Stimulation of progesterone production in human granulosa-lutein cells by lipoproteins: evidence for cholesterol-independent actions of high-density lipoproteins

J Ragoobir¹, D R E Abayasekara², K R Bruckdorfer¹
and A E Michael^{1,2}

¹Department of Biochemistry and Molecular Biology, Royal Free and University College Medical School, University College London, Rowland Hill Street, London NW3 2PF, UK

²Reproduction & Development Group, Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU, UK
(Requests for offprints should be addressed to J Ragoobir who is now at the Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU, UK; Email: jragoobir@rvc.ac.uk)

Abstract

Low-density lipoproteins (LDL) have been consistently reported to stimulate ovarian steroidogenesis, apparently by the provision of cholesterol as a steroidogenic substrate. Recent studies suggest that high-density lipoproteins (HDL) can also deliver cholesterol to support progesterone synthesis in human granulosa-lutein cells. Therefore, this study investigated the contributions of (i) cholesterol delivery, (ii) cyclic AMP and (iii) protein kinase C (PKC) in the steroidogenic responses of human granulosa-lutein cells to HDL and LDL. Over a 24-h treatment incubation, HDL stimulated a larger increase in progesterone output than did LDL at equivalent cholesterol concentrations. Moreover, at equal protein concentrations (100 µg protein/ml), HDL doubled progesterone production by cells co-treated with a maximally effective concentration

of 22R-hydroxycholesterol, whereas LDL had no effect on the progesterone response to this membrane-permeable sterol. These observations indicate that the progesterone response to HDL is not solely due to the delivery of cholesterol as a steroidogenic substrate. Over 24 h, the stimulation of progesterone synthesis by HDL was additive with the response to a maximally effective concentration of dibutyl-AMP, but was unaffected by the down-regulation of PKC activity (by chronic pre-treatment with a tumour-promoting phorbol ester). We have concluded that HDL appears to stimulate progesterone production in human granulosa-lutein cells by a mechanism not solely reliant on cholesterol delivery.

Journal of Endocrinology (2002) **173**, 103–111

Introduction

Vascularized ovarian tissues, such as the theca interna of developing ovarian follicles and the corpus luteum (CL), are exposed to circulating plasma lipoproteins. Moreover, follicular fluid from pre-ovulatory follicles has been shown to contain high-density lipoproteins (HDL) (Simpson *et al.* 1980, Volpe *et al.* 1991, Jaspard *et al.* 1996, 1997) which may have a bearing on the function of the surrounding granulosa cells.

In granulosa cells and luteal cells obtained from a range of non-primate species, both low-density lipoproteins (LDL) and HDL have been shown to stimulate steroidogenesis in granulosa and luteal cells (Savion *et al.* 1981, 1982, Schuler *et al.* 1981, Hwang & Menon 1983, Veldhuis *et al.* 1984, Wiltbank *et al.* 1990, Bao *et al.* 1995, Brannian 1997). In human and in non-human primates, the effects of lipoproteins are less clearly defined. Studies in human and non-human primate CL have suggested that

LDL serve as the main source of lipoprotein-derived cholesterol for luteal progesterone biosynthesis (Carr *et al.* 1981, Tureck & Strauss 1982, Brannian *et al.* 1992, Brannian & Stouffer 1993). However, while a number of studies have consistently reported the ability of LDL to stimulate progesterone production by the human CL and in human granulosa-lutein cells *in vitro* (e.g. Carr *et al.* 1981, Tureck & Strauss 1982, Soto *et al.* 1984, Golos *et al.* 1985), the effects of HDL have varied dramatically between different studies. Initial studies found that HDL had no significant effect on progesterone synthesis in the human CL (Simpson *et al.* 1980, Carr *et al.* 1981, Tureck & Strauss 1982), while more recent studies have reported increased progesterone synthesis by luteinized cells treated with HDL (Enk *et al.* 1987, Parinaud *et al.* 1987, Volpe *et al.* 1991, Azhar *et al.* 1998).

