Phorbol ester-induced low density lipoprotein receptor gene expression in HepG2 cells involves protein kinase C-mediated p42/44 MAP kinase activation

Amit Kumar, Timothy C. Chambers, Billie A. Cloud-Heflin, and Kamal D. Mehta[1]

Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7199

Abstract The signaling pathway involved in low density lipoprotein (LDL) receptor gene expression induced by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was investigated in the human hepatoma HepG2 cell line. Treatment of HepG2 cells with 100 nM TPA resulted in an approximately 20-fold increase in LDL receptor mRNA level, as determined by RT-PCR, which peaked at 2–4 h of treatment and subsequently declined. The protein kinase C (PKC) inhibitors calphostin C and staurosporine prevented TPA-mediated LDL receptor mRNA induction. In contrast, TPA did not affect squalene synthase mRNA expression. Immunoblotting of cell extracts with isozyme-specific PKC antibodies revealed that HepG2 cells expressed PKCα, which was mainly cytosolic, and PKCβ, PKε, and PKCζ, all of which were present in both the cytosolic and particulate fractions. Treatment of HepG2 cells with 100 nM TPA resulted in translocation of cytosolic PKCα to the particulate fraction, with a maximum at 30 min–2 h of treatment, but was without effect on the subcellular distribution of the other isozymes. TPA treatment also led to activation of the mitogen-activated protein kinase (MAPK) ERK cascade. The specific MAPK pathway inhibitor PD98059 blocked TPA-induced ERK activation. Furthermore, pretreatment of cells with PD98059 inhibited TPA-induced LDL receptor mRNA induction. Moreover, pretreatment of cells with calphostin C inhibited TPA-mediated ERK activation and LDL receptor mRNA induction in a dose-dependent fashion. Based on a close kinetic correlation between PKCα translocation and ERK activation, and the effects of specific inhibitors, these findings suggest that translocation/activation of PKCα, and subsequent activation of the Raf-1/MEK/ERK MAPK cascade, represent key events in the transcriptional induction of LDL receptor gene by TPA in HepG2 cells.—**Kumar, A., T. C. Chambers, B. A. Cloud-Heflin, and K. D. Mehta.** Phorbol ester-induced low density lipoprotein receptor gene expression in HepG2 cells involves protein kinase C-mediated p42/44 MAP kinase activation. *J. Lipid Res.* 1997. **38:** 2240–2248.

Supplementary key words cholesterol • LDL receptor expression • phorbol esters • protein kinase C isozymes • mitogen activated protein kinase • HepG2 cells

The mammalian low density lipoprotein (LDL) receptor mediates the clearance from plasma of lipoproteins that contain apolipoprotein (apo) B-100 and/or apoE (1). Transcriptional regulation of the LDL receptor gene is a major mechanism by which dietary and hormonal agents control plasma concentrations of cholesterol (2). The LDL receptor is ubiquitously expressed but certain specialized cells that require large amounts of cholesterol, such as the hepatocytes involved in bile acid synthesis, express particularly high numbers of cell surface LDL receptors. Approximately 70% of the LDL receptors in humans are present on liver cells, and feedback regulation of the receptor gene by cellular sterol levels is most important in the liver (1, 3). The ingestion of dietary cholesterol has been shown to reduce hepatic levels of the LDL receptor transcript; and the subsequent decline in hepatic LDL receptors resulted in plasma accumulation of LDL (4–6).

The specific features of the human LDL receptor promoter include the use of multiple transcription initiation sites, the absence of characterized tissue-specific transcription factor binding sites, and the presence of an indispensable but ubiquitous transcription factor Sp1 binding site neighboring a sterol responsive element-1 (SRE-1) and an FP1 site (7–10). SRE-1 binds to SRE-1 binding proteins (SREBP-1 and SREBP-2), tran-

Abbreviations: PKC, protein kinase C; LDL, low density lipoprotein; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SRE-1, sterol response element 1; SREBP, SRE-1 binding protein; RT-PCR, reverse-transcriptase polymerase chain reaction; MEK, mitogen/extracellular regulated protein kinase kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; bHLH-ZIP, basic-helix-loop-helix-leucine zipper.

