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Oxidative Stress and Phthalate-Induced Down-Regulation of Steroidogenesis in MA-10 Leydig Cells*

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Abstract

Previous studies have shown that phthalate exposure can suppress steroidogenesis. However, the affected components of the steroidogenic pathway, and the mechanisms involved, remain uncertain. We show that incubating MA-10 Leydig cells with mono-(2-ethylhexyl) phthalate (MEHP) resulted in reductions in luteinizing hormone (LH)-stimulated cAMP and progesterone productions. cAMP did not decrease in response to MEHP when the cells were incubated with cholera toxin or forskolin. Incubation of MEHP-treated cells with dibutyl-cAMP, 22-hydroxycholesterol or pregnenolone inhibited the reductions in progesterone. Increased levels of reactive oxygen species (ROS) occurred in response to MEHP. In cells in which intracellular glutathione was depleted by buthionine sulfoximine pretreatment, the increases in ROS and decreases in progesterone in response to MEHP treatment were exacerbated. These results indicate that MEHP inhibits MA-10 Leydig cell steroidogenesis by targeting LH-stimulated cAMP production and cholesterol transport, and that a likely mechanism by which MEHP acts is through increased oxidative stress.

Keywords

Phthalate; MEHP; Leydig Cell; Oxidative Stress; Steroidogenesis

1. Introduction

Epidemiological studies have shown strong correlations between exposures to endocrine disruptors, which are defined as chemicals that mimic hormones or alter their function, and testicular dysgenesis syndrome [1, 2]. Thus, decline in sperm production, poor semen

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quality, and increased incidences of cryptorchidism, hypospadias and testicular cancer have been attributed in part to exposures to environmental antiandrogenic compounds [1, 3–7]. Identifying such compounds, and determining the developmental stage(s) that they affect and their mechanism(s) of action, have become high public and research priorities [8–11].

Phthalic acid esters are used in the production of plastics [12, 13]. The global production of phthalic acid esters is approximately 6.0 million metric tons per year [14]. Humans are exposed to di-(2-ethylhexyl) phthalate (DEHP), the most widely used phthalate, through ingestion (food, infant formula, and breast milk), contact with consumer products (cosmetics and toys), inhalation, and medical procedures and devices [8, 9, 15, 16]. Typical human exposure is about 30 µg DEHP/kg/day [17]. Medical exposures are through intravenous, oral and inhalational routes [11, 18]. Dialysis patients can receive an additional 36 µg/kg/day, and newborn infants undergoing transfusion receive 1–10 mg/kg/day [8, 9, 19–22]. Phthalates have been shown to exhibit antiandrogenic properties in men [23], and to cause developmental and reproductive toxicity in rodents [24–27]. The *in vivo* exposure of steroidogenic cells to DEHP, or their *in vitro* exposure to mono-(2-ethylhexyl) phthalate (MEHP), the major DEHP metabolite, can result in the suppression of steroid formation [28–35]. As yet, the specific components of the steroidogenic pathway that are compromised by DEHP/MEHP, and the mechanisms by which DEHP and MEHP act to cause steroidogenic suppression, remain uncertain.

MA-10 Leydig tumor cells often are used as a model of Leydig cell steroidogenic function. A major advantage of these cells is that, unlike primary cells, they do not lose steroidogenic function when cultured long-term [36]. We and others reported that culturing MA-10 cells or isolated rat primary Leydig cells with MEHP resulted in changes in the expression of genes involved in regulating reactive oxygen species (ROS) balance, and in ROS generation [34, 35, 37]. Exposure to ROS has been reported to affect steroidogenic function in various steroid-producing cells, including adrenal, Leydig and luteal cells [38–42], and to mediate age-related decline in steroid hormone production [43]. These studies, taken together, led us to hypothesize herein that the induction of ROS by MEHP mediates the suppressive effects of MEHP on critical steps in the steroidogenic pathway, resulting in reduced steroidogenesis.

