Two Antiatherogenic Effects of Progesterone on Human Macrophages; Inhibition of Cholesteryl Ester Synthesis and Block of Its Enhancement by Glucocorticoids*

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ABSTRACT

The effects of progesterone and estradiol on cholesteryl ester (CE) formation by monocyte-derived human macrophages were examined. Formation was assessed from incorporation of 14C-cholesterol during a 20-h incubation with hormone and from that of 3H-oleate (3 h) after hormone removal. Progesterone inhibited cholesterol into CE and decreased CE cellular levels. Inhibition: 1) was reversed by progesterone removal; 2) was independent of the progesterone receptor (not blocked by the receptor antagonist RU40555); and 3) exhibited specific structural requirements: 11b-OH-progesterone was inhibitory, whereas its isomer 11b-OH-progesterone was not. In contrast to progesterone, estradiol was ineffective. We had reported that dexamethasone enhanced CE accumulation by human macrophages (1). In this study, we describe similar effects of the endogenous steroid, cortisol, and of the most widely prescribed glucocorticoid, prednisolone. Both steroids increased CE formation from two folds, in the presence of cholesterol-liposomes, to five folds, in the presence of modified low-density lipoprotein. Progesterone (0.1–1 μmol/L), added during glucocorticoid treatment, blocked this increase. The progesterone block: 1) was duplicated by the steroid receptor inhibitor RU40555; 2) was not reversed by hormone removal; and 3) reflected inhibition of glucocorticoid-induced increases in messenger RNA for acyl-CoA-cholesterol:acyl transferase. Thus, progesterone exerted two effects on macrophages: it acutely inhibited CE formation, and it prevented glucocorticoid-induced increases in acyl-CoA-cholesterol:acyl transferase gene expression and CE synthesis. (J Clin Endocrinol Metab 84: 265–271, 1999)

FEMALE SEX hormones exert a protective effect against the development of atherosclerosis. Women, before the onset of menopause, are at a much lower risk (ratio of 1:10) of cardiovascular disease than men of the same age. The risk gradually increases after menopause until cardiovascular disease becomes the main cause of death in women above the age of 60 (for a review, see Ref. 2). The protective effect of female sex hormones has generally been ascribed to estrogens (2–4), which were shown to lower blood levels of atherogenic modified low-density lipoprotein (LDL) to increase those of antiatherogenic high-density lipoprotein. Progestins opposed the beneficial action of estrogen to increase high-density lipoproteins (5, 6). However, despite this effect, postmenopausal women, using a combination of estrogens and progestins, had a significantly lower incidence of cardiovascular disease than those using estrogens alone. The adjusted risks were decreased to 0.39 for the first group vs. 0.60 for the second group (7). This suggested that progesterone had beneficial effects on parameters related to coronary disease, although, the nature and site of these effects were unknown.

The first aim of this study was to examine effects of progesterone and estradiol on cholesteryl ester (CE) synthesis by human macrophages. Macrophages internalize natural and modified LDL and recycle the cholesterol generated from lysosomal hydrolysis of LDL-lipid into cytoplasmic CE. Excessive deposition of CE by macrophages, present in the vascular wall, yields the rounded, lipid-filled foam cells that constitute a crucial early step in pathogenesis of vascular lesions (reviewed in Ref. 8).

The second aim of this study was to examine whether progesterone, known for its ability to exert selective anti-glucocorticoid effects (9), can antagonize glucocorticoid action to increase cholesterol esterification by human macrophages. We recently documented that the synthetic glucocorticoid, dexamethasone, enhanced CE deposition by macrophages severalfold by increasing expression and activity of acyl-coA-cholesterol: acyl transferase (ACAT) (1). This helped explain how the proatherogenic action of glucocorticoids (10–17) could be independent of changes in plasma lipids (18–20) and may not be prevented by controlling plasma cholesterol (20). Antagonism by progesterone of glucocorticoid effects on macrophage lipid synthesis may be an important site for a protective effect against the risk of coronary disease. In addition, it could have significant clinical potential, because glucocorticoids are widely used for treating conditions such as rheumatoid arthritis, inflammation, allergic reactions, tissue rejection after transplantation, and others.

