Decreased prostaglandin production by cholesterol-rich macrophages

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Abstract The regulation of prostaglandin production by macrophages enriched in cholesterol was examined. Mouse peritoneal macrophages were incubated for 18 h with 25 μg/ml of human acetyl-LDL (low density lipoprotein) and trace amounts of labeled arachidonic acid. After cholesterol enrichment, the cells were incubated with phorbol 12-myristate 13-acetate (PMA), calcium ionophore, or zymosan to stimulate endogenous arachidonic acid metabolism. A high performance liquid chromatography profile of the eicosanoids released revealed no qualitative differences between unmodified and modified macrophages. Cholesterol-rich cells, however, released less prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) compared to unmodified cells, and products from the lipoxygenase pathway became the predominant metabolites. A decrease in the synthesis of PGI_2 and PGE_2 by cholesterol-rich macrophages was confirmed by radioimmunoassay and radiolabeled experiments. The activity of prostaglandin synthetase was modestly increased in the cholesterol-modified macrophages compared to controls. As an estimation of phospholipase activity, the release of labeled arachidonic acid from membrane phospholipids, however, was significantly decreased in cholesterol-rich macrophages. The phosphatidylinositol fraction was particularly resistant to arachidonate release in response to calcium ionophore and PMA in the modified cells. The measurement of membrane phospholipid fatty acid composition before and after calcium ionophore supported the observation that less arachidonate was released by cholesterol-enriched cells in response to the ionophore. Based on these observations, we propose that prostaglandin synthesis from endogenous arachidonate stores is decreased in the cholesterol-rich macrophage. A decrease in agonist-induced activation of the phospholipase activity is proposed as a mechanism for this effect. —**Mathur, S. N., E. Albright, and F. J. Field.** Decreased prostaglandin production by cholesterol-rich macrophages. *J. Lipid Res.* 1989. **30:** 1385–1395.

Supplementary key words eicosanoids • arachidonic acid • phospholipase

An important early event in the pathogenesis of atherosclerosis is the adherence of monocytes to the endothelial surface and their eventual migration into the subendothelial space (1). Once they have taken residence within the vessel wall, these monocyte-macrophages accumulate lipid taking on the appearance of foam cells (1,2). Although macrophages, particularly cholesterol-rich macrophages, constitute the earliest recognizable lesion in atherosclerosis, i.e., the fatty streak, their role in the initiation and propagation of an atherosclerotic plaque is unknown.

It has long been recognized that the cells that are involved in atherosclerosis, such as macrophages, platelets, endothelial cells, and smooth muscle cells, are rich sources of eicosanoids (3–12). It has been postulated by several investigators that interactions between cells in a developing atheroma may be regulated by the localized synthesis and release of certain eicosanoids (3,5,9,11). For example, some studies have shown that atherosclerotic vessels produce less prostacyclin than normal vessels (5,8,9). This could cause an imbalance between the levels of platelet-derived thromboxane and vessel-derived prostacyclin which could contribute to a thrombotic event. Not all reports, however, support the view that atherosclerotic arteries release less prostacyclin or that prostacyclin levels are decreased in atherosclerosis per se (5,8,13,14).

Because it is the cholesterol-rich macrophage that resides in the subendothelial space of an atheroma, we have been interested in the regulation of eicosanoid production by macrophages that have been modified by cholesterol. We have previously shown that, compared to unmodified macrophages, the cholesterol-rich macrophage synthesizes and secretes significantly more 12-hydroxyeicosatetraenoic acid from exogenous arachidonic acid (4). The mechanism for this increase in 12-HETE production is closely related to the metabolism of acetyl-LDL, the lipoprotein used to modify the macrophage (15). In the present study, we investigated the synthesis and release of prostaglandins from endogenous arachidonic acid by the cholesterol-rich macrophage. The data suggest that mouse peritoneal macrophages, enriched in cholesterol by acetyl-LDL, release less prostacyclin (PGI_2) and prosta-

Abbreviations: RIA, radioimmunoassay; HPLC, high performance liquid chromatography; PMA, phorbol 12-myristate 13-acetate; LDL, low density lipoprotein; PGI_2, prostacyclin; PGE_2, prostaglandin E_2; HETE, hydroxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid.

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glandin E_2 (PGE_2) from endogenous stores of arachidonic acid compared to the amount of prostaglandins released by unmodified cells. We propose that there is a decrease in the release of arachidonic acid from phospholipids by phospholipase in response to an agonist as a mechanism for the observed decrease in PGI_2 and PGE_2 production in the cholesterol-rich macrophage.

MATERIALS AND METHODS

Arachidonic acid (5,6,8,9,11,12,14,15-[^{3}H(N)], 100 Ci/mmol) and tritium-labeled PGE_2 and PGI_2 were purchased from New England Nuclear, Boston, MA. [1-^{14}C]Arachidonic acid was purchased from Amersham, Arlington Heights, IL. Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, polyvinylpyrrolidone, dextran (mol wt 72,200), the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), and activated charcoal were from Sigma Chemical Co., St. Louis, MO. Zymosan was purchased from ICN Pharmaceuticals, Inc., Plainview, NY. To prepare serum-treated zymosan, 10 mg of zymosan was added to 1 ml fresh human serum and incubated for 30 min at 37°C. The zymosan particles were then sedimented by centrifugation at 100 *g* for 10 min and washed twice with 10 ml of 0.1 M phosphate buffer, pH 7.4. Antibody for PGE_2 and 6-keto-prostaglandin $F_{1\alpha}$ were from Advanced Magnetics Inc., Cambridge, MA. The antibodies showed less than 1% cross-reactivity between the two prostanoids and all other arachidonic metabolites.

Cell culture

Peritoneal cells from male Swiss Webster mice weighing 25–30 g (Harlan, Indianapolis, IN) were collected by washing the peritoneal cavity with 10 ml of sterile saline containing 10 U heparin/ml. The primary cultures were established as described by Edelson and Cohn (16). Cells from six to eight mice were pooled and distributed in a 24-well (16 mm diameter each well) Costar cluster dish (Costar, Cambridge, MA). In the experiment described in Table 1, cells were plated in a 12-well (22.6 mm diameter) cluster dish. The cells were cultured in Medium 199 containing 10 mM HEPES buffer (pH 7.4) and 2.5% fetal bovine serum. The control cells were incubated with this medium for 18 h at 37°C in an atmosphere of 5% CO_2. To enrich the macrophages with cholesterol, the medium was supplemented with 25 μg cholesterol, in human acetyl-LDL, per ml. After an 18-h incubation, the monolayers were washed four times with 2 ml of fresh Medium 199 containing 10 mM HEPES buffer, pH 7.4 (referred to as medium). All procedures were done under sterile conditions and the cells were incubated in an atmosphere of 5% CO_2/95% air at 37°C. The amount of cellular protein per dish usually was 40 μg for 24-well plates or 80 μg for 12-well plates. Within a particular experiment, the number of cells per dish for control and cholesterol-rich groups were identical.