[1]To whom correspondence should be addressed.

scription factors of the basic-helix-loop-helix-leucine zipper (bHLH-ZIP) family (11, 12). The proximal Sp1 site promotes transcription but requires SRE-1 for high level expression in response to low intracellular levels of sterol (13).

Exogenous factors other than ambient sterol levels, many of which increase LDL receptor expression through transcriptional activation (14–20), play a role in regulating LDL receptor expression in vivo. In HepG2 cells, LDL receptor gene transcription is induced by various non-sterol stimuli including activators of protein kinase C (PKC), increases in intracellular calcium, arachidonic acid metabolites, and cAMP. Regulation by PKC has received particular attention but this is complicated because PKC comprises a large family of serine–threonine protein kinases of which eleven isozymes have been cloned and characterized to date (21). These fall into three major categories; conventional cPKC [α, βI, βII, and γ subspecies], novel nPKC [δ, ϵ, η(L) and θ subspecies], and atypical aPKC [ζ and ι(λ) subspecies]. Another isoform, PKCμ, has been characterized recently and has a unique structure and properties distinct from other groups (21). cPKCs are calcium-dependent whereas nPKCs are calcium-independent; both groups are activated by phorbol esters (e.g., TPA, 12-*O*-tetradecanoylphorbol-13-acetate) and/or diacylglycerol. aPKCs are neither calcium nor TPA/diacylglycerol dependent. Specialized functions for individual PKC isozymes are suggested by tissue-specific and cell-type-specific expression patterns (21). PKC plays a key role in signal transduction and changes in subcellular location can occur upon activation. For example, in response to phorbol esters or signals that promote lipid hydrolysis, translocation of the enzyme to the plasma membrane, nucleus, or other compartment is often observed (21, 22). The range of target substrates includes receptors, enzymes, cytoskeleton proteins, proto-oncogene products, and certain transcription factors, reflecting the broad role of PKC in diverse cellular processes such as growth, differentiation and secretion (21). Several reports have indicated that PKC may play a role in regulation of LDL receptor expression. For example, induction of LDL receptor mRNA by TPA has been observed in the Jurkat leukemia T cells, THP-1 cells, and HepG2 cells (23–25). Evidence has also been presented that activation of PKC by phorbol ester in arterial smooth muscle cells results in transcriptional activation of the LDL receptor involving the 5′ upstream promoter region (26).

Although these studies suggest that LDL receptor gene expression can be induced by a PKC-dependent signal transduction pathway, little is known of the specific isozymes of this multigene family that may be involved. In addition, steps in the signaling pathway downstream of PKC activation have not been investigated. PKC can induce changes in transcription by several different mechanisms including functioning directly in the nucleus or initiating a nucleus-directed kinase cascade (22). In this study, we examined the extent and kinetics of LDL receptor induction by TPA, and we monitored under similar conditions the subcellular distribution of specific PKC isozymes by immunoblotting with isozyme-specific antibodies in HepG2 cells. We also examined MAPK activation in HepG2 cells by TPA, and the effect of PKC inhibitors and MEK (mitogen/extracellular regulated protein kinase kinase) inhibitors on TPA-mediated LDL receptor induction. Our findings show that activation of TPA-responsive, calphostin C-sensitive PKC isoform(s), possibly PKCα, and subsequent activation of the Raf-1/MEK/ERK MAPK cascade, represent key events in the mechanism of LDL receptor induction by TPA.

MATERIALS AND METHODS

Materials

Taq DNA polymerase and restriction enzymes were purchased from Perkin Elmer Cetus. 25-Hydroxycholesterol, staurosporine, calphostin C, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Company, and cholesterol was from Steraloids, Inc. TRIzol, Superscript II reverse transcriptase, anti-PKC antibodies, and all tissue culture supplies were purchased from GIBCO-BRL Life Technologies. RNase free DNase was purchased from Ambion. Antibodies for Raf-1 kinase and ERK1/2 were obtained from Santa Cruz Biotechnology and antibody specific for the phosphorylated forms of ERK1/2 was obtained from New England Biolabs. Zeta probe blotting membrane and the protein assay reagent were purchased from Bio-Rad. PD98059 was from Research Biochemicals International. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from ICN and the enhanced chemiluminescence (ECL) detection kit was obtained from Amersham Corp. Purified rat brain PKC was purchased from Calbiochem.