2. Materials and Methods

2.1. Chemicals and reagents

MEHP (P1073) was purchased from Tokyo Kasei Kogyo Co., LTD (Japan). Dibutylryl cAMP (dbcAMP), L-buthionine-sulfoximine (BSO), 22-hydroxycholesterol (22HC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Waymouth's MB/752 medium, poly-L-lysine, horse serum, cholera toxin and forskolin were from Sigma-Aldrich (St. Louis, MO). 2,7-Dichlorodihydrofluorescein diacetate (DCF) was from Invitrogen (Carlsbad, CA). [1,2,6,7-³H(N)]-progesterone was from Perkin Elmer Life Sciences, Inc (Boston, MA). Progesterone antibody was from ICN (Costa Mesa, CA). STAR antibody was from ABR, Inc. (Golden, CO). Stimulatory G protein subunit (Gs) antibody was from Enzo Life Sciences (formerly Biomol, Plymouth Meeting, PA). The HRP-conjugated anti-rabbit donkey IgG and [³H]-cAMP assay kits were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). Bovine LH (USDA-bLH-B-6) was provided by the USDA Animal Hormone Program (Beltsville, MD). Pregnenolone (P5) and progesterone were from Steraloids (Newport, RI).

2.2. MA-10 cell culture

The MA-10 mouse Leydig tumor cell line was a generous gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA). The cells were cultured in Waymouth's MB/752

medium containing 15% horse serum and 5% CO₂ at 34 °C [36]. For MEHP treatment, cells with 70% confluence were incubated with the reagent (0–400 µM) for 2–24 hours. In some studies, cells were pre-incubated with BSO (100 µM, 24h) to deplete glutathione (GSH) and thus alter the intracellular redox environment, and then with increasing concentrations of MEHP. In studies in which progesterone was assayed, cells were incubated in MEHP (0–400 µM), and then switched to serum-free M-199 medium containing 0.1% BSA and incubated for 2 hours with luteinizing hormone (LH, 100 ng/ml), dibutyryl cyclic AMP (dbcAMP, 2 mM), 22-hydroxycholesterol (22HC, 25 µM), or pregnenolone (P5, 25 µM). After treatment, the medium was collected and stored at –80 °C for subsequent radioimmunoassay (RIA) analysis, and the cells were lysed for Western blots. The viability of cells was estimated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (see section 2.6.).

2.3. Progesterone and cAMP assays

Medium collected at the end of each experiment was stored at –80 °C. Progesterone concentration in the medium was determined by RIA and expressed as micrograms per milligram cellular protein. For cAMP assays, the cells were cultured in a 24-well plate with MEHP (0–400 µM) for 24 hours, then switched to phenol red-free M-199 medium containing LH (100 ng/ml), cholera toxin (10 µg/ml) or forskolin (500 µM) for 2 hours. The medium also contained isobutyl-methylxanthine (IBMX, 100 µM) to inhibit phosphodiesterase activity. At the end of the incubation, an equal volume of Tris-HCl buffer (0.05 M, pH 7.5) containing 4 mM EDTA and 2 mg/ml theophylline was added to the culture medium to block cAMP degradation. The plates were frozen on dry ice and stored at –80 °C before cAMP and progesterone assays. cAMP levels were measured using the [³H]cAMP assay system from GE Healthcare Bio-Sciences Corp (Piscataway, NJ) according to the manufacturer's instructions. The sensitivity of the assay was 0.05 pmoles per assay tube.

2.4. Glutathione (GSH) assay

GSH was measured as previously described [44], with minor modifications. In brief, cells were lysed in 5% metaphosphoric acid and sonicated. Protein was precipitated by centrifugation at 13,000g for 30 minutes. The supernatant was diluted 10 times with sodium phosphate buffer (0.1 M, pH 8.0, with 5 mM EDTA). Diluted sample (10 µl) was incubated with 10 µl of o-phthalaldehyde (in methanol) and 180 µl of phosphate buffer for 15 minutes at room temperature. Fluorescence was read with a BioRad luminescence spectrometer at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The cellular GSH content was calculated using a concurrently run GSH standard curve.

