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Gonadotropin- and lipoprotein-supported progesterone production by primate luteal cell types in culture*

Sheryl L. Sanders & Richard L. Stouffer

Division of Reproductive Sciences, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, Oregon 97006, USA

This study examined the ability of gonadotropin and lipoproteins to support progesterone (P) production during long-term culture of luteal cell types obtained from rhesus macaques at midluteal phase of the menstrual cycle. Mixed (unsorted) luteal cells and small and large cells sorted by flow cytometry were cultured with human LDL, acetylated (ac)LDL or high density lipoprotein (HDL) (0–100 μg protein/ml) with or without hCG (100 ng/ml). In mixed cells, daily P levels declined during culture, although treatment with hCG alone increased P levels on all days of culture. Treatment with LDL, acLDL or HDL alone had no effect on P levels. However, hCG + LDL sustained P levels through day 4 at or above day 1 control values. Treatment with hCG + acLDL also increased P production above that of hCG alone, but hCG + HDL only modestly enhanced P production (180%). Although hCG stimulated P production by freshly-harvested large, but not small, cells during acute (3h) incubation, both cell types responded to hCG with up to an eightfold increase in P production on days 1–4 of culture. P levels were essentially nondetectable in both sorted cell groups by day 4. Small cells did not respond to any of the three lipoprotein treatments; large cells responded to LDL or acLDL on day 1, but this response was not apparent later in culture. Treating small or large cells with hCG + lipoprotein was no different from hCG alone. Thus, (1) LDL, and to some extent modified LDL, supports gonadotropin-stimulated steroidogenesis by mixed cell populations in the monkey corpus luteum; (2) the lack of LDL response by sorted cell types suggests that the culture conditions or absence of other cell types renders lipoprotein treatment ineffective; and (3) small luteal cells develop the cellular components necessary for gonadotropin-stimulated steroidogenesis within 24 h of culture.

Keywords: luteal cells; lipoprotein; progesterone

Introduction

Classical studies on luteal tissue slices (Carr *et al.*, 1982) and luteinized granulosa cells (Strauss *et al.*, 1981; Richardson *et al.*, 1992) support the concept that progesterone (P) synthesis by the human corpus luteum (CL) is mainly dependent upon cholesterol derived from circulating low density lipoprotein (LDL) (Illingworth *et al.*, 1982). LDL may also be an important source of cholesterol for P production by the non-human primate CL (Brannian *et al.*, 1992b). LDL-promoted P production by luteinizing granulosa cells from rhesus monkeys correlated positively with a marked increase in fluorescent-tagged LDL (DiI-LDL) uptake after the midcycle gonadotropin surge (Brannian *et al.*, 1992a). However, recent data from our laboratory suggest that other lipoproteins could also regulate macaque CL function. Notably, chemically-modified LDL [e.g., acetylated (ac) LDL] which does not bind to the LDL receptor, altered P production and the number of viable luteinized GCs during culture (Brannian & Stouffer, 1993). However, the effects of LDL *vs* its modified forms (i.e. acLDL) on luteal tissue/cells of the primate corpus luteum are unknown.

Within the corpus luteum reside at least two distinct populations of steroidogenic cells – small and large luteal cells – which have been examined in several species (Wilkinson *et al.*, 1976; Lemon & Loir, 1977; Ursely & Leymarie, 1979; Fitz *et al.*, 1982; Ohara *et al.*, 1987; Hild-Petito *et al.*, 1989). This laboratory recently isolated and characterized populations of small and large luteal cells from the rhesus macaque by flow cytometry based on differences in light scatter properties and DiI-LDL uptake (Hild-Petito *et al.*, 1989; Brannian *et al.*, 1991). Large luteal cells have a greater capacity to produce P than small cells (Hild-Petito *et al.*, 1989; Brannian & Stouffer, 1991). Additionally, aromatase activity primarily resides within the large cells (Hild-Petito *et al.*, 1989). At midluteal phase of the menstrual cycle, freshly-harvested small and large luteal cell subpopulations differed in gonadotropin sensitivity and LDL uptake during acute (3 h) incubations (Brannian *et al.*, 1991). Large luteal cells were responsive to gonadotropin treatment in terms of enhanced P production, whereas small luteal cells did not respond. A larger percentage of the large luteal cell population bound DiI-LDL, and the positive cells exhibited greater staining intensity than small cells. Greater DiI-LDL uptake by large luteal cells correlated positively with the 40-fold higher basal P production by large cells compared to small cells, but the effects of exogenous LDL and its modified forms (acLDL) on steroidogenesis by primate luteal cell types remain unknown.

