Post-transcriptional regulation of apolipoprotein E expression in mouse macrophages by phorbol ester

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Phorbol ester-mediated differentiation of THP-1 cells (a human monocytic cell line) into mature macrophages is associated with a transcriptional induction of apolipoprotein E (apoE) expression [Auwerx, Deeb, Brunzell, Peng and Chait (1988) *Biochemistry* **27**, 2651–2655]. Endotoxin, on the other hand, which may also act through activation of protein kinase C, is a potent inhibitor of apoE expression in mouse macrophages [Werb and Chin (1983) *J. Biol. Chem.* **258**, 10642–10648]. The present experiments examine the effect of phorbol ester, an activator of protein kinase C, on the apoE expression in mouse thioglycollate-elicited peritoneal macrophages. Phorbol ester inhibits apoE expression in a specific, time- and dose-dependent manner. A 75% inhibition in the rate of apoE secretion, but not that of total protein, was observed following a 4.5 h incubation with 160 nM phorbol ester, although nearly full inhibition was obtained with 40 nM. The changes in apoE secretion were paralleled by similar changes in apoE synthesis, indicating synthesis as the primary site of

action. The decreased rates of apoE synthesis are shown not to be due to increased apoE degradation. The profound inhibition of apoE synthesis was not accompanied by significant changes in apoE mRNA levels at any concentration of phorbol ester (up to 16 μ M), or length of treatment (up to 24 h), suggesting a post-transcriptional locus of regulation of apoE expression. Although the early changes in apoE synthesis correlate with increased microsomal protein kinase C activity, the suppression of apoE expression persists even during conditions of nearly complete (> 95%) loss of protein kinase C activity, suggesting that the direct or indirect effect of protein kinase C on apoE expression is mediated by a stable phosphorylated protein, or that the observed effects are mediated through a protein kinase C species that is not readily downregulated by phorbol esters. The presented studies clearly demonstrate the potential importance of the translational regulation of apoE expression through the protein kinase C signal transduction pathway.

INTRODUCTION

Apolipoprotein E (apoE) is an abundant constituent of circulating plasma lipoproteins [1]. It serves as a recognition ligand for the receptor-mediated removal of cholesterol-rich lipoproteins by the low-density lipoprotein (LDL) or chylomicron remnant receptors [2–4]. Although the liver is the major source of circulating apoE [5], a number of other tissues, including macrophages, adipocytes, adrenal glands, kidneys, Ito cells, brain and thymus have been shown to synthesize apoE [6–10]. ApoE derived from peripheral tissues may function in reverse cholesterol transport [11,12] and in mediating localized transport of cholesterol during repairs in areas of tissue injury [13,14]. Intracellular retention of a variable but substantial amount of apoE and its specific organelle localization is also suggestive of an intracellular function of apoE [15].

The regulation of apoE expression is not fully understood. Sterol- or differentiation-induced transcriptional up-regulation of apoE expression in hepatocytes, macrophages and adipocytes is well documented [10,16–18]. In addition, recent work in this laboratory has demonstrated post-transcriptional regulation of apoE secretion by high-density lipoprotein₃ (HDL₃); HDL₃ is able to redirect a substantial portion of apoE from intracellular degradation to a secretory pathway [18]. Endotoxin (a lipopolysaccharide from Gram-negative bacteria), on the other hand, is a potent inhibitor of apoE secretion by macrophages [19]. The nature of the interaction of endotoxin, or of lipid A (the active component of endotoxin [20]), with cells is not clear, but specific saturable binding to proteinase-sensitive sites on