Reverse transcription-polymerase chain reaction (RT-PCR)

HepG2 cells were routinely grown in DMEM with 10% fetal bovine serum (FBS) in 5% CO_2/95% air. Cells were treated with TPA and total cellular RNA was isolated using the TRIzol reagent followed by acid phenol:chloroform extraction and isopropanol precipita-

tion. Total RNA was subjected to DNase I treatment. Equivalent quantities (2 μg) of DNase-treated total RNA were converted to cDNA by Superscript II reverse transcriptase (10). The reaction was treated with RNase H, followed by phenol:chloroform extraction and ethanol precipitation. Two oligonucleotide primers specific for the human LDL receptor gene sequence were used to amplify its cDNA using the Taq polymerase as described earlier (10). The number of cycles and concentration of cDNA used were optimized to ensure that the reaction was in the logarithmic phase. Control RNA (without converting to cDNA) was also amplified in parallel to eliminate the possibility of genomic DNA contamination in the cDNA preparation. The PCR products were resolved by 5% polyacrylamide gel electrophoresis and electroblotted onto a Zeta-Probe membrane. The filter was probed with a ^{32}P-labeled oligonucleotide with a sequence complementary to the middle region of the amplified product as described previously (10). The oligonucleotide primers used for amplification and probing of the human LDL receptor were: Primer LDLR1, 5′-GGCTGGGTGATGTTGTGGAA-3′; Primer LDLR2, 5′-GGCCGCCTCTACTGGGTTGA-3′; Primer LDLR3 (Probing), 5′-GAAGCCATTTTCAGTGCCAA-3′. β-actin cDNA was amplified as an internal control. Oligonucleotides used for amplifying β-actin cDNA were: Primer ACI, 5′-TACAATGAGCTGCGTGT-3′; Primer AC2, 5′-TGAAGGTCTCAAACATGA-3′; Primer AC3 (Probing), 5′-AAGGCCAACCGCGAGAAGAT-3′. Squalene synthase (SS) primers were: Primer SS1, 5′-AATGGCCATTACCTGTGGAAT-3′; Primer SS2, 5′-ATACCAAACAGTGATTGCCG-3′. Primer SS2 was used for probing the blots.

Protein extraction

Cell cultures in 75-cm^2 flasks in the exponential phase of growth (60–70% maximum density) were treated with TPA and harvested by rinsing and scraping into ice-cold PBS. All steps were performed at 4°C. After brief sedimentation, the cell pellet was suspended in 0.3 ml of buffer A (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 20 μg/ml aprotinin, 50 μg/ml leupeptin, 10 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Cell suspensions were sonicated with a microprobe (3 × 10 s at 1-min intervals) and unbroken cells and nuclear debris removed by centrifugation for 10 min at 1,000 *g*. The homogenate was then centrifuged for 45 min at 100,000 *g* to obtain a cytosolic fraction and a pellet that was suspended in 0.3 ml buffer A and termed the particulate fraction. Whole cell extracts (for Raf and ERK immunoblots) were prepared as described previously (27). Protein concentrations were determined with bovine serum albumin as the standard.

Immunoblot analysis

Proteins were fractionated by SDS-PAGE with an 8% acrylamide separation gel and aliquots containing 15 μg of sample protein were electrophoresed. Proteins were transferred to nitrocellulose in 25 mM Tris-HCl, 192 mM glycine, and 10% methanol at 4°C for 12–16 h at a constant current of 50 mA, or for 2 h at 300 mA with similar results. Nitrocellulose membranes were processed as described previously (28). Briefly, membranes were incubated in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.2% v/v Tween-20 (Tris/NaCl/Tween-20) with 5% w/v non-fat dried milk for 1 h, washed in Tris/NaCl/Tween-20 (3 × 5 min), and incubated for 1 h with primary antibody in Tris/NaCl/Tween-20 containing 1% milk at room temperature. The following dilutions were used for individual antibodies against the different proteins: PKCα, 1:1000; PKCβ, γ, δ, and ϵ, 1:500; PKCζ, 1:3000; Raf-1, 1:1000; phospho-ERK1/2, 1:2500. After further washing in Tris/NaCl/Tween-20, membranes were incubated for 1 h with horseradish peroxidase-linked anti-IgG secondary antibody (Bio-Rad, diluted 1:5000), and immunoreactive proteins was detected by ECL as described by the supplier. Purified rat brain PKC, composed mainly of the calcium-dependent isozymes, was used as a positive control during immunoblotting.

