Role of phospholipase A₂ in expression of the scavenger pathway in cultured aortic smooth muscle cells stimulated with phorbol 12-myristate 13-acetate

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We have demonstrated that cultured intimal smooth muscle cells (SMC) from thickened intima can metabolize acetylated low-density lipoprotein (LDL) by a scavenger pathway, but medial SMC from normal arteries cannot. In this study we investigated the expression mechanism of the scavenger pathway in medial SMC using a phorbol ester. Medial SMC were incubated with 10⁻¹⁰–10⁻⁷ M phorbol 12-myristate 13-acetate (PMA) for 1–24 h and then their degradation of ¹²⁵I-labelled acetylated LDL was assayed. Unstimulated SMC degraded little acetylated LDL, but incubation for 24 h with PMA dose-dependently stimulated its degradation by SMC, the optimal PMA concentration being 1 × 10⁻⁸ M. Induction of expression of the scavenger pathway required more than 4 h of incubation with PMA and was completely inhibited by cycloheximide. In addition expression of the scavenger pathway was not transient but stable. Induction of

expression of the scavenger pathway by PMA was not inhibited by protein kinase C inhibitors, but was inhibited about 50 % by phospholipase A₂ inhibitors. The study, using various phorbol esters, indicated that induction of the scavenger pathway was well correlated with their ability to stimulate phospholipase A₂ in medial SMC but not with their ability to activate protein kinase C. Moreover, incubation with exogenous phospholipase A₂ (0.1–10 units/ml) or its product, lysophosphatidylcholine (0.01–100 µg/ml) dose-dependently increased degradation of ¹²⁵I-labelled acetylated LDL in medial SMC. Lysophosphatidylcholine was most effective in various lysophospholipids. These results suggest that PMA induced the scavenger pathway in part by stimulating phospholipase A₂ in medial SMC, and that a product, lysophosphatidylcholine, is a mediator of expression of the scavenger pathway.

INTRODUCTION

Mesenchymal cells such as aortic smooth muscle cells (SMC) and fibroblasts are reported to metabolize low-density lipoproteins (LDLs) through LDL receptors by the LDL pathway [1,2], but not acetylated LDLs, which are metabolized through scavenger receptors by the scavenger pathway [3]. This scavenger pathway was reported to be located mainly in macrophages and endothelial cells [4,5]. However, foam cells are derived not only from macrophages but also from SMC [6]. The LDL pathway in SMC does not explain the accumulation in these cells of a large amount of cholesterol ester, as there is strict regulation of intracellular cholesterol content [1,2,7]. We and others have demonstrated that certain types of SMC, or SMC stimulated by certain factors, metabolize acetylated LDL [8–11]. We found that cultured arterial SMC from thickened intima (intimal SMC) metabolize acetylated LDL, whereas those from normal media (medial SMC) did not [8,11]. As intimal SMC are derived from medial SMC *in vivo*, some factor(s) may induce the medial SMC to acquire the scavenger pathway. Pitas reported that stimulation of rabbit fibroblasts with phorbol esters resulted in expression of the scavenger pathway, although he did not report detailed data [10]. In the present study, using a phorbol ester, phorbol 12-myristate 13-acetate (PMA), we extended our previous studies and those of others on the mechanism of expression of the scavenger pathway.

EXPERIMENTAL

Materials

PMA and other phorbol esters, staurosporine, cycloheximide, phospholipase A₂ from *Naja mocambique* venom, indomethacin, nordihydroguaiaretic acid (NDGA), *p*-bromophenacyl bromide and mepacrin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Lysophospholipids (lyso-PLs) were from Serdary Research Laboratories (London, Canada). H-7 was from Seikagaku Kogyo (Tokyo, Japan). Arachidonic acid was from Nu-Chek Prep (Elysian, MN, U.S.A.). Vitamin E was kindly supplied by Eisai Co. (Tokyo, Japan). 1,1'-Diocadecyl-1,3,3,3',3'-tetramethylindocarbocyanine perchlorate-acetylated LDL (DiI-a-LDL) was from Paesel and Lorei (Frankfurt, Germany). The sources of fetal bovine serum (FBS, Lot no. 37k2305) and other materials for tissue culture were as reported previously [12]. J-774 macrophages were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.).

Tissue culture

Primary cultures of medial SMC were established from the medial layer of the thoracic aorta of male Japanese White rabbits (*Oryctolagus cuniculus* var. *domesticus*) weighing about 2 kg by an explant method [12,13]. Intimal SMC were obtained from thickened intima of the thoracic aorta of rabbits that had

Abbreviations used: SMC, smooth muscle cells; PMA, phorbol 12-myristate 13-acetate; LDL, low-density lipoprotein; NDGA, nordihydroguaiaretic acid; lyso-PL, lysophospholipid; FBS, fetal bovine serum; DME, Dulbecco's modified Eagle's medium; LPDS, lipoprotein-deficient serum; aLDL, acetylated LDL; PGE₂, prostaglandin E₂; M-CSF, monocyte colony stimulating factor; DiI-a-LDL, 1,1'-Diocadecyl-1,3,3,3',3'-tetramethylindocarbocyanine perchlorate-acetylated LDL.