Materials and Methods

Materials

Leukocyte-packs (less than 18 h old) were obtained from the Long Island Blood Service. Cholesterol, cholesteryl oleate, oleic acid, diolein, triolein, egg phosphatidylcholine, antibiotics for tissue culture, BSA
(fraction V, fatty acid-free), cortisol, prednisolone, progesterone, 11a-OH-progesterone, 11β-OH-progesterone, cholesterol oxidase, horseradish peroxidase, sodium cholate, and p-hydroxyphenylacetic acid were from Sigma Chemical Co. (St. Louis, MO). CE hydrolysis was from Boehringer Mannheim (Indianapolis, IN). Human male serum type AB was from Gemini (Calabasas, CA) and RPMI-1640 was from Gibco BRL (Gaithersburg, MD). [9,10(n)-3H]-oleic acid, [1,2,6,7(n)-3H]-cholesteryl linoleate, [1-14C]-oleic acid, and [4-14C]-cholesterol were purchased from New England Nuclear (Boston, MA). Silica G plates were from Alltech (Deerfield, IL) and solvents from Fisher (Pittsburgh, PA). RU40555 was a gift from Roussel-UCLAF (Romainville, France).

Cell isolation and culture

Mononuclear cells were isolated as previously described (1). Briefly, blood cells were diluted (2:1, vol/vol) with PBS, layered on an equal volume of Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), and centrifuged (20 min, 2,500 rpm, room temperature). Mononuclear cells were collected and washed three times at 4°C to remove platelets (50 mL, PBS, centrifugation at 1,000 rpm for 10 min). Any remaining red blood cells in the pellet were lysed by treatment with 10 mL 0.2% NaCl for 45 sec, followed by sequential addition of 10 mL 1.6% NaCl and of 30 mL cold PBS. The pelleted cells were suspended (5 × 10^6 cells/mL) in cold RPMI-1640 with 10% human AB serum and plated onto 60-mm diameter culture dishes. After 90 min at 37°C, the dishes were washed three times with warm RPMI-1640 to remove unattached cells. Adherent cells were greater than 95% monocytes, as determined by the α-naphthyl acetate esterase stain (Sigma Chemical Co.). Monocytes were cultured under 20 mmol/L Hepes and 10% human AB serum, with a medium change every fourth day.

Lipoproteins

Human LDL were purchased from Sigma Chemical Co. and were radiolabeled with [3H]cholesteryl-linoleate (15,600 cpm/mg protein) (21). LDL were acetylated (Ac-LDL) or oxidized (Ox-LDL) (22, 23) and used immediately after modification.

Uptake and incorporation of 14C-cholesterol/3H-oleate

Macrophages were preincubated (20 h) with cholesterol (250 μg/mL) in phospholipid dispersions (molar ratios greater than 1.8) (24) which, when indicated, contained 14C-cholesterol (2,000 cpm/nmol). In some experiments, cholesterol was supplied as a component of Ac-LDL or Ox-LDL (25 μg protein/mL). Steroids were added during the preincubation, unless indicated otherwise. At the end of preincubations, the cells were washed and analyzed, or assayed for 3H-oleate uptake and incorporation during a subsequent 3-h incubation. For this, the cells were washed once with warm RPMI-1640 with 0.5% albumin and twice with buffer without albumin; and isotopic solution (2 mL) consisting of RPMI-1640 with 150 μmol/L 3H-oleate (20,000 cpm/nmol), complexed to albumin at a molar ratio of 1:5:1 was added. Incubations (3 h, 37°C, under 95% air-5% CO2 for 10 days in RPMI-1640 medium supplemented with 20 mmol/L Hepes and 10% human AB serum, with a medium change every fourth day.