2.5. Analysis of intracellular ROS concentrations

Intracellular ROS concentration was assessed by measuring the ability of the cells to oxidize the redox sensitive dye 2,7-dichlorodihydrofluorescein diacetate (DCF). In brief, cells were cultured in black 96-well fluorescence assay plates (BD Falcon, Franklin Lakes, NJ). After their preincubation with or without BSO for 24 hours, the cells were treated with MEHP (0–400 µM) with or without the antioxidant vitamin E (40 µg/ml) for 2 hours. The cells then were incubated in 200 µl M199 medium containing 20 µM DCF for 30 minutes. After washing 3 times with PBS, 50 µl PBS was added, and the plates were read on a DTX800 Multimode Detector (Beckman Coulter, Fullerton, CA) with excitation of 485 nm and emission of 535 nm. DCF fluorescence also was examined by fluorescence microscopy. The cells were cultured in poly-L-lysine-coated six well chamber slides, treated with MEHP, and then incubated with DCF as above. Images were obtained with a Nikon Eclipse 800 microscope equipped with a Princeton Instruments 5-Mhz cooled CCD camera, custom CRI

color filter, and IP-Lab digital image analysis software (Scanalytics Inc., Fairfax, VA). All photos were taken at the same magnification and with the same exposure times.

2.6. Cell viability assays

Cell viability was estimated by MTT reduction, mitochondrial membrane potential, or plasma membrane permeability. The MTT assay measures the reduction of MTT to blue formazan in viable cells [45]. After their incubation with MEHP (up to 400 μ M, 24h), cells were incubated with MTT (0.5 mg/ml) for one hour. The medium was then removed and reduced formazan was dissolved in 100 μ l acidified (0.04 N HCl) isopropanol at room temperature for 20 minutes. The dissolved formazan then was transferred to a new 96-well plate and read by a DTX800 Multimode Detector (Beckman, Coulter, Inc., Fullerton, CA) at 562 nm wavelength. Control (blank) wells contained cells without MTT but with isopropanol. Cells from three different experiments were analyzed for each treatment.

2.7. Western blot analysis

Gs, STAR and α -actin proteins were analyzed by Western blots. Gs was analyzed after treatment of cells with MEHP (0–400 μ M) for 24 hours. For STAR and α -actin protein analysis, the MEHP treated cells were further stimulated with LH (100 ng/ml) for two hours. Thirty microgram total proteins were separated on 10% SDS PAGE and blotted by primary and then secondary (HRP-conjugated anti-rabbit donkey IgG) antibodies. For three independent studies, STAR and α -actin were quantified by densitometric scanning of film by NIH Image (ImageJ) software. The data then were analyzed statistically.

2.8. Statistical analyses

Data are expressed as the mean $\pm$ standard error of the mean (SEM). Group means were evaluated by one-way ANOVA. If group differences were revealed by ANOVA ($P < 0.05$), differences between individual groups were determined with the Student-Neuman-Kuels-test, using SigmaStat software (Systat Software Inc., Richmond, CA). Values were considered significant at $P < 0.05$.

3. Results

3.1. Effect of MEHP on steroidogenesis in MA-10 Leydig cells

The effect of MEHP on MA-10 Leydig cell steroidogenesis was examined by incubating the cells with increasing concentrations of MEHP for 24 hours, and then with maximally stimulating LH for 2 hours. Dose-dependent reductions in progesterone production were seen in response to MEHP (Fig. 1A) without effect on the ability of the cells to reduce MTT (Fig. 1B). These observations indicate that the effects of MEHP on progesterone production were not a consequence of altered cell viability.