macrophage membranes has been demonstrated [21]. Endotoxin treatment initiates enhanced phosphatidylinositol bisphosphate hydrolysis, inositol 1,4,5-trisphosphate (IP₃) production, Ca²⁺ influx and a pattern of protein phosphorylation similar to that induced by phorbol esters [22], known activators of protein kinase C (PKC) [23]. The potentially inhibitory effects of PKC activation on apoE expression in endotoxin-treated cells is in contrast to the effects of phorbol ester treatment (and activation of PKC) used to induce differentiation of THP-1, a human monocytic cell line isolated from a patient with acute monocytic leukaemia [24]. Exposure of these cells to phorbol 12-myristate 13-acetate (PMA) is accompanied by progressive induction of apoE expression (increases in both apoE mRNA and apoE secretion [25–27]). In the light of this discrepancy, and of the increased use of this cell line to study the expression of various genes, it was of interest to examine the effect of phorbol ester on apoE expression in primary murine macrophages. The results demonstrate that the effect of phorbol ester in primary macrophages is opposite to that seen in THP-1 cells; PMA induces a profound inhibition of apoE expression in primary macrophages at a post-transcriptional locus.

EXPERIMENTAL

Macrophage isolation and culture

Mouse peritoneal macrophages were collected from male Swiss-Webster mice (14–18 g; Harlan) 4 days after an intraperitoneal injection of 1.5 ml of sterile 4% thioglycollate broth. Cells were harvested and cultured as previously described

[28]. On the second day of culture, the cells were routinely incubated with acetylated LDL (acLDL) (100 $\mu\text{g/ml}$) for 24 h in order to load them with cholesterol. In some experiments cells were treated with 25-hydroxycholesterol (0.5 $\mu\text{g/ml}$) or plain Dulbecco's modified Eagle's medium (DMEM) instead of acLDL; these treatments did not change the cellular sterol content or distribution, as previously shown [18]. Following the loading period, the cells were washed and incubated in DMEM for an additional 24 h period prior to the experiments. When indicated, stock solutions of PMA in ethanol (160 μM or 16 mM) were used to obtain the indicated final PMA concentrations. Equivalent volumes of ethanol alone were used in control wells and never exceeded 0.1% of the total volume (2 μl /2 ml of medium).

Labelling studies

Apparent rates of total protein and apoE synthesis and secretion were determined, as previously described [18], using labelling media composed of methionine-free DMEM (Gibco) supplemented with [^{35}S]methionine (50 $\mu\text{Ci/ml}$). Cells were pre-incubated in methionine-free DMEM for 10 min before addition of the labelling media. Briefly, apparent rates of secretion were determined by long-term labelling experiments (1.5 or 3 h labelling period), followed by immunoprecipitation of medium apoE [18] and trichloroacetic acid precipitation of an aliquot of total medium proteins. Previous work in this laboratory demonstrated linear incorporation of isotope into apoE and other proteins for over 5 h [18]. Apparent rates of synthesis were initially determined (1) from the slope of the linear portion of isotope incorporation into cellular apoE and total protein, and subsequently (2) by the extent of isotope incorporation into cellular apoE following a 30 min labelling period (at this time, the amount of isotope incorporated is proportional to the rate of synthesis, as shown below). In some experiments a pulse-chase design was used: cells were pulsed with the labelling medium for 60 min and chased in regular DMEM for 0, 20, 40 and 80 min. Cellular and medium apoE recoveries at each time point were determined by immunoprecipitation. Apparent rates of total protein synthesis and secretion were determined by trichloroacetic acid precipitation of an aliquot of cell lysate or medium respectively on a Whatman #5 filter paper disc, followed by extensive washes in ice-cold 10% trichloroacetic acid and then washes in 100% ethanol and 100% ether. Dried filter paper discs were counted for radioactivity by scintillation spectroscopy. ApoE immunoprecipitates were resolved by SDS/PAGE, and the apoE band was identified, cut out, solubilized and subjected to scintillation counting as previously described [18]. In most experiments the immunoprecipitates were also quantified by densitometric scanning of the fluorographs of SDS gels.

Media collection and cell lysis were performed as previously described [18].