mRNA decay analysis

HepG2 cells grown in the presence or absence of PD98059 for 30 min were treated with TPA for 2 h. Actinomycin D (5 μg/ml) was added and total RNA was isolated using TRIzol at different time intervals. Northern blotting was done essentially as described earlier (5). Briefly, 20 μg RNA was fractionated on 1% formaldehyde agarose gel and transferred to Zeta Probe membrane by capillary blotting. RNA blots were hybridized with LDL receptor and actin specific single-stranded M13 probes labeled with [α-^{32}P]dCTP. Hybridized filters were washed and exposed to Kodak X-ray film. The relative intensities of specific bands were determined densitometrically within the linear range of film on a model 300A laser densitometer (Molecular Dyanamics, CA) and Image Quant software. LDL receptor mRNA was normalized to actin mRNA levels, and data for each point were plotted as the percentage of LDL receptor mRNA remaining at that point as compared to zero actinomycin treatment time.

RESULTS

The kinetics of TPA-induced LDL receptor gene expression in HepG2 cells was examined by treating cells

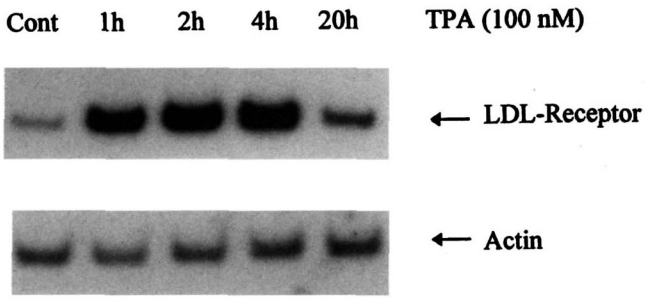

Fig. 1. Time course of LDL receptor mRNA accumulation in HepG2 cells treated with TPA. Cells were cultured in DMEM with 10% FBS and treated with 100 nM TPA for the times indicated; RNA was prepared, and mRNA levels were determined by RT-PCR analysis, as described under Materials and Methods. Actin was used as an internal control.

with 100 nM TPA for different periods from 1 h to 20 h and determining LDL receptor mRNA expression levels by RT-PCR, as described in Materials and Methods (**Fig. 1**). Unlike actin mRNA, LDL receptor mRNA started accumulating after 1 h of addition of TPA, reached a maximum at 2–4 h later, and then decreased to the preinduction level at 20 h.

To obtain additional evidence for a role of PKC activation in TPA-mediated LDL receptor gene expression, protein kinase C inhibitors were used. The non-selective PKC inhibitor staurosporine and the PKC-specific inhibitor calphostin C were both extremely effective in preventing TPA-induced LDL receptor mRNA expression (**Fig. 2**). In contrast, TPA or PKC inhibitors did not affect expression of the house-keeping actin gene or another gene of the cholesterol biosynthetic pathway, squalene synthase (Fig. 2).

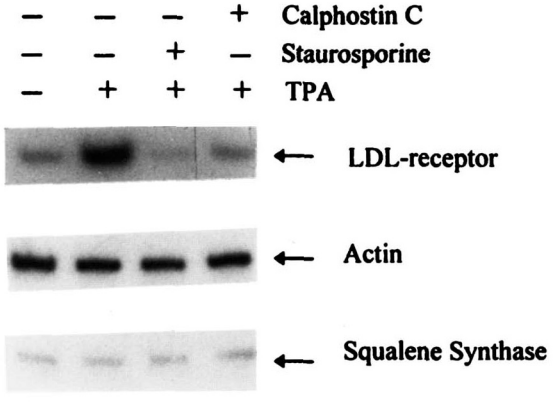

Fig. 2. Effect of staurosporine and calphostin C on TPA-induced LDL receptor mRNA accumulation. HepG2 cells were either untreated or treated with 100 nM TPA for 2 h in the absence or presence of either 1 μM staurosporine or 1 μM calphostin C (activated in the light). The inhibitors were added 30 min prior to TPA. RNA was prepared and mRNA levels of LDL receptor, squalene synthase, and actin were determined by RT-PCR.