As seen in Figure 2A, the ability of the cells to produce cAMP in response to LH also was reduced significantly by exposing the cells to increasing MEHP concentrations. These results suggested that components of the LH receptor/G-protein-adenylyl cyclase cascade might be affected by MEHP. To test this possibility, we compared the ability of cells that had been cultured with MEHP for 24 hours to produce cAMP in response to LH, cholera toxin (which activates Gs protein directly), and forskolin (which activates adenylyl cyclase directly). For these studies, MEHP-treated cells were switched to medium containing LH (100 ng/ml), cholera toxin (10 μ g/ml), or forskolin (500 μ M) for 2 hours. In comparison to the decreases in LH-stimulated cAMP, there were no decreases in cAMP production when MEHP-treated cells were incubated either with cholera toxin or forskolin (Fig. 2A). Gs protein content, as assessed by Western blot, also did not change in response to MEHP (Fig. 2B).

The effects of MEHP on progesterone production in response to LH, cholera toxin and forskolin also were examined (Fig. 2C). Progesterone production was reduced in LH-stimulated cells that had been pretreated with 200–400 μ M MEHP. In cells pretreated with 200 μ M MEHP, cholera toxin and forskolin prevented the reductions in progesterone production seen when the cells were incubated with LH. However, neither cholera toxin nor forskolin completely prevented the reduced progesterone production that resulted from exposure of the cells to higher (300 μ M or 400 μ M) MEHP concentrations (Fig. 2C). These results suggested that in addition to its effects on cAMP production, MEHP exposure might also affect downstream steps in the steroidogenic pathway.

With this in mind, we examined the effects of MEHP on specific components of the steroidogenic pathway beyond cAMP production. To this end, cells treated overnight with a high dose of MEHP (400 μ M) were incubated with LH, dbcAMP, 22-hydroxycholesterol (22HC), or pregnenolone (P5) for two hours, and progesterone was measured in the medium. As seen in Figure 3A, a significant, greater than 80%, reduction in LH-stimulated progesterone production was seen in the MEHP-treated cells. In MEHP-treated cells incubated with dbcAMP, which by-passes the LH receptor/G-protein-adenylyl cyclase cascade, progesterone formation also was reduced significantly from the control, by about 50%. In MEHP-treated cells incubated with the mitochondrial membrane-permeable 22HC or pregnenolone, however, progesterone production was maintained at control levels. These results, taken together with the effects of MEHP on cAMP production, support the conclusion that in addition to the effects of MEHP exposure on LH-stimulated cAMP production, cholesterol transport mechanisms may be affected by MEHP exposure. Consistent with the latter, MEHP treatment of the cells resulted in significant reductions in STAR protein (Fig. 3B).

3.2. Effect of MEHP on ROS production in relationship to steroidogenesis in MA-10 Leydig cells

We hypothesized that increased oxidative stress in response to MEHP might be responsible for the reduced cAMP and progesterone production by MEHP-treated MA-10 cells. To begin to test this hypothesis, we first assessed the effects of exposure of MA-10 cells to MEHP on intracellular ROS levels, using 2,7-dichlorodihydrofluorescein (DCF), a dye that fluoresces upon its oxidation by ROS. As seen in the micrographs shown in Figure 4A, intracellular DCF fluorescence, which was minimal in control cells, increased after treatment of the cells with MEHP. Quantification of the DCF fluorescence (Fig. 4B) indicated dose-dependent increases in response to MEHP treatment. These results point to an association between increased ROS and decreased cAMP and progesterone productions.

