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Stimulation of Human Placental Lactogen Release by Arachidonic Acid

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SUMMARY

Arachidonic acid at concentrations between 20 and 300 μM stimulated human placental lactogen (hPL) release from placental explants in a dose-dependent manner ($R = 0.90$, $p < 0.001$). Explants incubated for 2 hr in medium containing 20 μM arachidonic acid released 97.0 ± 12.1% (mean ± SE, $p < 0.05$) more hPL than did control explants, whereas explants incubated in medium containing 300 μM arachidonic acid released 514.2 ± 18.7% ($p < 0.001$) more hPL. Phospholipase A_2 (0.11 unit/ml), which cleaves arachidonic acid from the 2-acyl position of phospholipids, stimulated hPL release by 2272.6 ± 219.6% ($p < 0.001$). Neither arachidonic acid nor phospholipase A_2 affected the release of human chorionic gonadotropin, lactic dehydrogenase, or alkaline phosphatase, and arachidonic acid had no effect on the release of trichloroacetic acid-precipitable ^{35}S-labeled proteins. The fatty acid precursors of arachidonic acid—linoleic, γ-linolenic, and dihomo-γ-linolenic acids—also stimulated hPL release, but 11,14,17-icosatrienoic, oleic, and palmitic acids had no statistically significant effects. The cyclo-oxygenase inhibitors indomethacin (14 μM) and flufenamic acid (18 μM) had no effects on either the basal release of hPL or the stimulatory affect of arachidonic acid. In addition, the cyclo-oxygenase products prostaglandins E_1, E_2, and $F_{2\alpha}$ had no effects on hPL release. The lipoxygenase inhibitors 5,8,11,14-icosatetraynoic acid (82 μM) and BW755C (20 μM) had no effects on basal hPL release but potentiated the stimulatory effect of arachidonic acid. These results suggest that arachidonic acid may stimulate hPL release via a non-cyclo-oxygenase, non-lipoxygenase pathway.

INTRODUCTION

Arachidonic acid, which is cleaved from the 2-acyl position of membrane phospholipids by phospholipase A_2 (1), can be metabolized to prostaglandins and thromboxanes through the cyclo-oxygenase pathway and to hydroperoxy fatty acids through the lipoxygenase pathway (2, 3). In addition, arachidonic acid can be oxidized directly to other lipids such as the chemotactic fatty acids (4). In some tissues, arachidonic acid and its metabolites have been demonstrated to stimulate secretion (5, 6). Since phospholipids, esterified and unesterified arachidonic acid, and prostaglandins E and F are present in relatively large amounts in the placenta (7, 8), we have undertaken experiments to determine whether arachidonic acid, phospholipase A_2, and prostaglandins E and F affect the release of hPL.[4] Prostaglandin $F_{2\alpha}$ has been reported to inhibit the release of hPL and placental proteins (9), but the effects of arachidonic acid, phospholipase A_2, and prostaglandin E have not been reported.

MATERIALS AND METHODS

Arachidonic (C20:4), linoleic (C18:2), γ-linolenic (C18:3), dihomo-γ-linolenic (C18:3), 11,14,17-icosatrienoic (C20:3), and 11,14-icosadienoic (C20:2) acids and phospholipase A_2 were obtained from P-L Biochemicals, Inc. (Milwaukee, Wisc.). Palmitic (C16:0) and oleic (C18:1) acids were obtained from Eastman Kodak Company (Rochester, N. Y.). Prostaglandins E_1, E_2, and $F_{2\alpha}$ were gifts from Dr. John Pike, Upjohn Company (Kalamazoo, Mich.). Antiserum to prostaglandin E was a gift from Dr. F. Kohen, Weizmann Institute (Rehovot, Israel). [^{3}H] prostaglandin E_2 (120–170 Ci/mmole) was obtained from Amersham/Searle Corporation (Arlington Heights, Ill.). Indomethacin was obtained from Sigma Chemical Company (St. Louis, Mo.). Flufenamic acid was a gift from Dr. U. Zor, Weizmann Institute. ETYA was obtained from Hoffmann-La Roche, Inc. (Rensselaer, N. Y.), and

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[4] The abbreviations used are: hPL, human placental lactogen; ETYA, 5,8,11,14-icosatetraynoic acid; hCG, human chorionic gonadotropin; LDH, lactic dehydrogenase; TCA, trichloroacetic acid.