Lipoprotein preparation

Human lipoproteins were isolated by differential ultracentrifugation and acetyl-LDL was prepared as described by Basu et al. (17).

Utilization of endogenous arachidonic acid for prostaglandin synthesis in response to agonsist by mouse macrophages

Metabolism of radiolabeled endogenous arachidonic acid. To label the cellular lipids with radioactive arachidonic acid, the appropriate amount of the sodium salt of the radiolabeled fatty acid was complexed with Medium 199 containing 2.5% fetal bovine serum. This was added to the dishes at the beginning of the 18-h incubation period in the presence or absence of acetyl-LDL. Control cells were treated in the same manner except that acetyl-LDL was omitted from the medium. The medium was then removed and the monolayers were washed five times with fresh medium to remove loosely adherent arachidonic acid (20:4) from the cell surface. The radioactive fatty acid was allowed to equilibrate with the cellular lipids for 1 h by further incubation with 1 ml of medium. This medium was replaced with 1 ml of serum-free medium containing 2 μM ionophore, 0.1 μM PMA, or 200 μg serum-treated zymosan/ml. The cells were stimulated with zymosan and PMA for 60 min. Because the release of eicosanoids was linear for only 10 min after stimulation with calcium ionophore, a 10-min time period was used for this agonist. The ionophore and PMA were added to the medium in ethanol so that the final concentration of the ethanol was less than 0.1%. In all experiments, sets of control and cholesterol-rich cells were treated in the same manner except the agonists were omitted to determine the basal metabolism of the 20:4. The amount of products released into the medium by this group of cells was subtracted from the values obtained for agonist-treated cells to determine the products formed due to the stimulus. The radioactivity in the phospholipid fraction in cells treated without the agonist was considered to be 100%.

Estimation of PGE_2 and PGI_2 by radioimmunoassay (RIA). The control and cholesterol-rich cells were prepared as described above except that radiolabeled arachidonic acid was not added to the medium. The medium was collected after treatment with the agonist. PGE_2 and the stable product of PGI_2, 6-keto-prostaglandin $F_{1\alpha}$, were assayed by RIA as described by Campbell and Ojeda (18). The terms PGI_2 and 6-keto $PGF_{1\alpha}$ will be used interchangeably in these studies. Briefly, antiserum, tritiated prostaglandin, and dextran-coated charcoal solutions were prepared in RIA buffer (0.15 M sodium chloride, 0.1% so-

dium azide, 0.1% polyvinylpyrrolidone, 0.1 M phosphate buffer, pH 7.4). One hundred μl of either standard or sample were mixed with 50 μl of the [^{3}H]prostaglandin and 50 μl of the antiserum at a dilution that bound 50% of the radioactivity in the absence of the standard. After a 4 h incubation at 4°C, 0.2 ml of cold dextran-coated activated charcoal was added. The contents of the tube were thoroughly mixed and after centrifugation at 200 *g* for 15 min at 4°C, the radioactivity contained in the supernatant was measured in a liquid scintillation spectrometer (Packard, Tricarb 4000 MINAXI). The amount of prostaglandin in the sample was calculated by plotting (B/B_o) versus ligand concentration by C-fit algorithm described by Brooker et al. (19). The values were corrected for the small amount of prostaglandin that was secreted in the absence of the agonist (less than 3 pmol of PGE_2 or PGI_2 per 60 min for both control and cholesterol-rich macrophages).

Lipid extraction

Cellular lipids were extracted with 20 volumes of chloroform–methanol 2:1 (v/v) at pH < 3.0. The individual phospholipids and neutral lipids were separated by thin-layer chromatography on silica gel G (Fisher Scientific, Chicago, IL). After application of the sample at 1.5 cm from the bottom of the plate, phospholipids were resolved by developing the plate to 13 cm with a solvent system of chloroform–methanol–water–acetic acid 50:37.5:3.5:2 (v/v) (20). The plate was then developed to 19 cm with hexane–diethyl ether–acetic acid 90:10:1 (v/v) to separate the neutral lipids. The radioactivity in the lipid bands was determined by using a Vanguard radioactivity scanner (IN/US, Fairfield, NJ) or by scraping the lipid bands and counting them. The R_f values were: lysophosphatidylcholine, 0.02; sphingomyelin; 0.1; phosphatidylcholine, 0.21; phosphatidylserine, 0.34; phosphatidylinositol, 0.40; phosphatidylethanolamine, 0.47; diacylglycerol, 0.59; fatty acid, 0.63; triacylglycerol, 0.71; and cholesteryl esters, 0.87. Standards were visualized by exposure of the plate to iodine vapors.

Lipids from the medium were extracted by mixing 1 ml of the medium with 1 ml of acetone and adjusting the pH to <3 by the addition of 10 μl of 1.2 M citric acid (21). The aqueous phase was extracted twice with 2 ml of chloroform. Greater than 98% of the oxygenated products was recovered in the chloroform phase. The chloroform was evaporated under nitrogen and the lipids were dissolved in 50 μl of 30% methanol and used for HPLC.

Separation of the oxygenated products by HPLC

Reverse phase HPLC was performed on an Ultrasphere ODS, 5 μm, 4.6 × 25 cm column using the Gilson 41 gradient HPLC. The elution solvent was that described by Powell (22): solvent A was water–acetonitrile–phosphoric acid 72:28:0.025; solvent B was methanol–acetonitrile–water–trifluoroacetic acid 60:40:0.08:0.0016. The column was eluted with 100% solvent A for 6 min. A gradient was formed by increasing solvent B to 4% at 7 min and keeping this composition up to 15 min. Solvent B was raised to 40% at 16 min and maintained for 22 min. The level of solvent B from 23 to 32 min was 56% and from 33 to 36 min was 95%. Between 36 and 40 min the percent of B was lowered to 0%. The flow rate was 1.6 ml/min. Radioactivity in the eluate was monitored by a flow-one radioactivity detector (Radioactivity Instruments and Chemical Co., Tampa, FL). Radioactivity was determined every 0.2 min. Total radioactivity eluted from the column represented 100% of the counts. The percent of the radioactivity found in the various oxygenated compounds was used to calculate pmoles of the product secreted into the medium by the cells. The retention times for the standard compounds were: 6-keto-prostaglandin $F_{1\alpha}$, 6.4 min; thromboxane B_2, 10.3; prostaglandin $F_{2\alpha}$, 12.4 min; PGE_2, 13.7 min; 15-HETE, 29.2; 12-HETE, 30.1; 5-HETE, 31.4 min; and 20:4, 36.8 min.

