17β-Estradiol, progesterone, and testosterone inversely modulate low-density lipoprotein oxidation and cytotoxicity in cultured placental trophoblast and macrophages

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OBJECTIVES: We have previously shown that low-density lipoprotein oxidation is diminished by 17β-estradiol and enhanced by progesterone and testosterone. In these experiments we wished to learn whether sex hormone effects on low-density lipoprotein oxidation alter placental cell viability in primary tissue culture.
STUDY DESIGN: Primary tissue culture of human term placental cells was performed.
RESULTS: Addition of 17β-estradiol decreased low-density lipoprotein oxidation (measured as lipid peroxides, thiobarbituric acid–reacting substances, and low-density lipoprotein electrophoretic mobility) and placental cell toxicity (measured as chromium 51 release) with maximum reductions of 28% (macrophages) ($p < 0.05$) and 26% (trophoblasts) ($p < 0.01$). Conversely, progesterone and testosterone increased low-density lipoprotein oxidation and chromium 51 release, the latter a maximum of 28% and 18%, respectively, for progesterone and testosterone in macrophages ($p < 0.05$ in both instances) and 23% in trophoblasts ($p < 0.05$, testosterone only). Collectively, cytotoxicity was proportional to low-density lipoprotein oxidation and estradiol, progesterone, and testosterone concentrations.
CONCLUSIONS: Estradiol inhibits placental macrophage- and trophoblast-mediated low-density lipoprotein oxidation and cytotoxicity, whereas progesterone and testosterone promote these effects. Sex steroid hormones may modulate the effects of oxidative stress on placental function in pregnancy. (Am J Obstet Gynecol 1997;177:196-209.)

Key words: Placental cells, low-density lipoprotein oxidation, cytotoxicity, sex steroids

Estrogens oppose the effects of progestins and androgens in endocrine and vascular physiologic mechanisms. Estrogens up-regulate endometrial estrogen and progestin receptors, whereas progestins down-regulate these receptors.[1] With respect to lipoprotein metabolism, estrogens enhance hepatic very-low-density lipoprotein production, up-regulate the low-density lipoprotein (LDL) receptor, and raise plasma high-density lipoprotein concentrations. Progestins and androgens oppose these effects.[2] Regarding the arterial wall, estrogens reduce arteriosclerosis risk in animal models[3] and epidemiologic surveys.[2] Although there is no evidence that natural progesterone favors arteriosclerosis,[4] androgens and androgenic progestins have unfavorable effects on lipoprotein levels[2] and testosterone, levonorgestrel, and medroxyprogesterone acetate favor atherosclerosis development in cholesterol-fed animal models.[5-7]

LDL oxidation, another mechanism of atherogenesis, is also influenced by sex steroid hormones. Estrogen diminishes LDL oxidation in the absence and presence of cells.[8-11] We have shown that this effect encompasses the physiologic range of estrogen concentrations in plasma,[12] and Sack et al.[13] have demonstrated an antioxidant effect on LDL in vivo in women treated with estrogen in postmenopausal replacement doses. Finally, we have found that progestins and androgens promote LDL oxidation, opposite to the effect of estrogen.[12]

LDL oxidation may be important in human pregnancy. Plasma lipids are more susceptible to oxidation toward the end of pregnancy[14] in insulin-dependent diabetic pregnancy[15] (Bonet B. Unpublished observations), and products of lipid oxidation are increased in toxemia of pregnancy.[14] Lipoprotein oxidation may impair placental function, as suggested by the presence of lipid peroxides in placentas and plasma from toxemic pregnancies.[14, 16] In addition, placentas may be small and functionally impaired in toxemia,[17] some diabetic pregnancies,[18] and in smoking,[19] a condition of dramatically increased oxidative stress. At least one defense mecha-

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nism against this process exists in the form of scavenger receptors that can take up and degrade modified and oxidized LDL.[20]

Because LDL oxidation injures placental trophoblasts and macrophages and because sex steroid hormones influence the rate of LDL oxidation, we tested the hypothesis that estrogen prevents and progesterone and testosterone promote LDL oxidation and conjoint cytotoxicity in primary cultures of placental macrophages and trophoblasts. The results show that estrogens inhibit and progesterone and testosterone accelerate LDL oxidation and conjointly placental cell cytotoxicity.

Material and methods

Materials. 17β-Estradiol, progesterone (4-pregne-3,20-dione), and testosterone (4-androsten-17β-ol-3-one) were purchased from Sigma (St. Louis). Hydrogen peroxide solution was obtained from Mallinckrodt (Paris, Ky.), thiobarbituric acid from Eastman Kodak (Rochester, N.Y.), trichloroacetic acid from J.T. Baker (Philipsburg, N.J.), and tetramethoxypropane and cupric chloride from Sigma. The cholesterol color reagent (CHOD-iodide) was obtained from E. Merck, Diagnostica, Gibbstown, N.J. The Paragon lipoprotein electrophoresis system was purchased from Beckman Instruments (Brea, Calif.). Cell culture medium Ham's F-10 and AIM-5 were obtained from Gibco Laboratories (Grand Island, N.Y.). Radioactive sodium chromate (^{51}Cr) was obtained from Amersham (Arlington Heights, Ill.).

Placental cell culture. Placentas were obtained from healthy normal pregnant women at elective cesarean section delivery with intact fetal membranes. Placental trophoblast and macrophages were isolated according to a modification of procedures described previously by Lasunción et al.[21] Fetal cotyledons were dissected from the placenta under sterile conditions. Placental cells were obtained by digestion of minced cotyledons at 37° C for 60 minutes in buffered Ham's F-10 medium containing collagenase type II, hyaluronidase, deoxyribonuclease I, and 2% fetal bovine serum. Identifiable trophoblast cells (presumably cytotrophoblast) were separated from red blood cells and macrophages by isopycnic centrifugation on a linear gradient of 40% Percoll. The majority of cells in the middle band (about 85%) were trophoblasts and stained positively with monoclonal antibody 35βH11. In the bottom band the majority of cells were macrophages (about 95%) and stained positively with HAM-56. Before resuspension in AIM-5 medium cells collected from each band were washed several times to eliminate Percoll. Then cells were plated in AIM-5 in 24-well culture plates at an amount of 10^6 cells per well. After 24 hours macrophages attach to the wells whereas trophoblast continue to float. At this point, a further purification is achieved by decanting and discarding trophoblast from the wells consisting primarily of macrophages and decanted and replating trophoblast from the trophoblast-rich cultures to new culture wells. Cells were maintained at 37° C in a humidified atmosphere of air (95%) and carbon dioxide (5%). AIM-5 growth media were changed on the following day. On the fourth day cells were ready for experiments in Ham's F-10 medium, which contains transition metal ions and lacks phenol red, conditions appropriate to study LDL oxidation.