Isolation of RNA and Northern blot analysis

Total RNA was extracted by the method of Chomczynski and Sacchi [29]. Various amounts of total RNA (1–2 μg) were separated by agarose electrophoresis using formaldehyde-containing 1.2% agarose. The separated RNA was probed with nick-translated ^{32}P -labelled probes for apoB [30] and β -actin [31], as previously described [18]. The autoradiographs were evaluated by densitometric scanning, and changes in apoE mRNA were normalized to the levels of β -actin mRNA in the same sample.

Assay of PKC activities

PKC activities were assayed using reagents supplied in an assay kit (Gibco BRL; cat. no. 3161SA). The assay is based on the phosphorylation of an acetylated myelin basic protein peptide (amino acids 4–14), a specific substrate for PKC [32]. A peptide from a pseudosubstrate region of PKC (amino acids 19–36) was used as a specific inhibitor of PKC [32]. Macrophages were homogenized in an extraction buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA and 25 $\mu\text{g/ml}$ each of aprotinin and leupeptin) and fractionated into cytosolic and microsomal fractions by centrifugation (100 000 g for 30 min). Membrane-bound PKC was solubilized by treatment of the microsomal fractions with a Triton X-100-containing extraction buffer (0.5% final concentration), prior to DEAE affinity chromatography (Whatman DE52). PKC was eluted with the extraction buffer containing 0.2 M NaCl and 10 mM β -mercaptoethanol, and assayed according to the manufacturer's instructions.

Analytical procedures

Protein determinations were carried out according to Lowry et al. [33]. SDS/PAGE was by the method of Laemmli [34]. acLDL was prepared as previously described [18]. Media components, including DMEM with or without methionine, fetal bovine serum, antibiotics and phosphate-buffered saline, were obtained from Gibco. All other chemicals (including PMA and other phorbols) were obtained from Sigma. Trans- ^{35}S -label ($^{\text{L}}\text{[}^{35}\text{S]methionine/L-[}^{35}\text{S]cysteine}$; sp. radioactivity > 1000 Ci/mmol) and [α - ^{32}P]dCTP (sp. radioactivity $\sim$ 3000 Ci/mmol), for the labelling experiments and nick-translations respectively, were obtained from ICN. [γ - ^{32}P]ATP (sp. radioactivity 3000 Ci/mmol) for PKC assays was obtained from NEN.

RESULTS

Effect of phorbol ester on apoE secretion

Induction of differentiation of THP-1 cells by prolonged exposure to PMA (160 nM) is accompanied by a profound increase in apoE expression [25,26]. It was therefore of interest to see the effect of a similar treatment on apoE expression in cholesterol-loaded primary macrophages. Table 1 shows the apoE secretion by control macrophages, macrophages treated with 160 nM PMA for 24 h and macrophages treated with HDL₃ during the administration of the labelling medium (as a positive control). ApoE secretion was assessed by switching all cells to a labelling medium of appropriate composition during the last 3 h of

Table 1 ApoE secretion by mouse peritoneal macrophages

Cholesterol-loaded macrophages were incubated for 21 h in the absence (control and HDL₃) or presence (PMA) of 160 nM PMA. At the end of this period all cells were switched to labelling media of identical composition for an additional 3 h. One of the groups (HDL₃) also received at this time HDL₃ (50 $\mu\text{g/ml}$) in the labelling medium. ApoE secretion was quantified by immunoprecipitation, as described in the Experimental section. Each value represents an average $\pm$ S.E.M. of three experiments of six wells each, assayed individually.