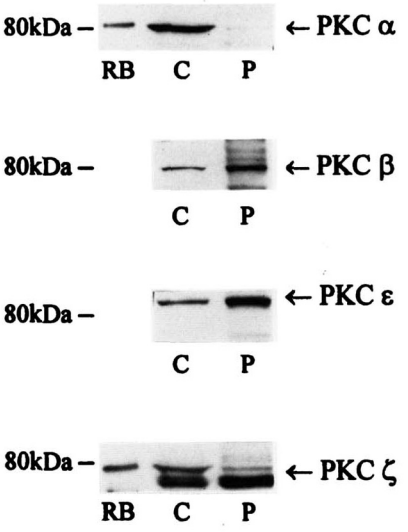

Fig. 3. Analysis of the subcellular distribution of PKC isozymes in HepG2 cells by cell fractionation and immunoblotting. Cytosolic (C) and particulate (P) fractions were prepared and 15 μg/lane was applied to SDS/PAGE and individual PKC isozymes detected by immunoblotting with isozyme-specific antibodies. RB, 5 ng purified rat brain PKC. The migration position of an 80 kDa molecular mass standard is indicated.

To identify specific PKC isozymes that may be involved in LDL receptor induction by TPA, cytosolic and particulate fractions were prepared from HepG2 cells and examined for the presence of PKC isozymes by immunoblot analysis. Four of the known PKC isozymes were detected: cPKCα, which was mainly present in the cytosolic fraction; and cPKCβ, nPKCε and aPKCζ, all of which were present in both the cytosolic and particulate fractions (**Fig. 3**). Each species migrated with its characteristic apparent molecular mass of 80 kDa for PKCα and PKCβ, ~90 kDa for PKCε, and ~70 kDa for PKCζ. The PKCζ antibody also reacted with PKCα, evident as a band of 80 kDa migrating slower than authentic PKCζ (Fig. 3). This known cross-reactivity does not influence data interpretation because of the electrophoretic separation of the two isozymes. Highly purified rat brain PKC (RB in Fig. 3), composed mainly of cPKCα, was recognized as a species of 80 kDa by both the anti-PKCα and anti-PKCζ antibodies.

Subcellular redistribution is a hallmark of PKC activation by TPA and three of the four detected isozymes in HepG2 cells are TPA-responsive, namely PKCα, β and ε (21). Cells were exposed to TPA under conditions established above to induce LDL receptor expression, and cytosolic and particulate fractions were prepared and examined for the presence of PKC isozymes by immunoblotting. TPA treatment resulted in translocation of a portion of cytosolic PKCα to the particulate fraction, which was maximal at 30 min to 2 h exposure to

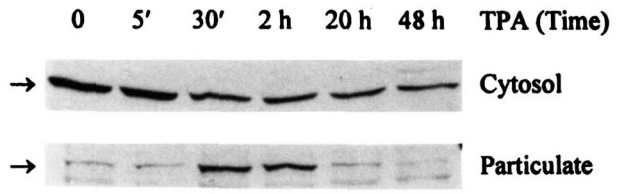

Fig. 4. Subcellular distribution of PKCα in HepG2 cells in response to TPA. Cells were treated with 100 nM TPA for the times indicated and cytosolic and particulate fractions (15 μg protein/lane) were analyzed for the presence of PKCα (arrow) by immunoblotting.

the phorbol ester (**Fig. 4**). Prolonged (24–48 h) TPA treatment led to a depletion of the newly recruited particulate PKCα, most likely due to the well-documented sensitivity of the membrane-associated form of the enzyme to proteolysis (29). In contrast, the subcellular localizations of the other isozymes detected, PKCβ, PKCε and PKCζ, were not affected by TPA treatment of the cells (data not shown). These results suggest that PKCα may mediate the effects of TPA on LDL receptor levels in HepG2 cells.