Prostaglandin synthase assay

The basal activity of prostaglandin synthase in whole homogenates of control and cholesterol-rich macrophages was determined as described by Tai, Yuan, and Wu (23). Macrophages from 12 mice were distributed into two T-75 flasks. The cells in one flask were enriched in cholesterol by incubation for 18 h with 10 ml of medium containing 2.5% fetal bovine serum and 25 μg of acetyl-LDL cholesterol/ml. Control cells were incubated in the same medium but in the absence of acetyl-LDL. The monolayers were washed five times with 1 ml of Medium 199. The cells were collected using a plastic spatula and taken up in 0.5 ml of 0.1 M phosphate buffer. The cell suspension was sonicated for 2 min at 10-sec bursts. The basal activity of prostaglandin synthase in cell-free whole homogenates was determined in a reaction mixture containing 0.2 ml of 0.1 M phosphate buffer, pH 7.4, 20 μM [^{3}H]20:4 (59.6 mCi/mmol), 1 μM NDGA, and 80 μg of macrophage protein. The assay mixture (0.15 ml) was preincubated for 5 min at 25°C before starting the reaction by adding the sodium salt of arachidonic acid in 0.05 ml of the phosphate buffer. After incubating for 1 min at 37°C, the reaction was terminated by adding 1 ml of acetone. The lipids were extracted by the addition of 0.5 ml water, 10 μl 1.2 M citric acid, and 1 ml of chloroform. The chloroform layer was removed and the aqueous phase was extracted with 2 ml of chloroform. The two chloroform extracts were combined, evaporated under nitrogen, and taken up in 50 μl of 30% methanol in water. The oxygenated metabolites were analyzed by HPLC as described in Methods.

Determination of radiolabeled 12-HETE in cellular phospholipids

Control and cholesterol-rich macrophages were prelabeled for 18 h with [^{3}H]arachidonic acid. Total lipids were extracted from the cells with chloroform–methanol 2:1 (v/v) and separated by thin-layer chromatography. The phospholipids were eluted from the silica gel by rinsing the gel four times with 1 ml of chloroform–methanol–acetic acid–water 60:50:1:4 (v/v) in a glass Pasteur pipette plugged with glass wool. Chloroform (1.5 ml) and water (1.1 ml) were added to separate the phases and the lower chloroform layer was dried under nitrogen. The phospholipids were then hydrolyzed for 1 h at 80°C in 2 ml of 0.5 N NaOH in methanol. After adding 1.5 ml of water and adjusting the pH to less than 3, the fatty acids were extracted twice with 2 ml of chloroform. The labeled 12-HETE and 20:4 were analyzed by HPLC.

Fatty acid analysis

The cellular phospholipid fraction was separated from the neutral lipids by thin-layer chromatography using hexane–diethyl ether–acetic acid 60:40:1 (v/v). The phospholipids were eluted from the plate as described above. After drying down the chloroform phase, the methyl esters of the fatty acids were prepared by adding 1 ml of 14% boron trifluoride in methanol and heating for 1 h at 80°C. The tubes were cooled and 1 ml of water was added before extracting the fatty acids twice with 2 ml of hexanes. The fatty acids were separated by gas–liquid chromatography using a glass column of GP 10%, SP-2330. The initial temperature was 165°C for 8 min, increasing by 3° per min thereafter until reaching 210°C. The percent area composition was determined using a Hewlett-Packard 3390A integrator.

Chemical analyses

In a given experiment, the number of cells and protein per dish were similar in control and cholesterol-rich preparations. Protein was estimated according to the method of Lowry et al. (24). Cholesterol was measured by gas–liquid chromatography using cholestane as an internal standard (4). Phospholipids were measured according to the method of Chalvardjian and Rudnicki (25).

Statistics

Data were expressed as the mean ± SE. Significance was determined by the unpaired Student's *t*-test.

RESULTS

Eicosanoids produced by control and cholesterol-rich macrophages

It has been shown that the predominant eicosanoids synthesized by mouse peritoneal macrophages from endogenous arachidonic acid are the prostaglandins PGE_2 and PGI_2 (26). Products of the lipoxygenase pathway, 5-, 12-, and 15-hydroxyeicosatetraenoic acids, are synthesized to a lesser extent. **Fig. 1** shows a representative HPLC profile of labeled products of arachidonic acid metabolism released into the medium by control and cholesterol-rich macrophages after a 1 h incubation with PMA. HPLC profiles from cells stimulated with calcium ionophore and zymosan gave similar results (data not shown). In support of previous observations (26–28), PGE_2 was the dominant prostaglandin released by unmodified macrophages followed by PGI_2 and the monohydroxy-fatty acids. In contrast, cholesterol-rich macrophages released less PGE_2 and PGI_2. On a percent basis, the lipoxygenase products, 12- and 15-HETE became the predominant metabolites. Inducing cholesterol-rich foam cell formation, however, did not qualitatively alter the eicosanoids produced. Similar metabolic products were seen with both macrophage populations.