Group	ApoE secretion	
	(c.p.m./3 h per mg)	(% of control)
Control	284 000 $\pm$ 34 000	100 $\pm$ 12
PMA (160 nM for 24 h)	64 000 $\pm$ 7000	23 $\pm$ 2
HDL ₃ (0.5 μM for 3 h)	770 000 $\pm$ 66 000	271 $\pm$ 11

Figure 1 Time- and dose-dependent inhibition of apoE secretion by macrophages

Cells were incubated with 160 nM PMA for indicated time periods, and switched to the labelling medium for the last 1.5 h of incubation. ApoE secretion is expressed as a percentage of that in the absence of PMA (a), or as a percentage of total secreted protein (b). ApoE secretion by cells incubated with various concentrations of PMA for 3 h is shown in (c). ApoE secretion was quantified as described in the Experimental section. Each point in the panels represents an average $\pm$ S.E.M. of two experiments, with six individual wells for each time point.

incubation. As previously reported [18], addition of HDL₃ to cholesterol-loaded macrophages stimulates a nearly 3-fold increase in apoE secretion. In contrast, treatment of macrophages with the phorbol ester for 24 h resulted in a profound (> 75%) inhibition of apoE secretion.

The time course and dose-dependence of the PMA-mediated inhibition of apoE secretion are shown in Figure 1. As shown in Figure 1(a), the effect of 160 nM PMA on apoE secretion is rapid and powerful: a 25% inhibition was observed after a 1.5 h exposure. Maximal inhibition (~75%) was achieved after 4.5–7 h of exposure to PMA. This level of inhibition persisted for up to 24 h of PMA treatment, as shown in Table 1. The effect of PMA on apoE secretion is specific (Figure 1b). PMA at this concentration had little effect on total protein secretion (a slight stimulation of total protein secretion was observed; results not shown). This is reflected on expressing apoE secretion as a percentage of total protein secretion. Under normal conditions, apoE represented $15 \pm 3\%$ of the proteins secreted by cholesterol-loaded macrophages; treatment with PMA reduced the contribution of apoE to 4% or less. The extent of inhibition of apoE secretion by PMA is also dose-dependent, as shown in Figure 1(c). Cholesterol-loaded macrophages were treated with indicated amounts of PMA for 3 h, and the extent of apoE secretion was determined by immunoprecipitation of [³⁵S]apoE. The full extent of inhibition (50% for this length of treatment) was achieved with a PMA concentration of 80 nM; a nearly full inhibition was observed at 40 nM. Qualitatively similar results were obtained in cells not loaded with cholesterol or cells treated with 25-hydroxycholesterol.

Effect of phorbol ester on apoE synthesis

The experiments presented above established that PMA specifically inhibits apoE secretion. Since previous studies in this laboratory have shown that secretory rates can vary widely without any changes in synthetic rates [18], it was important to establish the effects of PMA on rates of apoE synthesis. The initial approach was to establish apparent rates of apoE synthesis by examining the extent of [³⁵S]methionine incorporation into

Figure 2 Time course of [³⁵S]methionine incorporation into cellular apoE in control (○) and PMA-treated (●) cells

PMA-treated cells were pre-incubated with 160 nM PMA for 1 h prior to the addition of the labelling media. At indicated times, cells were washed with ice-cold saline and lysed as described in the Experimental section. Cell lysates were used for immunoprecipitation of apoE. The 30 min time point was used for all subsequent experiments to estimate relative synthetic rates for apoE. The extent of apoE labelling at this point is proportional to the slope of the linear portion of label incorporation into apoE. Each point represents an average $\pm$ S.E.M. of two experiments, with six individual wells for each time point.

cellular immunoprecipitable apoE at various time points after isotope administration. The results of these experiments are shown in Figure 2. Isotope incorporation into immunoprecipitable apoE was linear for at least 30 min in both control and PMA-treated cells. The apparent rate of apoE synthesis in PMA-treated cells (pretreated with PMA at 160 nM for 1 h prior to isotope administration) was one-half of that observed in untreated control cells. The lower plateau in the extent of apoE labelling achieved in PMA-treated cells is a reflection of the decreased apoE pool size in these cells. The extent of isotope

Figure 3 Time- and dose-dependent inhibition of apoE synthesis by PMA

Cells were incubated with 160 nM PMA for the indicated times (a), or for 3 h at the indicated concentrations (b). The label was added for the last 30 min, following which cell lysate apoE was immunoprecipitated and quantified, as described. Each point in both panels represents an average $\pm$ S.E.M. of two experiments, with six individual wells for each time point (a) or each PMA concentration value (b).