LDL preparation. Pooled plasma was obtained from the blood drawn in ethylenediaminetetraacetic acid (EDTA) (1 to 2 mg/ml) after a 12-hour fast from healthy donors. LDL was isolated by sequential ultracentrifugation in a Beckman L5-50 ultracentrifuge, in the density range of 1.019 to 1.063 gm/ml.[21] The LDL fraction was then exhaustively dialyzed against sodium chloride–EDTA (1 mmol/L) buffer, pH 7.4, for 24 hours and sterilized by passing through a Millipore 0.45 μm filter and stored under nitrogen at 4° C until the time of experiment (usually within 10 days). Before it was added to cultured cells, LDL was dialyzed against phosphate-buffered saline solution for 24 hours to remove EDTA and was then sterilized again by a Millipore 0.45 μm filter. The LDL protein concentration was determined by the Lowry method.[21]

Experimental procedures. Placental macrophages and trophoblast from six human placentas were used in the experiments. After 3 days of culture in growth-promoting AIM-5 medium, the medium was replaced with 1 ml of AIM-5 containing radioactive ^{51}Cr, 0.6 μCi per well. After incubation with ^{51}Cr for 2 to 3 hours, the radioactive medium was removed and cells were gently washed three times, the medium replaced with Ham's F-10, and LDL was added to the medium at a final concentration of 150 μg/ml protein. A solution of cupric chloride was sterilized by passing through a Millipore 0.45 μm filter and added to the medium at a final concentration of 2 μmol/L. Cells were then incubated at 37° C for the indicated times (see Figs. 1 through 9). Controls consisted of wells without addition of LDL.

At the conclusion of the experimental incubations, the following procedures were done on ice or in the cold room. Media were collected from culture plates at indicated times and EDTA–butylated hydroxytoluene solution added into the collected medium at a concentration of 0.1 mmol/L to inhibit any further LDL oxidation. Cells remaining in the wells were gently washed three times with cold saline solution and the wash fluid containing ^{51}Cr was collected. The medium and washes were centrifuged at 1000 revolutions/min for 10 minutes and supernatants and pellets were collected and counted separately. One milliliter of 0.1 N sodium hydroxide was added to each tissue culture well and incubated at room

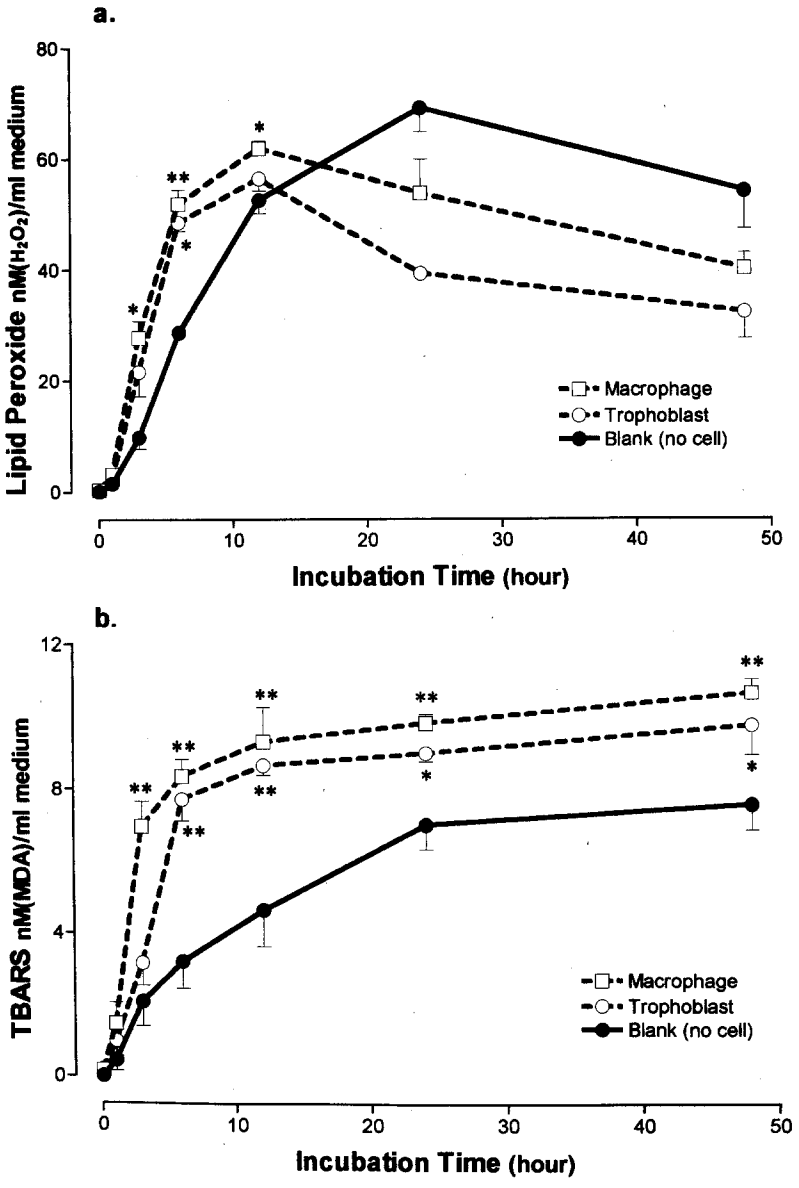

Fig. 1. Oxidation of LDL by placental macrophages and trophoblast. Placental cells were incubated with 150 μg/ml LDL protein from 0 to 48 hours at 37° C in Nunc petri dishes in fresh HAM's F-10 (serum free) medium containing 2 μmol/L cupric chloride. Controls *(blank)* consisted of LDL incubated in HAM's F-10 medium in absence of placental cells under same conditions. Lipid peroxides **(a)** and thiobarbituric acid–reacting substances *(TBARS)* **(b)** were measured at incubation times indicated. Values are means ± SE of triplicate determinations from one of three experiments. *Asterisks* denote significant differences from blank: *one asterisk*, $p < 0.05$; *two asterisks*, $p < 0.01$.

temperature for 30 minutes, cell solution was collected, and an aliquot was taken for cell protein analysis by the Lowry method.[21] The percent of ^{51}Cr release was determined according to the method of Henriksen et al.[22] by counting (1) supernatant of the medium and wash fluid (Scpm), (2) pellets of the medium and wash fluid (Pcpm), and (3) cells remaining in the plate well after wash (Ccpm). The percent of ^{51}Cr release (% ^{51}Cr R) was calculated by following formula:

$$\%\ ^{51}Cr\ R = Scpm/(Scpm + Pcpm + Ccpm) \times 100$$

Quantification of LDL oxidation. Lipid hydroperoxides in the incubation medium were measured by incubating 100 μl of the medium with 1 ml of potassium iodide color reagent containing cholesterol esterase and oxidase for 30 minutes at room temperature and the absorbencies of the sample solution measured in a spectrophotometer at wavelength of 365 nm. Lipid per-

oxide products are expressed as nanomoles of hydroperoxide equivalents per milligram of LDL protein. Hydrogen peroxide solution was freshly diluted and used as a standard.

Thiobarbituric acid–reacting substances were assayed by incubating 300 μl of medium with 1 ml of thiobarbituric acid–reacting substances reagent (0.4% thiobarbituric acid, 15% trichloroacetic acid, and 2.5% hydrochloric acid) for 20 minutes in a water bath at 95° C to 100° C. The reaction tubes were then cooled and centrifuged at 2000 revolutions/min for 10 minutes, and the absorbance of the supernatant was measured spectrophotometrically at 532 nm. Thiobarbituric acid–reacting substances content is expressed as nanomoles of malondialdehyde equivalents per milligram of LDL protein compared with freshly diluted tetramethoxypropane standards.