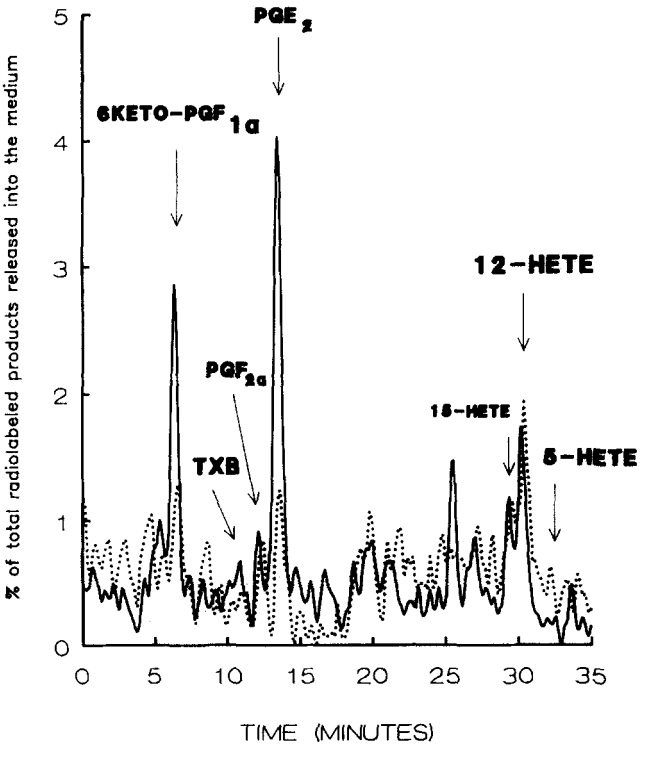

Fig. 1. Reversed-phase HPLC of [^{3}H]20:4 metabolites released by control and cholesterol-rich macrophages in response to PMA. Cellular lipids of mouse macrophages were labeled with radioactive 20:4 by incubation with 1 ml of medium supplemented with fetal bovine serum, 0 or 25 μg of cholesterol in acetyl-LDL, and 2 μCi, 20 nM [^{3}H]20:4 for 18 h at 37°C. The monolayers were washed four times with serum-free medium and stimulated by adding 0.1 μM PMA for 1 h. The medium was collected and the oxygenated products of arachidonic acid were analyzed by HPLC as described in Methods. The percent radioactivity eluted per 0.2 min is shown on the y axis. The elution times for standard radioactive eicosanoids are indicated by the arrows; (—) control cells; (....) cholesterol-rich cells.

PGI_2 and PGE_2 production as determined by RIA

Macrophages were incubated for 18 h in the presence or absence of 25 μg/ml of acetyl-LDL cholesterol. Macrophages incubated with the modified lipoprotein contained 508 nmol of cholesterol per mg protein, of which 70% was cholesteryl ester. Control macrophages contained 56 nmol of cholesterol per mg protein and the amount of esterified cholesterol was negligible (4). The cells were then incubated with either zymosan, PMA, or calcium ionophore, three agents known to stimulate endogenous arachidonic acid metabolism. PGE_2 and the stable product of PGI_2, 6-keto-prostaglandin $F_{1\alpha}$ were measured in the medium by radioimmunoassay. **Fig. 2** shows that, of the three agents, the calcium ionophore was the most potent stimulator of prostaglandin production followed closely by zymosan and then PMA. Irrespective of the agent used, cholesterol-rich macrophages produced less immunoreactive PGI_2 and PGE_2 than unmodified cells.

PGI_2 and PGE_2 production as determined by radiolabel

To investigate potential mechanisms for the observed decrease in prostaglandin production by cholesterol-rich macrophages, unmodified cells and cells enriched in cholesterol were incubated for 18 h with trace amounts of [^{3}H]arachidonic acid to label the intracellular lipids. Both control and modified cells incorporated similar amounts of arachidonic acid into total lipids (**Fig. 3A**). Compared to control cells, however, less label was observed in total phospholipids in cholesterol-rich cells as more of the radiolabel was diverted into triglycerides and cholesteryl esters by the modified cells. As shown in Fig. 3B, the decrease in the incorporation of the label into total phospholipids of cholesterol-rich cells was not secondary to a decrease occurring in any one particular phospholipid class. The incorporation of the arachidonate label was decreased similarly in each phospholipid fraction. Fig. 3C illustrates that cholesterol modification of the cells did not change the relative percent distribution of the radiolabel in the individual phospholipids.

Compared to control cells, cholesterol-rich macrophages contained less labeled 20:4 (Fig. 3) and had more 20:4 (**Table 1**) in their phospholipids. Cholesterol-rich cells contained 180 ± 5 nmol phospholipid/mg protein compared to 143 ± 5 nmol/mg protein in control macrophages. A lower specific activity of 20:4 in cholesterol-rich cells could, therefore, result in the underestimation of prostaglandins produced by these cells compared to prostaglandins produced by control cells. To estimate the actual specific activity of 20:4 in both cell populations, gas–liquid chromatography was performed using a stream splitter which permitted the determination of radioactivity and area for each individual fatty acid peak. The specific activity of 20:4 in phospholipids was determined by dividing the radioactivity found in each peak by the area under the peak. The estimated specific activity of 20:4 in phospholipids of control and cholesterol-rich macrophages was 64 ± 3 dpm/unit area and 53 ± 2, respectively (n = 6 determinations). Both control and cholesterol-rich macrophages contained similar amounts (67–72%) of the radiolabel as arachidonic acid in phospholipids. The remaining 28–33% of the radiolabel represented elongation and desaturation products of arachidonate.