To determine whether the effects of PKC on LDL receptor gene expression were mediated via MAPK activation, the Raf/MEK/ERK pathway was examined. First, Raf-1 kinase and ERK activities were evaluated in control and TPA treated cells. Raf-1 activation can be measured by immunoblot analysis due to a discrete retardation of electrophoretic mobility upon phosphorylation (30). As shown in **Fig. 5A,** treatment of cells with TPA led to a decreased electrophoretic mobility of Raf-1 immunoreactivity, consistent with phosphorylation and apparent activation of the enzyme. To assay ERK, we subjected cell extracts from control and TPA-treated cells to immunoblot analysis with an antibody that specifically recognizes the activated, phosphorylated forms of ERK1/2. This antibody does not recognize inactive, unphosphorylated enzyme. As shown in Fig. 5B, phospho-ERK1/2 immunoreactivity was very low in control cells but readily detected in TPA treated cells. Furthermore, pretreatment of the cells with PD98059, a specific inhibitor of MEK (31), the kinase upstream of ERK, significantly inhibited TPA-mediated activation of ERK (Fig. 5B). Thus, TPA activates ERK via Raf-1 and MEK activation, and ERK activation can be inhibited by PD98059. Neither TPA nor PD98059 affected ERK protein expression, as indicated by immunobloting of the same cell extracts with a phosphorylation-independent anti-ERK1/2 antibody (Fig. 5B).

To determine whether PKC-mediated induction of LDL receptor mRNA expression depended on activation of the MAPK pathway, the effect of PD98059 on TPA-mediated LDL receptor mRNA expression was investigated. HepG2 cells were pretreated with or without

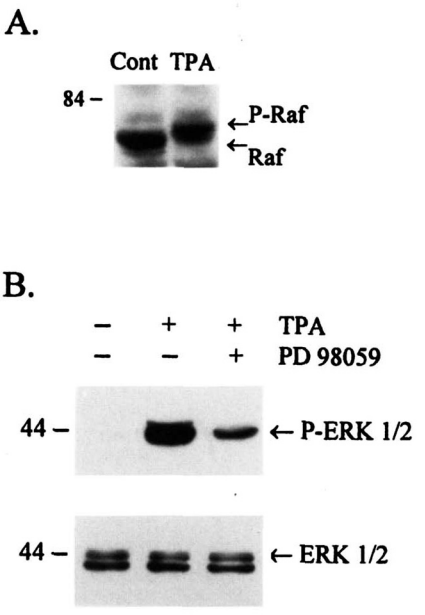

Fig. 5. Activation of Raf-1 and ERK MAPK by TPA in HepG2 cells. A. Cells were untreated or treated with 100 nM TPA for 30 min, cell extracts were prepared, and 50 μg protein/lane was applied to SDS-PAGE and Raf-1 detected by immunoblotting. The mobility shift due to phosphorylation (P-Raf) is indicated and the migration of a molecular mass standard of 84 kDa is shown on the left. B. Cells were untreated, or treated with 100 nM TPA for 30 min in the absence or presence of 100 μM PD98059, as indicated. The inhibitor was added 30 min prior to TPA. Cell extracts were prepared and subject to SDS-PAGE and immunoblotting with either anti-phospho-ERK1/2 antibody (upper panel) or phosphorylation-independent anti-ERK1/2 antibody (lower panel). Protein loadings were 56 μg/lane and 10 μg/lane for the upper and lower panels, respectively.

100 μM PD98059 for 30 min and then treated with 100 nM TPA for an additional 2 h. RNA was prepared, and LDL receptor mRNA levels were determined by RT-PCR as above. As shown in **Fig. 6,** the MEK inhibitor blocked TPA-mediated LDL receptor mRNA induction. The inhibition was dose dependent and was maximal at 100 μM PD98059 (data not shown). Identical results

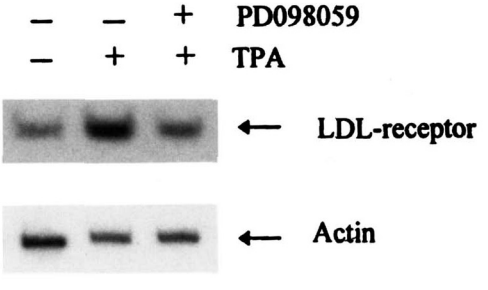

Fig. 6. Effect of PD98059 on TPA-induced LDL receptor mRNA accumulation. HepG2 cells were either untreated or treated with 100 nM TPA for 2 h either in the absence or presence of 100 μM PD98059 added 30 min prior to TPA. RNA was prepared and mRNA levels of LDL receptor and actin were determined by RT-PCR.

have been observed in two repeats of this experiment. These results indicate that induction of LDL receptor mRNA by TPA requires activation of the Raf-1/MEK/ERK MAPK cascade.