Agarose gel lipoprotein electrophoresis was performed with a Beckman horizontal electrophoresis unit by use of the Beckman Paragon lipoprotein electrophoresis kit. Gels were placed in the running buffer for about 30 minutes, fixed in fixation solution, stained about 10 minutes, and dried in a dryer unit.

Hormonal effects on cellular modification of LDL and subsequent cytotoxicity. Hormones dissolved in ethanol were added to final medium hormone concentrations of 0.001, 0.01, 0.1, and 1.0 μmol/L and a final ethanol concentration at 0.5%. Hormones were added at the beginning of the incubation at the time of adding LDL, 150 μg/ml, and 2.0 μmol/L cupric chloride. Controls consisting of cell cultures with LDL and 0.5% ethanol alone were incubated over the same length of time. After 24 hours of incubation LDL oxidation and the percent ^{51}Cr release were determined. No effect was found at 0.5% ethanol and 2μmol/L copper on ^{51}Cr release.

To determine whether prior hormone exposure influenced placental cell susceptibility to the toxicity of LDL oxidation, cells were preincubated with each hormone at the same concentrations as above for 24 hours. Cells were then washed two times with fresh medium containing no hormone. After washing the cytotoxicity assay described above was performed, consisting of labeling placental cells with ^{51}Cr, washing the cells, adding 150 μg/ml LDL and 2 μmol/L cupric chloride but no hormone and incubating for 24 hours.

In all experiments manipulations were performed in duplicate. Statistical analysis was performed with the Student *t* test. Correlation was obtained by Spearman correlation analysis. Hormone concentration effect was assessed with two-way analysis of variance. A significant difference was chosen as the $p \leq 0.05$ level.

Results

The first experiments were performed to confirm that placental macrophages and trophoblasts accelerate LDL oxidation. In the presence of Cu^{++} (Fig. 1) both lipid peroxide and thiobarbituric acid–reacting substances formation increased with time, as shown by media sampled at 2, 6, 12, 24, and 48 hours of incubation. The rates of formation of both substances were greater in the presence of both placental macrophages and trophoblasts than in their absence. Also noteworthy is that lipid peroxide content declined after 12 to 24 hours of incubation whereas content of thiobarbituric acid–reacting substances did not. LDL electrophoretic mobility increased markedly when LDL was incubated in the presence of copper with both placental cell types (shown in Fig. 2) compared with nonoxidized LDL and the LDL oxidized with copper in HAM's F-10 medium without cells. LDL electophoretic mobility increased only slightly when incubated with cells in the absence of copper (data not shown). These data indicate that lipid peroxidation products accumulate in the incubation medium of placental cells incubated with LDL. The rise and fall of lipid peroxide levels with time are consis-

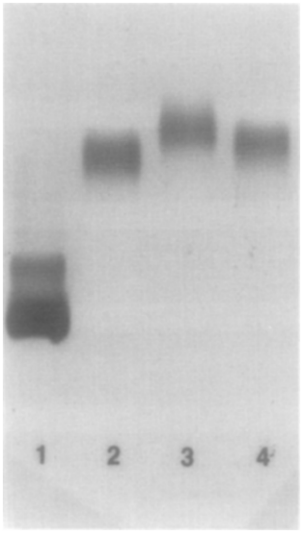

Fig. 2. LDL electrophoretic mobility in presence of placental cells. LDL samples were obtained from same experiment described in Fig. 1 after 24 hours and agarose gel electrophoresis performed. Nonincubated LDL was used as blank, and control was LDL incubated in HAM's F-10 medium in absence of cells under same condition.

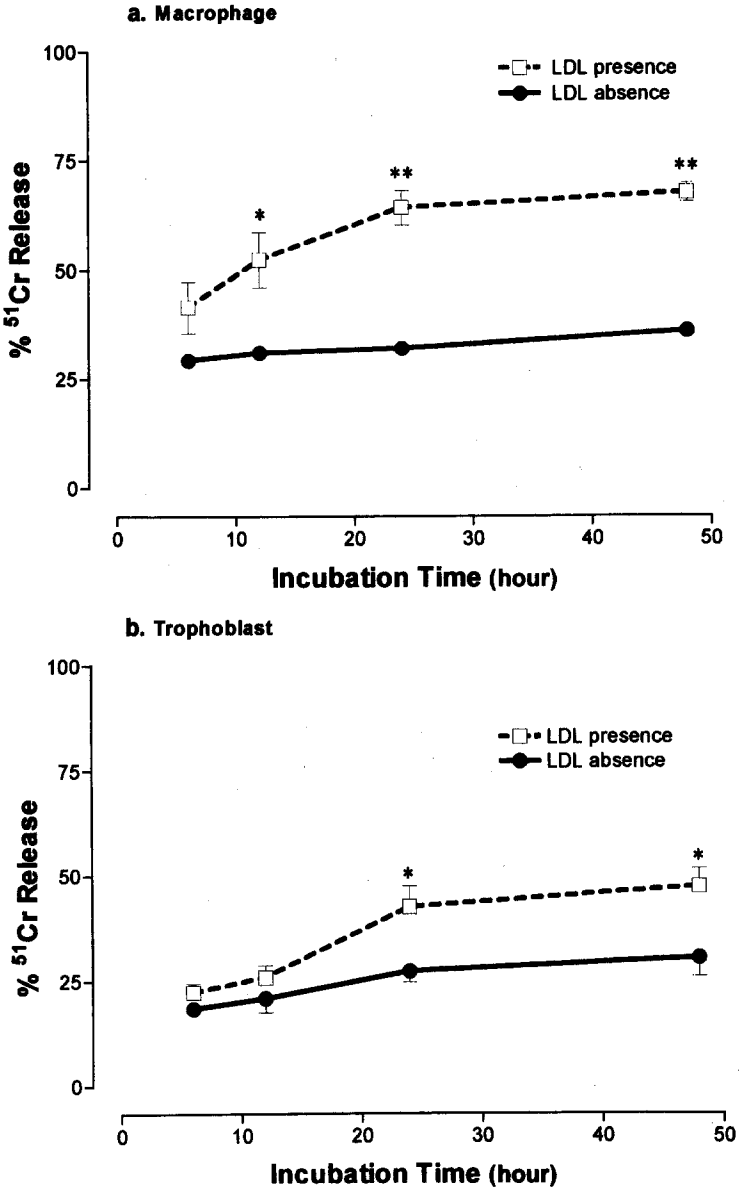

Fig. 3. Effect of LDL oxidation on placental macrophage (**a**) and trophoblast (**b**) cytotoxicity. Placental cells were preincubated with ^{51}Cr (about 0.6 μCi/well) for at least 2 hours. Cells were then carefully washed three times with fresh isotope-free HAM's F-10 medium, and 1 ml/well of fresh HAM's F-10 medium was added to the dish. LDL was incubated with cells at final concentration of 150 μg/ml protein in presence of 2 μmol/L cupric chloride. At end of 6, 12, 24, and 48 hours of incubation, cytotoxicity of LDL was determined by ^{51}Cr release assay: *Asterisk*, $p < 0.05$; *two asterisks*, $p < 0.01$, versus LDL absence. Controls consisted of each cell type incubated in absence of LDL under same condition. Data are means ± SE of triplicates from one of three experiments.

tent with their intermediate place in the sequence of lipid oxidation. In contrast, the continuous rise of thiobarbituric acid–reacting substances reflects the more terminal place of this product in the sequence of LDL oxidation.