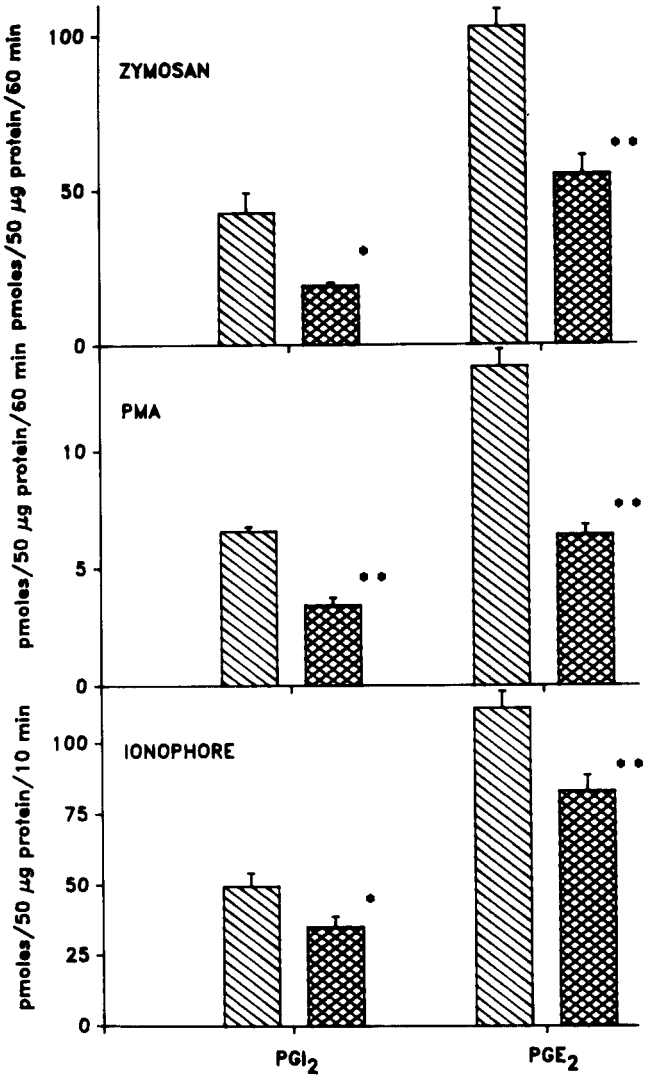

Fig. 2. Prostaglandin production by control and cholesterol-rich macrophages in response to zymosan, PMA or ionophore. Mouse peritoneal macrophages were enriched in cholesterol by incubating them for 18 h with 25 μg of human acetyl-LDL/ml of medium 199 containing 2.5% fetal bovine serum. Control macrophages were incubated in medium in the absence of acetyl-LDL. The monolayers were washed five times with 1 ml of medium 199 and then exposed for 1 h to 1 ml of serum-free medium 199 containing 200 μg zymosan, 0.1 μM phorbol 12-myristate 13-acetate (PMA), or 2 μM calcium ionophore A23187 for 10 min at 37°C. The medium was collected and 6-ketoprostaglandin $F_{1\alpha}$ and prostaglandin E_2 were assayed by radioimmunoassay. Two macrophage preparations were used for the zymosan and PMA experiments and the values represent the mean ± SE of 7 dishes. The values for the ionophore experiment represent the mean ± SE of 15 dishes from five separate macrophage preparations. $^*P < 0.05$ versus control cells; $^{**}P < 0.01$ versus control cells; ▨ control; ▩ cholesterol-rich.

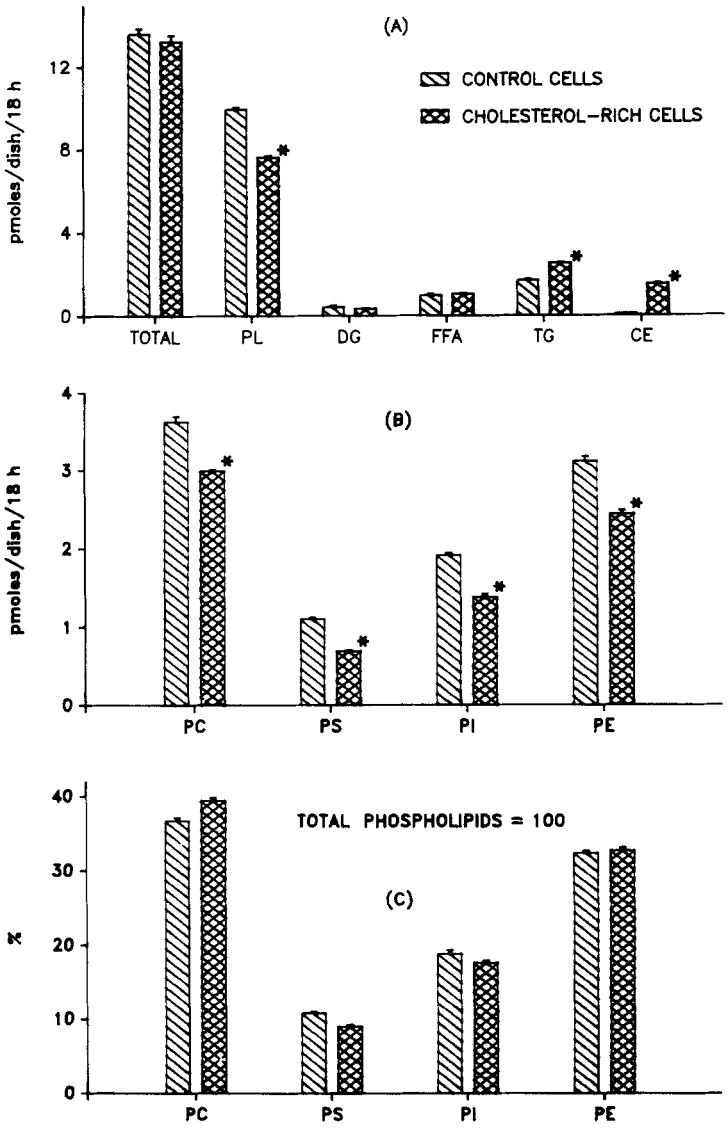

Fig. 3. Incorporation of [^{3}H]20:4 into macrophage cellular lipids. Cellular lipids of macrophages were labeled with [^{3}H]20:4 during an 18-h incubation at 37°C with 1 ml of medium containing 0 or 25 μg acetyl-LDL and 2 μCi, 20 nM, [^{3}H]20:4 complexed to 2.5% fetal bovine serum. The monolayers were washed five times with 1 ml of medium 199 and incubated for 10 min in 1 ml of serum-free medium. The medium was removed and cellular lipids were extracted as described in Methods. The lipids were separated by thin-layer chromatography. The plates were developed first with chloroform-methanol-water-acetic acid 50:37.5:3.5:2 (v/v) to resolve the phospholipids. This was then followed by developing the plate in hexane-diethyl ether-acetic acid 90:10:1 (v/v) to separate the neutral lipids. The amount of protein in each dish was 30 μg. The values are the mean ± SE of 16 dishes from five separate macrophage preparations. *$P < 0.001$ versus control macrophages. (A) Amount of radiolabeled 20:4 incorporated into total phospholipids and neutral lipids. (B) Amount of radiolabeled 20:4 incorporated into the major individual phospholipid fractions. (C) Relative amount of radiolabeled 20:4 incorporated into the individual phospholipid fractions. ▨ control cells; ▩ cholesterol-rich cells.

Macrophages that were prelabeled for 18 h with arachidonic acid were incubated with the calcium ionophore or PMA. The amounts of PGI_2 and PGE_2 released into the medium were measured by HPLC (**Fig. 4**). To adjust for the decreased amount of arachidonate label found in the total phospholipids of cholesterol-rich cells, the results are expressed as a percent of the radiolabel that was present in the intracellular phospholipid fraction of cells treated with buffer but without the agonist. Less PGI_2 and PGE_2 were produced by cholesterol-rich macrophages in response to both calcium ionophore and PMA compared to control cells.