In order to show that LDL receptor induction by TPA required sequential activation of PKC and ERK, we studied the effects of the PKC-specific inhibitor calphostin C on ERK activation and LDL receptor induction. As shown in **Fig. 7,** pretreatment of cells with calphostin C led to a dose-dependent inhibition of TPA-mediated ERK activation without affecting ERK protein expression. Furthermore, under essentially identical conditions, calphostin C inhibited TPA-mediated LDL receptor induction.

In view of the recent demonstration that TPA induces LDL receptor mRNA expression (~50-fold) both at the transcriptional and post transcriptional levels (32), we tested whether PD98059 exerts its effect at the level of transcription or stability. The rates of degradation of LDL receptor mRNA were compared in the absence and presence of this inhibitor in TPA-treated HepG2 cells. RNA synthesis was blocked with actinomycin D after induction with TPA, and the relative rates of disappearance of LDL mRNA were determined. As shown in **Fig. 8,** rates of degradation of LDL receptor mRNA were similar in both the conditions, although PD98059 inhibited mRNA induction approximately 80%. Thus, PD98059 did not change LDL receptor mRNA stability appreciably to account for the loss of TPA-induced LDL receptor gene expression. These results are consistent with the notion that PD98059 inhibits TPA-induced LDL receptor expression mainly at the transcription level.

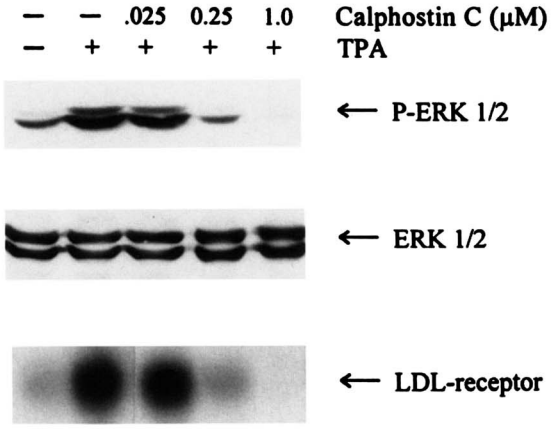

Fig. 7. Effects of calphostin C on TPA-mediated ERK1/2 activation and induction of LDL receptor mRNA expression. HepG2 cells were pretreated with the indicated concentrations of calphostin C and induced with 100 nM TPA for 30 min. Cell extracts were prepared and subjected to SDS-PAGE and immunoblotting with either anti-phospho-ERK1/2 antibody (top panel), or phosphorylation-independent anti-ERK1/2 antibody (middle panel). In parallel, HepG2 cells were pretreated with the same concentrations of calphostin C and induced with TPA for 2 h and total RNA was subjected to Northern analysis for measurement of LDL receptor mRNA levels (lower panel).

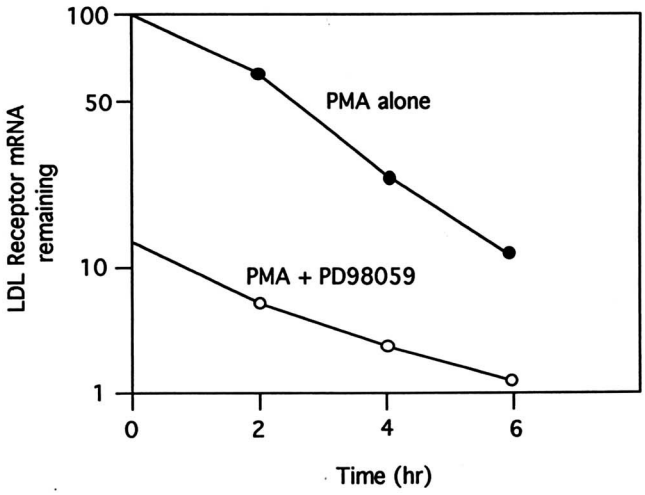

Fig. 8. Stability of LDL receptor mRNA is unaffected on treatment with PD98059. HepG2 cells pretreated with 100 μM PD98059 were subjected to TPA (100 nM) treatment, and after 2 h, actinomycin D (5 μg/ml) was added. At indicated time points, total RNA was isolated and LDL receptor and actin mRNAs were quantified by Northern analysis as described in Materials and Methods. Data at each time point represent percentage of normalized LDL receptor mRNA remaining as compared to TPA treated HepG2 cells at zero actinomycin time point (set at 100%). Each point represents the mean value from two separate experiments.

