types, including fibroblasts and aortic endothelial cells (Ruiz-Gines et al., 2000). Antioxidants, including \(\alpha\)-tocopherol, inhibit proliferation of many types of cells including vascular smooth muscle, fibroblasts and various cancer cell lines (Ivanov et al., 1997; Azzi et al., 1998; Nesaretnam et al., 1998; Onat et al., 1999).

This study was designed to compare the effects of antioxidants and oxidative stress on proliferation of T-I cells.

Materials and methods

Reagents

The following reagents were purchased from Sigma Chemical Co. (St Louis, MO): medium-199 with Hank’s buffered salt solution (HBSS)
and NaHCO₃, medium 199 with HBSS (×10), McCoy’s 5a medium (modified, without serum), t-glutamine, bovine serum albumin (BSA), trypsin-EDTA (0.05%/0.02%), nitroblue tetrazolium grade III crystalline, hydroxyurea, 5β-androstan-3β-ol-17-one, β-NAD⁺, sesame oil, paraformaldehyde, Percoll, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-di-phenyl-tetrazolium bromide (MTT), superoxide dismutase (SOD), vitamin E succinate (VES), ebselen, hypoxanthine (HX) and xanthine oxidase (XO). Trypan blue stain (0.4%; w/v), antibiotic-antimycotic preparation (penicillin, 10 000 IU/ml; streptomycin, 10 000 μg/ml; amphotericin B, 25 μg/ml) and Dulbecco’s phosphate-buffered saline (PBS, ×1, pH 7.2, without MgCl₂ and CaCl₂) were obtained from Grand Island Biological Co. (Grand Island, NY). Collagenase type I (Clostridium histolyticum, CLS1: 146 U/mg) and DNase I (bovine pancreas; 2298 U/mg) were obtained from Worthington Biochemical Co. (Freehold, NY). Radiolabelled [³H]thymidine (92.0 mCi/mmol) was purchased from Amersham Life Science Inc. (Arlington Heights, IL).

Animals
Sprague–Dawley female rats were obtained on day 25 of age from Taconic Farms (Germantown, NY) and housed with a 12 h light:12 h dark photoperiod in an air-conditioned environment. Standard rat chow and water were given ad libitum. Starting on day 28 of age, the animals were injected with 17β-estradiol (1 mg/0.3 ml sesame oil s.c.) daily for 3 days in order to stimulate ovarian development and development of antral follicles. Approximately 24 h after the last injection (day 31 of age), the animals were anaesthetized with ketamine and xylazine (i.p.) and sacrificed by intracardiac perfusion with 0.9% saline. All treatments and procedures were in accordance with accepted standards of humane animal care as outlined in the NIH Guide for the Care and Use of Laboratory Animals and a protocol approved by the Yale University Animal Care Committee.

Cell culture
Ovarian T-I cells were obtained as follows. Following saline perfusion described above, ovaries were dissected, and T-I cells were purified using discontinuous Percoll gradient centrifugation as described previously (Magoffin and Erickson, 1988; Duleba et al., 1997). The immunohistochemical purity of this cell preparation has been demonstrated previously (Duleba et al., 1997). The cells were counted and viability was routinely in the 85–95% range. T-I cells were incubated for up to 96 h at 37°C in an atmosphere of 5% CO₂ in humidified air, in serum-free McCoy’s 5a medium (with antibiotics, 0.1% BSA and 2 mmol/l t-glutamine). In cultures carried out for >48 h, media and treatments were replaced at 48 h; in order to minimize detachment of cells, these cultures were carried out on fibronectin-coated plates. The cells were incubated without or with VES (1–100 μmol/l), ebselen (0.3–30 μmol/l), SOD (100–1000 U/ml) or HX/XO (1 mmol/l of HX and 1–1000 μU/ml of XO).

Thymidine incorporation assay
T-I cells were incubated for 48 h in 96-well culture plates with or without individual additives. Assay of DNA synthesis was carried out using the thymidine incorporation assay. Radiolabelled [³H]thymidine (4 μCi/ml) was added to T-I cells during the last 24 h of culture. At the end of the culture period, the cells were harvested using a multwell cell harvester (PHD Harvester, Model 290; Cambridge Technology, Inc., Watertown, MA). Radioactivity was measured in a liquid scintillation counter, SL 4000 (Intertechnique, Fairfield, NJ). Each treatment was carried out in at least eight replicates.

MTT assay
T-I cells were incubated for up to 96 h with or without individual additives in 96-well fibronectin-coated plates. The media and additives were replaced after 48 h. At the end of the culture period, MTT (125 μg/well) was added for 4 h, then the supernatants were removed and 96 μl of isopropanol + 4 μl of HCl (1 mol/l) was added to each culture well. Optical density at 570 nm was determined.