Although the principal function of HDL is to mediate the reverse transport of cholesterol from peripheral tissues to the liver (Pieters *et al.* 1994, Barter & Rye 1996), HDL

is also capable of delivering cholesterol into steroidogenic cells, including human granulosa-lutein cells (Azhar *et al.* 1998). While LDL delivers cholesterol via a receptor-mediated endocytic pathway (Brown *et al.* 1979, Golos & Strauss 1985, Golos *et al.* 1985, 1986, Foster *et al.* 1993), the delivery of cholesterol from HDL particles is mediated through a selective uptake pathway via the scavenger receptor, class B, type I (SR-BI) (Fielding & Fielding 1995, Acton *et al.* 1996, Babitt *et al.* 1997, Reaven *et al.* 1998, Graf *et al.* 1999). Hence, the recent localization of SR-BI expression to ovarian tissues (Acton *et al.* 1996, Reaven *et al.* 1998), together with the identification of mRNA encoding the apolipoprotein components of HDL in the human ovary (Pascher & Sturm 1997), supports a possible role for HDL in the control of human ovarian steroidogenesis.

While the uptake of lipoprotein-derived cholesterol is important in providing steroidogenic cells with a sterol substrate from which to synthesize steroid hormones, the primary endocrine stimulus to ovarian and testicular steroidogenesis is provided by the gonadotrophins. Within steroidogenic gonadal cells, luteinizing hormone (LH), follicle-stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) each appear to act predominantly via the cyclic adenosine-3',5'-monophosphate (cAMP)-protein kinase A (PKA) transmembrane signalling pathway (reviewed by Cooke 1990, Leung & Steele 1992, Davis 1994, Cooke 1999). Additional signalling pathways implicated in the steroidogenic actions of the gonadotrophins include the phosphoinositide-phospholipase C (PI-PLC) pathway and activation of protein kinase C (PKC) pathway (reviewed by Cooke 1990, Leung & Steele 1992, Davis 1994, Cooke 1999).

Given that luteinized human ovarian cells are exposed to both classes of lipoproteins *in vivo*, this study compared the effects of HDL and LDL on progesterone production by human granulosa-lutein cells, and assessed the potential cellular mechanisms by which HDL may increase progesterone output. Specifically, the contributions of cholesterol delivery, cAMP and PKC to the steroidogenic actions of HDL and LDL were determined.

Materials and Methods

Materials

Progesterone antiserum was supplied by the Central Veterinary Laboratories (Surrey, UK) while [1,2,6,7-³H]-progesterone was purchased from Amersham International (Aylesbury, Buckinghamshire, UK). Dulbecco's modified Eagles medium containing Ham's F₁₂ medium (1:1 (v/v) DMEM:F₁₂), FCS, L-glutamine, penicillin, PBS and streptomycin were each obtained from GIBCO-BRL Life Technologies (Cheshire, UK) and plastic culture dishes from Costar (High Wycombe, Buckinghamshire, UK). hCG (CR127) was provided by NIDDK (National

Institutes of Health, Bethesda, MD, USA). Percoll, dibutyryl-cAMP (db-cAMP), 4 α -phorbol-12-myristate-13-acetate (PMA), 4 β -PMA, 22R-hydroxycholesterol (22ROHC) and assay kits for the determination of cholesterol content in lipoproteins were purchased from Sigma Chemical Co. (Poole, Dorset, UK). All other chemicals and organic solvents were supplied by Merck (Poole, Dorset, UK).

22ROHC was dissolved at a stock concentration of 30 mM in ethanol, whereas 4 α - and 4 β -PMA were each prepared at concentrations of 10 mM in DMSO. 22ROHC, 4 α - and 4 β -PMA were each diluted into culture medium to the stated concentrations immediately prior to use, ensuring that the final concentration of organic solvent remained = 0.1% (v/v).

Isolation and preparation of human granulosa cells

Follicular aspirates were obtained from women undergoing controlled ovarian hyperstimulation for assisted conception at the Lister Private Hospital (Chelsea, London) and Hallam Women's Clinic (Harley Street, London). (All patients gave informed consent for collection of cells in accordance with the Declaration of Helsinki, and all procedures were approved by the relevant local ethics committee.) Pituitary down-regulation was achieved by the administration of a gonadotrophin-releasing hormone (GnRH) analogue (buserelin acetate; Suprefact, Hoechst, Hounslow, Middlesex, UK) for either 3 or 10 days, commencing on the first day of the menstrual cycle (corresponding to the short and long down-regulation protocol respectively). Human menopausal gonadotrophin (hMG; Pergonal, Serono, Feltham, Middlesex, UK) was co-administered for up to 10 days. After assessment of follicle development by ultrasonography and plasma oestradiol assays, hCG (10 000–20 000 IU; Pregnyl, Organon, Cambridge, UK) was administered approximately 36 h prior to oocyte retrieval. During oocyte retrieval, the oocytes were aspirated transvaginally and flushed with Earl's balanced salt solution supplemented with antibiotics. Aspirates were transported in sterile flasks to the Royal Free Campus of Royal Free and University College Medical School within 3 h of oocyte retrieval.