The present study examined the ability of lipoproteins and gonadotropin (hCG) to support long-term progesterone production by luteal cell populations isolated from functional CL of rhesus monkeys at midluteal phase of the normal menstrual cycle. Mixed luteal cells and luteal cell subpopulations sorted by flow cytometry were treated *in vitro* with LDL, acLDL or HDL (high density lipoprotein) alone or in com-

Correspondence: R.L. Stouffer
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bination with hCG to ascertain their effects on steroidogenesis. Additionally, this study evaluated the response of small luteal cells following acute (3 h) or chronic (24 h) exposure to hCG.

Results

Cultures of mixed luteal cells

Figure 1 depicts the daily P levels in cultures of mixed luteal cells exposed to gonadotropin and/or LDL. P levels declined during culture under control conditions in the absence of hCG or lipoprotein (53 *vs* 0.2 ng/ml; day 1 *vs* day 6). Treatment with hCG (100 ng/ml) alone increased ($P<0.05$) P levels on all days of culture; however, a similar pattern of decline as observed in control cultures occurred through day 6. Although LDL (25 μg/ml) treatment alone had no effect on P secretion, exposure to hCG + LDL synergistically increased ($P<0.05$) P production above that of hCG alone on days 3–6. Treatment with hCG + LDL sustained P levels through at least days 3–4 at or above that of day 1 control values. At 25 μg/ml, acLDL or HDL alone also had no effect on P secretion (data not shown). The combination of hCG + acLDL did not further enhance P production on days 1–3. However, on days 4–6, hCG + acLDL increased ($P \leq 0.05$) P production above that of hCG alone. Treatment with hCG + HDL did not increase P production above that of hCG alone at any time during culture.

Figure 2 demonstrates the time- and dose-dependency of LDL, acLDL and HDL effects on hCG-stimulated luteal cell steroidogenesis in three additional experiments. On day 1, none of the lipoproteins had an effect on P production above that of hCG alone. However, by day 4, all three lipoproteins in combination with hCG enhanced P production above hCG treatment alone. LDL at concentrations of 10–100 μg/ml significantly increased ($P<0.05$) P synthesis. Likewise,

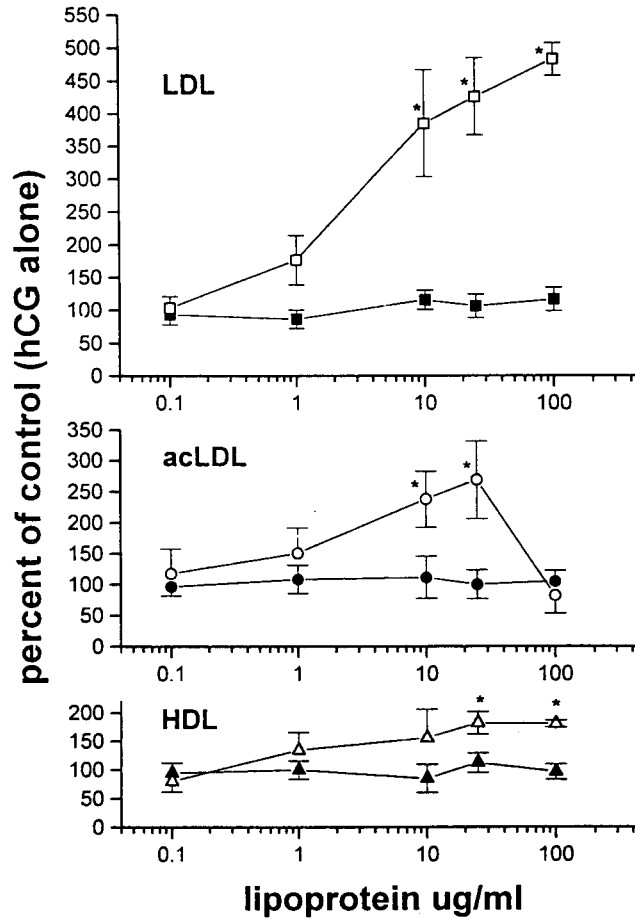

Figure 2 Relative change (percentage of control) in hCG-stimulated P production by mixed luteal cells during continuous exposure to various concentrations (0.1–100 μg/ml) of LDL, acLDL and HDL for 1 or 4 days of culture. Closed symbols indicate day 1 samples. Open symbols indicate day 4 samples. Asterisks denote doses of lipoproteins different ($P \leq 0.05$) from control values. Control (hCG alone) values: day 1, 858.2 ± 369.7; and day 4, 56.6 ± 24.3 ng/ml 2×10^4 cells/day. Values are the means ± SEM ($n = 3$ experiments)