Cell counting and identification of steroidogenically active cells
T-I cells were cultured in fibronectin-coated 24-well plates for 96 h. Media and treatments were replaced after 48 h of culture. Each treatment was carried out in four replicates. At the end of the cell culture period, the cells were washed with calcium-free and magnesium-free PBS (×1, pH 7.2). Trypsin-EDTA (0.05 and 0.02%, respectively; 0.3 ml/2 cm²) solution was dispensed into cell cultures to completely cover the monolayer of cells and the culture dish was placed at 37°C for 2–3 min. When cells were in suspension and appeared rounded, McCoy’s 5a medium was added to inhibit trypsin activity. The T-I cells were then washed with PBS (×1, pH 7.2) and fixed in 1% paraformaldehyde for 20 min. Steroidogenically active T-I cells were identified histochemically by detection of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity as described by others (Bao et al., 1995). Briefly, fixed T-I cells were reconstituted in histochemical staining solution containing PBS pH 7.2 supplemented with 0.1% BSA, 1.5 mmol/l β-NAD⁺, 0.25 mmol/l nitroblue tetrazolium and 0.2 mol/l β-androstane-3β-ol-17-one. The cells were incubated overnight in a shaker at 37°C in the dark, spun down and resuspended in PBS (pH 7.2). The number of stained cells (3β-HSD⁺; steroidogenically active) and non-stained cells (3β-HSD⁻; steroidogenically inactive) was determined by counting 10 squares from each sample using a haemocytometer. In separate experiments, the viability of T-I cells at the end of cultures and following trypsinization was assessed using the trypan blue exclusion test and was found to be 94–98%.

Statistical analysis
Values represent means ± SEM. Statistical analysis was performed using analysis of variance followed by pairwise comparisons using Bonferroni correction.

Results
Figure 1 summarizes the effects of VES on T-I DNA synthesis. VES at concentrations of 10–100 μmol/l induced a dose-dependent inhibition of T-I cell proliferation, decreasing DNA synthesis by 52–98% (P < 0.01). Succinate alone (at concentrations of up to 100 μmol/l) did not inhibit cell proliferation (not shown).

Ebselen is a seleno-organic compound with antioxidant glutathione peroxidase-like activity (Muller et al., 1984). Figure 2 demonstrates that ebselen inhibited DNA synthesis in a dose-dependent fashion at all tested concentrations (0.3–30 μmol/l) by 26–100% (P < 0.01). Similarly (Figure 3), SOD at concentrations of 300–1000 IU/ml decreased DNA synthesis by 61–97% (P < 0.01).

Superoxide radical generation using the HX/XO system resulted in a biphasic effect on DNA synthesis (Figure 4). At lower concentrations of XO (3–30 μU/ml), DNA synthesis was significantly stimulated by up to 2.15-fold (P < 0.01). In contrast, at the highest concentration of XO (1 mU/ml), the
proliferation of T-I cells was profoundly inhibited by 85% ($P < 0.01$).

The above experiments evaluated the rate of DNA synthesis and were carried out during 48 h cultures. Subsequent experiments evaluated the cell number (indirectly by MTT assay and directly by cell counting) and were carried out during 96 h culture. Longer cultures were selected in order to characterize the cumulative effect of changes in proliferation on cell number. Estimation of T-I cell number using the MTT assay resulted in findings consistent with those of thymidine incorporation studies. Figure 5 presents the effects of antioxidant and oxidative stress. VES (10 and 30 μmol/l) induced a dose-dependent inhibition by up to 90% ($P < 0.01$) below control. Similarly, the MTT signal was inhibited by ebselen (10 and 30 μmol/l) by up to 98% ($P < 0.01$), and by SOD by up to 44% ($P < 0.01$). In contrast, HX/XO at a dose of 30 μU/ml increased the MTT signal by 24% ($P < 0.01$).

Figure 6 summarizes the findings of direct cell counting at the end of 96 h culture. When compared with control cultures, the total cell count was decreased in the presence of VES (100 μmol/l) by 28% ($P < 0.01$), in the presence of ebselen (30 μmol/l) by 50% ($P < 0.01$), and in the presence of SOD (1000 IU/ml) by 41% ($P < 0.01$). In contrast, induction of modest oxidative stress by HX/XO using XO at 30 μU/ml increased the total cell count by a modest but statistically significant 16% ($P < 0.01$). The steroidogenic activity of T-I cells was identified by histochemical staining of the cells for 3β-HSD. Antioxidants had comparable inhibitory effects on...
the number of both steroidogenically active and inactive cells. Similarly, HX/XO (using XO at $3 \times 10^{-5}$ μU/ml) led to a significant increase of the number of both steroidogenically active and inactive cells.

**Discussion**

The present study, to our knowledge, is the first report demonstrating that antioxidants and oxidative stress modulate proliferation of ovarian T-I cells. We have shown that under serum-free conditions, antioxidants exert a potent inhibitory effect on DNA synthesis irrespective of the nature of the tested antioxidant. The antiproliferative effects of antioxidants were confirmed using the MTT assay and direct cell counting. Furthermore, inhibition of proliferation occurred among steroidogenically active and inactive cells.