Granulosa cells were isolated from follicular aspirates using 60% (v/v) Percoll as previously described (Webley *et al.* 1988). For each individual experiment, cells from multiple follicles (3–14), all from the same patient, were pooled. However, on no occasion were cells harvested from different patients combined. The number of granulosa-lutein cells isolated from each patient ranged from 1.2×10^6 cells to 9.4×10^6 cells, dependent upon the precise number of follicles aspirated.

Granulosa cells were cultured for 72 h at a density of 5×10^4 viable cells/ml in DMEM:F₁₂ medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, penicillin (87 000 IU/l) and streptomycin (87 mg/l). Cells

were plated in 96-well sterile culture plates and incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ in air. As stated below, all experiments were initiated on day 3 of culture under serum-free conditions unless stated otherwise.

Lipoprotein isolation

Blood from healthy fasted human volunteers was drawn into a syringe containing 63 mM EDTA and 42 µM diethylenediamino-pentaacetic acid (DTPA) at a ratio (by volume) of 1:20 anticoagulant: blood. LDL and HDL were isolated from human plasma by sequential ultracentrifugation as previously described (Havel *et al.* 1955). The isolated lipoproteins were dialysed at 4 °C against 12 mM Tris (pH 7.4) with three changes of dialysis buffer over 12 h. The protein concentration of each lipoprotein preparation was determined using the modified method of Lowry (Markwell *et al.* 1978), with stock solutions of BSA as standard. Oxidation of the lipoproteins was assessed by measuring thiobarbituric acid reactive substances (TBARS) using a spectrophotometric method as previously described (Janero 1990). The HDL and LDL preparations used in this study had values of <0.6 nmol TBARS/mg protein, indicating that no significant oxidation had taken place.

Experimental designs

To assess the concentration-dependent effects of lipoproteins on basal progesterone production, cells were treated for 24 h on day 3 of culture in serum-free DMEM:Ham's F₁₂ medium containing HDL or LDL either at equal protein concentrations (0–400 µg/ml) or at equivalent cholesterol concentrations (0–400 µg/ml).

Based on the results obtained in these studies, for all subsequent studies, both HDL and LDL were used at a fixed concentration of 100 µg protein/ml.

To establish whether each lipoprotein could enhance the steroidogenic action of 22ROHC, cells were incubated in serum-free medium for 24 h with a range of concentrations of 22ROHC (0–30 µM), each in the presence and absence of LDL or HDL.

To establish the time course of the progesterone response to each of the lipoproteins, cells were treated for 2, 4 and 24 h on day 3 of culture with HDL and LDL. Since trophic hormones are known to induce the expression of lipoprotein receptors (Cummings *et al.* 1982, Ohashi *et al.* 1982, Golos & Strauss 1985, Golos *et al.* 1985, 1986, Takagi & Strauss 1989, Rudling *et al.* 1990, Foster *et al.* 1993), prior exposure of luteinizing granulosa cells to hCG might be expected to accelerate or enhance the steroidogenic response to lipoproteins as compared with naive cells that have not been pre-exposed to hCG. To test this hypothesis, cells were pre-incubated for 24 h (starting on day 2 of culture) in serum-supplemented medium

containing hCG (100 ng/ml), and then challenged as above on day 3 of culture with HDL or LDL for 2, 4 and 24 h in serum-free medium.

To explore the signal transduction pathways involved in the steroidogenic action of the lipoproteins, cells were treated for 24 h on day 3 of culture in serum-free medium containing HDL and LDL, each in the presence and absence of a maximally effective concentration of the membrane-permeable cAMP analogue, db-cAMP (1 mM) (Abayasekara *et al.* 1993a). In a final series of experiments, designed to investigate the potential involvement of PKC in lipoprotein-stimulated progesterone production, cells were pre-treated for 20 h (commencing on day 2 of culture) in serum-supplemented medium with DMSO, 100 nM 4α-PMA or 100 nM 4β-PMA before being challenged for 24 h in serum-free medium on day 3 of culture with HDL and LDL. (Previous studies in our laboratories have confirmed that this treatment protocol with the active, tumour-promoting 4β-phorbol ester down-regulates PKC activity by more than 90% whereas the inactive 4α-phorbol stereoisomer has no effect on this serine/threonine kinase (Abayasekara *et al.* 1993b).)