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received a 1% high cholesterol diet for 4 months [12,14]. Cells from primary cultures were subcultured at a density of 1×10^6 /flask in T-75 flasks and grown to confluency (passage 2). Then they were harvested, seeded at a density of 3×10^4 cells/well in 1 ml of Dulbecco's modified Eagle's medium (DME) supplemented with 10% (v/v) FBS in 24-well plates and incubated in a CO₂ (5%) incubator for about 3 days until they reached confluency. These cells were incubated with various reagents for 24 h in 1 ml of DME containing 5% (v/v) human lipoprotein-deficient serum (LPDS). They were then tested for their ability to degrade acetylated LDL. For microscopic studies, cells were seeded into Labtek Chamber Slides (Nunc, Naperville, IL, U.S.A.) at the same density, cultured to subconfluency or confluency and incubated with various factors for 24 h as mentioned above. They were then examined microscopically.

PMA and other phorbol esters, and staurosporine were dissolved in dimethyl sulphoxide. The final concentration of this solvent in the medium for experiments was less than 0.5%. Indomethacin, NDGA, mepacrin, *p*-bromophenacyl bromide, arachidonic acid and vitamin E were dissolved in ethanol. The final concentration of ethanol in media for experiments was also less than 0.5%. Inhibitors were added to the culture 1 h before stimulation with PMA, and the same volume of solvent was added to the control cultures. Lyso-PL was sonicated in distilled water for 5 min.

J-774 macrophages were cultured in DME containing 10% (v/v) FBS.

Assay of acetylated LDL degradation

Degradation and binding of ¹²⁵I-labelled acetylated LDL (¹²⁵I-aLDL) or ¹²⁵I-LDL were assayed as reported [15,16]. Briefly, LDL was obtained from the serum of normal volunteers, and acetylated and iodinated as reported [17,18]. Confluent SMC in 24-well plates were incubated with 5–20 µg/ml of ¹²⁵I-aLDL or 25–150 µg/ml of ¹²⁵I-LDL in 1 ml of DME containing 5% (v/v) LPDS for 24 h and then degradation of ¹²⁵I-aLDL or ¹²⁵I-LDL was measured. For binding assays SMC were incubated with 5–20 µg/ml of ¹²⁵I-aLDL for 2 h.

Microscopic study

Subconfluent SMC or J-774 macrophages in Lab-Tek Chamber Slides were incubated with various factors for 24 h in DME containing 5% (v/v) LPDS and then with 5 µg/ml of DiI-aLDL in fresh DME containing 5% (v/v) LPDS for 12 h. The cells were photographed with an Olympus fluorescence microscope to determine uptake of a-LDL [19].

Other methods

Cell injury was estimated by vital dye (Trypan Blue, 0.15%) uptake and microscopic observation of the detachment of cells. Cellular proteins were measured by the method of Lowry et al. [20] as reported previously [12].

RESULTS

Microscopic studies on the uptake of DiI-a-LDL (photographs not shown)

All J-774 cells took up appreciable DiI-a-LDL, whereas unstimulated medial SMC did not. However, intimal SMC also

took up DiI-a-LDL. Comparison of the fluorescence photographs with light-microscopy photographs showed that all intimal cells became fluorescent. These results are consistent with our previous findings that intimal SMC metabolize ¹²⁵I-a-LDL whereas medial SMC do not [8,11].

Medial SMC stimulated with 1×10^{-8} M PMA for 24 h took up DiI-a-LDL markedly, all cells showing uptake as judged by comparison of fluorescence and light-microscopy photographs.

Dose-dependence of the effect of ¹²⁵I-aLDL on the degradation or binding activity of SMC stimulated with PMA

Medial SMC stimulated with 1×10^{-8} M PMA degraded ¹²⁵I-aLDL dose-dependently, as shown in Figure 1. On the other hand unstimulated medial SMC did not degrade aLDL at all at any of the concentrations tested. These results are consistent with the data on the uptake of DiI-a-LDL. In the following experiments 10 µg/ml of labelled aLDL was used. Binding assays also showed that medial SMC stimulated with 1×10^{-8} M PMA bound ¹²⁵I-aLDL dose-dependently (Figure 1, ○), whereas unstimulated medial SMC bound little ¹²⁵I-aLDL (Figure 1, ●), being consistent with degradation results.

