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The Influence of Exogenous PMS and HCG on the Arachidonic Acid Content of the Immature Rat Ovary (38944)

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(Introduced by R. H. Gadsden)

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The present report describes the first definitive evidence of a regulatory effect by gonadotropins on the uptake and incorporation of arachidonic acid (C-20:4ω6) into ovarian lipids. The implications of this hormonal response are evident, since arachidonic acid is known to be the immediate biosynthetic precursor of prostaglandin E_2 (PGE_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1, 2). More recent evidence indicates that these same two prostaglandins may play a role in mammalian ovulation (3), as well as in the regulation of the corpus luteum by stimulating adenylate cyclase (4–6). Previous studies in this laboratory indicated that PMS and HCG produced marked changes in both morphology and lipid content of the immature rat ovary *in vivo* (7).

The present study was undertaken in order to learn whether levels of individual lipids or fatty acids are regulated by gonadotropins in the ovary. Accordingly, we have evaluated the effects of exogenous gonadotropins on ovarian arachidonic acid levels *in vivo* in immature female rats. At appropriate intervals throughout the 12-day experimental period, we have assessed the PMS and HCG induced changes in the arachidonic acid content of ovarian total lipid extracts, as well as of several neutral and phospholipid fractions.

Materials and Methods. Animals. Immature 29-day-old female Holtzman (Expt I) or Sprague–Dawley rats (Expts II, III) were used. The presence of a closed vagina indicated that the animals had not commenced estrous prematurely. Pseudopregnancy was induced by intraperitoneal administration (ip) of PMS (125 IU FSH activity)[1] followed by HCG (50 IU LH activity)[2] administered 60–65 hr (3 days) later (8). The animals were sacrificed by cranial fracture at 29, 32, 33, 34, 36, 38, and 42 days. The ovaries were quickly removed, dissected free of sheathe fat, and placed in dry ice to prevent autolysis. Ovaries from 10 animals per experimental or 70 animals per control group were pooled and stored at −10° prior to the extraction of lipids.

Isolation of lipids. Prior to extraction, the tissue wet weight was determined for ovaries in each group. The pooled organs for each group were then homogenized and the total lipid extracted in chloroform:methanol 2:1 (9). The lipid residue, determined gravimetrically following evaporation of solvent is reported as total lipid. All of the solvents employed in the lipid analyses were freshly redistilled.

Experiment I. In the initial experiments, we studied changes in the relative percentages of individual fatty acids in several lipid classes. Fractionation of the total lipid extract was accomplished by utilizing three different silicic acid column chromatographic systems. The extract was first separated into neutral and polar fractions by elution with pure chloroform and pure methanol, respectively (10). The neutral lipids were further separated by stepwise elution from a second silicic acid column with increasing amounts of benzene in hexane (11). Although five neutral lipid fractions were obtained, ovarian arachidonate values (rela-

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[1] PMS, Gonadogen, supplied through the courtesy of the Upjohn Company, Kalamazoo, Michigan.

[2] HCG, Antuitrin-S, supplied through the courtesy of the Parke-Davis Company, Detroit, Michigan.

tive %) for only sterol esters, and partial glycerides (mono, diglycerides) are reported here. Elution of phospholipids from a third silicic acid column with increasing amounts of methanol in chloroform produced four phospholipid fractions (12). The relative percentage of arachidonic acid for only fractions I (serine and ethanolamine phosphatides, cardiolipin) and II (primarily phosphatidyl choline, with inositol phosphatides) are presented here.

GLC of fatty acids. The methyl esters of the total fatty acids from each of the lipid fractions of interest were prepared by the hydrolysis and transmethylation of 1–2 mg of the lipid in 6% sulfuric acid in methanol (13). A Barber-Coleman gas chromatograph (Nuclear-Chicago Corp., Chicago) was used for all analyses of fatty acid methyl esters. The instrument was equipped with a flame-ionization detector, and a 4 mm × 12 ft glass column packed with 8% ethylene glycol adipate[3] coated on 80/100 mesh gas chrom P (Analabs). The column, injector port, and detector temperatures were 185°, 225°, and 250°, respectively. Nitrogen flow through the column was maintained at 30 ml/min.

Experiment II. A separate experiment measuring absolute amounts of arachidonic acid by GLC was performed to verify (in μg/pair of ovaries), the changes in arachidonic acid (relative %) observed in Expt I. A fatty acid internal standard (C21:0) of known concentration was added to each lipid prior to preparation of fatty acid methyl esters for GLC analysis, to ensure accurate quantitation.