The association of LDL oxidation with placental macrophage and trophoblast cytotoxicity was investigated next. The percent ^{51}Cr release into the incubation medium (Fig. 3) was determined in the same experiments described in Fig. 1. Incubation of placental macrophages or trophoblast with LDL significantly increased ^{51}Cr release after 24 and 48 hours compared with cultures in the absence of LDL. When the increase in percent of ^{51}Cr release was compared with the increase in LDL oxidation (Fig. 1), a significantly positive linear correlation was found between the percent ^{51}Cr release

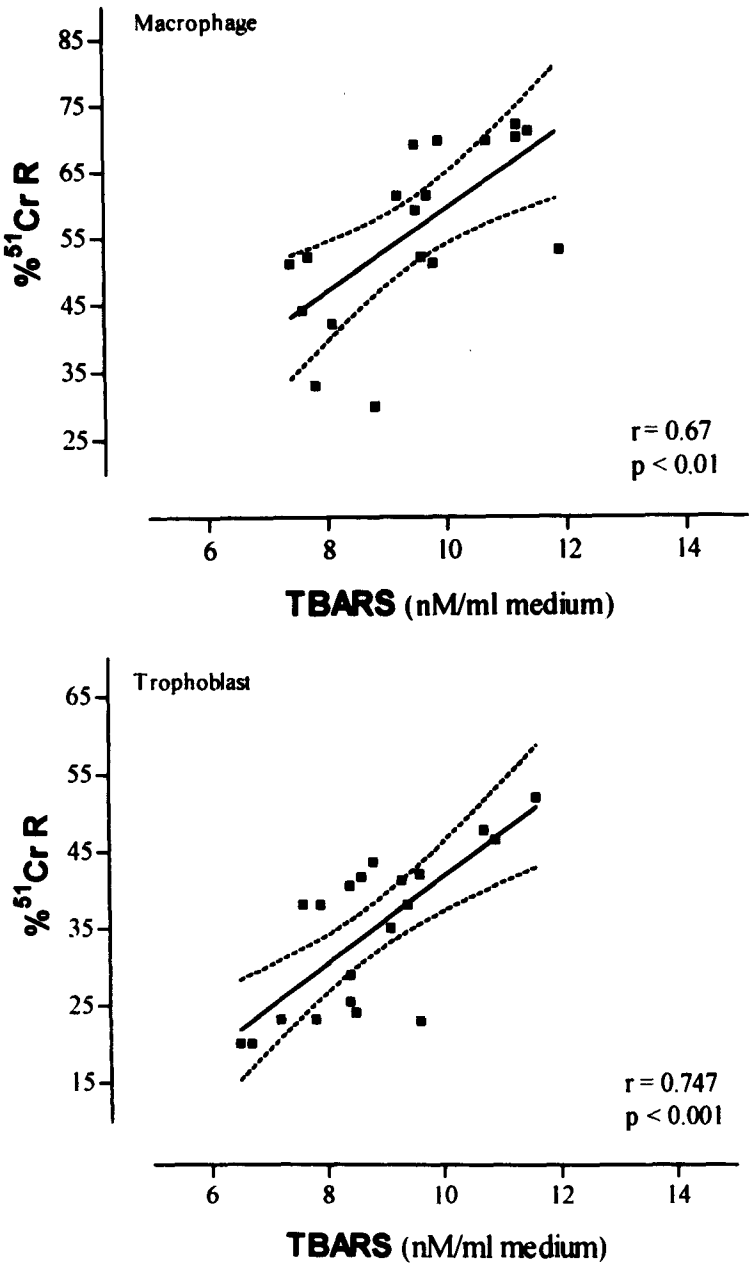

Fig. 4. Association of macrophage and trophoblast cytotoxicity as measured by ^{51}Cr release *(^{51}CrR)* versus LDL oxidation measured as thiobarbituric acid–reacting substances *(TBARS)* formation. Data are from individual experiments presented in Fig. 3. Regression coefficients (Pearson's *r*) are statistically significant for macrophages and trophoblast ^{51}Cr release versus thiobarbituric acid–releasing substances formation. Main point is that extent of LDL oxidation is linearly proportional to amount of cytotoxicity occurring.

and thiobarbituric acid–reacting substances in cultures of both trophoblast and macrophages (Fig. 4).

To further explore the relationship between LDL oxidation and placental cell cytotoxicity, Cu^{++} was added in increasing concentrations to culture wells. As shown in the upper panel of Fig. 5, greater formation of thiobarbituric acid–reacting substances was observed in the wells containing higher concentrations of Cu^{++}. A proportional increase in the percent of ^{51}Cr release was also observed in the presence of increasing concentrations of Cu^{++}. There were no significant effects of higher concentrations of copper observed on ^{51}Cr release in the absence of LDL (data not shown). Thus increasing amounts of copper added to cell culture in the presence of LDL were related to an increased oxidation rate, with more oxidation yielding more cytotoxicity.