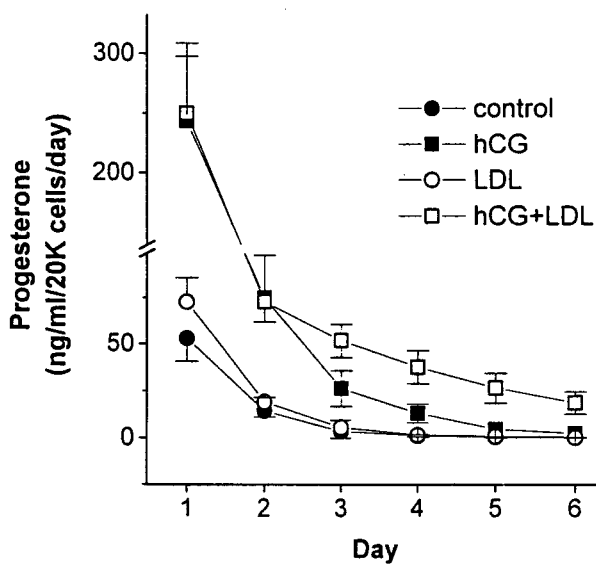

Figure 1 Progesterone production by mixed luteal cells during 6 days of culture. Cells were treated (days 1–6) with or without hCG (100 ng/ml) and with or without LDL (25 μg/ml). Values are the means ± SEM of three experiments

acLDL at 10 and 25 μg/ml stimulated ($P \leq 0.05$) P production; however, the highest concentration of acLDL (100 μg/ml) had no effect. HDL only enhanced modestly ($P \leq 0.05$) P production at the highest doses (25 and 100 μg/ml). Thus, in the presence of gonadotropin, the order of maximal response of luteal cells to lipoproteins is: LDL>>acLDL>HDL.

None of the treatments had a detrimental effect on cell survival. As shown in Figure 3, only hCG + LDL at the highest doses enhanced the number of cells present at termination of culture. Neither acLDL or HDL altered cell number.

Cultures of sorted luteal cells

Large luteal cells produced up to 36-fold more P than small cells during culture (180 *vs* 5 ng/ml: day 1 control) (Table 1). Both large (Figure 4) and small (Figure 5) cell types responded ($P<0.05$) to hCG with up to an eightfold increase in P above control levels on days 1–4. However, P levels were almost nondetectable in both large (0.5 ng/ml) and small (0.1 ng/ml) cells by day 4 of culture. The large luteal cell subpopulation

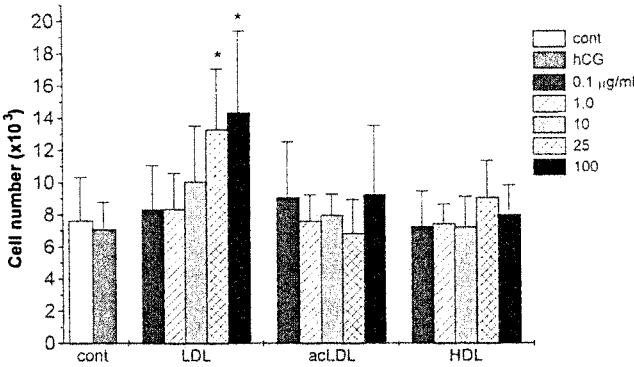

Figure 3 Number of mixed luteal cells present per well after 6 days of culture. Cells were treated, beginning on day 0, with hCG (100 ng/ml) and with various concentrations (0.1–100 μg/ml) of LDL, acLDL or HDL. Values are the means ± SEM ($n = 3$ experiments). Asterisks denote differences ($P < 0.05$) from control or hCG treatment

Table 1 Progesterone production by small and large luteal cells under basal conditions or hCG treatment during one day of culture

	Progesterone production (ng/ml/20K cells)[1]	
Luteal cell type	*Basal*	*hCG (100 ng/ml)*
Small	4.6 ± 0.6[a]	27.6 ± 5.7[b]
Large	180.1 ± 31.8[a]*	775.9 ± 147.0[b]*

[1]Mean ± SEM, $n = 6$ experiments (small cells); $n = 7$ experiments (large cells). [a,b]Superscripts denote differences ($P < 0.05$) between treatments within cell type. *Denotes differences ($P < 0.05$) within treatment between cell types