We reasoned that if reduced progesterone production in response to MEHP was *caused by* increased ROS, it should be possible to increase the deleterious effects of MEHP on progesterone production by first experimentally creating an increased intracellular oxidative environment. To test this, MA-10 cells were treated with buthionine sulfoximine (BSO), a specific inhibitor of γ -glutamylcysteine synthetase, to reduce intracellular glutathione (GSH) and thus alter the intracellular redox environment. As seen in Figure 5A, BSO treatment for 24 hours resulted in greater than 80% reduction in intracellular GSH concentration compared to controls. This reduction in GSH was unaffected by subsequent treatment of the BSO-treated cells with the antioxidant vitamin E (40 μ g/ml, 2h). When BSO pre-treated cells were incubated with increasing concentrations of MEHP for two hours, there was increased DCF fluorescence at every MEHP concentration compared to the cells that had not been pre-treated with BSO (Fig. 5B).

When the cells were incubated with antioxidant vitamin E along with MEHP, these increases in DCF fluorescence in BSO-treated cells were blunted (Fig. 5B). We then

examined the effect of pre-treating cells with BSO on progesterone production in response to MEHP (300 μ M, 24 h) (Fig. 5C). Treating the cells with MEHP without pretreatment with BSO resulted in a 60% reduction in progesterone production in response to LH. Pretreatment with BSO resulted in a greater reduction in progesterone production in response to MEHP, of about 80%. In cells pre-treated with BSO and then incubated with MEHP plus the antioxidant vitamin E, there was a decrease in progesterone production, but only by about 40% rather than 60–80%.

4. Discussion

In the present study, we used MA-10 Leydig cells to ask which components of the steroidogenic pathway are affected by MEHP exposure, and the mechanism(s) involved in steroidogenic inhibition. MA-10 cells, a transformed tumor cell line, retain all key components of the steroidogenic pathway from LH signaling through progesterone production, and unlike primary Leydig cells, the cells can be cultured long-term without losing steroidogenic function [36]. Incubating MA-10 cells overnight with increasing concentrations of MEHP resulted in dose-dependent decreases in the ability of the cells to produce progesterone in response to LH stimulation. These results are consistent with previous studies reporting that the exposure of primary or tumor Leydig cells to MEHP or DEHP can result in the suppression of steroid formation [28–35]. LH-stimulated cAMP production also was reduced in response to MEHP. In light of the critical role played by cAMP in steroidogenesis, we hypothesized that reduced cAMP production might be responsible for reductions in progesterone formation by MEHP-treated MA-10 cells, and reasoned that if this was the case, by-passing the defective cAMP-producing cascade might result in increased levels of steroid formation in the face of exposure to MEHP. Indeed, incubating MEHP-treated cells with dbcAMP largely overcame the MEHP-induced reductions in progesterone formation, though not entirely. These results suggested that LH-stimulated cAMP production is an important target of MEHP, but also that there may be targets in the steroidogenic pathway that are downstream from LH-stimulated cAMP production.

Indeed, when the cells were incubated with 22HC or with pregnenolone, both of which bypass cAMP-dependent cholesterol transport into mitochondria, the inhibitory effect of MEHP on LH-stimulated progesterone production was overcome fully. This observation is consistent with previous results showing that 22HC can overcome the MEHP-induced reductions in testosterone production in primary Leydig cells [29]. This, together with the observed suppressive effect of MEHP on cAMP production, suggest that the sensitive targets of MEHP involve one or more steps in the sequence from LH binding to its receptor through cAMP production, as well as cholesterol transport into the mitochondria. The import of cholesterol into the mitochondria, the rate-determining step of steroidogenesis, is facilitated by the steroidogenic acute regulatory protein (STAR), a LH-responsive protein that contains an N-terminal mitochondrial signal sequence [46–48]. In the testis, STAR de novo synthesis parallels LH-induced steroid formation [49, 50]. Thus the decrease in STAR protein shown herein further supports the contention that the cholesterol transport step of steroidogenesis is affected by MEHP exposure.