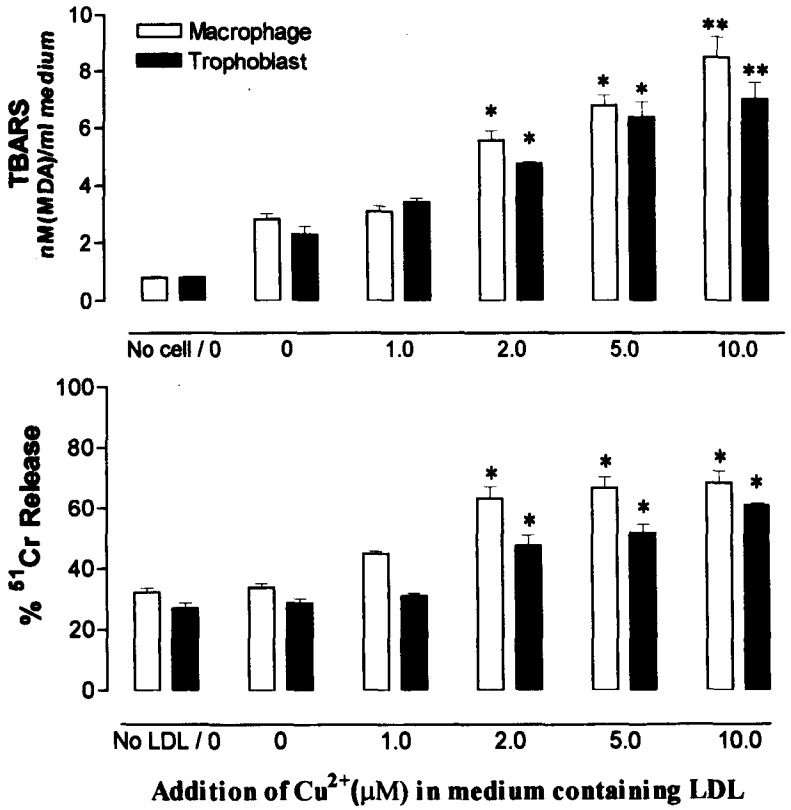

Fig. 5. Effects of Cu^{++} on placental cell–mediated LDL oxidation and cell ^{51}Cr release. Procedures are same as described in Figs. 1 and 3. At day 4 cells were preloaded with ^{51}Cr (0.5 to 1.0 μCi/ml AIM-5). After 2 to 3 hours of incubation, cells then were washed three times with fresh HAM's F-10 medium containing no radioactivity. One milliliter of Ham's F-10 medium containing a fixed concentration of LDL (150 μg/ml protein) was placed in each well and Cu^{++} was added at increasing concentrations (1, 2, 5, 10 μmol/L). After 24 hours of incubation, medium samples were taken for thiobarbituric acid–reacting substances testing and ^{51}Cr release assay was processed as described in Material and Methods. Controls consist of each cell type incubated in absence of LDL (for ^{51}Cr release assay) and no addition of Cu^{++}. Data present mean ± SD of triplicate. *Asterisk*, $p < 0.05$, and *two asterisks*, $p < 0.01$, versus no Cu^{++} *(top panel)* and versus no LDL *(bottom panel)*.

To determine whether sex steroid hormones modified LDL oxidation in the presence of cells, 17β-estradiol, progesterone, and testosterone were added at increasing concentrations of 0.001 to 1.0 μmol/L to placental cell culture medium containing fixed amounts of LDL protein and Cu^{++}. Assays for lipid peroxides, thiobarbituric acid–reacting substances, and ^{51}Cr release were performed after 24 hours of incubation. Data are expressed as percent change from non-hormone-containing controls. Hormone effects on LDL oxidation in macrophage cultures are shown in Fig. 6. 17β-Estradiol decreased formation of both lipid peroxide and thiobarbituric acid–reacting substances in a concentration-dependent manner (analysis of variance, $p < 0.01$) in the macrophage culture medium. At the highest concentration of 17β-estradiol, 1.0 μmol/L, formation of lipid peroxide and thiobarbituric acid–reacting substances decreased 62% and 59% below control, respectively. These 17β-estradiol–induced reductions were statistically significant at all concentrations except at the 0.001 μmol/L level in the lipid peroxide assay (Fig. 6). Conversely, progesterone and testosterone increased formation of lipid peroxide and thiobarbituric acid–reacting substances in a dose-related trend, most prominently in the lipid peroxide assay. At the highest concentrations of progesterone and testosterone of 1.0 μmol/L, lipid peroxide formation increased 20% and 43%, respectively, and thiobarbituric acid–reacting substances increased 28% and 19%, respectively ($p < 0.05$).

Similar effects of sex steroids on LDL oxidation were observed in trophoblast culture, as shown in Fig. 7. 17β-Estradiol decreased both lipid peroxide and thiobarbituric acid–reacting substances formation in a concentration-dependent fashion (analysis of variance, $p < 0.01$), with a maximum reduction of 65% and 36%, respectively, at the highest 17β-estradiol concentration of

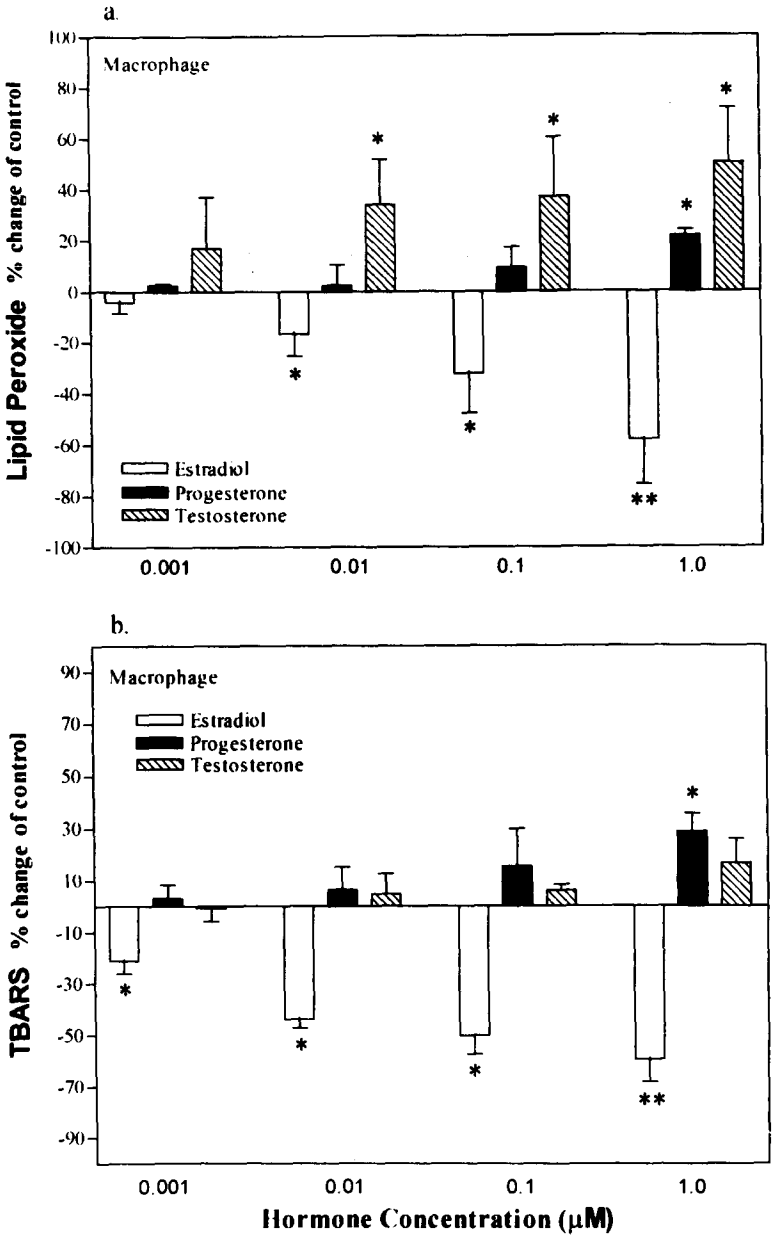

Fig. 6. Effects of sex steroids on LDL oxidation in cultures of placental macrophages. After 3 days of incubation medium was changed and 150 μg/ml LDL protein was incubated for 24 hours with placental cells in HAM's F-10 medium containing increasing concentrations of 17β-estradiol, progesterone, or testosterone at concentrations indicated. LDL oxidation in cell medium was monitored by lipid peroxide **(a)** and thiobarbituric acid–reacting substances *(TBARS)* **(b)** assays. Controls consisted of LDL incubated with cell in absence of hormone. Hormone effects on LDL oxidation are expressed as percent change from control. Values represent means ± SE of five experiments. Original control values were lipid peroxide, 54.5 ± 13.2 nmol/L (hydrogen peroxide) per milliliter of medium; thiobarbituric acid–reacting substances, 9.1 ± 3.6 nmol/L (malondialdehyde) per milliliter of medium.

1.0 μmol/L ($p < 0.01$). Conversely, progesterone and testosterone, respectively, increased lipid peroxide formation 19% and 42% and thiobarbituric acid–reacting substances 31% and 27% at the maximum hormone concentrations of 1.0 μmol/L ($p < 0.05$). Effects of sex steroids on LDL electrophoretic mobility are shown in Table I. 17β-Estradiol decreased and progesterone and testosterone increased LDL electrophoretic mobility in a concentration-dependent manner in the presence of both cell types.