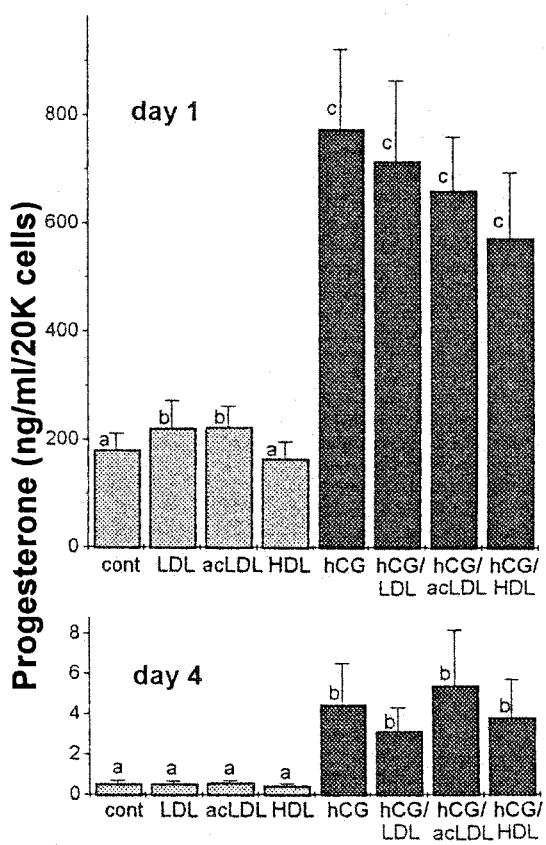

Figure 4 Progesterone production by large luteal cells on days 1 and 4 of culture. Cells were treated daily with or without hCG (100 ng/ml) and with or without LDL, acLDL or HDL (25 μg/ml). Values are the means ± SEM ($n = 7$ experiments). P production decreased over time in culture (note the differences in y-axes). Bars with different superscript letters denote differences between treatments within day ($P < 0.05$)

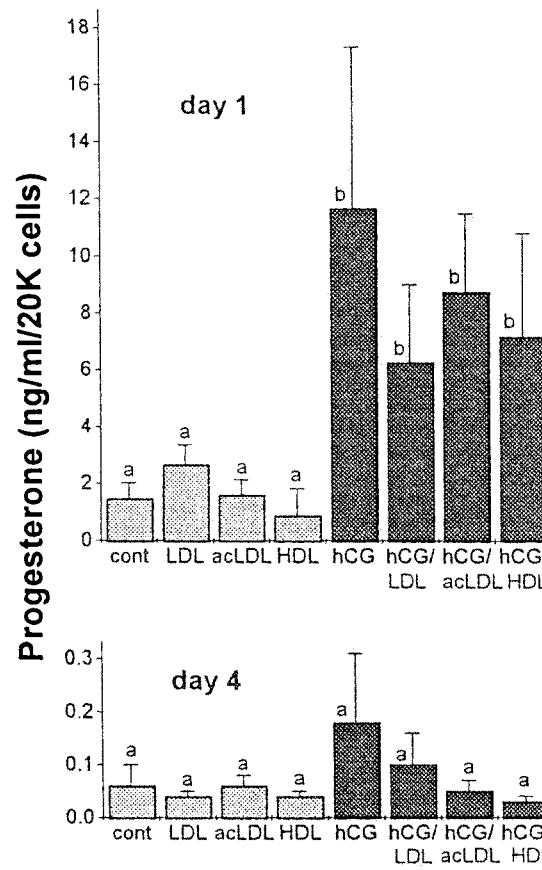

Figure 5 Progesterone production by small luteal cells on days 1 and 4 of culture. Cells were treated daily with or without hCG (100 ng/ml) and with or without LDL, acLDL or HDL (25 μg/ml). Values are the means ± SEM ($n = 6$ experiments). P production decreased over time in culture (note the differences in y-axes). Bars with different superscript letters denote differences between treatments on a given day ($P < 0.05$)

responded ($P < 0.05$) modestly to LDL and acLDL, but not HDL, on day 1 of culture only. None of the lipoprotein treatments in combination with hCG was different from hCG alone on day 4. In contrast, the small luteal cell subpopulation did not respond significantly to any lipoprotein treatment on day 1–4; although the LDL treatment group tended to increase P ($P \leqslant 0.06$). Also, P levels following treatment with hCG + lipoprotein were not different from those of hCG alone.