LH receptors are coupled to adenylyl cyclase through G proteins [51]. To begin to address the mechanism by which MEHP affects cAMP production, we examined the cell membrane LH receptor/G-protein/adenylyl cyclase cascade by culturing MEHP-exposed cells with forskolin, which directly stimulates adenylyl cyclase, or with cholera toxin, which directly activates the stimulatory G protein (Gs protein). MEHP-treated cells that were cultured with forskolin produced high levels of cAMP regardless of MEHP dose, indicating that adenylyl cyclase was not affected by MEHP. Incubation of MEHP-treated cells with cholera toxin

likewise over came the reductions in cAMP production seen in response to LH, indicating that Gs protein and its coupling to adenylyl cyclase also is well maintained in MEHP-treated cells. Additionally, Western blot analysis of Gs protein α -subunit indicated that Gs protein did not change quantitatively with MEHP treatment. We conclude from these results that Gs protein, adenylyl cyclase, and their coupling were unaffected when cells were exposed to MEHP, and therefore are unlikely to be the explanation for MEHP-induced decreases in the LH-stimulated cAMP production. Rather, we suggest that reduction in cAMP most likely results from defects of the LH receptor itself and/or the coupling of the LH receptor to adenylyl cyclase through Gs proteins. This has yet to be tested directly.

The mechanisms by which MEHP and other environmental toxicants affect targets in the steroidogenic pathway and thereby suppress steroidogenesis remain uncertain. In a previous study, we reported that culturing MA-10 Leydig cells with MEHP resulted in changes in the expression of genes involved in the regulation of ROS balance and in ROS generation [34]. We and others have shown that MEHP affects ROS generation in both primary and tumor Leydig cells [34, 35, 37]. In the present study, MEHP treatment resulted in dose-dependent increases in ROS levels, as shown by increases in DCF fluorescence. The MEHP concentrations that resulted in increased ROS production also resulted in reduced steroidogenesis. Numerous studies have shown that when the levels of ROS overcome the antioxidant capacity of a cell, DNA damage, lipid peroxidation, and protein oxidations can occur, leading to progressive declines in the functional efficiency of various cellular processes [52, 53]. With this in mind, it is tempting to speculate from the correlations between MEHP-induced increase in ROS and decrease in steroidogenic function that increased ROS may, at least in part, be the cause of steroidogenic deficits. However, correlations do not prove cause-and-effect.

In an effort to begin to ascribe cause-effect to the correlations among ROS, cAMP and progesterone, we addressed the effect of altering the intracellular oxidant-antioxidant balance in MA-10 cells by experimentally decreasing glutathione levels (with BSO) on the subsequent responses of the cells to MEHP. We reasoned that if reduced progesterone production in response to MEHP was the result of increased ROS, it should be possible to increase the suppressive effects of MEHP on progesterone production by experimentally creating an increased intracellular oxidative environment. As predicted, progesterone production was decreased in BSO pre-treated cells as a function of intracellular ROS content. Treatment of the cells with the antioxidant vitamin E blocked ROS increases resulting from BSO/MEHP, and thus largely protected the cells from MEHP-induced progesterone decrease. Thus, depletion of GSH sensitized the response of the cells to MEHP treatment, resulting in greater reductions in progesterone formation than obtained with MEHP alone. These results strongly support the hypothesis that MEHP affects Leydig cell steroidogenesis through increased oxidative stress in the cells. The specific signaling pathways that mediate increased oxidative stress and decreased steroidogenesis in MEHP-treated cells are under investigation.

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Abbreviations

DEHP	Di-(2-ethylhexyl) phthalate
MEHP	mono-(2-ethylhexyl) phthalate

LH	luteinizing hormone
dbcAMP	dibutyl cAMP
22HC	22-hydroxycholesterol
P5	pregnenolone
BSO	L-buthionine-sulfoximine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
DCF	2,7 -dichlorodihydrofluorescein diacetate
GSH	glutathione
ROS	reactive oxygen species
Gs	stimulatory G protein subunit
IBMX	isobutyl-methylxanthine
STAR	steroidogenic acute regulatory protein

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MEHP induces two defects in steroidogenic pathway of MA-10 Leydig cells.