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BW755C was a gift from Burroughs-Wellcome Company (Research Triangle Park, N. C.). ETYA was dissolved in dimethyl sulfoxide, and arachidonic and other fatty acids were dissolved in 100 mM sodium carbonate-absolute alcohol (2:1). Stock solutions of each of the compounds were prepared in absolute ethanol. The final concentration of the solvent did not exceed 0.5%. In each instance, an equal volume of solvent was added to the control media.

Placentas from normal uncomplicated pregnancies of 36–40 weeks' gestation were obtained within a few minutes of delivery, excised into explants, and incubated as previously described (10). Permission for these studies was approved by the Human Investigation Committee of Duke University, and informed patient consent was given beforehand in each instance. Twelve explants, each approximately 1 × 1 × 1 mm, with a combined wet weight of 30–40 mg, were placed into Falcon tissue culture flasks containing 5 ml of Krebs-Ringer buffer with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, penicillin-G (50 IU/ml), and streptomycin (50 μg/ml). The flasks were incubated at 37° in a water bath shaking at a rate of 50–60 cycles/min in an atmosphere of 95% O_2-5% CO_2. The medium in each flask was changed 18–24 hr later, and incubation was continued for another 2 hr in 3 ml of fresh medium of identical composition. Following this preincubation, the medium was changed to control medium or to medium containing one of the variables to be tested. In the experiments with indomethacin, flufenamic acid, ETYA, and BW755C, the explants were exposed to these agents for 1–2 hr after the preincubation period. These media were then changed to media of identical composition with or without arachidonic acid. Two hours later, each incubation was stopped by decanting the supernatant. In selected instances, 200 μl of medium were removed from control and experimental flasks at various times during the incubation period. The media were processed, stored at −20°, and assayed for hPL and hCG by specific homologous radioimmunoassays as previously described (11, 12). Prostaglandin E was measured in selected media by a radioimmunoassay in which prostaglandins E_1 and E_2 were detected with identity (13). LDH and placental alkaline phosphatase were determined by automated enzyme analysis using a Centrifichen analyzer (Union Carbide Corporation, New York, N. Y.).

The effects of arachidonic acid on the release of newly synthesized TCA-precipitable proteins were studied in an experiment in which explants were incubated for 4 hr in control medium containing 2 μCi of [^{35}S]methionine (Amersham/Searle Corporation, 600 μCi/mmole). The medium in each flask was then changed to methionine-supplemented medium (1 mM) with or without 75 μM arachidonic acid. The ^{35}S-labeled proteins in the media were precipitated with 10% TCA, and the [^{35}S]hPL was precipitated with specific antiserum to hPL as previously described (10).

In each experiment the variables as well as the controls were tested in triplicate incubation flasks, and each variable was tested in at least three separate experiments using different tissue. To account for slight variability in cell number among experimental flasks, the amount of hPL released into the medium in any flask was expressed as a percentage of the amount released in the same flask during the preincubation period. In selected instances, the percentage of hormone released in the experimental flasks was expressed as a percentage of the hormone released in control flasks. The statistical differences between sample means were tested by analysis of variance.

RESULTS

As shown in Fig. 1, arachidonic acid at concentrations of 20–300 μM stimulated the release of hPL, and the magnitude of the increased release was directly proportional to the arachidonic acid concentration ($R = 0.90$, $p < 0.005$). After 2 hr of exposure, 20 μM arachidonic acid stimulated hPL release by 97.0 ± 12.1% (mean ± SE, $p < 0.005$), and 300 μM arachidonic acid stimulated by 514.2 ± 18.7% ($p < 0.001$). As shown in Fig. 2, the stimulation of hPL release occurred within 20 min of exposure to arachidonic acid and persisted for the remainder of the 2-hr incubation. When explants which had been exposed to arachidonic acid were subsequently incubated in control medium, hPL release returned to basal levels (Fig. 3). Re-exposure of the explants to arachidonic acid resulted in a further stimulation of hPL release.

Despite its stimulatory effect on hPL release, arachidonic acid had no effects on the release of hCG and TCA-precipitable ^{35}S-labeled proteins or the release of the cytoplasmic enzymes LDH and alkaline phosphatase (Table 1). In contrast, the detergents deoxycholic acid and Triton X-100 caused significant release of hPL, hCG, and LDH, and Triton X-100 caused release of alkaline phosphatase (Table 1).