Experiment III. ^{3}H-labeled arachidonic acid (^{3}H-5, 6, 8, 9, 11, 12, 14, 15-eicosatetraenoic acid, New England Nuclear, Boston) was administered (ip), 5 μCi/rat, 5 rats/group. After 20 min, the animals were sacrificed, and the *in vivo* uptake of radioactivity into ovarian total lipid was measured by liquid scintillation spectrometry of the unfractionated total lipid.

Results. Values for relative amounts of arachidonate in total fatty acids (relative %) from several lipid fractions throughout the 12-day experimental period (Expt I) are shown in Figs. 1 and 2. In the sterol ester fraction (Fig. 1A) PMS alone produced a transient increase initially, followed by a marked reduction in relative percentage of arachidonic acid content by the 34th day compared to controls. Addition of HCG to PMS stimulation was associated with a return to control levels at 38 days not found with PMS alone.

In the partial glycerides (mono and diglycerides) shown in Fig. 1B a marked reduction in relative amounts of arachidonic acid are observed by 33 days with PMS either alone or with HCG. The depletion of arachidonate accompanying added HCG appears to last longer than with PMS alone. Significant relative amounts (10–12%) of arachidonic acid are present even in control animals in the partial glyceride fraction of ovarian neutral lipids.

Marked changes in relative amounts of arachidonic acid associated with gonadotropin administration are clearly evident in both phospholipid fractions I and II (Fig. 2). These changes appear particularly important

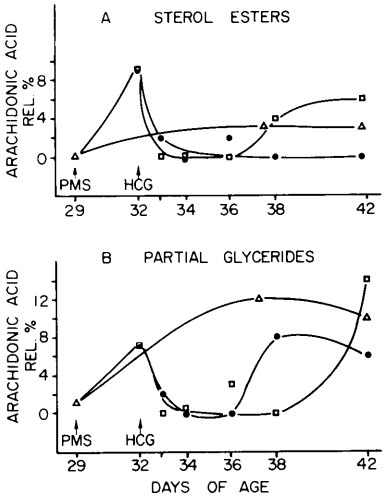

FIG. 1. Effects of exogenous PMS and/or HCG on the relative percentage of arachidonic acid contained in the total fatty acids from neutral lipid fractions, isolated from total lipids from immature rat ovaries by silicic acid column chromatography; hydrolyzed, transesterified, and measured by GLC. (△—Unstimulated; ●—PMS (ip) at 29 days of age; ⊡—PMS at 29 days of age, HCG (ip) at 32 days of age.) Figure 1A—Sterolester fraction. Figure 1B—Partial glyceride (mono- and diglyceride) fraction.

[3] Kindly given by Doctor Evan C. Horning, Director, Institute for Lipid Research, Baylor College of Medicine, Houston, Texas.

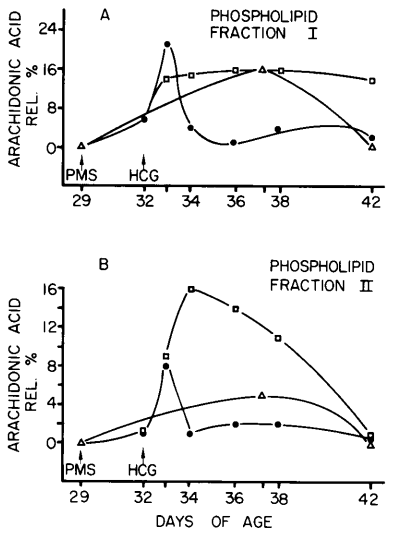

FIG. 2. Effects of exogenous PMS and/or HCG on the relative percentage of arachidonate in the total fatty acids from phospholipid fractions, isolated from total lipids from immature rat ovaries by silicic acid column chromatography; hydrolyzed, transesterified, and measure by GLC. (△—Unstimulated; ●—PMS (ip) at 29 days of age; ⊡—PMS at 29 days of age, HCG (ip) at 32 days of age.) Figure 1A—Phospholipid fraction I; noncholine containing, phosphatidyl ethanolamine, phosphatidyl serine, cardiolipin. Figure 1B—Phospholipid fraction II; primarily phosphatidyl choline, with inositol phosphatides.

since arachidonic acid is a major constituent of the fatty acids from ovarian phospholipids, even in unstimulated animals in fraction I (Fig. 2A). Here, it appears that adding HCG to PMS priming reduced the initial surge associated with PMS alone, and led ultimately to arachidonic acid levels comparable to controls, but which are consistently much higher than those with PMS alone between 33 and 38 days.