TABLE 1. Effect of ionophore on fatty acid composition

Fatty Acid	Control Cells + Ionophore		Cholesterol-Rich Cells + Ionophore	
	0 μM	10 μM	0 μM	10 μM
		%		
16:0	20 ± 0.9	20 ± 0.2	18 ± 1.2	15 ± 2.0
16:1	2 ± 0.2	2 ± 0.2	1 ± 0.1	2 ± 0.3
18:0	26 ± 0.5	29 ± 1.7	28 ± 1.2	34 ± 2.6
18:1	18 ± 1.1	18 ± 0.7	11 ± 0.4	11 ± 0.7
18:2	5 ± 0.1	4 ± 0.3	13 ± 0.5	12 ± 1.0
20:3	2 ± 0.1	1 ± 0.1	2 ± 0.2	1 ± 0.2
20:4	12 ± 0.5	7 ± 0.3*	14 ± 0.5	13 ± 1.7
20:5	1 ± 0.8	1 ± 0.3	1 ± 0.1	1 ± 0.2
22:4	3 ± 0.7	2 ± 0.5	2 ± 0.6	1 ± 0.6
22:5	2 ± 0.1	1 ± 0.1	1 ± 0.0	1 ± 0.3
22:6	2 ± 0.3	1 ± 0.2	2 ± 0.1	2 ± 0.3
Others	8 ± 0.9	13 ± 2.2	8 ± 0.6	8 ± 0.4
Saturated	48 ± 1.2	51 ± 1.6	46 ± 0.9	49 ± 3.2
Monoenoic	22 ± 0.6	22 ± 0.9	13 ± 0.5	13 ± 0.7
Polyenoic	31 ± 0.7	27 ± 1.8	41 ± 0.6	37 ± 2.8

Control and cholesterol-rich macrophages were cultured in 12-well culture plates. Each well contained 80 μg protein. They were then incubated for 10 min with 1 ml of serum-free medium 199 containing 0 or 10 μM ionophore. Cellular lipids were extracted as described in Methods. The total phospholipid fraction was isolated by TLC using the solvent system of hexane–diethyl ether–acetic acid 60:40:1 (v/v). The total phospholipid fraction was eluted from the gel, transesterified with 14% boron trifluoride in methanol, and the fatty acid composition was determined by GLC. The values represent the mean ± SE of four dishes. *, $P < 0.001$ versus without ionophore.

Loss of arachidonic acid from cellular phospholipids

The release of arachidonic acid from membrane phospholipids is catalyzed by the phospholipases A_2 and C (29–33). Cellular phospholipase activity is thought to regulate the amount of arachidonate substrate provided to the cyclooxygenase or lipoxygenase enzymes (29). To estimate the total phospholipase activity in control and modified macrophages, the loss of arachidonic acid label from cellular phospholipids by calcium ionophore and PMA was determined (**Fig. 5**). In cholesterol-rich cells, an increase in phospholipase activity that resulted from the addition of both agents was significantly attenuated. Only 3–5% of the radiolabeled arachidonic acid observed in cellular phospholipids was released by cholesterol-rich macrophages, compared to 10–15% of arachidonic acid which was released by unmodified macrophages. In response to the two agonists, cholesterol-rich cells lost a significantly smaller fraction of the label from the phosphatidylinositol fraction compared to control cells. There was a definite trend, however, for less arachidonate label to be lost from all phospholipid fractions in the modified macrophages. The data suggest that phospholipase activity is attenuated in the cholesterol-enriched macrophage.

The data presented in Figs. 4 and 5 underestimate the release of arachidonic acid from phospholipids and hence total phospholipase activity. Only a small fraction of the arachidonate released becomes available for eicosanoid production. Most of the arachidonate will be reacylated, either back into phospholipids or into neutral lipids. In the next experiment, reuptake and reutilization of released arachidonic acid by the macrophage was minimized by adding ibuprofen, NDGA, unlabeled arachidonic acid, and albumin to the medium prior to the addition of calcium ionophore. Under these experimental conditions, more than 92% of the radioactivity released into the medium was unesterified arachidonic acid. **Fig. 6** shows the results. The data support the observations that were made in the previous experiment. Significantly less arachidonic acid was released into the medium by cholesterol-rich cells compared to control cells in response to calcium ionophore.

Fatty acid composition

There are data to suggest that certain fatty acids, particularly linoleic acid, regulate phospholipase activity (34). To investigate the effects of cholesterol enrichment and calcium ionophore stimulation on membrane fatty acid composition, phospholipids were isolated from control and modified macrophages before and after stimulation with calcium ionophore. The fatty acid compositions were then determined. The data are shown in Table 1. Cholesterol-rich cells contained significantly more arachidonic and linoleic acids in phospholipids compared to the amount found in control cells. As expected, the ionophore caused no significant alteration in phospholipid linoleic acid content in either cell population. In contrast, the calcium ionophore resulted in a significant decrease in the percent of arachidonate in

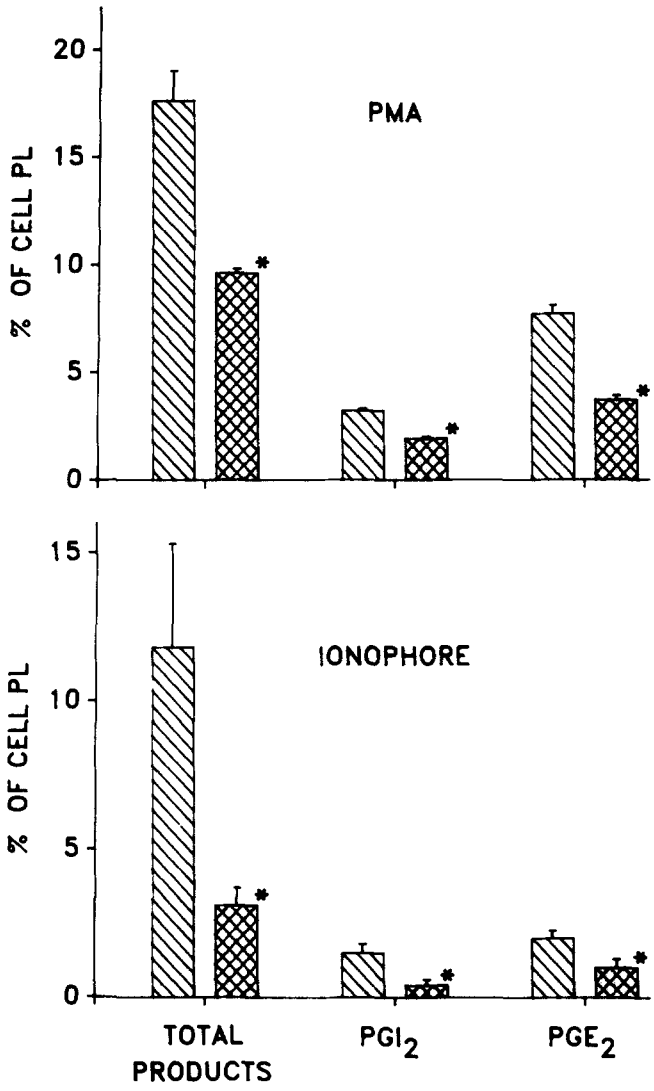

Fig. 4. Prostaglandins released by macrophages in response to ionophore and PMA. Macrophage cellular lipids were labeled for 18 h with [^{3}H]20:4. The oxidation products were analyzed as described in Fig. 1 except that cells were stimulated to produce prostaglandins by adding 1 ml of 2 μM ionophore for 10 min or 0.1 μM PMA for 1 h at 37°C. The values represent the mean ± SE of six dishes from two macrophage preparations. The amount of oxygenated products released into the medium is expressed as the percent of the radiolabeled 20:4 that was found in the total phospholipid fraction of the cell. The values have been corrected for the small amount of radioactivity released into the medium by cells incubated with buffer but without the agonist. *$P < 0.001$ versus control cells; ▨ control; ▩ cholesterol-rich.