Extraction and separation of lipids

Washed and resuspended cells were extracted according to Bligh and Dyer (25). Lipids in the extract were separated by chromatography on silica G with hexane-diethyl ether-acetic acid (80:20:1) and identified with standards run on one side of the plate and visualized with iodine vapors. The silica band corresponding to each lipid fraction was added with standards run on one side of the plate and visualized with iodine vapors. The silica band corresponding to each lipid fraction was added to vials with 4 mL Safety solve, (RPI, Mount Prospect, IL), shaken for 20 min, and counted in an LS 8901 counter (Beckman Coulter, Inc., Palo Alto, CA). Free and total cholesterol were determined as previously described (26), and CE mass was calculated by subtraction of free cholesterol from total cholesterol.

Isolation and analysis of ACAT messenger RNA (mRNA)

Total RNA, prepared using RNA STAT-60 (Tel-Test “B” Inc., Friendswood, TX), was electrophoresed on 1% agarose/formaldehyde and transferred to a nylon-supported nitrocellulose membrane (Hybond-N+, Amersham, Arlington Heights, IL). Membranes were prehybridized and hybridized at 42°C and washed at 55°C. Autoradiographs were scanned with a laser densitometer (1). Signal for ACAT-mRNA [probed using 32P-labeled human ACAT cDNA C1 (1 kilobase), a gift from Dr. T. Y. Chang] was standardized to that of β-actin (27).

Statistical analysis

Differences between group mean values were compared by two-tailed Student’s t test or by ANOVA, when appropriate.

Results

Inhibition by progesterone of CE formation by human macrophages

Incubation of macrophages for 20 h with progesterone reduced by 50% 14C-cholesterol esterification into CE during that period (Fig. 1, open circle). The effect was detected at 0.1 μmol/L, reached statistical significance at 0.5 μmol/L, and reached a maximum at about 3 μmol/L. Cholesterol uptake was unaltered, as shown in Table 1. When the cells were washed, after the preincubation to remove the steroid, and then assayed for oleate incorporation into CE, no effect of progesterone was observed. Progesterone-treated and washed cells incorporated the same amount of oleate into CE as untreated controls (Fig. 1, filled circles), which indicated that the inhibitory effect required the continued presence of progesterone.

Fig. 1. Inhibition by progesterone of CE formation by human macrophages. Macrophages were preincubated for 20 h at 37°C with phospholipid-14C-cholesterol dispersions (250 μg cholesterol/mL; 2000 cpm/nmol cholesterol) and 0–10 μmol/L progesterone. At the end of the preincubation, the cells were washed and processed for measurement of cell-associated 14C-cholesterol and its incorporation into lipids or were assayed for 3H-oleate uptake and incorporation during an additional 3-h incubation. Oleate uptake was started by addition of 2 mL warm loading medium with 150 μmol/L 3H-oleate (20,000 cpm/nmol) complexed to 100 μmol/L albumin. The cells were then washed three times in ice cold PBS, scraped, and resuspended in 0.7 mL of the same buffer. Aliquots were taken for determination of cell-associated radioactivity (total uptake), 14C and for protein measurement. The remainder was extracted for lipids and subjected to thin-layer chromatography. Data, expressed as percent of control (values for control: 0.63 ± 0.03 nmol/mg protein·3 h and 0.7 ± 0.03 nmol/mg protein·20 h for oleate and cholesterol incorporation in CE, respectively), are means ± SEM from two monocyte macrophage cultures studied in triplicate.
progesterone. This was confirmed by experiments, where the effect of progesterone on incorporation of $^{3}$H-oleate was examined during a short (3-h) incubation. Incorporation rates during this time period are linear and reflect ACAT activity (1), because hydrolysis of labeled CE is negligible as a result of their long half-life (12 h, reviewed in Ref. 8). As shown in Fig. 2, inclusion of progesterone at 1 and 5 $\mu$mol/L, decreased $^{3}$H-oleate incorporation into CE by 25 and 50%, respectively. Oleate uptake (Table 1) and incorporation into phospholipids (PL) and triglycerides (TG) (data not shown) were unaffected, indicating that inhibition was exerted specifically on oleate esterification into CE.