Experiments were routinely terminated by the addition of 107 mM perchloric acid to each well prior to transfer of the culture plates to be stored at –20 °C pending assay. Immediately prior to assay of progesterone, samples were thawed and neutralized by the addition of 154 mM tripotassium orthophosphate.

Radioimmunoassay

Progesterone concentrations were determined in lipoprotein samples and in spent culture medium as previously described (Pallikaros *et al.* 1995). Intra- and inter-assay coefficients of variation were 6.7% and 10.8% respectively at 38% binding, with a working range of 0.5–15.9 nM. HDL and LDL had endogenous progesterone concentrations of <0.5 nM.

Statistics

The majority of experimental results are presented as mean ± S.E.M. values internally referenced with the relevant experimental control standardized to 100%. However, all statistical analyses were performed on non-referenced data either using one-way ANOVA with repeated measures (followed by Dunnett's multiple comparison, Bonferroni's multiple *t*-tests or the Tukey *post hoc* test) or using two-way ANOVA, as appropriate to the experimental design. All statistical analyses were performed using GraphPad Prism2 software (San Diego, CA, USA) and *P* values of less than 0.05 were accepted as significant in each case. Each experiment was repeated at least three times using cells from a different patient on each occasion.

Figure 1 The concentration-dependent effects of HDL and LDL on progesterone production at equal protein concentrations. Granulosa-lutein cells were incubated on day 3 of culture for 24 h in serum-free medium containing 0–400 µg protein/ml of HDL (●) or LDL (○). Data are presented as mean ($\pm$ S.E.M.) of eight independent experiments. ** $P < 0.01$ and *** $P < 0.001$ for each lipoprotein relative to progesterone production at 0 µg protein/ml.

Results

Concentration-dependent effects of HDL and LDL

In the first series of experiments, human granulosa-lutein cells were treated for 24 h on day 3 of culture with LDL and HDL at equal concentrations of apolipoprotein (25–400 µg protein/ml). Both lipoproteins stimulated comparable concentration-dependent increases in progesterone synthesis which were maximal at 100 µg protein/ml ($n=8$; $P < 0.01$; Fig. 1). In the second experimental series, cells were again stimulated with a range of concentrations of LDL and HDL, but in this second experimental series, plasma lipoproteins were presented to cells at concentrations where the cholesterol contents of each were equal. At concentrations equivalent to 50–400 µg cholesterol/ml, HDL stimulated progesterone production by up to 2.4 ± 0.3 -fold ($n=5$; $P < 0.001$) while LDL stimulated progesterone synthesis by up to 1.9 ± 0.2 -fold ($n=5$; $P < 0.01$; Fig. 2). The progesterone responses to HDL were consistently and significantly greater than those to LDL at equal cholesterol concentrations of 100, 200 and 400 µg/ml ($P < 0.05$; Fig. 2).

Effects of HDL and LDL on the steroidogenic response to 22ROHC

In the absence of additional lipoproteins, 22ROHC elicited a concentration-dependent increase in progesterone synthesis which was maximal (2.6 ± 0.2 -fold) at a

Figure 2 The concentration-dependent effect of HDL and LDL on progesterone synthesis at equal cholesterol concentrations. Granulosa-lutein cells were incubated on day 3 of culture for 24 h in serum-free medium containing 0–400 µg cholesterol/ml of HDL (●) or LDL (○). Data are presented as mean ($\pm$ S.E.M.) of five independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for each lipoprotein relative to progesterone production at 0 µg protein/ml.

concentration of 10 µM 22ROHC ($n=8$; $P < 0.01$; Fig. 3). Co-treatment with a maximally effective concentration of HDL significantly enhanced the progesterone responses to 22ROHC at concentrations of 3, 10 and 30 µM ($n=8$; $P < 0.01$; Fig. 3). In contrast, while treatment with a maximally effective concentration of LDL doubled progesterone production in the absence of 22ROHC, progesterone synthesis in the presence of ≥ 0.3 µM 22ROHC was unaffected by co-treatment with LDL.