Dose-dependence of the effect of PMA on degradation of ¹²⁵I-aLDL

Medial SMC were incubated with various concentrations of PMA for 24 h and then with 10 µg/ml of ¹²⁵I-aLDL for 24 h. PMA stimulated ¹²⁵I-aLDL degradation by SMC dose-dependently, with a maximal effect at 1×10^{-8} M PMA. The

Figure 1 Concentration-dependence of ¹²⁵I-aLDL on degradation and binding by medial SMC stimulated with PMA or phospholipase A₂ (binding only)

Medial SMC were incubated with 1×10^{-8} M PMA or 1 unit/ml phospholipase A₂ for 24 h and after changing the medium were incubated with various concentrations of ¹²⁵I-aLDL for 24 h or 2 h for assay of degradation (open symbols) or binding activity (closed symbols) respectively. Points and bars for degradation activities are means $\pm$ S.D. for values from four separate cultures. Key to symbols: □, cells not incubated with PMA; ○, cells incubated with PMA. Points for binding activities were means of values from two separate cultures which did not differ from each other by more than 10%. ■, Cells not incubated with PMA or phospholipase A₂; ●, cells incubated with PMA; ▲, cells incubated with phospholipase A₂.

Figure 2 Effects of PMA on degradation of ^{125}I -LDL

Medial SMC were incubated with 1×10^{-8} M PMA for 24 h and after changing the medium were incubated with various concentrations of ^{125}I -LDL for 24 h for assay of degradation activity. Points are means of values from two separate cultures which did not differ from each other by more than 10%. ●, Cells not incubated with PMA; ○, cells incubated with PMA.

Figure 3 Effect of length of stimulation period with PMA on degradation of ^{125}I -aLDL by medial SMC

Medial SMC were incubated with 1×10^{-8} M PMA for the indicated periods and then after changing the medium with $10 \mu\text{g/ml}$ ^{125}I -aLDL as described in the legend of Figure 1. Points and bars are means $\pm$ S.D. of values from three separate cultures.

degradation activities were as follows; without PMA, 0; 1×10^{-10} M PMA, 2.81 ± 0.16 ($n = 3$); 1×10^{-9} M PMA, 2.76 ($n = 2$); 1×10^{-8} M PMA 5.39 ± 0.12 ($n = 3$); 1×10^{-7} M PMA, 0.62 ± 0.28 ($n = 3$) $\mu\text{g/mg}$ of cell protein (mean $\pm$ S.D.). PMA at 1×10^{-7} M had less effect on the degradation activity of SMC,

although it caused no injury of the cells detectable by microscopy or by the Trypan Blue exclusion test. The degradation activity of medial SMC stimulated with 1×10^{-8} M PMA and incubated with $10 \mu\text{g/ml}$ ^{125}I -aLDL ranged from 3 to 6 $\mu\text{g/mg}$ of cell protein in experiments with different samples of medial SMC. In the following experiments 1×10^{-8} M PMA was used for cell stimulation.

Effect of PMA on degradation of ^{125}I -LDL

As shown in Figure 2, unstimulated medial SMC degraded a substantial amount of ^{125}I -LDL. Medial SMC stimulated with 1×10^{-8} M PMA for 24 h increased degradation activity over that of unstimulated SMC only by 10–30 % at 25–150 $\mu\text{g/ml}$ of ^{125}I -LDL. On the other hand, the degradation activity of ^{125}I -aLDL at a concentration of $10 \mu\text{g/ml}$ increased from 0.21 to 2.56 ($\mu\text{g/mg}$ of cell protein) in the same SMC strain. These results suggest that marked induction of lipoprotein metabolism by PMA is specific to the scavenger pathway.

Effect of PMA on degradation of ^{125}I -aLDL as a function of the stimulated period

Incubation of SMC with 1×10^{-8} M PMA for 1–4 h did not enhance degradation activity by SMC. After incubation for 9 h with PMA the activity markedly increased and it reached a plateau after 15 h of incubation (Figure 3). The results suggested that the induction of degradation activity of PMA needs a relatively long period of stimulation and the gene for the scavenger pathway is not a so-called 'early-response gene' to PMA. In the following experiments a 24 h incubation period was used for cell stimulation.

Effect of cycloheximide on degradation of ^{125}I -aLDL stimulated by PMA

To clarify whether protein synthesis is necessary for the induction of degradation activity, SMC were preincubated with cycloheximide. Medial SMC were incubated with 1×10^{-8} M PMA in the presence or absence of $10 \mu\text{g/ml}$ cycloheximide for 24 h. Cycloheximide was added to the culture 1 h before stimulation with PMA. The degradation activities of ^{125}I -aLDL were as follows: without PMA, 0.112 ± 0.044 ($n = 3$); 1×10^{-8} M PMA 3.44 ± 0.28 ($n = 3$); 1×10^{-8} M PMA plus $10 \mu\text{g/ml}$ cycloheximide 0.051 ± 0.026 ($n = 3$) $\mu\text{g/mg}$ of cell protein (mean $\pm$ S.D.). The complete blockage of the PMA effect by cycloheximide suggests that protein synthesis is essential in order to induce the degradation activity.