In addition to arachidonic acid, the unsaturated fatty acid precursors of arachidonic acid—linoleic, γ-linolenic, and dihomo-γ-linolenic acids—also stimulated hPL

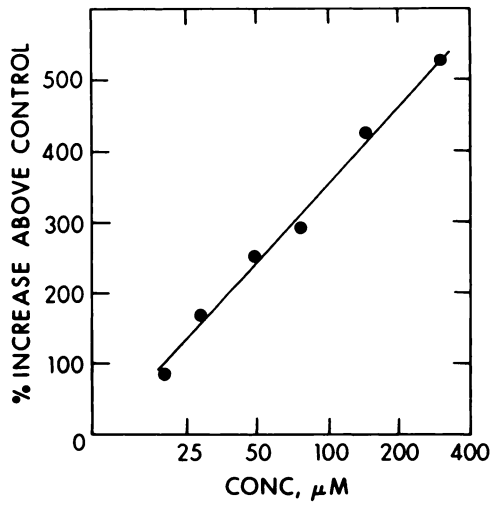

FIG. 1. *The effect of different concentrations of arachidonic acid on the release of hPL*

Triplicate cultures of placental explants were exposed for 2 hr to arachidonic acid at concentrations between 20 and 300 μM. The amounts of hPL released during the 2 hr is expressed as the percentage increase above control values. In each instance, the standard error was less than 8% of the mean. For each arachidonic acid concentration, the amount of hPL released was statistically greater ($p < 0.01$) than that secreted by control explants. The regression coefficient (R) = 0.90, $p < 0.005$.

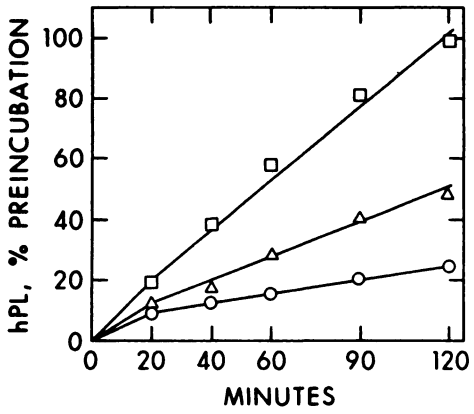

FIG. 2. *Time course of the stimulatory effect of arachidonic acid on hPL release*

Triplicate cultures of placental explants were incubated for 2 hr in either control medium (○—○), medium with 37.5 μM arachidonic acid (△—△), or 75 μM arachidonic acid (□—□). Each *point* represents the mean hPL release expressed as a percentage of the hPL released by the same explants during a 4-hr preincubation in control medium. In each instance, the standard error was less than 8% of the mean. Both concentrations of arachidonic acid significantly stimulated hPL release at each time point ($p < 0.05$–< 0.001).

release. However, the unsaturated fatty acids 11,14-icosadienoic, 11,14,17-icosatrienoic, and oleic acids and the saturated fatty acid palmitic acid had no significant stimulatory effects on hPL release.

Phospholipase A_2 caused a marked stimulation of hPL release. As shown in Fig. 4, placental explants exposed for 2 hr to phospholipase A_2 at concentrations of 0.11, 1.10, and 11.00 units/ml released 2272.6 ± 219.6, 4286.1 ± 277.8, and 7261.7 ± 160.4% more hPL, respectively, than did control explants. Phospholipase A_2 (11.10 units/ml), however, had no effects on the release of hCG or the release of LDH and alkaline phosphatase (Table 1).