Acute vs *chronic hCG treatment*

Since our previous studies (Hild-Petito *et al.*, 1989; Brannian *et al.*, 1991) indicated that small luteal cells did not respond acutely to hCG, whereas this study demonstrated the ability of small cells to respond to hCG within 24 h, experiments were performed to compare directly hCG responsiveness of small cells following acute (3 h) incubation versus chronic ($\geqslant$24 h) culture. As previously reported, treatment with hCG for 3 h did not alter P production by small luteal cells (Table 2). However, after 24 h of hCG treatment, P increased ($P < 0.05$) over control values (89.4 ± 72.2 *vs* 33.9 ± 27.9 ng/ml). In contrast, freshly-harvested large luteal cells responded during both a 3 h hCG incuba-

Table 2 Progesterone production by small luteal cells with acute *vs* chronic hCG treatment

	Progesterone production (ng/ml/20K cells)[1]	
Time	*Basal*	*hCG (100 ng/ml)*
3 h	18.5 ± 16.0^{a}	25.3 ± 19.0^{a}
24 h	33.9 ± 27.9^{b}	$89.4 \pm 72.2^{b*}$

[1]Mean ± SEM, $n = 3$ experiments. [a,b]Superscripts denote differences ($P < 0.05$) within treatments between times. *Denotes differences ($P < 0.05$) between basal and hCG treatment within time

tion (previously shown) and after 24 h in culture (Figure 5) with enhanced P production compared to control.

Discussion

This is the first report on the long-term culture of monkey luteal cells in chemically-defined media for the purpose of examining the effects of gonadotropin and lipoproteins on primate luteal steroidogenesis. Treatment with hCG alone increased P production by mixed luteal cells on all days of culture, but did not prevent the general decline in P over time. Whereas lipoprotein treatment alone did not increase P production, the combination of hCG + lipoprotein was more effective than hCG alone in promoting steroidogenesis after the first 2 days of culture. In the presence of gonadotropin, the ability of various lipoproteins to stimulate P production by mixed cell cultures ranked as LDL>>acLDL>HDL. The combination of LDL + hCG was most effective and sustained P production through day 4 at levels comparable to those observed on day 1 in control cultures. The data are similar to that from earlier studies on slices of human luteal tissue (Carr *et al.*, 1982), where lipoprotein alone had little effect, but LDL markedly increased P release after 3 days of culture in the presence of hCG. The presence of high-affinity, low capacity binding sites for LDL in human luteal tissue (Carr *et al.*, 1982; Bramley *et al.*, 1987) and the uptake of fluorescent-tagged (DiI)-LDL by macaque luteal cells (Brannian *et al.*, 1991) is consistent with a classical LDL receptor-mediated pathway for binding and internalization in target cells (Brown & Goldstein, 1983). Collectively, the data support the concept that circulating LDL is the primary source of cholesterol for steroidogenesis in human and macaque luteinized granulosa cells (Strauss *et al.*, 1981; Richardson *et al.*, 1992; Brannian & Stouffer, 1993) and luteal cells (Carr *et al.*, 1982; Brannian *et al.*, 1991).

These experiments also provide evidence that modified (i.e., acetylated LDL) lipoproteins can influence P production by primate luteal cells. By day 4 of culture, exposure to acLDL + hCG increased production by mixed cells above that of hCG alone. We reported recently that the presence of 10–25 μg acLDL/ml initially enhanced P production by luteinized granulosa cells from rhesus monkeys (Brannian & Stouffer, 1993). Moreover, Chen & Menon (1993) observed that acLDL was as effective as LDL in promoting P production by rat luteal cells under basal and hCG-stimulated conditions. It is generally regarded that acLDL does not interact with the LDL receptor; rather, target cells [such as macrophages and endothelial cells (Goldstein *et al.*, 1979; Brown & Goldstein, 1983)] have 'scavenger' receptors that bind modified LDL moieties, e.g., acLDL, and mediate cholesterol metabolism (Freeman *et al.*, 1991). Such a scavenger receptor pathway was recently observed in rat luteal cells (Chen & Menon, 1993). High-affinity [^{125}I]acLDL binding sites detected in the plasma membrane were specific for scavenger receptor ligands (e.g., acLDL, but not LDL) and up-regulated by hCG exposure. Evidence from this laboratory that acLDL does not competitively inhibit DiI-LDL uptake by luteinized granulosa cells, but that DiI-acLDL readily binds to luteinized granulosa cells (Brannian & Stouffer, 1993) and macaque luteal cells (unpublished data), supports the hypothesis that an alternative mechanism, such as a scavenger receptor pathway, exists in the primate ovary. Whether the effect of acLDL on P production by mixed cells in the current study is due to direct effects on luteal cells or to indirect effects via nonsteroidogenic (e.g., endothelial) cells is unknown. Nevertheless, the data suggest that the cells within the periovulatory follicle and corpus luteum possess diverse mechanisms for the uptake of native and modified LDL moieties that can influence steroidogenesis.