In line with the reversibility of the inhibitory effect of progesterone on CE synthesis, it was not blocked by the steroid receptor inhibitor, RU40555 (Fig. 2), indicating that it did not involve the progesterone receptor, which generally mediates transcriptional effects of progesterone.

To examine whether inhibition by progesterone was related to hydrophobicity of the molecule or whether it had specific structural requirements, we tested the effect of a hydroxyl substitution on carbon 11. The analog 11$\alpha$-OH-progesterone, at a concentration of 1 $\mu$mol/L, inhibited oleate incorporation into CE (25%, $P < 0.05$), as well as 1 $\mu$mol/L progesterone. In contrast, the same concentration of its stereo-isomer 11$\beta$-OH-progesterone was not inhibitory (data not shown).

In contrast to progesterone, estradiol, tested over a range (0.02–1 $\mu$mol/L) of concentrations, did not exhibit any effect on cholesterol incorporation into CE during the 20-h preincubation or on oleate incorporation during the short 3-h incubation (data not shown). These findings are in line with those reporting no effect of estrogens on ACAT activity (28, 29).

**Antagonism by progesterone of glucocorticoid enhancement of CE formation**

We had reported that dexamethasone, a synthetic glucocorticoid, enhanced CE formation by human macrophages, after an 18- to 20-h incubation (1). A major aim of this study was to examine whether progesterone could block this effect and be potentially beneficial as adjunct therapy in conditions of high glucocorticoid. However, because the synthetic dexamethasone is not the most relevant glucocorticoid clinically, we examined the ability of progesterone to antagonize the actions of cortisol (the endogenous hormone) and of prednisolone (the most widely prescribed glucocorticoid).

Preincubation of macrophages for 20 h with either cortisol or prednisolone (Fig. 3) enhanced incorporation of $^{14}$C-cholesterol into CE. The effect was not reversed by hormone removal, because incorporation of $^{3}$H-oleate into CE was also increased. There was no increase in oleate incorporation into PL or TG. The effect of cortisol was detected at the lowest concentration (0.5 $\mu$mol/L) tested, and a maximum was reached at about 2 $\mu$mol/L. That of prednisolone reached statistical significance at 100 nmol/L and was maximal at about 500 nmol/L. Neither cortisol (data not shown) nor prednisolone altered cellular uptakes of oleate or cholesterol (Table 1).

To determine whether progesterone can antagonize the effects of cortisol and prednisolone on CE accumulation, it was added with these steroids to macrophages during the 20-h preincubation with $^{3}$H-oleate-loading medium plus the indicated steroid(s) for 3 h, and assayed for uptake and incorporation into CE, as described in the legend to Fig. 1. Results are means ± SEM from three independent experiments for CE mass. Data for uptakes are from one experiment (four determinations per point) that is typical of at least six other experiments.

* $P < 0.001$ (compared with control cells).
* $P < 0.05$ (compared with control cells).
* $P < 0.001$ (compared with prednisolone-treated cells).

![Fig. 2. Effect of the steroid receptor-inhibitor RU40555 on CE accumulation and on its inhibition by progesterone. Macrophages were preincubated with phospholipid-cholesterol dispersion for 20 h. The cells were then washed, incubated with $^{3}$H-oleate-loading medium plus the indicated steroid(s) for 3 h, and assayed for uptake and incorporation into CE, as described in the legend to Fig. 1. Results are means ± SEM from two macrophage cultures studied in triplicate.](Image 267)
incubated for an additional 3 h with \(^{3}\)H-oleate. Figure 4 shows that progesterone blocked prednisolone enhancement of CE formation in a dose-dependent manner (Fig. 4). In cells preincubated with prednisolone and progesterone, oleate incorporation (during 3-h incubation after hormone removal) was identical to that in untreated cells. \(^{14}\)C-cholesterol into CE (during 20-h preincubation with hormones) was reduced 50% below control levels, reflecting the acute, glucocorticoid-independent effect of progesterone.