Long-term effects of PMA

The above results indicated that medial SMC express the so-called scavenger pathway when stimulated with PMA. Next we examined whether this effect was transient or stable by the following two experiments.

First, confluent SMC were stimulated with 1×10^{-8} M PMA for 24 h as described above, and then PMA was removed and the SMC were further cultured for up to 3 days in DME containing 5% (v/v) LPDS. Each day aliquots of cells were assayed for ability to degrade ^{125}I -aLDL. As shown in Figure 4, the degradation activity in SMC stimulated with 1×10^{-8} M PMA remained high (about 5 $\mu\text{g/mg}$ of cell protein) for at least 3 days after removal of PMA.

Secondly, confluent medial SMC stimulated with 1×10^{-8} M PMA for 24 h were subcultured at a 1:2 split ratio and grown to

Figure 4 Effect of long-term culture on degradation of ^{125}I -aLDL

Medial SMC were incubated with 1×10^{-8} M PMA for 24 h, washed to remove PMA, and cultured for 1–3 days. Each day cells were assayed for degradation activity. Points and bars are means $\pm$ S.D. of values from three separate cultures.

Figure 5 Degradation of ^{125}I -aLDL by medial SMC stimulated with 1×10^{-8} M PMA for 24 h and then subcultured three times

See the legend for Figure 1 and the text for details. Points and bars are means $\pm$ S.D. of values from three separate cultures.

confluency in DME containing 10% (v/v) FBS in the absence of PMA. This process was repeated three times. At each passage, the scavenger pathway was examined by measuring DiI-a-LDL uptake. Results showed that SMC stimulated with PMA took up DiI-a-LDL at passages 1–3 after PMA removal (results not shown). Control cultures did not take up DiI-a-LDL. Figure 5 shows quantitative results of the assay of degradation of ^{125}I -

Table 1 Effects of various inhibitors on degradation of ^{125}I -labelled acetylated LDL in medial SMC

Medial SMC were incubated with PMA for 24 h in the presence or absence of various inhibitors and then their activity was measured. Inhibitors were added to the culture 1 h before PMA stimulation. Degradation activities are shown as percentages of that with PMA alone (100%) and are means of the values for two or three separate cultures, which did not differ from each other by more than 10%.

Treatment	Concentration	Relative activity (%)
PMA alone	1×10^{-8} M	100
PMA + PKC inhibitors		
H-7	10 μM	105
25 μM		105
Staurosporine	0.1 μM	110
1.0 μM		95
PMA + phospholipase A_2 inhibitors		
Mepacrin	1 μM	69
10 μM		51
<p>-Bromophenacyl bromide</p>	1 μM	72
10 μM		55
25 μM		52
Vitamin E	1 μM	91
10 μM		107
25 μM		61
50 μM		53
100 μM		53
PMA + cyclooxygenase inhibitor		
Indomethacin	10 μM	114
PMA + lipoxygenase inhibitor		
Nordihydroguaiaretic acid	5 μM	93
10 μM		96

aLDL as a function of the concentration of ^{125}I -aLDL in SMC stimulated with PMA and subcultured three times after its removal. Even after three passages in the absence of PMA, these SMC still degraded ^{125}I -aLDL (about 4 $\mu\text{g}/\text{mg}$ of cell protein with 10 $\mu\text{g}/\text{ml}$ ^{125}I -aLDL). During these cultures the SMC were estimated to divide at least three times. The results indicate that expression of the scavenger pathway in medial SMC stimulated with PMA was not transient, but stable for at least 3 days or three passages after PMA removal.

Effects of inhibitors of protein kinase C (PKC) or phospholipase A_2

Next the mechanism of expression of the scavenger pathway was investigated. As PMA is known to stimulate PKC [21], we tested the effects of the putative PKC inhibitors H-7 and staurosporine [22,23] on PMA induction of the scavenger pathway in SMC. As shown in Table 1, these inhibitors, at concentrations up to 25 μM and 1 μM respectively, had little effect on the degradation of ^{125}I -aLDL in SMC stimulated with 1×10^{-8} M PMA. H-7 at above 50 μM and staurosporine at above 10 μM were toxic to the cells, as judged by microscopic observations of cell detachment and the Trypan Blue exclusion test. These results were confirmed by fluorescence-microscopy observations of the uptake of DiI-a-LDL by SMC, using 1, 10, 25, 50, and 100 μM concentrations of H-7 and 0.01, 1.0, 10 and 50 μM staurosporine (results not shown). These results raised the possibility that PKC may not be related to the expression of the scavenger pathway in medial SMC induced by PMA.