phospholipids of unmodified macrophages. In cholesterol-rich macrophages, however, the decrease in the percent of arachidonic acid that occurred after calcium ionophore stimulation was not significant, further evidence that phospholipase activity is attenuated in the modified macrophage.

Prostaglandin synthetase

Another step that may be regulatory for prostaglandin production in the macrophage is the activity of cyclooxygenase. To investigate whether cholesterol enrichment inhibited this step in arachidonate metabolism, prostaglandin synthetase activity was determined in cell-free whole homogenates prepared from control and cholesterol-rich macrophages. There was a modest but significant increase in prostaglandin synthetase activity in macrophages enriched in cholesterol compared to the activity observed in unmodified cells, 370 ± 30 pmol prostaglandins formed/mg per min versus 250 ± 30. Thus production of PGE_2 and PGI_2 in cholesterol-rich macrophages is not decreased secondary to a decrease in the activity of cyclooxygenase.

DISCUSSION

The results from this study clearly demonstrate that production of PGI_2 and PGE_2, is significantly decreased in cholesterol-enriched mouse peritoneal macrophages. The proposed mechanism for this phenomenon is an attenuation of

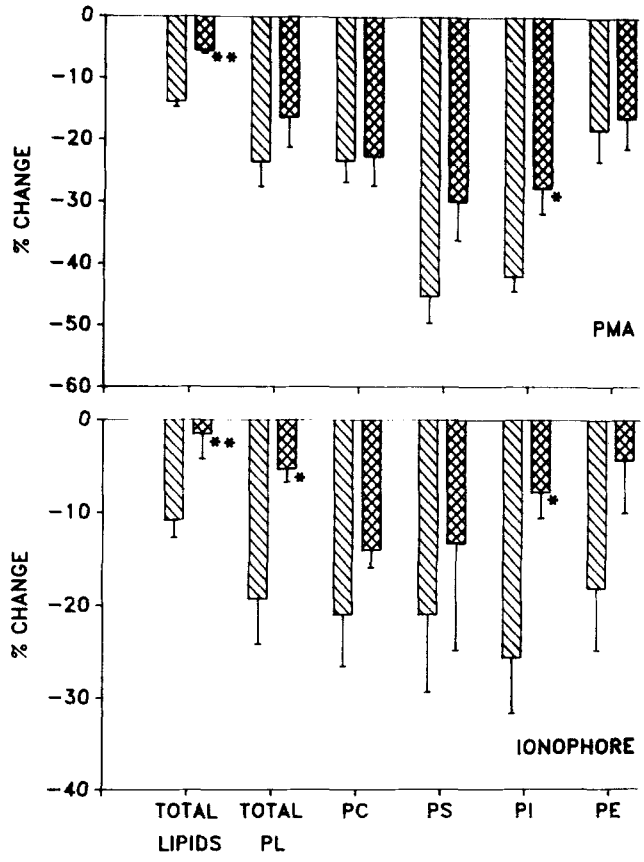

Fig. 5. Loss of [^{3}H]20:4 from cellular phospholipids in response to ionophore and PMA. The experimental conditions were the same as described in Figs. 1, 3, and 4. The values represent the mean ± SE of six dishes from two macrophage preparations. The percent change in the individual phospholipid fractions that was secondary to the agonists was calculated by taking the amount of the radiolabel in the phospholipid fractions in cells not exposed to the agonists as 100%. *$P < 0.05$ versus control cells; **$P < 0.001$ versus control cells; ▨ control; ▩ cholesterol-rich.

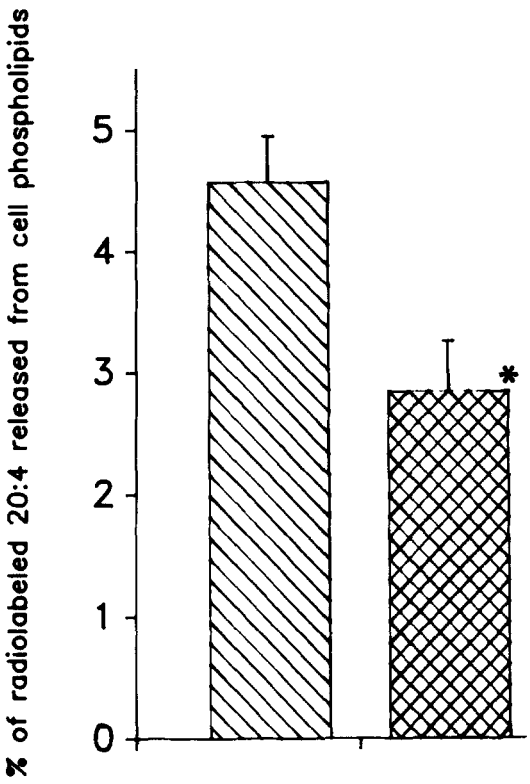

Fig. 6. Loss of radiolabeled 20:4 from cellular phospholipids by ionophore in the presence of unlabeled 20:4, bovine serum albumin, ibuprofen and NDGA. To label the cellular lipids, macrophages were incubated for 6 h with 0.55 μCi [^{14}C]20:4 (59.6 mCi/mmol) complexed to 2.5% fetal bovine serum. The medium was then replaced with medium containing 2.5% fetal bovine serum and supplemented with 0 or 25 μg acetyl-LDL cholesterol. The incubation was continued for another 12 h. At the end of this period, the cells were washed and treated with 10 μM ionophore for 20 min in the presence of 100 μM unlabeled 20:4, 100 μM bovine serum albumin, 100 μM ibuprofen, and 10 μM NDGA. The amount of the radiolabeled compound released into the medium is expressed as the percent of the labeled 20:4 found in the total phospholipid fraction of the cell. The values have been corrected for the radioactive compounds released by cells incubated with buffer containing all of the above compounds except ionophore. Each dish contained 48 μg protein. The values are the mean ± SE of triplicate dishes. *P < 0.05 versus control cells; ▨ control; ▩ cholesterol-rich.

the activation of phospholipase by an agonist in the modified macrophage compared to control cells.