In contrast to progesterone, estradiol was ineffective in antagonizing the effect of glucocorticoid to enhance CE formation (Fig. 5).

The effects observed on oleate and cholesterol incorporation into CE were confirmed by enzymatic measurements of cellular CE mass (Table 1). At the end of a 20-h preincubation, cellular CE was increased 2-fold by prednisolone, and the effect was blocked by RU-40555. In contrast, progesterone alone had no significant effect on oleate incorporation into CE, measured after hormone removal, whether cholesterol was delivered by Ox-LDL or Ac-LDL (data not shown).

The effects observed on oleate and cholesterol incorporation into CE were confirmed by enzymatic measurements of cellular CE mass (Table 1). At the end of a 20-h preincubation, cellular CE was increased 2-fold by prednisolone, and the effect was blocked by RU-40555. On the other hand, CE levels in progesterone-prednisolone-treated cells were decreased below those of controls.
Modulation of ACAT mRNA levels

The effect of prednisolone and cortisol on CE formation was mediated by an increase in ACAT expression, as previously shown for dexamethasone (1). ACAT mRNA, detected as a doublet at 3 and 4 kilobases, was increased after preincubation with cortisol (Fig. 7). A similar effect was observed with prednisolone (data not shown). In contrast, no increase was observed in cells treated with cortisol and progesterone or with cortisol and RU-40555.

Discussion

Our studies with human macrophages demonstrated two effects of progesterone that may provide protection against coronary disease: 1) reduction of macrophage CE accumulation; and 2) inhibition of the action of glucocorticoids to enhance the process.

Several characteristics of the acute inhibitory effect of progesterone on CE formation were established. First, the effect does not survive removal of the hormone, and so is not mediated by transcriptional modulation. Second, it does not involve the progesterone receptor, as evidenced by the negative results obtained with RU40555. Third, it exhibits distinct structural requirements, because 11α-OH-progesterone is inhibitory, whereas 11β-OH-progesterone is not. This last finding strongly suggests that progesterone action involves binding to a target protein and that the binding site has spacial constrictions. It remains to be determined whether the target is the ACAT enzyme, which would be in line with the reported progesterone inhibition of ACAT activity in homogenates of mouse macrophages (30). Progesterone has also been reported to inhibit lysosomal cholesterol transport in cultured fibroblasts (31). However, very high concentrations (33 μmol/L), greatly exceeding those we used, were required for this effect. In addition, lysosomal cholesterol transport was not a factor in experiments where liposomes were used to load cells with cholesterol.

The second effect of progesterone in macrophages reflected its antiglucocorticoid property. Cortisol and prednisolone increased CE synthesis between 2- to 5-fold, at con-
centrations (100–1,000 nmol/L) that can be observed in human serum (11, 32). These effects were receptor-mediated (blocked by RU-40555) and caused, at least in part, by an increase in ACAT gene expression, as evidenced by the increase in ACAT mRNA levels. Although increases in mRNA levels may not always lead to corresponding increases in translation, the enhanced rate of oleate incorporation into CE, which reflected ACAT activity after steroid removal, argued for an increase in ACAT protein levels. The increase in ACAT activity was observed whether cholesterol was delivered in liposome dispersions or in modified LDL (Figs. 3 and 6) and when cholesterol and serum were omitted from the overnight preincubation (80% increase with 2 μmol/L cortisol, P < 0.01). These data would argue that changes in ACAT expression, independent of serum cholesterol, can contribute to long-term regulation of CE deposition by macrophages. Increases in ACAT mRNA have been observed during monocyte-macrophage differentiation (33) (Cheng and Abumrad, unpublished observation) and were reported in the aorta of rabbits fed a high-fat high-cholesterol diet (34).