Figure 4 Effect of PMA on rates of apoE synthesis and apoE mRNA levels

A typical fluorograph is shown of immunoprecipitated cellular apoE following a 30 min pulse (as described in Figure 2), after treatment with 160 nM PMA for the indicated times. ApoE and β -actin mRNA levels at corresponding times from identically treated cells are also shown. The Northern blot assay is described in the Experimental section.

incorporation into cellular apoE at 30 min thus faithfully reflects the differences in relative rates of apoE synthesis; consequently, data relating the dose- and time-dependence of changes in rates of apoE synthesis effected by PMA (Figures 3a and 3b) were determined by the extent of label incorporation into cellular apoE at 30 min after isotope administration. Maximal inhibition of apoE synthesis (75%) was seen in cells treated with PMA for 3 h (Figure 3a), and at a concentration of 40 nM (Figure 3b). These results are similar to those seen for the effects of PMA on apoE secretion, and suggest that PMA affects primarily apoE synthesis; the effects on apoE secretion are secondary to the effects on synthesis. A typical experiment in which rates of apoE synthesis were correlated with levels of apoE mRNA is shown in Figure 4. Cells were pulsed for 30 min after treatment with 160 nM PMA for 0, 3, 4.5 and 6 h, and cell lysate apoE was immunoprecipitated. Parallel incubations of cells in the absence of [35 S]methionine were carried out to examine changes in apoE

Figure 5 Effect of PMA on apoE turnover in cholesterol-loaded macrophages

Cells were pulsed for 60 min with the labelling media alone (a and b) or labelling media containing 160 nM PMA (c). At the end of the pulse period, all cells were quickly switched to chase medium (regular DMEM) and chased for indicated periods of time in the absence (a) or presence (b, c) of PMA. Cell lysate and media apoE was immunoprecipitated and quantified, and percentage recoveries are shown for each compartment (▨, cells; ▤, medium). Numbers at the top of the bar representing cellular apoE at 0 time show the absolute incorporation of [35 S]methionine into apoE ($\times 10^{-4}$ c.p.m./mg of protein). Bars in each panel represent averages $\pm$ S.E.M. of two experiments, each having six individual wells for each time point. Values for the zero time bars in panels (a) and (b) were obtained from the same set of wells, pulsed in the absence of PMA. Asterisks by the media apoE columns in (b) and (c) indicate values that are statistically significantly different (at $P < 0.05$) from the corresponding values in control cells (a).

mRNA. It is apparent that, despite the significant decrease in the rate of apoE synthesis (also quantified in Figure 3a), levels of apoE mRNA did not change. When normalized to the levels of β -actin mRNA in the same sample, apoE mRNA levels were, expressed as percentages of control, $89 \pm 9\%$, $85 \pm 8\%$ and $116 \pm 16\%$ for the 3, 4.5 and 6 h treatment periods respectively (average $\pm$ S.E.M. of three separate experiments). Similar results were obtained in cells not loaded with acLDL-derived cholesterol and cells treated with 25-hydroxycholesterol.

The observation that PMA treatment results in a profound decrease in the apparent rate of apoE synthesis without corresponding changes in apoE mRNA levels raised the possibility that PMA treatment may stimulate increased degradation of apoE within the cells, thus giving rise to the apparent decrease in synthetic rate. To investigate this possibility further, pulse-chase experiments were carried out as follows. Cholesterol-loaded cells were pulsed with [35 S]methionine for 60 min in the absence or presence of 160 nM PMA, after which they were chased in regular medium ($\pm$ PMA, as indicated) for 0, 20, 40 and 80 min. Cell-associated and medium [35 S]apoE were quantified at each time point and recoveries were calculated (Figure 5). As shown in Figure 5(a), chase of labelled control cells in the absence of PMA resulted in a gradual accumulation of labelled apoE in the medium, accompanied by a loss of cellular apoE. The loss in cellular apoE was substantially greater than could be recovered in the medium at all time points, indicating significant intracellular degradation (previous work in this laboratory has shown negligible degradation or re-uptake of labelled apoE in the medium [18]). Under these conditions, 30% of labelled apoE present in the cells at zero time was recovered after 80 min of