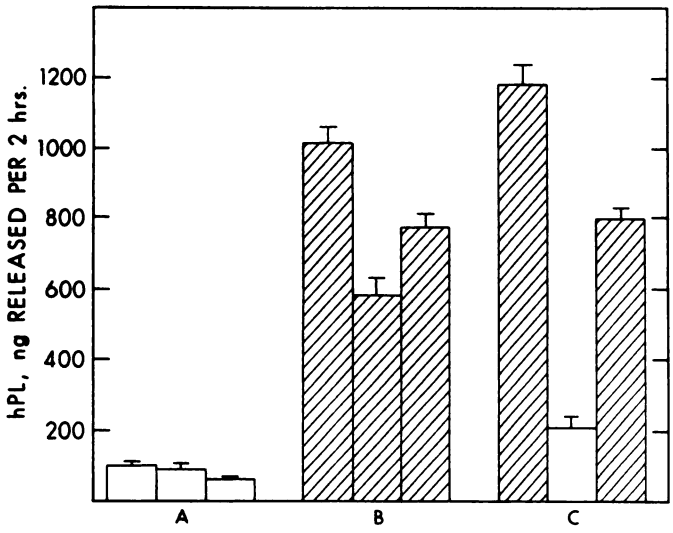

FIG. 3. *Reversibility of the stimulatory effect of arachidonic acid*

Placental explants were incubated for 6 hr with medium changes at 2 and 4 hr. *Group A* was exposed to control medium during each 2-hr period. *Group B* wasexposed to 75 μM arachidonic acid during each 2-hr period. *Group C* was exposed to both control medium and medium containing 75 μM arachidonic acid. The *open bars* represent intervals during which explants were exposed to control medium, and the *striped bars* represent intervals during which explants were exposed to arachidonic acid. The amounts of hPL released during each 2-hr period are expressed as nanograms of hPL ± standard error. In each instance the amount of hPL released in the presence of arachidonic acid was statistically greater ($p < 0.01$) than that released by control explants at a comparable time.

TABLE 1

Effects of arachidonic acid, deoxycholic acid, and Triton X-100 on the release of hPL, hCG, TCA-precipitable ^{35}S-labeled proteins, LDH, and alkaline phosphatase

In A, triplicate cultures of placental explants were incubated for 2 hr in medium containing arachidonic acid, deoxycholic acid, or Triton X-100 (300 μM each) or phospholipase A_2 (11.1 units/ml). In B, triplicate cultures of explants were preincubated for 4 hr in control medium containing 2 μCi of [^{35}S]methionine. The medium in each flask was then changed to methionine-supplemented (1 mM) control medium with our without 75 μM arachidonic acid, and incubation was continued for an additional 1 hr. The results are expressed as means ± standard error. In the control flasks, the explants were incubated in Krebs-Ringer buffer alone (see Methods).

A

Variable	hPL	hCG	LDH	Alkaline phosphatase
	mg/flask	*mIU/flask*	*mIU/flask*	*mIU/flask*
Control	137.4 ± 7.8	16.8 ± 1.0	2.0	45.0 ± 3.3
Arachidonic acid	276.9 ± 33.1[a]	12.9 ± 1.6	2.0	54.0 ± 0.3
Deoxycholic acid	435.8 ± 59.3[b]	32.1 ± 2.5[a]	69.0 ± 3.0[b]	52.5 ± 1.5
Triton X-100	1499.7 ± 143.7[b]	75.3 ± 10.5[b]	147.0 ± 24.0[b]	82.5 ± 7.5[a]
Control	115.0 ± 20.5	18.7 ± 2.2	2.0	37.8 ± 2.1
Phospholipase A_2	8351.0 ± 184.5[b]	19.4 ± 2.7	2.0	36.4 ± 5.1

B

Variable	hPL	hCG	^{35}S-Labeled proteins	^{35}S-labeled hPL
	mg/flask	mIU/flask	cpm/flask	cpm/flask
Control	80.2 ± 4.8	7.2 ± 2.0	5917 ± 105	284 ± 27
Arachidonic acid	284.5 ± 47.2[b]	7.3 ± 0.9	6269 ± 344	560 ± 41[b]

[a] $p < 0.05$ versus control.
[b] $p < 0.001$ versus control

The cyclo-oxygenase inhibitors indomethacin (14 μM) and flufenamic acid (18 μM) had no significant effects on either the basal release of hPL or the stimulatory effect of 75 μM arachidonic acid (Table 3). Both inhibitors, however, decreased prostaglandin E synthesis by greater than 90%. In addition, prostaglandins E_1, E_2, and $F_{2\alpha}$ (3.3 and 33.0 μM each) had no significant effects on hPL release over a 2-hr period (Table 4). The lipoxygenase inhibitors ETYA (82 μM) and BW755C (20 μM) had no significant effects on basal hPL release, but each inhibitor potentiated the stimulating effect of arachidonic acid (Table 3).