PMA is also known to stimulate phospholipase A_2 [24–26]. Addition of phospholipase A_2 inhibitors, such as mepacrin (1–10 μM) [27], *p*-bromophenacyl bromide (1–25 μM) [28], and high concentrations of vitamin E (25–100 μM) [29] to cultures

Table 2 Effects of various phorbol esters on PGE₂ synthesis and degradation of ¹²⁵I-aLDL by medial SMC

Medial SMC were incubated with PMA (1×10^{-9} M– 1×10^{-7} M) or other phorbol esters (1×10^{-8} M) for 24 h in 5% (v/v) LPDS/DME. The medium was collected and PGE₂ content was measured. After changing the medium the cells were incubated with 10 μ g/ml ¹²⁵I-aLDL for 24 h for assay of degradation activity. Values are means of values from three separate cultures, which did not differ from each other by more than 15%.

Experimental group	PGE ₂ (pg/well)	Degradation of ¹²⁵ I-aLDL (μ g/mg of protein)	PKC activation
Control	2340	0	—
PMA (1×10^{-9} M)	14120*	0.84	+
PMA (1×10^{-8} M)	87100*	3.44	+
PMA (1×10^{-7} M)	8550*	0.72	+
Phorbol 12,13-didecanoate	75500*	3.46	+
Phorbol 12,13-dibutyrate	3300	0	+
Phorbol 12,13-dibenzoate	2750	0	+
4 α -Phorbol 12,13-didecanoate	2230	0	—
Phorbol 13-monoacetate	3880*	0.20	—
Phorbol 12-monomyristate	4320*	0.25	—

* Significantly increased ($P < 0.05$ versus control).

Figure 6 Effect of phospholipase A₂ from venom on degradation of ¹²⁵I-aLDL by medial SMC

Medial SMC were incubated with venom phospholipase A₂ for 24 h and then with 10 μ g/ml ¹²⁵I-aLDL as described in the legend for Figure 1. Points and bars are means $\pm$ S.D. of values in three separate cultures.

1 h before stimulation of medial SMC with PMA resulted in about 50% inhibition of the degradation of ¹²⁵I-aLDL induced by 1×10^{-8} M PMA. Higher concentrations of mepacrin and *p*-bromophenacyl bromide were toxic, as judged by microscopic observations of cell detachment and the Trypan Blue exclusion test. The effects of vitamin E at 50 and 100 μ M were almost the

same. Vitamin E at 1–10 μ M, which is sufficient for antioxidant activity in SMC [30,31], had little effect, suggesting that vitamin E acted as a surfactant in this experiment. These results suggest that expression of the scavenger pathway in medial SMC induced by PMA is partly mediated by phospholipase A₂.

Effects of various phorbol esters on phospholipase A₂ reaction and degradation of ¹²⁵I-aLDL by medial SMC

To confirm the above conclusion, we used various kinds of phorbol esters with or without potencies to activate phospholipase A₂ or PKC in medial SMC. Prostaglandin E₂ (PGE₂) synthesis was used as an indicator of phospholipase A₂ reaction. Two phorbol esters with potency to activate PKC, PMA and phorbol 12,13-didecanoate [32], also strongly stimulated both PGE₂ synthesis and degradation activities of ¹²⁵I-aLDL in medial SMC. But two phorbol esters with the same potency to activate PKC, phorbol 12,13-dibutyrate and phorbol 12,13-dibenzoate [32], did not stimulate PGE₂ synthesis and failed to induce the scavenger pathway. Two phorbol esters without potency to activate PKC, phorbol 13-monoacetate and phorbol 12-monomyristate [32], stimulated both PGE₂ synthesis and degradation activities of ¹²⁵I-aLDL although the stimulation was weaker than that by PMA and phorbol 12,13-didecanoate. A phorbol ester, 4 α -phorbol 12,13-didecanoate, did not stimulate PGE₂ synthesis nor degradation activities of ¹²⁵I-aLDL. These results clearly show that induction of the scavenger pathway in medial SMC is well correlated with activation of phospholipase A₂ but not that of PKC. Moreover, the correlation was also observed when medial SMC were stimulated with various concentrations of PMA (Table 1).

Effects of phospholipase A₂ and lysophosphatidylcholine (lyso-PC)

The above data indicate the importance of phospholipase A₂ in expression of the scavenger pathway. We next tested the effects of exogenous *Naja* venom phospholipase A₂ and its product, lyso-PC. The phospholipase A₂ used was active against phospholipids of SMC as incubation of SMC with 1 unit/ml of phospholipase A₂ for 24 h resulted in an increase of PGE₂ synthesis from 2340 to 13850 pg/well (as with PMA; Table 2). Stimulation of medial SMC for 24 h with 0.1–10 units/ml of phospholipase A₂ increased the degradation activities (Figure 6). The binding activity of ¹²⁵I-aLDL was also increased in medial SMC by stimulation with 1 unit/ml of phospholipase A₂ (Figure 1, $\blacktriangle$). The effect on degradation activity was low at 20 units/ml. This was not due to toxicity of a high concentration of phospholipase A₂, as judged by microscopic observation of cell detachment and the Trypan Blue exclusion test. Stimulation of medial SMC with lyso-PC at concentrations of between 10 ng/ml and 10 μ g/ml also increased the degradation activity dose-dependently (Figure 7). Lyso-PC at 100 ng/ml had less effect, probably not due to its toxicity, as judged by microscopic observation of cell detachment and the Trypan Blue exclusion test. Its maximal effect was, however, about one-third of that of 1×10^{-8} M PMA.