- Defect in cAMP production may be due to LH receptor or its coupling to Gs protein.
- Reduction in STAR may be involved in the defect in cholesterol transport.
- MEHP increases oxidative stress in MA-10 Leydig cells.
- Glutathione redox affects the susceptibility of MA-10 cells to MEHP treatment.

Figure 1.

Dose effects of MEHP on MA-10 cell steroidogenic function and cell viability. A) Cells were incubated with MEHP (0–400 μM) for 24 h and then stimulated with LH (100 ng/ml) for 2 h. Progesterone production was assayed in the medium. B) After 24h of MEHP treatment, the cells were incubated with MTT dye (0.5 mg/ml) for one hour. The reduction of the MTT dye by viable cells was read on plate reader at 562 nm wavelength. The data are expressed as mean ± SEM of 4 experiments. * Significantly different from control cells cultured without MEHP, P<0.05.

Figure 2.

Effect of MEHP on MA-10 cell signal transduction in relationship to steroid formation. A) The cells were treated with MEHP (0–400 μM) for 24 h, after which their ability to produce cAMP in response to LH (100 ng/ml, 2h), cholera toxin (3 μg/ml, 2h) or forskolin (200 μM, 2h) in the presence of isobutyl-methylxanthine (IBMX, 100μM) was assessed. cAMP was assayed in the cells plus medium. B) Western blots of Gsα content of cells incubated with increasing concentrations of MEHP. C) Progesterone production by MEHP-treated cells incubated with LH, cholera toxin or forskolin. Data are expressed as mean ± SEM of 3 experiments. * Significantly different from the respective control cell values (i.e. cells cultured without MEHP), $P < 0.05$.

Figure 3.

Effect of MEHP (0–400 μ M) on steps of the steroidogenic pathway. A) The cells were first treated without (control, C) or with MEHP (400 μ M, 24h), and the cells then were incubated with LH (100 ng/ml), dbcAMP (2 mM), 22-hydroxycholesterol (22HC; 25 μ M) or pregnenolone (P5; 25 μ M) for 2h. Progesterone was measured in the medium. * Significantly different from the control, $P < 0.05$. B) Three separate sets of cells were treated with MEHP (0–400 μ M, 24h) and then with LH (100 ng/ml) for 2h. STAR and -actin protein were analyzed by Western blot. Significant decreases in STAR/ -actin ratios were seen at 200, 300 and 400 μ M MEHP.

Figure 4. Effect of MEHP treatment on ROS production. Cells were treated with MEHP (0–400 μM) for two hours, and then incubated in M199 medium containing 20 μM DCF for 30 minutes. Control cells were incubated with DCF without MEHP pre-treatment. A) Upper: phase contrast images of control and MEHP (400 μM)-treated cells. Lower: fluorescence microscope images of DCF-stained control and MEHP-treated cells. B) Quantification of DCF fluorescence after MEHP treatment (0–400 μM), determined by plate reader. The data (mean $\pm$ SEM of 3 experiments) are expressed as the percentage of the cells without MEHP. * Significantly different from control cells, $P < 0.05$.

Figure 5.

Effects of MEHP on cells with altered intracellular GSH levels. A) GSH content of control cells (C), cells 24 hours after BSO treatment (100 μ M, 24h), and cells incubated with vitamin E (40 μ g/ml, 2h) after BSO treatment (24h). B) Effect of MEHP (0–400 μ M, 2h) on DCF fluorescence by control cells (C), cells after pretreatment with BSO (24h), and cells after pretreatment with BSO with vitamin E in the medium during the MEHP treatment (2h). C) Effect of MEHP (300 μ M, 24h) on progesterone production by LH-stimulated cells that had been incubated with vehicle (c), BSO (24h), or BSO with vitamin E in the medium during MEHP treatment. Progesterone was assayed in the media. The data are expressed as mean $\pm$ SEM of 3 experiments. *Significantly different from control cells, $P < 0.05$.