To test the consistency of the effects of increasing hormone concentrations on formation of lipid oxidation

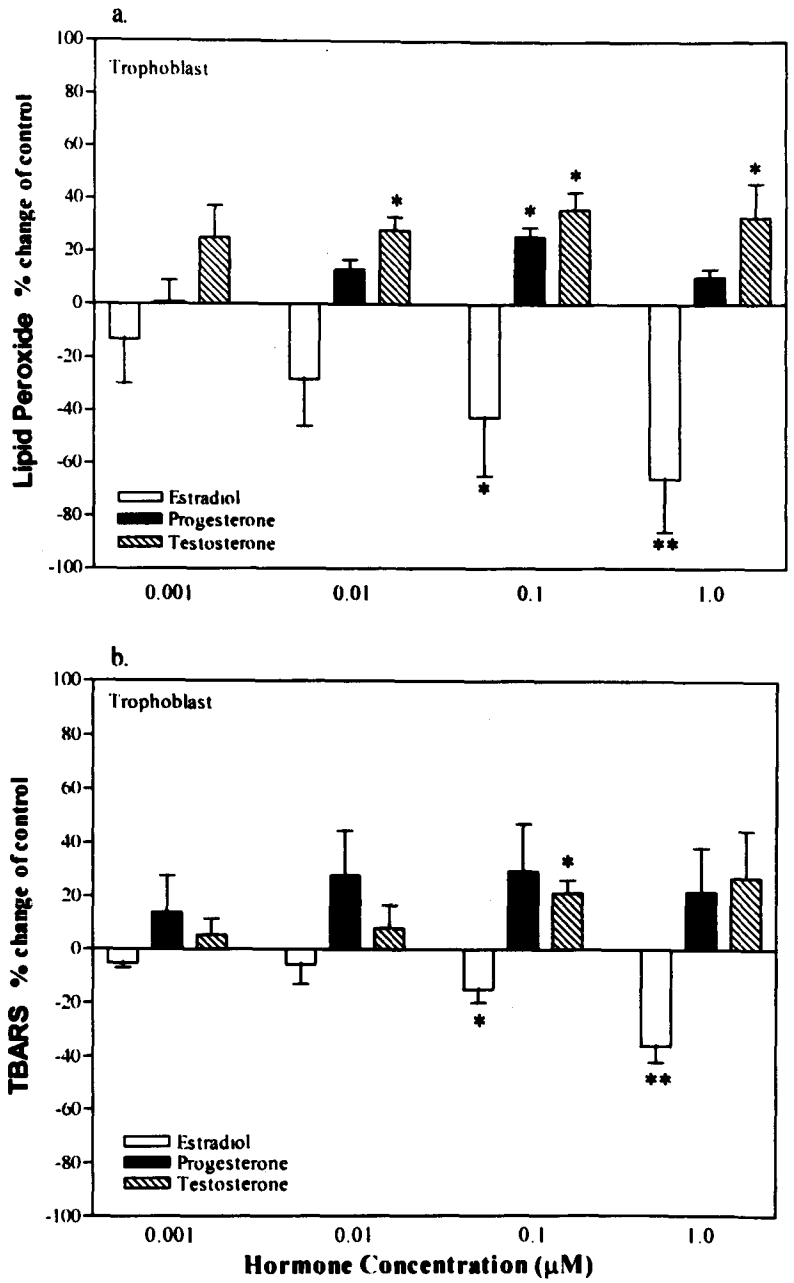

Fig. 7. Effects of sex steroids on LDL oxidation in cultures of placental trophoblast. Experimental details are same as in Fig. 6. LDL oxidation in trophoblast cell culture medium was monitored by lipid peroxide **(a)** and thiobarbituric acid–reacting substances *(TBARS)* **(b)** assays. Controls consisted of LDL incubated with cell in absence of hormone. Hormone effects on LDL oxidation are expressed as percent change from control. Values represent mean ± SE of five experiments. Original control values were lipid peroxide, 37.2 ± 12.8 nmol/L (hydrogen peroxide) per milliliter of medium; thiobarbituric acid–reacting substances, 8.8 ± 2.3 nmol/L (malondialdehyde) per milliliter of medium.

products, a two-way analysis of variance was performed to determine the relationship between lipid oxidation products and added hormone concentration in macrophage and trophoblast cultures. A negative correlation was observed between the 17β-estradiol concentration and medium thiobarbituric acid–reacting substance levels in macrophages ($p < 0.01$) and both medium lipid peroxide and thiobarbituric acid–reacting substance levels in trophoblast ($p < 0.01$). A statistically positive correlation was observed between formation of thiobarbituric acid–reacting substances and progesterone and testosterone concentrations, respectively, in macrophage

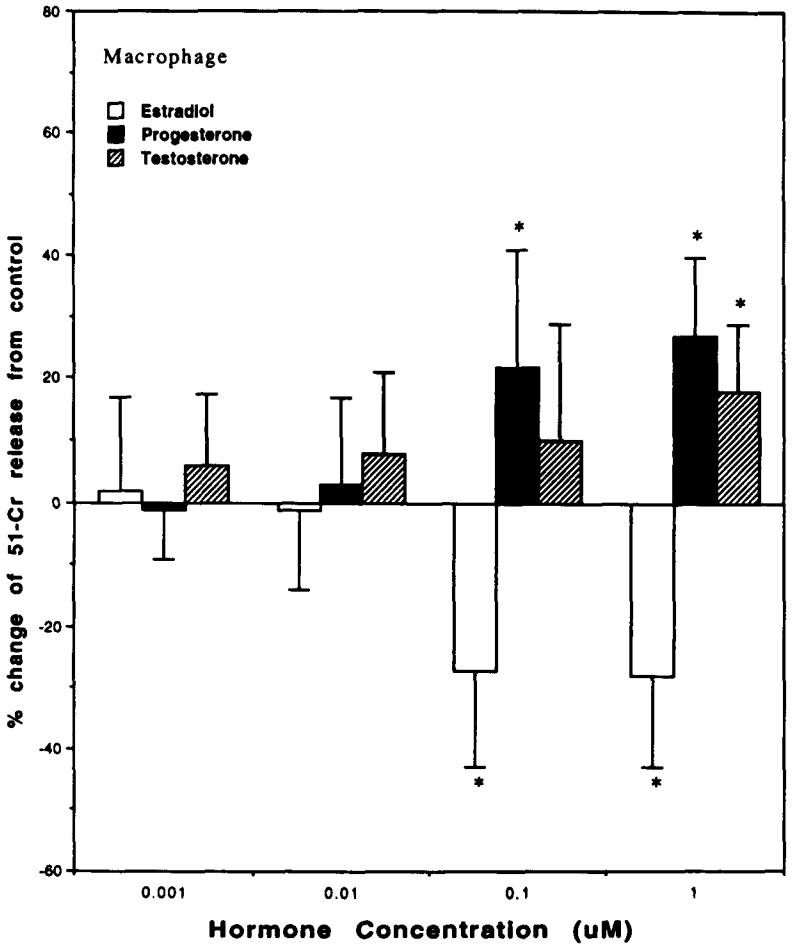

Fig. 8. Effects of sex steroids on placental macrophage cytotoxicity. Increasing concentrations of each hormone (0.001, 0.01, 0.1, and 1.0 μmol/L) were added to medium at beginning of incubation with LDL (0.15 mg/ml protein). Controls were incubated in absence of hormone under same conditions. Hormone effects on cytotoxicity are expressed as percent change of ^{51}Cr release from control. Values represent mean ± SE from five experiments. Original control values were 32% ± 3.4%.

cultures ($p < 0.05$). No statistically significant association was seen between progesterone and testosterone concentrations and thiobarbituric acid–reacting substances or lipid peroxide formation in trophoblast culture.