Figure 8 shows specificity of various lyso-PL. Lyso-PC stimulated degradation activity of SMC in a similar manner, as shown in Figure 7. However, lysophosphatidylinositol or lysophosphatidylserine showed little effect on the activity. Lysophosphatidylethanolamine slightly stimulated the activity at 1 μ g/ml. These results indicate that induction of degradation activity in SMC by lyso-PL was specific to lyso-PC.

Addition of a cyclo-oxygenase inhibitor, 10 μ M indomethacin, or a lipoxygenase inhibitor, 5–10 μ M NDGA, to the cultures 1 h before stimulation of medial SMC with 1×10^{-8} M PMA had

Figure 7 Effect of lyso-PC on degradation of ^{125}I -aLDL by medial SMC

Medial SMC were incubated with various amounts of lyso-PC for 24 h and then with 10 $\mu\text{g/ml}$ ^{125}I -aLDL as described in the legend for Figure 1. Points and bars are means $\pm$ S.D. of values in three separate cultures.

Figure 8 Substrate specificity of lyso-PL for stimulation of degradation of ^{125}I -aLDL

Medial SMC were incubated with various kinds of lyso-PL for 24 h and then with 10 $\mu\text{g/ml}$ ^{125}I -aLDL as described in the legend for Figure 1. Points are means for values of two separate cultures. Values were not different from each other by more than 10%. ○, Control (no addition); ●, lyso-PC; ■, lysophosphatidylethanolamine; ▼, lysophosphatidylinositol; ▲, lysophosphatidylserine.

little influence on degradation activity (Table 1) or on the uptake of DiI-a-LDL, compared with the effect of PMA alone (results not shown), indicating that neither cyclo-oxygenase- nor lipox-

genase products of released arachidonic acid, such as PGE_2 , prostaglandin I_2 and hydroxyeicosatetraenoic acids are mediators of expression of the scavenger pathway. Moreover, stimulation of medial SMC with 1, 10 and 50 μM arachidonic acid did not induce the scavenger pathway (results not shown). Taken together, these data suggest that the phospholipase A_2 reaction is important for expression of the scavenger pathway, and that a product, lyso-PC, is a mediator of expression of this pathway.

DISCUSSION

Previously we have shown expression of the scavenger pathway by intimal SMC, and also their expression of a proto-oncogene, *c-fms* [11], which is the gene for a receptor of the monocyte colony stimulating factor (M-CSF) [33], and found that their expression of the scavenger pathway increased in response to M-CSF [11]. On the other hand, *c-fms* was not expressed in cultured SMC from the normal media (medial SMC) and the scavenger pathway was not expressed on stimulation with M-CSF [11]. Intimal SMC are derived from medial SMC *in vivo*. Therefore, some factors must be responsible for expression of the scavenger pathway. The above data indicate that M-CSF itself is not the factor responsible.

In the present study the scavenger pathway was expressed by PMA stimulation in medial SMC, which otherwise do not, or only slightly, express it [8,11]. PMA is not a physiological substance, but it was used for convenience in this study to elucidate the mechanism of activation. It is known to be a PKC stimulator [21] and expression of the scavenger pathway in rabbit fibroblasts has been suggested to be through activation of PKC because another phorbol ester that stimulates PKC induced expression of the scavenger pathway, whereas a phorbol ester that did not had no effect [10]. However, our results with PKC inhibitors such as H-7 or staurosporine suggested that the effect of PMA was not due to activation of PKC. Recently various isoenzymes of PKC have been reported such as PKC α , β I/II, γ (conventional PKC) and PKC δ , ϵ , η , θ , ζ , λ (novel PKC) in a variety of cells and tissues including SMC [34,35]. The relationship between types of isoenzymes and function for signal transduction is not well understood. PMA stimulates both conventional PKC and novel PKC (δ , ϵ) [36–39]. H-7 and staurosporine are effective inhibitors against both conventional PKC and novel PKC (δ , ϵ) [40]. According to these reports, it is highly possible that all kinds of PKC activities stimulated by PMA were blocked by H-7 or staurosporine in the present study. However, there is a limitation on the interpretation of the data using these inhibitors. These inhibitors could inhibit other protein kinases such as S6 kinase, phosphorylase kinase and *src*-kinase [41], and the conclusion that PKC is not involved in expression of the scavenger pathway in SMC can not necessarily be reached if such kinases have a role in downregulation of the pathway. In spite of such limitations, the conclusion is possible because expression of the scavenger pathway was not correlated with potency of PKC activation by various phorbol esters (Table 2).