Time-dependent effects of lipoproteins; influence of pre-treatment with hCG

While the effects of HDL and LDL on basal progesterone production (on day 3 of culture) were insignificant at 2 h and 4 h of treatment, both HDL and LDL stimulated comparable increases in progesterone synthesis at 24 h of 5.0 ± 1.0 -fold and 6.5 ± 2.3 -fold, respectively, relative to control progesterone production in the absence of lipoproteins at the matched time-point ($n=3$; $P < 0.05$; Fig. 4).

Pre-incubation of granulosa-lutein cells for 24 h on day 2 of culture with hCG (100 ng/ml) enhanced subsequent progesterone output on day 3 of culture (by 2.6 ± 0.7 -fold relative to naive cells from the same patients), but had no effect on the subsequent abilities of LDL and HDL to stimulate progesterone production. In cells pre-exposed to hCG, HDL and LDL exerted no significant effects on progesterone output at 2 h and 4 h, but stimulated

Figure 3 The effects of HDL and LDL on 22R-hydroxycholesterol (22ROHC) stimulated progesterone production. Granulosa-lutein cells were incubated on day 3 of culture for 24 h in serum-free medium containing 0–30 μ M 22ROHC plus lipoprotein vehicle (□), HDL (100 μ g protein/ml; ●) or LDL (100 μ g protein/ml; ○). Data are presented as mean ($\pm$ S.E.M.) of eight independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for each lipoprotein relative to progesterone production at 0 μ M 22ROHC in the presence of the appropriate lipoprotein. ^a $P < 0.05$, ^b $P < 0.01$ and ^c $P < 0.001$ relative to progesterone production at the appropriate concentration of 22ROHC without lipoproteins.

Figure 4 The time-dependent effects of HDL and LDL on progesterone production. Granulosa-lutein cells were incubated on day 3 of culture for 2, 4 and 24 h in serum-free medium containing lipoprotein vehicle (C: open bar), HDL (100 μ g protein/ml; H: hatched bar) or LDL (100 μ g protein/ml; L: solid bar). Data are presented as mean ($\pm$ S.E.M.) of three independent experiments. * $P < 0.05$ for lipoprotein-stimulated progesterone production versus progesterone production in the absence of lipoprotein at the same time point.

Figure 5 The time-dependent effects of HDL and LDL on progesterone production following 24 h pre-exposure of cells to hCG. Granulosa-lutein cells were incubated for 24 h on day 2 of culture with hCG (100 ng/ml) before being treated on day 3 of culture for 2, 4 and 24 h with lipoprotein vehicle (C: open bar), HDL (100 μ g protein/ml; H: hatched bar) or LDL (100 μ g protein/ml; L: solid bar). Data are presented as mean ($\pm$ S.E.M.) of three independent experiments. * $P < 0.05$ for each treatment versus basal progesterone production in the absence of lipoprotein at the same time point.

progesterone production at 24 h by 3.3 ± 0.7 -fold and 3.1 ± 0.7 -fold, respectively, relative to the untreated control cells at 24 h ($n=3$; $P < 0.05$; Fig. 5). Hence, at 24 h, the fold-responses to each of the lipoproteins in cells pre-treated with hCG did not differ significantly from those in cells that had not been pre-exposed to this gonadotrophin.

Interactive effects of HDL and cAMP

In the absence of lipoproteins, treatment of cells for 24 h with 1 mM db-cAMP stimulated progesterone production by $88 \pm 19\%$ ($n=6$; $P < 0.05$; Fig. 6). In the absence of db-cAMP, HDL and LDL increased progesterone output in the same experiments by 2.2 ± 0.1 -fold ($P < 0.01$) and 2.0 ± 0.2 -fold ($P < 0.05$) respectively (Fig. 6). In cells co-treated with HDL+db-cAMP and with LDL+db-cAMP, progesterone production was stimulated by 4.2 ± 0.4 -fold and 4.0 ± 0.5 -fold, respectively, relative to the untreated control cells ($P < 0.001$; Fig. 6). Hence, co-treatment with either HDL or LDL enhanced progesterone production by 2.3 ± 0.2 -fold relative to cells treated with a maximally stimulatory concentration of db-cAMP alone ($P < 0.001$; Fig. 6).