Because we had observed that placental cell cytotoxicity was related to oxidation rate in the absence of added hormone (Figs. 4 and 5), we hypothesized that cytotoxicity should be inhibited by 17β-estradiol because it inhibits LDL oxidation in the absence of cells.[12] Conversely, placental cell toxicity should be enhanced by progesterone and testosterone because of their prooxidant effects on LDL.[12] The effects of 17β-estradiol, progesterone, and testosterone on placental cell toxicity are shown in Figs. 8 and 9 for macrophages and trophoblasts, respectively. In macrophage cultures (Fig. 8) 17β-estradiol decreased the percent of ^{51}Cr release at concentrations at and above 0.1 μmol/L, with a maximum reduction of 28% at the highest estrogen concentration of 1.0 μmol/L. At lower concentrations the percent ^{51}Cr release did not differ significantly from non-hormone-containing control cultures. Conversely, progesterone and testosterone increased the percent ^{51}Cr release in a concentration-dependent manner to a maximum of 26% and 20%, respectively. Positive correlations were obtained between percent ^{51}Cr release and thiobarbituric acid–reacting substances ($r = 0.523$, $p < 0.05$) and lipid peroxide formation ($r = 0.399$, $p < 0.05$) in the presence of the three hormones (data not shown).

Similar hormonal effects were seen on trophoblast toxicity as shown in Fig. 9. 17β-Estradiol produced a concentration-dependent decrease in the percent ^{51}Cr release with a maximum reduction of 27% ($p < 0.05$), whereas testosterone significantly increased the percent ^{51}Cr release a maximum of 24% ($p < 0.05$) at the highest concentration of 1.0 μmol/L. Progesterone produced only a slight, nonsignificant increase in cytotoxicity of about 6%. A positive correlation ($r = 0.343$, $p < 0.05$) was

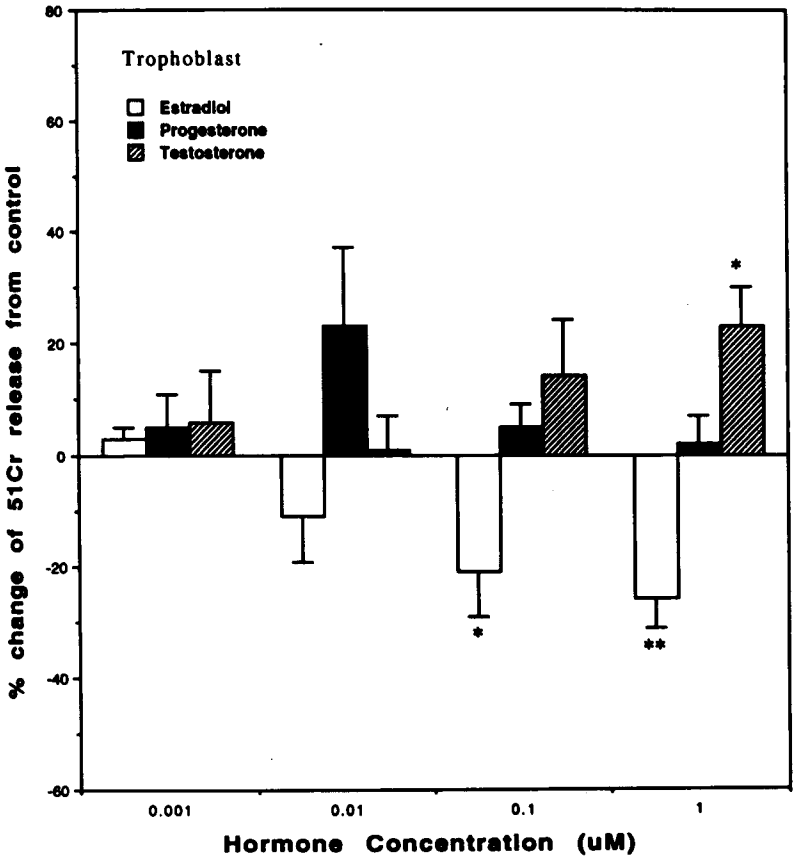

Fig. 9. Effect of sex steroids on placental trophoblast cytotoxicity. Increasing concentrations of each hormone (0.001, 0.01, 0.1, and 1.0 mmol/L) were added to medium at beginning of incubation with LDL (0.15 mg/ml protein). Controls were incubated in absence of hormone under same conditions. Hormone effects on cytotoxicity are expressed as percent change of ^{51}Cr release from control. Values represent mean ± SE from five experiments. Original value of percent ^{51}Cr release of controls was 28% ± 1.4%.

again observed between LDL oxidation, lipid peroxidation, and ^{51}Cr release in the presence of the three hormones (data not shown). These correlations indicate that the percent ^{51}Cr release is significantly influenced by the rate of LDL oxidation, which is in turn influenced by the concentration of hormone in the medium.

It is noteworthy that the effect of progesterone on the percent of ^{51}Cr release differs in cultures of macrophages and trophoblast. In the case of macrophages the percent ^{51}Cr release was significantly increased 23% and 26% at progesterone concentrations of 0.1 and 1.0 μmol/L, respectively ($p < 0.05$), whereas the percent ^{51}Cr release was not significantly increased at any progesterone concentration in trophoblast cultures. This lack of effect in trophoblast is not explained by an overall lack of susceptibility because 17β-estradiol equally inhibited and testosterone equally exaggerated cytotoxicity in cultured macrophages and trophoblasts, respectively (compare Figs. 8 and 9).

To determine whether the beneficial effect of 17β-estradiol and the adverse effects of progesterone and testosterone on placental cell toxicity were associated with a direct effect on the cultured cells in the absence of LDL, cells were preincubated with hormone for 24 hours. Cells were then washed free of hormone and incubated with LDL and 2 μmol/L Cu^{++} in the absence of hormone. The results showed no carryover effect of prior incubation of cells with 17β-estradiol, progesterone, or testosterone on the subsequent cytotoxic response of cultured cells incubated in the presence of LDL and copper (data not shown).

Comment

The current study demonstrates four major points. First, placental cells oxidatively modify LDL and are, in turn, damaged in the process. Placental cell-mediated LDL oxidation was amplified by increasing duration of exposure and by increasing copper ion concentration (Fig. 4).