Therefore, we focused attention on the phospholipase A_2 reaction as PMA is also known to be a potent stimulator of phospholipase A_2 , resulting in release of arachidonic acid from membrane phospholipids and formation of prostanoids such as PGE_2 and prostaglandin I_2 [24,25]. Moreover, PMA stimulates the phospholipase A_2 reaction in a cell-free vesicle system without PKC, which clearly shows that stimulation of the phospholipase A_2 action by PMA is not due to activation of PKC [26]. The importance of the phospholipase A_2 reaction in the expression of the scavenger pathway in medial SMC was shown by the present finding that inhibitors of phospholipase A_2 blocked the ex-

pression of the scavenger pathway induced by PMA, exogenous phospholipase A₂ had a similar effect to that of PMA and expression of the scavenger pathway correlated well with phospholipase A₂ action. Moreover, a product of the phospholipase A₂ reaction, lyso-PC, had a similar effect to those of PMA and phospholipase A₂, suggesting that this product itself was the mediator of expression of the scavenger pathway. Other metabolites of arachidonic acid, such as cyclo-oxygenase- or lipoxygenase-products (eicosanoids), are not likely to be mediators because neither indomethacin nor NDGA inhibited expression of the scavenger pathway. Two types of phospholipase A₂, the 14 kDa secretory type (type II) and the 85 kDa arachidonic-acid-preferring phospholipase A₂ have been reported in mammalian cells [42]. Exogenous *Naja* venom phospholipase A₂ is structurally closely related to type II and was actually active against SMC in the present study. It is not known which type of phospholipase A₂ is activated by PMA. Both types of enzymes could be activated by PMA according to data in other cell types [43]. If type-II phospholipase A₂, which is not arachidonic acid specific, is important for expression of the scavenger pathway in SMC the possibility cannot be excluded that fatty acids other than arachidonic acid or their metabolites are mediators besides lyso-PC.

The present study suggests that the source of the lyso-PC that stimulates expression of the scavenger pathway is not only endogenous. Phospholipase A₂ activity is associated with LDL [43] and its activation plays an important role in oxidation of LDL, probably by releasing peroxidized free fatty acids from the 2-position of LDL-PC and thus stimulating further free-radical-initiated peroxidation by favouring propagation reactions [43]. These processes generate lyso-PC derived from LDL, which may interact with SMC in the intima derived from the media and express the scavenger pathway. The expression of this pathway may play some role in scavenging the oxidized LDL, the formation of which triggers expression of the scavenger pathway, because oxidized LDL may be an *in vivo* ligand of scavenger receptors [44]. If so, expression of the scavenger pathway seems reasonable and purposeful.

Lyso-PC has membrane-perturbing properties and interacts with many membrane-bound enzymes including Na,K-ATPase, the sodium pump by PKC, guanylate cyclase and adenylate cyclase [45–49]. However, it is unknown whether the effect of lyso-PC is through one of these known interactions. Further studies are needed to elucidate these points.