Figure 6 Effect of PMA on macrophage microsomal PKC activity

Cells were incubated with 160 nM PMA for indicated periods of time. At the end of the incubations cells were rapidly washed in ice-cold Ca^{2+} -free phosphate-buffered saline, and processed for assay of PKC as described in the Experimental section. Each point represents an average $\pm$ S.E.M. of three experiments in which cells from six wells were pooled for each time point.

Figure 7 Effect of down-regulation of PKC activity on apoE mRNA, and on apoE synthesis and secretion

Cells were incubated in the presence or absence of 16 μM PMA for 24 h. This treatment resulted in a $> 95\%$ decrease in total cellular PKC activity (results not shown). At the end of this period some cells were labelled for 30 min (to assess relative rates of synthesis) or for 3 h (to assess rates of secretion). Identically treated cells were also used for RNA preparation, and relative amounts of apoE and β -actin mRNA were assessed by Northern blot assay. (a) Northern blot assay of RNA obtained from untreated cells (lanes 1 and 2) and cells exposed to 16 μM PMA for 24 h (lanes 3 and 4). Lanes 1 and 3 contain 2 μg and lanes 2 and 4 contain 1 μg of total RNA. The Northern blot was co-hybridized with a probe for apoE and β -actin, as indicated. (b) Relative rates of apoE synthesis, determined as described in the legend for Figure 2 (lanes 1–4), and the corresponding rates of apoE secretion, determined by immunoprecipitation of medium [^{35}S]apoE and after a 3 h labelling period (lanes 5–8). Duplicate aliquots were assayed in each case, from cell lysates (lanes 1 and 2, untreated cells; lanes 3 and 4, PMA-treated cells) and medium (lanes 5 and 6, untreated cells; lanes 7 and 8, PMA-treated cells). The autoradiogram shown is typical of three separate experiments.

chase (approx. 30% of the recovered material remaining in the cells). When identically labelled cells were chased in the presence of PMA (Figure 5b), a striking acceleration of apoE secretion was noted. Much of the pre-labelled apoE was removed by 40 min of chase (as shown by the plateau in medium apoE after 40 min). Thus when PMA was present in the chase medium, over 40% of the labelled apoE present in the cells at zero time was recovered, with less than 25% of the recovered material being in

the cells. These results are inconsistent with the notion that PMA treatment of cells leads to enhanced degradation of newly synthesized apoE. On the contrary, the data suggest that PMA, independent of its effect on apoE synthesis, appears to accelerate the secretion of pre-synthesized apoE. Indeed, the $t_{1/2}$ of cellular apoE decreased from 24 ± 3 min to 16 ± 4 min when PMA was present in the chase medium (results not shown). Qualitatively similar results were obtained when PMA was present throughout the pulse and chase (Figure 5c). Under these conditions the pre-labelled amount of apoE at the end of the pulse period was reduced by over 60% (due to the above-noted inhibition of apoE synthesis), but was chased out of the cells by PMA-containing medium more rapidly and with higher recoveries than in control cells.