In phospholipid fraction II (Fig. 2B) it can be seen that HCG, added to PMS, produced little change in the relative percentage of arachidonic acid up to 33 days, but subsequently (33–38 days), levels in PMS primed animals fell, remaining below control values. HCG added to PMS consistently produced striking increases in the relative percentage of arachidonate compared to ultimate (38 days) controls (two- to threefold), or to PMS primed animals (five- to eightfold). Furthermore, these changes in the relative amounts of arachidonate accompanied increases up to tenfold in ovarian total lipid levels (μg/pair) (Fig. 3C).

Since our earlier experiments had indicated that marked changes in arachidonic acid percentage composition in ovarian lipids on days 32 and 33 (Expt I), we performed additional studies (Expt II) to measure arachidonic acid concentration (μg/pair of ovaries) in ovarian total lipid on these 2 days. These data (Fig. 3A) indicate that exogenous PMS, given with or without HCG, is associated with a threefold increase in the arachidonic acid concentration in total lipid. These experiments which measure absolute tissue concentrations confirm our previous findings that PMS and HCG, are associated with marked changes in the levels of arachidonic acid in ovarian total lipid.

In Expt III ^{3}H-labeled arachidonic acid utilized as radioactive precursor further confirmed our unlabeled studies regarding the effects of PMS and HCG on ovarian arachidonate levels. The latter data (Figs. 3B, 4B) demonstrate a marked increase in the uptake of ^{3}H from arachidonic acid into ovarian total lipids with PMS alone or with added HCG.

Discussion. As previously stated, there is new evidence that prostaglandins are involved in mammalian ovulation. In rabbits, the levels of both PGE and PGF, measured by radioimmunoassay rose only slightly during the first 5 hr after mating. While the LH surge occurs within 2 hr, prostaglandins did not rise until just prior to ovulation at 9 hr (3). Moreover, our own studies in the rabbit (6) and those of others in the mouse (4), and in the bovine (5) clearly demonstrated the stimulation of adenyl cyclase *in vitro* by PGE_1 and PGE_2 in the corpus luteum of these species.

Results of the present studies indicate that arachidonic acid, the biosynthetic precursor of PGE_2 and $PGF_{2\alpha}$ is first transiently elevated, then markedly depleted from ovarian neutral lipid following administration of PMS. The depletion appears to be more rapid and more complete when HCG is added to PMS stimulation. PMS alone is associated with pronounced effects on several phospholipid fractions manifested in a pattern of arachidonate levels clearly different from that observed in animals receiving

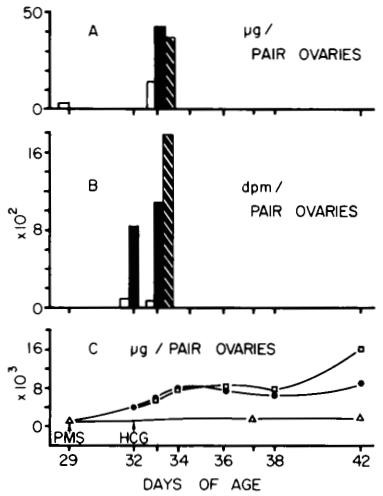

FIG. 3. Effects of exogenous PMS and/or HCG on the uptake of arachidonic acid into ovarian total lipid extracted from immature rat ovaries and analyzed without fractionation. Figure 3A—Arachidonic acid content (μg/pair of ovaries) measured by GLC with an internal standard for quantitation. Figure 3B—tritium uptake (dpm/pair of ovaries) into ovarian total lipid, 20 min after pulse labeling with 5 μCi/rat ^{3}H-labled C-20:4ω6 (arachidonic acid), 6 animals per group. (□—Unstimulated; ■—PMS (ip) at 29 days; ▨—PMS at 29 days, HCG (ip) at 32 days.) Figure 3C—Changes in ovarian total lipid content (μg/pair of ovaries) determined gravimetrically (△—unstimulated; ●—PMS at 29 days; ⊡—PMS at 29 days, HCG at 32 days.