Role for PKC in the steroidogenic actions of HDL and LDL

Pre-treatment of cells for 20 h with 100 nM 4α -PMA had no effect on basal progesterone production relative to control cells incubated in the presence of 0.1% (w/v)

Figure 6 Effects of HDL and LDL on dibutyl cAMP-stimulated progesterone production. Granulosa-lutein cells were incubated on day 3 of culture for 24 h in serum-free medium in the presence and absence of 1 mM db-cAMP (as indicated) plus lipoprotein vehicle (Cont: open bars), HDL (100 µg protein/ml; hatched bars) or LDL (100 µg protein/ml; solid bars). Data are presented as mean ($\pm$ S.E.) of six independent experiments. * P <0.05, ** P <0.01 and *** P <0.001 for each treatment versus basal progesterone production in the absence of db-cAMP or lipoproteins. ^a P <0.001 relative to progesterone production in the presence of db-cAMP alone. ^b P <0.001 relative to progesterone production in the presence of the appropriate lipoprotein without db-cAMP.

Figure 7 The effects of PKC down-regulation on the progesterone responses to HDL and LDL. Granulosa-lutein cells were incubated on day 2 of culture for 20 h in serum-free medium containing (i) 0.1% (v/v) DMSO, (ii) 100 nM 4α-PMA or (iii) 100 nM 4β-PMA (as indicated), followed by a 24-h incubation with lipoprotein vehicle (C: open bars), HDL (100 µg protein/ml; H: hatched bars) or LDL (100 µg protein/ml; L: solid bars). Data are presented as mean ($\pm$ S.E.M.) of four independent experiments. * P <0.05 and ** P <0.01 for each treatment versus basal progesterone production in the appropriate control cells.

DMSO without phorbol esters (Fig. 7). Moreover, chronic pre-exposure to 4α-PMA had no significant effect on the subsequent progesterone responses to either HDL or LDL (Fig. 7). In cells pre-treated with 100 nM 4β-PMA, basal progesterone concentrations were enhanced by $44 \pm 9\%$ ($n=4$), but the fold-responses to HDL and LDL did not differ significantly from those measured in cells pre-treated with either 4α-PMA or DMSO vehicle (Fig. 7).

Discussion

The results obtained in this study confirm that HDL can stimulate progesterone synthesis in human granulosa-lutein cells and indicate that this steroidogenic action of HDL (unlike LDL) is probably not solely due to the delivery of cholesterol as a steroidogenic substrate.

This study provides evidence that at equal protein concentrations, freshly prepared, non-oxidized HDL and LDL are equipotent in stimulating progesterone production by human granulosa-lutein cells. While the stimulation of progesterone production by HDL is consistent with a number of previously published studies (e.g. Enk *et al.* 1987, Parinaud *et al.* 1987, Volpe *et al.* 1991), prior studies have all reported HDL to be less potent than LDL in increasing basal and gonadotrophin-stimulated

progesterone output from human luteinized ovarian cells. To the best of our knowledge, this is the first study to report that HDL can stimulate a comparable steroid response to that elicited by LDL at the same protein concentration.

Because of their differing compositions in terms both of apolipoproteins and cholesterol/cholesteryl esters (Rice-Evans & Bruckdorfer 1992), at equal protein concentrations, the cholesterol concentration of LDL would be expected to be approximately fourfold higher than the cholesterol concentration of HDL. Hence, the fact that HDL and LDL evoked similar progesterone responses at equal protein concentrations suggested that either HDL may be more efficient than LDL at delivering cholesterol substrate to human granulosa-lutein cells, or that HDL may stimulate progesterone production by a cholesterol-independent mechanism. Certainly, the delivery of fluorescent cholesterol from HDL into human granulosa-lutein cells has been previously documented (Azhar *et al.* 1998) and one might expect delivery of cholesterol via SR-BI to be more efficient than uptake via the receptor-mediated endocytosis of LDL. However, this does not exclude the possibility that HDL might also act via a cellular mechanism(s) independent of the delivery of cholesterol.

To determine whether HDL is a more efficient source of cholesterol than LDL, or whether HDL might act via a cholesterol-independent mechanism(s), cells were first challenged with HDL and LDL at concentrations normalized for their cholesterol content. In these experiments,