Much of the data pertaining to the effects of atherosclerosis on eicosanoid production has been generated from experiments performed in intact vessels or vessel rings. The results are conflicting. Dembinska-Keic et al. (35) observed a decrease in PGI_2 production in atherosclerotic vessels prepared from rabbits on a high cholesterol diet. In contrast, Mehta et al. (14) observed the opposite effect. These investigators found an increase in PGI_2 production in atherosclerotic aortic rings prepared from rabbits fed a diet enriched in cholesterol. In yet another study (8) done in rabbits fed an atherogenic diet for 10 weeks, aortic intimal PGI_2 production was initially increased at 2 weeks but was significantly decreased by 6 weeks, suggesting that PGI_2 production was biphasic during progression of atherosclerosis. The results of such studies perhaps become more clear when specific cell types are used as models to investigate the effects of cholesterol enrichment on eicosanoid production. In aortic smooth muscle cells prepared from rabbits fed cholesterol for 6 months, Larrue et al. (9) found a decrease in the production of PGI_2 and PGE_2 from exogenous arachidonic acid. In cultured arterial smooth muscle cells that were enriched in cholesterol by incubation with acetyl-LDL, Pomerantz and Hajjar (12) found a decrease in prostaglandin synthesis in response to a calcium ionophore or arachidonic acid. In another study (11), prostaglandin production was decreased in blood monocytes isolated from hypercholesterolemic rabbits. In contrast, platelets respond differently to cholesterol enrichment. Stuart, Gerrard, and White (10) observed an increase in thromboxane synthesis in platelets that had been enriched in cholesterol. Lastly, in experiments with mouse peritoneal macrophages, Hartung et al. (7) observed that prostaglandin synthesis was stimulated in cells incubated with acetyl- or malondialdehyde-treated LDL. The data from that study suggested that the binding of the modified lipoprotein to its receptor on the macrophage membrane was sufficient to trigger endogenous arachidonic acid metabolism. In the present study, experiments were performed in macropages that had been incubated with acetyl-LDL for 18 h. At this time, the cells were extensively modified and cholesterol-enriched. In contrast, in the study by Hartung et al. (7), prostaglandin production was actually measured during a 6-h incubation with the modified lipoprotein. No stimulatory agent, other than the lipoprotein, was used to promote prostaglandin production. Whereas, in our study, endogenous arachidonic acid metabolism was stimulated by PMA, calcium ionophore, or zymosan. Together, the results of the two studies suggest that during the very early period of atherosclerosis, at a time when lipoproteins are binding to the macrophage, prostaglandin synthesis may be stimulated. Over time and with progression of the lesion, however, cholesterol modification of the macrophage results in a decrease in PGI_2 and PGE_2 synthesis. This can be likened to the observations of Beetens et al. (8). These investigators found prostaglandin synthesis to be increased in intima of thoracic aorta obtained from rabbits fed cholesterol for 2 weeks. After 6 weeks of hypercholesterolemia, however, intimal prostaglandin synthesis was observed to be decreased and it remained low throughout the remaining 4 weeks of the study.

The results of the present study also agree with the observations made in arterial smooth muscle cells (12). In both macrophages and smooth muscle cells, prostaglandin synthesis was inhibited by cholesterol enrichment. In another study done with aortic smooth muscle cells from cholesterol-fed rabbits, a decrease in the activity of prostaglandin synthetase was observed (9). We did not observe this in the macrophage. In fact, a modest increase in prostaglandin

synthetase was observed in cholesterol-modified macrophages. In agreement with the study by Pomerantz and Hajjar (12), we also observed a decrease in the release of arachidonic acid from membrane phospholipids, suggesting a relatively lower activation of phospholipase in cholesterol-rich cells. It is also possible that, in cholesterol-rich macrophages, more of the released arachidonic acid is being diverted from the oxidation pathway and is, instead, being reacylated into cellular lipids. Not only would less arachidonate be hydrolyzed from the phospholipids, but less of it would be available as substrate for prostaglandin synthetase. Obviously, if arachidonic acid were available to the enzyme in cholesterol-rich macrophages, these cells would be more than capable of producing significant amounts of prostaglandins due to the higher levels of prostaglandin synthetase activity. Ballou and Cheung (34) observed that unsaturated fatty acids were potent noncompetitive inhibitors of platelet phospholipase A_2. During the processes of purification, they found that the activity of phospholipase increased significantly when unsaturated fatty acids were removed from the membrane. The investigators also found that cholesterol modestly inhibited phospholipase activity. In another study, Lister et al. (36) demonstrated the inhibition of macrophage phospholipase activity by a variety of unsaturated fatty acids. Both of these studies suggested that phospholipase activity was regulated by membrane fat saturation and cholesterol content. In the present study, both of these membrane lipids could play a role in regulating phospholipase activity. The membranes of cholesterol-enriched macrophages were also highly enriched in linoleic acid, a fatty acid shown to inhibit phospholipase activity (34). In smooth muscle cells, it was postulated that linoleate was competing with arachidonic acid for the phospholipase enzyme. Our data would argue against that. Calcium ionophore did not significantly affect the release of linoleic acid from cellular phospholipids in cholesterol-rich macrophages, suggesting that it was not competing with arachidonate for release.

12-Hydroxyeicosatetraenoic acid (12-HETE), a product of the lipoxygenase pathway, has also been shown to inhibit phospholipase activity (37,38). In a previous study, we have demonstrated an increased production of 12-HETE by cholesterol-rich macrophages (4). Moreover, macrophages can incorporate 12-HETE back into their membrane phospholipids (39). To investigate the possibility that membrane 12-HETE could have contributed to the decreased phospholipase activity in modified macrophages, the amount of 12-HETE that accumulated in membrane phospholipids after an 18-h incubation with acetyl-LDL was determined. Essentially no or minimal 12-HETE was observed in the phospholipids of these cells.

The data of the present study suggest that prostaglandin production, particularly PGI_2 and PGE_2, is decreased in macrophages that have been modified to cholesterol-enriched foam cells. Whether this has pathophysiological consequences in the development of an atherosclerotic lesion is unclear. In view of increased thromboxane production by cholesterol-rich platelets (10), however, it can be speculated that these conditions could lead to an increased propensity for vessel spasm and thrombosis.

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