DISCUSSION

The results of this study indicate that arachidonic acid stimulates the release of hPL *in vitro*. The stimulation was dose-dependent, reversible, and occurred within 20

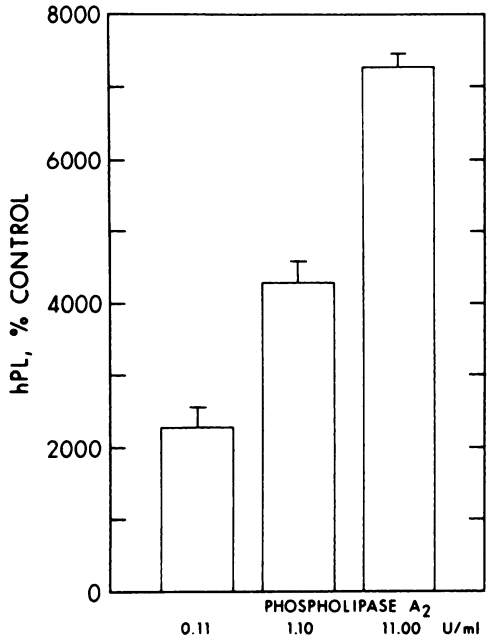

FIG. 4. *Effect of phospholipase A_2 on the release of hPL*

Triplicate cultures of placental explants were exposed for 2 hr to phospholipase A_2 at the indicated concentrations. The amount of hPL released is expressed as a percentage(± standard error) of the amount released by control explants. At each concentration of phospholipase A_2, the amount of hPL released was statistically greater ($p < 0.001$) than that released by control explants.

min of exposure to arachidonic acid. The unsaturated fatty acid precursors of arachidonic acid—linoleic, γ-linolenic, and dihomo-γ-linolenic acid—also stimulated hPL release, but other long-chain unsaturated fatty acids and the saturated fatty acid palmitic acid were without significant effects.

Phospholipase A_2, an enzyme which cleaves arachidonic acid from the 2-acyl position of membrane phospholipids, also stimulated the release of hPL. However, the magnitude of the secretory response to phospholipase A_2 was considerably greater than the response to extracellular arachidonic acid. Although the reason(s) for the greater response to phospholipase A_2 is unknown, the arachidonic acid released by phospholipase A_2 may be more readily available to conversion to the active substance(s) which mediates the arachidonic acid effect. Alternatively, phospholipase A_2 may have effects on the plasma membrane in addition to the release of arachidonic acid, such as the production of lysophospholipids and/or changes in membrane fluidity. Despite their stimulatory effects on hPL release, both phospholipase A_2

TABLE 2

Effects of various fatty acids on the release of hPL

Triplicate cultures of placental explants were incubated for 2 hr in media containing 75 μM of various fatty acids. The results are expressed as the mean percentage change from control ± standard error.

Variable	hPL secretion
	% control
Control	100.0 ± 6.9
Arachidonic acid	217.1 ± 10.7[a]
Linoleic acid	212.6 ± 9.8[b]
γ-Linolenic acid	229.3 ± 14.7[a]
dihomo-γ-linolenic acid	291.7 ± 16.8[c]
11,14-Icosadienoic acid	53.8 ± 11.4
11,14,17-Icosatrienoic acid	46.7 ± 8.6
Oleic acid	102.1 ± 6.4
Palmitic acid	94.4 ± 7.0

[a] $p < 0.05$ versus control.
[b] $p < 0.01$ versus control.
[c] $p < 0.001$ versus control.

TABLE 3

Effects of cyclo-oxygenase and lipoxygenase inhibitors on the basal release of hPL and the stimulation of hPL release by arachidonic acid

Following an overnight incubation in control medium, triplicate cultures of placental explants were incubated for 1 hr in control medium or medium containing one of the cyclo-oxygenase inhibitors (indomethacin and flufenamic acid) or lipoxygenase inhibitors (ETYA and BW755C). The medium was then changed to medium of identical composition for an additional 2 hr. The amounts of hPL secreted during these 2 hr are expressed as the mean ± standard error of triplicate cultures. In each instance, arachidonic acid stimulated hPL release ($p < 0.05$). Indomethacin caused a decrease in prostaglandin E release from 44.5 ± 2.9 to 4.4 ± 1.3 ng ($p < 0.001$), and flufenamic acid caused a decrease from 47.2 ± 3.4 to 3.8 ± 0.8 ng ($p < 0.0001$).