Stimulation of CE formation was larger in magnitude when lipoproteins were used instead of liposomes, suggesting better accessibility to ACAT of the cholesterol delivered by lipoproteins. However, this may also reflect other effects of glucocorticoids or of the glucocorticoid-LDL combination on macrophage scavenger receptors or on intracellular cholesterol transport.

Effects of progesterone were observed using concentrations (0.1–1 μmol/L), which overlap the range of plasma progesterone in premenopausal women (0.022–0.55 μmol/L) (35, 36). However, possible differences in progesterone sensitivity of cultured vs. tissue macrophages and lack of information on local tissue progesterone levels may limit the value of the above comparison.

The opposite effects of cortisol and progesterone on macrophage CE could contribute to the gender difference in incidence of coronary disease. A recent study of age- and weight-matched men (n = 90) and women (n = 87) showed that plasma cortisol was lower in women as a result of a smaller response to the circadian signal and a faster return to baseline (37). There is also evidence for sex differences in cortisol metabolism (38). For example, 11-hydroxy metabolites of cortisol are higher in men, suggesting that local cortisol bioavailability may be reduced in women, relative to men (38). In summary, our data indicate that the macrophage may be an important site for the opposite effects of cortisol and progesterone on incidence of coronary disease. A lack of correlation between the magnitude of changes in blood lipids and the incidence of coronary disease has been reported for progesterone, cortisol, and even estrogens (5, 6, 7, 19, 20, 29) and would suggest that other effects of the steroids, such as those we describe in this report, may be significant in vivo. Estrogen (but not progesterone) has been recently reported to reduce internalization and metabolism of lipoproteins by the macrophage scavenger receptor (29), which would suggest that estrogen and progesterone may have additive protective effects on macrophage CE. The increased incidence of the disease in women after menopause may reflect, in addition to a lack of estrogen, also a lack of progesterone, in the face of unaltered cortisol levels (36, 39). Interaction between stress and ovarian steroids in the incidence of atherosclerosis was suggested by findings that dominant female monkeys, which endured less social stress and exhibited high levels of estrogen and progesterone, had lower atherosclerosis (40).

The data may suggest beneficial effects of progesterone in subjects experiencing frequent or chronic glucocorticoid excess (16, 17, 20). Glucocorticoids are widely prescribed for their antiinflammatory action in patients with rheumatoid arthritis and asthma, and for their immunosuppressive properties in the treatment of allergic reactions and in the prevention of tissue rejection after transplantation (41). As a result, their proatherogenic action is a significant concern for a large number of subjects. Progesterone effectively antagonized the effect on CE formation of cortisol, prednisolone, and dexamethasone, despite their widely different antiinflammatory potencies. For example, dexamethasone is about 300-times more potent than cortisol and about 7-times more potent than prednisolone (42). However, it is hard to predict the relative potency of progesterone, in vivo, and its efficacy in opposing undesirable (antiinflammatory) vs. nondesirable (proatherogenic, insulin antagonistic) effects of glucocorticoids. Progesterone, in vivo, can be a precursor for cortisol.
In addition, it has antiinflammatory and immunosuppressive effects (43). For example, its administration intraarticularly to humans with rheumatoid arthritis has beneficial antiinflammatory effects (44, 45). During pregnancy, most studies report improvements of inflammatory conditions, such as rheumatoid arthritis, which may reflect the modest increase in levels of free cortisol, as well as the up-to-10-fold increase in progesterone (46, 47). However, some in vivo effects of progesterone may not be beneficial. For example, progesterone, like glucocorticoids, might promote insulin resistance (48, 49). In postmenopausal women, though our study and those of others (7, 40) suggest progesterone might help reduce the risk of coronary disease, there is evidence that it might increase the incidence of breast cancer (50).

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References