In contrast to LDL and acLDL, the effect of HDL on P production by macaque luteal cells was modest and variable. For example, the combination of hCG + HDL somewhat increased P production by mixed cells by day 4 of culture during dose-response studies (180% of hCG alone, Figure 2), but not during time course experiments (Figure 1). Corpora lutea of some species utilize HDL as a cholesterol source for steroidogenesis (Wiltbank *et al.*, 1990), and the recent characterization of an HDL binding protein and its mRNA in rat and/or cow luteal tissue (Ferreri & Menon, 1992; Chen & Menon, 1994) is consistent with a receptor-mediated process. A similar HDL receptor protein has not been reported in primate luteal tissue. Since a small percentage of plasma HDL in humans contains ApoE (Fielding, 1992), and, hence, is a ligand for the LDL receptor, we cannot rule out that the modest HDL effect in the present study is via the latter mechanism.

Although the effects of gonadotropin and lipoproteins on mixed luteal cells were generally similar to those reported previously on luteinizing granulosa cells (Brannian & Stouffer, 1993) during culture, there were some notable differences. First, luteal cells did not respond to lipoproteins alone, whereas LDL and acLDL increased P production by luteinizing granulosa cells as early as day 1 of culture. Second, whereas the combination of hCG and LDL helped sustain P levels in luteal cell cultures, this treatment markedly increased P levels over time in cultures of luteinizing granulosa cells. Third, unlike for mixed luteal cells, there were biphasic response(s) of luteinizing granulosa cells to lipoproteins, such that (a) high (100 μg/ml) concentrations of LDL and acLDL failed to enhance P levels, and (b) the stimulatory effect of lower (10–25 μg) concentrations of acLDL observed early in culture changed to an inhibitory effect by days 4–6. The biphasic effects observed with luteinizing granulosa cells correlated with a decreased number of viable cells after culture in the presence of 100 μg/ml LDL or 10–100 μg/ml acLDL. A similar change did

not occur in cultures of mixed luteal cells; if anything, higher doses (25–100 μg/ml) of LDL tended to increase the number of viable cells in culture (Brannian & Stouffer, 1993; current data). The drop in progesterone production by luteal cells from day 1 to day 2 could be related to suboptimal plating efficiency (with unattached cells removed in the first media change); however, on subsequent days that was probably not a major factor for the continued decline in progesterone since the remaining cells were attached to extracellular matrix. The disparate responses of mixed luteal cells and luteinized granulosa cells may be indicative of differences in the heterogeneity and developmental stages of these cell preparations. Mixed cell preparations consist of luteal cells derived from theca as well as granulosa cells of the periovulatory follicle, plus nonsteroidogenic (e.g., endothelial) cells found in the corpus luteum (Hild-Petito *et al.*, 1989). Many characteristics of the corpus luteum vary during its lifespan in the ovarian cycle. It is likely that aspects of LDL uptake and metabolism (Bramley *et al.*, 1987; Brannian & Stouffer, 1993) vary between the developing corpus luteum, which may be portrayed in part by luteinized granulosa cells from the periovulatory follicle (Brannian & Stouffer, 1993), and the mature corpus luteum, as depicted by luteal cells obtained at mid-luteal phase and cultured for 4–6 days.

Although many investigators have examined the activities and regulation of small and large luteal cells during acute incubations, few have examined the effects of lipoproteins on luteal cell types (Buhr, 1987; Pate & Condon, 1989; Wiltbank *et al.*, 1990; Fitz *et al.*, 1993) or maintained these cells chronically in culture (Nelson *et al.*, 1992; Fitz *et al.*, 1993). In the absence of exogenous gonadotropin or lipoproteins, large luteal cells from the macaque corpus luteum produced up to 36-fold more P than did small luteal cells during culture. The greater steroidogenic activity of large luteal cells is consistent with data from short-term (3 h) incubations of macaque luteal cell types (Brannian & Stouffer, 1991), and similar to findings with large and small luteal cells from domestic animals (Fitz *et al.*, 1982; Harrison *et al.*, 1987; Alila *et al.*, 1988) and rats (Smith *et al.*, 1989). It remains to be determined if the increased activity of large cells from the primate corpus luteum correlates positively, as in large luteal cells from the rat (McLean *et al.*, 1992), with the enhanced expression of proteins responsible for intracellular transport (e.g., sterol carrier protein-2) and processing (e.g, cytochrome P450 side chain cleavage enzyme) of cholesterol.