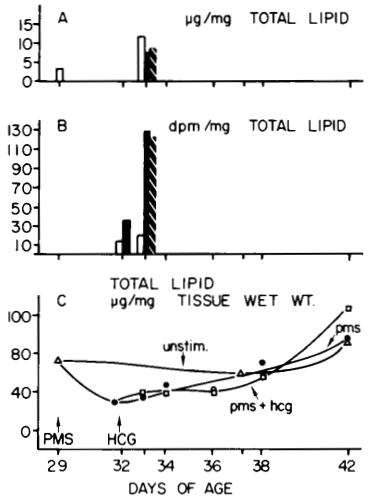

FIG. 4. Effects of exogenous PMS and/or HCG on the uptake of arachidonic acid into total lipid extracted from immature rat ovaries and analyzed without fractionation. Figure 4A—Arachidonic acid content (μg/mg ovarian total lipid) measured by GLC with an internal standard for quantitation. Figure 4B—tritium uptake (dpm/mg total lipid) into ovarian total lipid, 20 min after pulse labeling with 5 μCi/rat ^{3}H-labeled C-20:4ω6 (arachidonic acid), 6 rats/group. (⊡—Unstimulated; ■—PMS (ip) at 29 days ▨—PMS at 29 days, HCG (ip) at 32 days.) Figure 4C—Changes in ovarian total lipid content (μg/mg total lipid) determined gravimetrically (△—Unstimulated; ●—PMS at 29 days; ⊡—PMS at 29 days, HCG at 32 days.

both PMS and HCG. In the serine and ethanolamine containing phosphatides, and cardiolipins (phospholipid fraction I), the early accumulation of arachidonic acid associated with PMS administration, is apparently not sustained without added HCG. On the other hand, the choline and inositol containing phosphatides (phospholoid fraction II) appear to accumulate somewhat less arachidonic acid in PMS primed rats, whereas a more marked elevation is sustained by the addition of HCG to PMS stimulation.

It appears that PMS, with or without HCG, leads to consistently marked increases in arachidonic acid based on the amounts per pair of ovaries for unlabeled or radioactive fatty acid, or for total lipid as shown in Fig. 3A, 3B, and 3C, respectively. However, comparison of these whole organ values to those for tissue concentrations (Fig. 4) indicates that, while uptake of radioactivity from ^{3}H-labeled arachidonic acid increases either in the whole organ or in the total lipid, tissue concentrations of unlabeled arachidonate, or of total lipid actually decrease markedly, initially, and only after 38 days return to greater than control levels. Thus, it would appear that an immediate outpouring of lipid accompanied by a high turnover of arachidonic acid is associated with PMS administration, and is accentuated or modified temporarily by added HCG.

These findings provide the first quantitative evidence for the concept that gonadotropins PMS and HCG are associated with marked changes in ovarian levels of arachidonic acid, a major precursor of prostaglandins, and thus may act as mediators of prostaglandin biosynthesis by regulating the uptake, storage, or release of arachidonic acid from specific ovarian lipids. On the basis of our own work reported here, as well

as that of others (14), it would appear that there are several likely sites at which gonadotropins might exert their effect on ovarian prostaglandin biosynthesis. First, gonadotropins could act by regulating the release of precurosr arachidonate from phospholipids, triglycerides, or cholesterol esters *in situ*, via effects on the activity or level of the respective tissue esterases (phospholipase A, tissue lipase, or cholesterol esterase). The findings presented here, we feel, provide strong new evidence that gonadotropins may act at these sites in the immature rat ovary, but certainly do not rule out the possibility that they may act on prostaglandin synthetase and thus regulate the conversion of arachidonic acid to prostaglandins.

Summary. The influence of exogenous PMS and/or HCG, on the arachidonic acid (C 20:4ω6) content of the immature rat ovary was examined. Changes in ovarian arachidonate content associated with hormone administration were assessed in total lipid extracts, and in several neutral and phospholipid fractions. Both relative percentage and absolute amounts of arachidonic acid in several lipids were measured as well as uptake of radioactivity into total lipid resulting from the administration of ^{3}H-labeled arachidonic acid *in vivo*.

On the basis of these studies, we conclude (1) PMS, with or without HCG promotes increased uptake of exogenous arachidonic acid into ovarian total lipids; (2) Arachidonic acid is a major fatty acid constituent from noncholine containing phosphatides at the onset of normal estrous (ca. 38 days) even in the animals which received no PMS or HCG; (3) Changes in ovarian arachidonic acid levels following gonadotropin administration are more striking in the two phospholipid fractions than in the two neutral lipids examined; (4) PMS is associated with a rapid outpouring of ovarian lipid, accompanied by a high turnover of arachidonic acid which is enhanced or modified temporally by added HCG *in vivo*.

These results provide the first quantitative evidence that gonadotropins may regulate prostaglandin biosynthesis in the ovary by their effects on the uptake, storage, or release of arachidonic acid, a major PG precursor, from specific ovarian lipids. While the data strongly suggest that the regulation of one or more ovarian esterases (cholesterol esterase, lipase, phospholipase) is the mechanism by which gonadotropins regulate PG biosynthesis, a direct action on PG synthetase is not ruled out.

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