Table I. LDL electrophoretic mobility in presence of sex steroid hormones under oxidizing conditions

Hormone concentrations	*mm*			
	0.001 μmol/L	*0.01 μmol/L*	*0.1 μmol/L*	*1.0 μmol/L*
Macrophages (without hormone 15.5 mm)				
Estradiol	15.0	14.5	12.5	10.0
Progesterone	16.0	16.5	17.0	17.8
Testosterone	16.5	17.0	17.0	17.9
Trophoblast (without hormone 14.5 mm)				
Estradiol	14.1	13.2	12.0	10.0
Progesterone	14.2	14.2	15.0	16.0
Testosterone	14.5	14.7	15.2	16.3

Means of two experiments run on separate days.

Lipid peroxide formation, thiobarbituric acid–reacting substance formation, and electrophoretic mobility were all greater in the presence than in the absence of cells. Lipid peroxide levels rose and then fell with time because they are an intermediate step in LDL oxidation, whereas thiobarbituric acid–reacting substances and LDL electrophoretic mobility increased continuously because they are terminal steps in the oxidation process. Finally, LDL oxidation, as judged by thiobarbituric acid–reacting substance formation was positively associated with the degree of induced placental cell cytotoxicity.

Second, this study shows that sex steroid hormones influence the rate of LDL oxidation in the presence of cells. Estrogen significantly inhibited LDL oxidation in a dose-dependent manner, and progesterone and testosterone promoted LDL oxidation. The rate of LDL oxidation was linearly related to medium estrogen, progesterone, and testosterone concentrations in macrophage cultures and estrogen concentrations in trophoblast cultures. These data support the view that sex steroids can influence LDL oxidation in biologic systems as well as in the test tube.[12]

Third, increasing concentrations of estrogen inhibit LDL-mediated cytotoxicity of both cultured macrophages and trophoblast. Statistically significant decreases in cytotoxicity were observed at estrogen concentrations of 0.1 and 1.0 μmol/L in both placental cell types. Effects at these concentrations have in vivo relevance because the concentration of 17β-estradiol in maternal plasma approximates 0.1 μmol/L at term and is likely higher in the intervillous circulation. In addition, estrone and estriol circulate at concentrations approximating 0.050 and 0.025 μmol/L and again would be expected to be higher in the intervillous circulation. Although the antioxidant potential of various estrogens differs,[8, 11] 17β-estradiol is the most potent of the human estrogens.[8, 11] Additionally, hormonal antioxidation effects may be mediated through the formation of 17β-estradiol sulfate and its antioxidant metabolites.[23] 17β-Estradiol sulfate is significantly negatively correlated with plasma lipid peroxide concentrations in late gestation.[23] An in vivo antioxidant effect of 17β-estradiol has been reported by Sack et al.,[13] who gave estrogen postmenopausally and demonstrated a prolongation of LDL oxidation. These data suggest that estrogen may diminish trophoblast- and macrophage-mediated LDL oxidation and cytotoxicity in vivo.

Regarding the mechanism of estrogenic inhibition of LDL oxidation and cytotoxicity, prior exposure of bovine aortic endothelial cells to 17β-estradiol reportedly increased their ability to resist the cytotoxic effect of subsequent incubation with oxidized LDL.[10] In another study prior incubation of macrophage cells with estrogens inhibited subsequent LDL oxidation 40% to 60%.[8] To determine whether preincubation with hormone would alter LDL oxidation and conjoint cytotoxicity in our system, we preincubated macrophages and trophoblast with hormone for 24 hours, then LDL and copper for 24 hours in the absence of hormone. No consistent effect of preincubation of 17β-estradiol, progesterone, or testosterone was found on subsequent LDL oxidation or placental cell cytotoxicity. The lack of response could be due to the different cell types used or to the fact that our cells were incubated at physiologic rather than supraphysiologic hormone concentrations.[8, 10]

A fourth result is that progesterone and testosterone promote placental cell cytotoxicity. The amount of cytotoxicity is linearly related to the rate of LDL oxidation using both lipid peroxide and thiobarbituric acid–reacting substance end points. Therefore sex steroid hormones that modify the oxidation of LDL in the absence of cells[12] also do so in the presence of cells, and these modulations directly influence the extent of observed cytotoxicity.

Some investigators have looked for prooxidant, procytotoxic effects of progesterone and testosterone without result.[8-10] These investigators generally used higher hor-

mone concentrations, other cell lines, and different incubation media. The reasons for the differences between these reports and ours are unclear but deserve further investigation.

The potential for progesterone and testosterone to affect biologic oxidation in pregnancy can be considered in light of the plasma levels of both hormones at term. Progesterone concentrations reach 400 nmol/L at term in normal pregnancy.[24] Testosterone concentrations exceed 7 nmol/L. These concentrations are within the 1 to 1000 nmol/L concentration range that we studied. Statistically significantly increased cytotoxicity in macrophages was seen at 100 nmol/L progesterone and 1000 nmol/L testosterone, whereas trophoblast cytotoxicity was significant only for the 1000 nmol/L concentration of testosterone. Thus progesterone could be a procytotoxic hormone for macrophages but not trophoblast; however, any extrapolation to the in vivo setting is highly speculative. Because the levels of testosterone in pregnancy approximate 7 nmol/L at term, this level is well below the level of cytotoxicity observed in trophoblast or macrophage cultures. However, other androgens including those derived from the fetal adrenal in high concentrations, may have prooxidant effects. For instance, we have shown that both dehydroepiandrosterone and dihydrotestosterone promote LDL oxidation in the absence of cells.[12] Again, in vivo extrapolation is uncertain on the basis of the tissue culture model.

The mechanisms of cytotoxicity induced by LDL oxidation are still uncertain, but a self-sustaining process of lipid peroxidation, free radical generation, and cell toxicity is implicated.[25] The toxic components of oxidized lipids include peroxy radicals, malondialdehyde, and related compounds and free radicals.[25] The hydroxyphenol group of ring A of estrogen appears to act as a trap for free radicals.[10] Further studies are needed to understand how the chemical structures of the other hormones studied confer prooxidant effects.

An implication of the current data is that the prooxidant effects of progesterone and testosterone on LDL both in the absence[12] and presence of cells could have deleterious effects not only in the placenta but in other cell systems including the arterial wall where LDL oxidation has been demonstrated.[22] Until now, the protection of women from arteriosclerosis compared with men has been explained by the effect of estrogen.[2, 3] The conjoint presence of progestin with estrogen in women, progestin alone in women, or androgen in men might have an additional proatherosclerotic effect. We have recently demonstrated a lowering effect of testosterone on high-density lipoprotein cholesterol levels in young men,[2] and testosterone, levonorgestrel, and medroxyprogesterone all have proatherogenic effects largely independent of lipoprotein concentrations.[5-7] Thus more research needs to be directed toward the possibility of direct atherosclerotic effects of progestins and androgens.

In summary, our data demonstrate that placental cells exaggerate LDL oxidation and that LDL oxidation induces cytotoxicity in placental macrophages and trophoblast. Physiologic concentrations of 17β-estradiol inhibit placental macrophage- and trophoblast-mediated LDL oxidation and subsequent cytotoxic effects in a concentration-dependent manner. Progesterone and testosterone exaggerate LDL oxidation mediated by placental cells and increase cytotoxicity in relation to progesterone and testosterone concentration. We speculate that sex hormones may modulate oxidative stress in the plasma of the pregnant mother and in the placenta in vivo by their opposing actions on lipoprotein oxidation.

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