Effect of phorbol ester on PKC activity

PMA-mediated inhibition of apoE synthesis may be due to activation of PKC, or to PMA-mediated loss of PKC, as is reported to take place in some cells [23]. The rapidity of the observed actions in these experiments, and the effectiveness of relatively low PMA concentrations (40 nM), would suggest the former rather than the latter. Nevertheless, in an attempt to distinguish between these two possibilities, the effect of PMA on membrane-bound PKC activity over similar periods of time was examined. Figure 6 shows the time course of membrane-bound PKC activity as a result of treatment with PMA. PMA treatment led to a rapid and significant (nearly 3-fold) increase in membrane-bound PKC activity, followed by a gradual decrease, over an 18 h period, to 65% of control values. These experiments demonstrate that the initial inhibition of apoE synthesis (the first 6 h, at least) is correlated with a significant increase in membrane-bound PKC activity. It is thus clear that the initial PMA-mediated effect on apoE synthesis in macrophages is not due to down-regulation of PKC activity. On the other hand, it also should be noted that the effect of PMA on apoE expression persists well beyond the time of maximal PKC stimulation. PKC activity after 18 h of treatment with PMA was reduced by 35%, yet apoE expression continued to be suppressed. Indeed, complete down-regulation of cellular PKC activity (inhibition of $> 95\%$ of activity) by a 24 h treatment with 16 μM PMA was associated with nearly complete ($> 95\%$) suppression of apoE expression (Figure 7). Under these conditions a nearly complete inhibition of apoE synthesis (and secretion) was evident, yet levels of apoE mRNA (normalized to those of β -actin mRNA) did not change correspondingly. Although the levels of apoE mRNA decreased slightly (by an average of $24 \pm 5\%$), these changes are clearly inadequate to explain the profound inhibition of apoE synthesis, and suggest significant post-transcriptional regulation.

DISCUSSION

The presented experiments clearly demonstrate that PMA, an activator of PKC, is a powerful inhibitor of apoE expression in mouse macrophages. Its effect is rapid (within 30 min of exposure, the earliest time at which effects on synthesis were examined), and dose- and time-dependent. Although the majority of the experiments reported were carried out with 160 nM PMA, significantly lower concentrations (40 nM) were also effective. While the initial observations suggested that PMA inhibits apoE secretion, subsequent studies have shown that PMA inhibits apoE synthesis; the decrease in apoE secretion is secondary to the inhibition of synthesis. Indeed, pulse-chase experiments demonstrated that PMA treatment leads to accelerated secretion of the pre-labelled pool of cellular apoE. The inhibition of apoE synthesis on the one hand, and the stimulation of apoE secretion

on the other, illustrate the complexity of the regulatory pathways involving PKC.

An important finding is the absence of an effect of PMA on the levels of apoE mRNA, particularly during the first 6 h of treatment, a period during which the greatest decrease in apoE synthesis occurs. Even with longer periods of exposure (24 h at 16 μ M PMA) the profound inhibition of apoE synthesis was accompanied by only moderate decreases in apoE mRNA. These findings strongly suggest the occurrence of post-transcriptional regulation. These and earlier findings from this laboratory indicate that there are at least two post-transcriptional loci of regulation of apoE expression in macrophages: (1) HDL₃ stimulates apoE secretion in the absence of any changes in apoE mRNA levels or rates of apoE synthesis; it appears to divert a significant portion of synthesized apoE from an intracellular degradative pathway to a secretory pathway [18], a finding recently confirmed in J774 cells transfected with human apoE [35]; and (2) PMA inhibits apoE expression at the level of synthesis, without a significant effect on apoE mRNA levels, as shown in the present study. Between these two mechanisms of post-transcriptional regulation of apoE expression, > 10-fold changes in apoE secretion can be achieved in the absence of any changes in message levels (compare rates of apoE secretion shown in Table 1), pointing out potentially powerful regulatory mechanisms that may operate *in vivo*.

There is accumulating evidence for post-transcriptional and translational regulation of expression of a number of proteins important in lipid metabolism. The complex regulation of lipoprotein lipase expression in adipocytes by insulin has been described [36]. Translational regulation of expression of the LDL receptor and of hydroxymethylglutaryl-CoA reductase has also been recently demonstrated [37,38]. Steroid hormones appear to have a profound effect on the extent of association of apoA-I mRNA with polysomes in the rat liver, thus influencing rates of initiation and/or completion of translation, without significantly affecting total mRNA levels [39]. Whether PMA similarly affects the distribution of apoE mRNA between polysomes and unassociated message remains to be determined.