Unlike in acute incubations where small luteal cells from the macaque corpus luteum did not respond to gonadotropin with enhanced P production (Hild-Petito, *et al.*, 1989; Brannian *et al.*, 1991; current study), chronic exposure to hCG during culture enabled small cells to increase P production. The data suggest that small cells develop one or more components in the cellular pathway(s) for gonadotropin-stimulated steroidogenesis within 24 h of culture. It is possible that this phenomenon is an artifact of the cell preparation; for example, the small cells may have been damaged during enzymatic dissociation of luteal tissue or sorting by flow cytometry, resulting in a loss of hCG responsiveness. A 24 h time period permits cells to recover from this defect. However, it is noteworthy that large luteal cells prepared by the same techniques exhibit gonadotropin-responsiveness during both acute and long-term incubations. Alternatively, Farin and colleagues (1990) hypothesized that one of the actions of high levels of gonadotropins in the ewe is to promote differentiation of small luteal cells into large luteal cells. If such a process occurs in primates, it is possible that the high, pregnancy-like (Atkinson *et al.*, 1975) concentration of hCG employed in the current study conferred limited characteristics of large cells to small cells. Evidence that the gonadotropin-responsiveness of large and small luteal cells may vary between *in vitro* conditions (current study), the stage of the luteal phase (Koos & Hansel, 1981; Brannian & Stouffer, 1991), and between laboratories using different techniques to isolate and sort cell types (Koos & Hansel, 1981; Fitz *et al.*, 1982; Hoyer *et al.*, 1986; Hild-Petito *et al.*, 1987, 1989; Ohara *et al.*, 1987; Alila *et al.*, 1988; Smith *et al.*, 1989), suggests that caution is warranted in extrapolating results, particularly between species.

In contrast to the mixed cell cultures, sorted small and large cells were not markedly influenced by lipoprotein treatment. We previously observed that increased uptake of DiI-LDL by macaque granulosa cells after an ovulatory stimulus *in vivo* correlated with an increase in LDL-supported steroidogenesis (Brannian & Stouffer, 1993). We, therefore, hypothesized that the greater uptake of DiI-LDL by large cells (Brannian *et al.*, 1991) would also correlate with LDL-supported P production during culture. However, aside from a very modest effect on day 1, lipoproteins did not influence steroidogenesis by sorted cell types. The lack of lipoprotein response by sorted luteal cells, compared to that by mixed cells, suggests that the culture conditions rendered the lipoprotein treatment ineffective. The precipitous drop in P production within the first day of culture, which was most pronounced for large cells, may have masked any response to exogenous lipoprotein that is not apparent in primate luteal tissue (Carr *et al.*, 1982) or dispersed cells (current study) until after 3–4 days of culture. Aside from injury resulting from procedural techniques noted earlier, disruption of cell-extracellular matrix (Juengel *et al.*, 1993; Frisch & Francis 1994) or cell-cell interactions within luteal tissue could influence P production by sorted cell types. There are reports that recombining sorted small and large luteal cells from domestic animals (Lemon & Mauléon, 1982; Harrison *et al.*, 1987), and large luteal cells with nonsteroidogenic cells from the rat (Nelson *et al.*, 1992), can increase P secretion *in vitro*.

In summary, the study employing dispersed (mixed) cells from the macaque corpus luteum cultured in chemically-defined conditions provides evidence that both native and modified (i.e., acetylated) LDL can influence P production by primate luteal tissue. This model should be useful in examining the diverse receptor-mediated actions of LDL-related moieties in controlling luteal structure and function. Additionally, novel data indicate that small luteal cells acquire the cellular components necessary for gonadotropin-stimulated steroidogenesis within 24 h of culture. However, the lack of LDL response by large luteal cells suggests that the culture conditions or absence of other cell types renders lipoprotein treatment ineffective. Further studies are necessary to characterize the interac-

tions between different populations of cells comprising the primate corpus luteum and their potential role in controlling gonadotropin-stimulated, lipoprotein-supported steroidogenesis.

Materials and methods

Animals

The care and housing of rhesus macaques (Macaca mulatta) at the Oregon Regional Primate Research Center (ORPRC) was described previously (VandeVoort *et al.*, 1988). Corpora lutea ($n = 14$) were surgically removed from anesthetized, adult female rhesus monkeys at midluteal phase (days 7–8 post-LH surge) of spontaneous menstrual cycles as described previously (VandeVoort *et al.*, 1988).