	hPL released (mean ± SE)		
Arachidonic acid	Control	Flufenamic acid, 18 μM	ETYA, 82 μM
		ng/flask	
0 μM	21.5 ± 2.3	23.7 ± 0.3	39.3 ± 2.4
75 μM	87.3 ± 8.3	80.1 ± 4.3	174.0 ± 7.4[a]
300 μM	333.9 ± 35.9	315.3 ± 30.7	432.6 ± 10.6[b]
	Control	Indomethacin, 14 μM	
		ng/flask	
0 μM	21.3 ± 0.8	28.5 ± 1.4	
75 μM	53.4 ± 4.7	46.2 ± 1.0	
	Control	BW755C, 20 μM	
		ng/flask	
0 μM	24.6 ± 1.8	26.3 ± 2.4	
75 μM	52.4 ± 2.3	72.5 ± 7.6[b]	

[a] $p < 0.005$ versus control with the same amount of arachidonic acid.
[b] $p < 0.05$ versus control with the same amount of arachidonic acid.

TABLE 4

Effects of prostaglandins E_1, E_2, and $F_{2\alpha}$ on human placental lactogen release

After an overnight preincubation, placental explants were incubated for 2 hr in medium containing prostaglandins E_1, E_2, or $F_{2\alpha}$. The prostaglandins had no significant effects on hPL release.

Variable	hPL released
	ng/flask
Control	135.9 ± 12.1
Prostaglandin E_1	
3.3 μM	121.0 ± 6.1
33.0 μM	141.9 ± 20.4
Prostaglandin E_2	
3.3 μM	125.3 ± 2.4
33.0 μM	136.3 ± 15.7
Prostaglandin $F_{2\alpha}$	
3.3 μM	112.7 ± 4.8
33.0 μM	131.3 ± 11.5

and arachidonic acid had no effects on the release of hCG. Since phospholipase A_2 activity has been shown to be activated by many hormones and other factors, it is possible that some of the factors (as yet unidentified) which affect the secretion of hPL do so by activating the membrane bound phospholipase (14–17).

Although the mechanisms by which arachidonic acid stimulate hPL release is unknown, several lines of evidence indicate that the effect is not due to a disruption of the structural integrity of the plasma membrane. First, fatty acids other than the precursors of arachidonic acid had no effects on hPL release, even at concentrations 15 times greater than the least effective concentration of arachidonic acid. Second, arachidonic acid had no effects on the release of hCG, LDH, or alkaline phosphatase, whereas Triton X-100 caused the release of all three. These results therefore indicate that the release of hPL by the detergents was the result of a generalized release of cytoplasmic proteins. Third, the effect of arachidonic acid on hPL secretion was reversible but the effects of deoxycholic acid and Triton X-100 were not.

The effect of arachidonic acid also does not appear to be due to a cyclo-oxygenase product of arachidonic acid metabolism. Indomethacin and flufenamic acid had no effects on the basal release of hPL or the stimulatory effect of arachidonic acid, even at concentrations which inhibited prostaglandin E synthesis by greater than 90%. In addition, prostaglandins E_1, E_2, and $F_{2\alpha}$ have no effects on hPL release. These results are in contrast to those of Genbacev *et al.* (9), who noted that prostaglandin $F_{2\alpha}$ decreased total protein and hPL synthesis in placental fragments. The reason(s) for this discrepancy is unclear.

The effect of arachidonic acid also does not appear to be due to a lipoxygenase product of arachidonic acid metabolism, since ETYA and BW755C had no effects on basal hPL secretion. However, both inhibitors potentiated the stimulation of hPL release by arachidonic acid. The increased stimulation of arachidonic acid in the presence of ETYA or BW755C is most likely due to the increased availability of arachidonic acid for metabolism via pathways other than the cyclo-oxygenase and lipoxygenase pathways.

In a recent study (18), we demonstrated that the release of hPL *in vitro* is stimulated by a low extracellular calcium environment and by methoxyverapamil, a drug which blocks calcium channels. These results suggest that hPL secretion is stimulated by a change in calcium flux. Arachidonic acid and some of its metabolites have been shown to affect calcium flux across the plasma membrane (19) and the mitochondrial membrane (20). Consequently, the effects of arachidonic acid and phospholipase A_2 on hPL release may be mediated by changes in calcium flux.

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