REFERENCES

- Goldstein, J. L. and Brown, M. S. (1977) *Annu. Rev. Biochem.* **46**, 897–930
- Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russel, D. W. and Schneider, W. J. (1985) *Annu. Rev. Cell Biol.* **1**, 1–39
- Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P. and Krieger, M. (1990) *Nature (London)* **343**, 531–535
- Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 333–337
- Nagelkerke, J. F., Barto, K. P. and van Berkel, T. J. C. (1983) *J. Biol. Chem.* **258**, 12221–12227
- Iami, H., Lee, K. T., Pastori, S., Panlilio, E., Florentin, R. and Thomas, W. A. (1966) *Exp. Mol. Pathol.* **5**, 273–310
- Brown, M. S. and Goldstein, J. L. (1976) *Science* **191**, 150–154
- Saito, Y., Bujo, H., Morisaki, N., Shirai, K. and Yoshida, S. (1988) *Atherosclerosis* **69**, 161–164
- Jaakkola, O., Kallioniemi, O.-P. & Nikkari, T. (1988) *Atherosclerosis* **69**, 257–268
- Pitas, R. E. (1990) *J. Biol. Chem.* **265**, 12722–12727
- Inaba, T., Gotoda, T., Shimano, H., Shimada, M., Kadowaki, T., Momomura, K., Motoyoshi, K., Tsukada, T., Morisaki, N., Saito, Y., Yoshida, S., Yazaki, Y., Takaku, F. and Yamada, N. (1992) *J. Biol. Chem.* **267**, 5693–5699
- Morisaki, N., Kanzaki, T., Fujiyama, Y., Osawa, I., Shirai, K., Matsuoka, N., Saito, Y. and Yoshida, S. (1985) *J. Lipid Res.* **26**, 930–939
- Fischer-Dzoga, K., Jones, R. M., Vesselinovitch, D. and Wissler, R. W. (1973) *Exp. Mol. Pathol.* **18**, 162–176
- Morisaki, N., Shinomiya, M., Mizobuchi, M., Fujiyama, Y., Sasaki, N., Shirai, K., Matsuoka, N., Saito, Y. and Yoshida, S. (1982) *Artery* **11**, 225–237
- Mori, S., Morisaki, N., Saito, Y. and Yoshida, S. (1989) *Arteriosclerosis* **9**, 644–649
- Mori, S., Yodote, K., Morisaki, N., Saito, Y. and Yoshida, S. (1990) *Eur. J. Clin. Invest.* **20**, 137–142
- Basu, S. K., Goldstein, J. L., Anderson, R. G. W. and Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3178–3182
- McFarlane, A. S. (1958) *Nature (London)* **182**, 53
- Voyta, J. C., Via, D. P., Butterfield, C. E. and Zetter, B. R. (1984) *J. Cell Biol.* **99**, 2034–2040
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
- Shibanuma, M., Kuroke, T. and Kiyose, N. (1987) *Biochem. Biophys. Res. Commun.* **144**, 1317–1323
- Tamaoka, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402
- Ohuchi, K. and Levine, L. (1978) *J. Biol. Chem.* **253**, 4783–4790
- Morisaki, N., Tomei, L. D., Milo, G. and Cornwell, D. G. (1985) *Lipids* **20**, 602–610
- Nam, K.-Y., Morino, A., Kimura, S., Fujiki, H. and Imanishi, Y. (1990) *Biochem. J.* **268**, 169–173
- Marcus, H. B. and Ball, E. G. (1969) *Biochim. Biophys. Acta* **187**, 486–491
- Volwerk, J. J., Pieterse, W. A. and Dehaas, G. H. (1974) *Biochemistry* **13**, 1446–1454
- Panganamala, R. V. and Cornwell, D. G. (1982) *Ann. N.Y. Acad. Sci.* **393**, 376–390
- Gavino, V. C., Miller, J. S., Ikharabha, S. O., Milo, G. E. and Cornwell, D. G. (1981) *J. Lipid Res.* **22**, 763–769
- Morisaki, N., Lindsey, J. A., Stitts, J. M., Zhang, H. and Cornwell, D. G. (1984) *Lipids* **19**, 381–394
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
- Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T. and Stanley, E. R. (1985) *Cell* **41**, 665–676
- Ohno, S., Akita, Y., Hata, A., Osada, S., Kubo, K., Konno, Y., Akimoto, K., Mizuno, K., Saido, T. C., Kuroki, T. and Suzuki, K. (1991) *Adv. Enzyme Regul.* **31**, 287–303
- Nishizuka, Y. (1988) *Science* **258**, 607–614
- Konno, Y., Ohno, S., Akita, Y., Kawasaki, H. and Suzuki, K. (1989) *J. Biochem. (Tokyo)* **106**, 673–678
- Mizuno, K., Kubo, K., Saido, T. C., Akita, Y., Osada, S., Kuroki, T., Ohno, S. and Suzuki, K. (1991) *Eur. J. Biochem.* **202**, 931–940
- Saido, T. C., Mizuno, K., Konno, Y., Osada, S., Ohno, S. and Suzuki, K. (1992) *Biochemistry* **31**, 482–490
- Koide, H., Ogita, K., Kikkawa, U. and Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1149–1153
- Mizuno, K., Saido, T. C., Ohno, S., Tamaoki, T. and Suzuki, K. (1993) *FEBS Lett.* **330**, 114–116
- Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marm, D. and Schchtele, C. (1993) *J. Biol. Chem.* **268**, 9194–9197
- Mayer, R. J. and Marshall, L. A. (1993) *FASEB J.* **8**, 339–348
- Parthasarathy, S., Steinbrecher, U. P., Barnett, J., Witztum, J. L. and Steinberg, D. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3000–3004
- Aria, H., Kita, T., Yokode, M., Narmiya, S. and Kawai, C. (1989) *Biochem. Biophys. Res. Commun.* **159**, 1375–1382
- Weltzien, H. (1979) *Biochim. Biophys. Acta* **559**, 259–287
- Oishi, K., Zheng, B. and Kuo, J. F. (1990) *J. Biol. Chem.* **265**, 70–75
- Shier, W. T., Baldwin, J. H., Nilsen-Hamilton, M., Hamilton, R. T. and Thanassi, N. M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1586–1590
- Kolesnick, R. N. and Paley, A. E. (1987) *J. Biol. Chem.* **262**, 9204–9210
- Matsumoto, A., Naito, M., Itakura, H., Ikemoto, S., Asaoka, H., Hayakawa, I., Kanamori, H., Aburatani, H., Takaku, F., Suzuki, H., Kobara, Y., Miyai, T., Takahashi, K., Cohen, E., Wydro, R., Housman, D. E. and Kodama, T. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9133–9137