Luteal cell dispersion and flow cytometry

Luteal tissue was dissociated in Ham's F10 medium (Gibco, Grand Island, NY) + 1% BSA (Sigma) containing 0.16% collagenase (type IV, Worthington Biochemicals, Freehold, NJ) and 0.02% deoxyribonuclease I (Sigma, St Louis, MO) as described previously (Brannian & Stouffer, 1991). Dispersed cells were counted using a hemacytometer and cell viability was determined by trypan blue exclusion (Tennant, 1964). Isolated luteal cells were separated by size (small and large) based on forward and 90° light scatter properties with an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) as described previously (Hild-Petito *et al.*, 1989). The flow cytometer simultaneously isolated enriched populations of small luteal cells (96% of cells $\leqslant 15\,\mu m$ diameter) and large luteal cells ($\geqslant 20\,\mu g$ diameter; $\leqslant 5\%$ small cell contamination) from the cell suspension obtained from individual corpora lutea. In two of the seven experiments on sorted cells, an aliquot of mixed cells was reserved for a direct comparison to small and large luteal cells.

Cultures of mixed luteal cells

Mixed (unsorted) luteal cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; 1:1 vol/vol) medium supplemented with 1.2 g/L sodium bicarbonate, 10 mmol HEPES, gentamycin sulfate (10 μg/ml), insulin (2 μg/ml; Sigma), transferrin (5 μg/ml; Sigma), selenium (0.25 nmol; Sigma), and aprotinin (10 μg/ml; Sigma) (Brannian & Stouffer, 1993). Mixed luteal cells were plated (2×10^4 cells/0.2 ml) on 96-well culture plates coated with extracellular matrix from bovine endothelial cells (ECM) as previously described (Brannian *et al.*, 1992b). Mixed cell cultures were maintained for 6 days (day 0 = day of plating) with human LDL (0–100 μg protein/ml; Sigma), acLDL (0–100 μg protein/ml; Biomedical Technologies, Stoughton, MA), or HDL (0–100 μg protein/ml; Sigma) alone or in combination with hCG (CR-123; 100 ng/ml). Triplicate wells were utilized for each treatment in the mixed cell studies. Luteal cell cultures were maintained at 37°C under 95% air-5% CO_2, and media were changed daily starting on day 1.

Cultures of sorted luteal cells

Sorted (small and large) luteal cells were cultured in media as described above. Depending on cell yields, sorted luteal cells were plated at densities of 3000–5000 large cells or 9600–15 400 small cells/0.2 ml on 96-well ECM coated culture plates. Sorted cells were cultured for 4 days. Sorted cell subpopulations were treated with 25 μg protein/ml of hLDL, acLDL or hHDL alone or in combination with 100 ng/ml of hCG. Due to limited numbers of sorted cells, cell types were cultured as singlets, duplicates or triplicates for each treatment group. Luteal cell cultures were maintained at 37°C under 95% air-5% CO_2, and media were changed daily starting on day 1.

Acute vs chronic hCG treatment

Additionally, small luteal cells were incubated in media as described above for 3 h (acute) or 24 h (chronic) with or without hCG (100 ng/ml). Acute incubations were performed in test tubes in a 37°C shaking water bath (Brannian *et al.*, 1991). Cells incubated for 24 h were plated on 96-well ECM coated culture plates. Due to limited numbers of sorted cells, cell types were cultured as singlets, duplicates or triplicates for each treatment group. Luteal cell culture plates were maintained at 37°C under 95% air-5% CO_2 and media were removed 24 h after plating.

Measurement of progesterone

Media samples were stored at −20°C until RIA for P concentrations in the Hormone Assay Laboratory at ORPRC. Values were normalized to ng P/2×10^4 cells/day of culture. At the end of 6-day cultures, the total number of mixed cells remaining in each well was measured for DNA content by the method of Brasaemle & Attie (1988).

Statistics

P concentrations in media were log-transformed due to heterogeneity of variance. The transformed values were analysed by one- or two-way analysis of variance (ANOVA) with repeated measures. Means were compared using Newman-Keul's multiple range test. In selected instances, P data were analysed by paired *t*-test. For example, in the experiment with small luteal cells treated acutely or chronically with gonadotropin, P values were analysed by paired *t*-test (Table 2). For all tests, significance was assumed at $P \leqslant 0.05$.

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