The relationship between PMA-induced inhibition of apoE expression and the time-dependent biphasic effect of the phorbol ester on PKC activity is not clear. In contrast to other cell types [23], exposure of macrophages to PMA (up to 160 nM) for up to 24 h does not significantly down-regulate PKC activity. The earliest effect on apoE synthesis coincides with a significant increase in membrane-bound PKC activity. It should be noted, however, that the inhibition of apoE synthesis persists well beyond the time of peak PKC activity. Indeed, treatment of macrophages with 16 μ M PMA for 24 h leads to a complete (> 95%) loss of total PKC activity, yet the inhibition of apoE expression remains nearly complete. While these observations rule out the possibility that the effect of PMA is due to a rapid down-regulation of macrophage PKC, the mechanism of continued complete suppression of apoE expression remains to be determined. A number of possible mechanisms can be envisaged. The initial activation of PKC may result in the phosphorylation of a long-lived protein intermediate, which continues to suppress apoE expression by a mechanism yet to be determined. Activation of PKC may lead to a specific and irreversible (or slowly reversible) modification of the apoE mRNA, which inhibits translation. This may be mediated directly by PKC or by a phosphorylated protein intermediate. PKC activation may also result in the redistribution of apoE mRNA and its dissociation from polysomes. It is also possible that treatment of macrophages with PMA activates a PKC isoenzyme which is not down-regulated by PMA or readily detected by the assay system used.

An indirect effect of PMA on apoE expression should also be considered. PKC is known to play a role in macrophage cholesterol metabolism. Treatment of macrophages with endotoxin or with phorbol ester is accompanied by a 2–4-fold increase in cholesteryl ester formation, and thus a decrease in the pool of unesterified cholesterol available for efflux [40,41]. A recent report also suggests that treatment of mouse macrophages with PMA reduces cholesterol efflux in the presence of cholesterol acceptor [42]. Such observations would suggest that the reduction in apoE expression observed in the present studies may be related to decreased free cholesterol formation and decreased cholesterol efflux. This is unlikely, however, for two reasons: (1) in all of the reported experiments, apoE expression is measured in the absence of cholesterol efflux (cholesterol acceptor is not present in the incubation medium); the inhibitory effect of PMA on cholesterol efflux [42] is therefore irrelevant, and (2) any changes in sterol distribution due to PMA would be expected to affect apoE expression at a transcriptional level [18]. Nevertheless, it is interesting that activators of PKC inhibit both cholesterol efflux and apoE expression; this concerted action may lead to the inhibition of reverse cholesterol transport, promotion of peripheral lipid accumulation and formation of atherosclerotic lesions.

The physiological implications of these findings may be considerable. The PKC family of enzymes includes at least seven subspecies with differing characteristics with respect to Ca²⁺, phospholipid and diacylglycerol requirements and tissue distribution [23]. They are involved in a variety of cell processes, including proliferation, differentiation and metabolism. PKC involvement has been proposed in the response of various cells to growth factors, cytokines, neurotransmitters and hormones. There is a growing body of evidence for the involvement of PKC in the metabolic actions of insulin in adipose tissue and other cells [43,44]. In this respect, it is of interest that insulin has been shown to inhibit apoE secretion by HepG2 cells in the absence of measurable changes in apo mRNA levels [45]. PKC has also been implicated in the regulation of the macrophage response to γ -interferon and platelet-activating factor [46,47]. Significantly, γ -interferon has been shown to inhibit apoE secretion by macrophages [48]. Based on the presented results, the well-documented inhibitory effect of endotoxin on apoE expression in macrophages [19] may well be mediated through activation of PKC. It remains to be shown whether other physiological agents, such as platelet-activating factor, or hormones which activate the PKC signalling pathway, have an effect on the expression of apoE by macrophages, and potentially by other cells.

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