HDL was shown to be more potent than LDL in stimulating progesterone production at an equivalent concentration of cholesterol. If this observation reflects a difference in the efficiency of cholesterol provision as a steroidogenic substrate, then it should be possible to abolish the responses to both LDL and HDL by co-treating cells with high concentrations of a membrane permeable hydroxysterol, such as 20R-, 22R- or 25-hydroxycholesterol. The rate-determining enzymatic step in the synthesis of progesterone is the conversion of cholesterol to pregnenolone, catalysed by cytochrome P450 cholesterol side-chain cleavage (P450_{CSCC}) in the inner mitochondrial membrane (Burstein & Gut 1971, Voutilainen *et al.* 1986, Miller 1988). The rate-determining physical step in this reaction is the transport of cholesterol across the mitochondrial membranes (Burstein & Gut 1971, Hume & Boyd 1978, Stocco & Clark 1996, Culty *et al.* 1999). 22ROHC is a membrane permeable sterol which is one of the intermediates in the action of P450_{CSCC} and which is used routinely in studies of steroidogenic cells to bypass the rate-determining provision of cholesterol to the P450_{CSCC} enzyme complex (Miller 1988, Tuckey & Atkinson 1989). The fact that LDL was unable to influence progesterone production in cells treated with 22ROHC is consistent with the assumption that LDL stimulates steroidogenesis predominantly (if not solely) by the provision of cholesterol substrate. In contrast, the ability of HDL to further enhance progesterone biosynthesis in conditions where the availability of cholesterol substrate was no longer limiting suggests that HDL must be capable of acting by a mechanism or mechanisms other than by the net delivery of cholesterol to the steroidogenic pathway.

As noted above, trophic hormones, including hCG and adrenocorticotrophic hormone (ACTH), have previously been reported to induce the expression of lipoprotein receptors (Cummings *et al.* 1982, Ohashi *et al.* 1982, Golos & Strauss 1985, Golos *et al.* 1985, 1986, Takagi & Strauss 1989, Rudling *et al.* 1990, Foster *et al.* 1993). Moreover, it has been reported that pre-treatment of granulosa-lutein cells for 24 h with hCG (or db-cAMP) facilitates rapid uptake of HDL-cholesteryl ester within 1 h, followed by increased intracellular lipid staining at 3–6 h of subsequent HDL treatment (Reaven *et al.* 1995, 1998, Azhar *et al.* 1998). Based on these observations, we hypothesized that pre-treatment with hCG for 24 h might potentiate the subsequent steroidogenic response to HDL and, moreover, might facilitate an acute response to HDL. However, pre-treatment of granulosa-lutein cells with hCG neither enhanced the 24 h response to HDL and LDL, nor did it accelerate the ability of these plasma lipoproteins to elicit significant increases in progesterone output. The failure of hCG pre-treatment to potentiate the progesterone response to HDL under circumstances reported to enhance delivery of HDL-cholesterol into human granulosa-lutein cells further supports our hypothesis that the steroidogenic

response to HDL is not merely determined by the net delivery of cholesterol into these cells.

Having raised the possibility that HDL may not simply act as a source of cholesterol substrate, we designed experiments to look at the cellular signalling pathways via which HDL might be stimulating progesterone production. In the current study, the progesterone responses to HDL and db-cAMP were additive, consistent with prior observations by Azhar *et al.* (1998). This finding suggests that HDL cannot be stimulating steroidogenesis via the cAMP–PKA signalling pathway; if it were, then co-treatment with HDL should not have been able to stimulate progesterone production over and above that observed in the presence of a maximally effective concentration of db-cAMP.

As an alternative to the cAMP–PKA signal transduction pathway, the calcium–PKC pathway has been implicated in the stimulation of gonadal steroid biosynthesis (Brunswig *et al.* 1986, Lopez-Ruiz *et al.* 1992, Jamaluddin *et al.* 1994, Yuan & Connor 1997). Furthermore, PKC may also be involved in the efflux of cholesterol to HDL (Mendez *et al.* 1991). In the current study, down-regulation of PKC activity did not influence the fold-increase in progesterone production induced by either HDL or LDL. Hence, it seems highly unlikely that PKC either mediates or modulates the progesterone response to plasma lipoproteins in human granulosa-lutein cells.

In conclusion, based on several distinct lines of evidence reported in this study, HDL appears to be capable of stimulating ovarian steroidogenesis via a cellular mechanism(s) other than via the delivery of cholesterol as a steroidogenic substrate. This mechanism, which appears to act independently of the cAMP and PKC signalling pathways, is currently under investigation in our laboratories.

Acknowledgements

We wish to acknowledge the staff at the Lister Private Hospital and Hallam Medical Centre for the collection of ovarian cells. This work was supported, in part, by a BBSRC studentship to JR.

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Received in final form 30 November 2001

Accepted 5 December 2001