Importantly, VES exhibited potent inhibitory effects on proliferation at concentrations corresponding to physiological levels of $\alpha$-tocopherol. In a large survey of adults in the USA, the mean concentration of $\alpha$-tocopherol was 26.8 μmol/l (Ford and Sowell, 1999). $\alpha$-Tocopherol is a particularly important natural chain-breaking antioxidant inhibiting lipid peroxidation in membranes by scavenging peroxyl and alkoxyl radicals (Smith et al., 1993; Ham and Liebler, 1995).

Proliferation was also inhibited in a dose-dependent fashion by the glutathione peroxidase mimetic, ebselen, and by the scavenging of superoxide anions by SOD. The inhibitory effects of antioxidants occurred under baseline conditions, i.e. in the absence of induction of ROS, thus indicating that the source of ROS resides within T-I cells. Furthermore, our findings suggest that ongoing generation of ROS is required in order to maintain DNA synthesis and that the reduction of the level of ROS inhibits proliferation of T-I cells. The speculation that moderate oxidative stress promotes proliferation is supported by the observation that generation of superoxide radicals using HX/XO results in a significant increase of DNA synthesis (Figure 4). Indeed, growing evidence indicates that ROS may act as important mediators of mitogenic signalling, as demonstrated in several cell types including fibroblasts and smooth muscle (Murrell et al., 1990; Brar et al., 1999; Clement and Pervaiz, 1999; Kunsch and Medford, 1999).

While the findings of this study suggest a role for oxidative stress in the regulation of the proliferation of T-I cells, several important caveats should be noted. Most importantly, the *in vitro* and *in vivo* milieu of cells may differ greatly due to endocrine/paracrine effects as well as differences in other parameters such as the amount of available oxygen. In particular, oxygen tension in culture media is significantly greater than that in the theca compartment *in vivo*; thus, observations in our *in vitro* system may not accurately reflect effects occurring *in vivo*. Furthermore, an observation of different rates of DNA synthesis and cell number does not exclude the possibility that changes in oxidative stress may affect the rate of apoptosis. In this study, cell viability at the end of the culture was in the 96–98% range even after exposure to the highest concentrations of antioxidants; nevertheless, such findings do not exclude the possibility of changes in the rate of ongoing apoptosis.

The observation in this study of a biphasic effect of the generation of superoxide radicals on proliferation is consistent with the concept that while moderate oxidative stress may induce cell growth, very high levels of ROS are cytotoxic and lead to apoptotic cell death (Roberg and Ollinger, 1998; Takahashi et al., 2002). However, at moderate concentrations, ROS may act as intra- and intercellular messengers capable of promoting growth responses (Burdon et al., 1995, 1996; del Bello et al., 1999).
The above speculations raise interesting questions regarding the mechanisms regulating oxidative stress. It is possible that ROS may be induced by a broad range of agents, including insulin, insulin-like growth factors (IGFs) and TNF-α. Indeed, administration of insulin and IGF-I increases low-density lipoprotein (LDL) peroxidation, as measured by the production of thiobarbituric acid-reactive substances (TBARS), and other measures of oxidative stress (Riﬁci et al., 1994). Treatment of adipocytes with insulin leads to a rapid increase in hydrogen peroxide (Krieger-Brauer and Kather, 1995; Krieger-Brauer et al., 1997). Insulin-induced oxidative stress may explain the observation that insulin decreases circulating vitamin E levels in type II diabetics, as well as in healthy lean and obese subjects; this effect remains signiﬁcant even after accounting for insulin-induced changes in lipid levels (Krieger-Brauer et al., 1997). TNF-α may also be involved in oxidative stress. An association between the plasma level of TNF-α and lipid peroxidation has been observed (Chen et al., 1998). Injection of TNF-α into healthy animals results in changes in plasma lipoprotein lipid composition due to peroxidation (McDonagh et al., 1992). TNF-α also induces oxidative stress in vitro (Adamson and Billings, 1992). T-I cells possess receptors to insulin, IGFs and TNF-α, agents that have been shown to induce T-I proliferation (Duleba et al., 1997, 1998; Spaczynski et al., 1999). It is, therefore, possible that the actions of insulin, IGFs and TNF-α may be mediated, at least in part, by induction of oxidative stress.

The present ﬁndings may also have important clinical relevance in conditions such as PCOS. Recently, Sabuncu et al. (2001) found that women with PCOS have increased oxidative stress and decreased antioxidant reserve. PCOS is also characterized by hyperplasia of ovarian theca and stroma (Hughesdon, 1982), Thus, it is tempting to speculate that in PCOS, increased oxidative stress and insufﬁcient antioxidant activity contribute to excessive growth of ovarian mesenchyme. At present, the mechanisms involved in the generation of oxidative stress in PCOS remain elusive. However, since PCOS is associated with elevations of insulin, free bioavailable IGF-I and TNF-α, it is possible that these agents act through generation of excessive ROS. Our current studies are directed at evaluating possible interactions between insulin/IGF-I/TNF-α systems and oxidative stress.

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References

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