DISCUSSION

The LDL receptor gene is subject to complex regulation by both sterol and non-sterol factors. The induction of LDL receptor mRNA by mitogens, growth factors, and cytokines provides a critical link between cell growth and membrane biosynthesis and reflects the extra requirement of proliferating cells for cholesterol. Of the non-sterol stimuli known to induce LDL receptor expression, the PKC-activating phorbol ester TPA has been shown to activate the LDL receptor gene in many cell types. In HepG2 cells, TPA increased LDL receptor mRNA expression approximately 20-fold (Fig. 1), consistent with earlier findings (15). The effects of TPA are mediated through activation of TPA-responsive PKC isozymes of which at least 8 members have been characterized to date (21). We detected four major PKC isozymes in HepG2 cells including PKCα, PKCβ, PKCε, and PKCζ, similar in general to the findings recently reported by Ducher et al. (33). However, unlike Ducher et al., we did not observe any unusual lower molecular weight forms of the different PKC subtypes. The immu-

noreactive species we detected exhibited their expected electrophoretic mobilities, and we found no evidence of enzyme degradation or nonspecific immunoreactivity with the different antibodies.

Of the three TPA-responsive isozymes (PKCα, PKCβ, and PKCε) in HepG2 cells (Fig. 3), TPA treatment promoted the cytosol to membrane translocation of PKCα (Fig. 4), without affecting the subcellular distribution of the other isozymes. These results suggest that a signal transduction pathway involving activation of PKCα mediates TPA-induced LDL receptor mRNA expression in HepG2 cells. These findings represent the first analysis of this signal transduction pathway at the level of specific PKC isozymes. However, because PKC activation can occur without enzyme redistribution, these results do not rule out the possible involvement of other isoforms in this response. Additional experiments have suggested that PKCα may also be involved in TPA-mediated LDL receptor mRNA expression in non-hepatoma cell lines such as fibroblasts (unpublished observations).

Our results also have begun to address the mechanism of LDL receptor gene activation by PKC. Activated PKC can affect transcriptional activity directly or indirectly. Translocation of PKC to the nucleus and direct phosphorylation by the enzyme is known to modulate the properties of many DNA regulatory proteins including p53, myogenin, and the vitamin D3 receptor (22, 34, 35). Alternatively, activated PKC, in particular PKCα, can phosphorylate and activate Raf-1, leading to a nucleus-directed kinase cascade involving MEK and ERK, with subsequent phosphorylation and functional modification of specific transcription factors (30, 36–38). The results presented here are consistent with this latter mechanism and strongly suggest that LDL receptor mRNA induction by TPA in HepG2 cells requires PKC-mediated ERK activation. First, TPA treatment leads to a robust activation of ERK, and TPA causes an electrophoretic mobility shift in Raf-1 consistent with its activation (Fig. 5). Second, the MEK inhibitor PD98059 inhibits both ERK activation and LDL receptor mRNA expression induced by TPA. Third, the PKC inhibitor calphostin C inhibits both ERK activation and LDL receptor mRNA expression induced by TPA. Fourth, the kinetics of PKCα translocation, Raf-1 apparent activation, ERK activation, and LDL receptor mRNA induction are closely correlated. Together, these results suggest that TPA induces LDL receptor mRNA expression in HepG2 cells via a mechanism involving sequential activation of PKCα, Raf-1, MEK, and ERK. Further expriments will be required to provide evidence that the α-isoform of PKC is specifically involved and to show that the enzymatic activity of Raf-1 is increased under these conditions.

Events occurring downstream of ERK to activate LDL receptor gene transcription are unknown and presently under investigation. Candidate transcription factors responsible include SREBPs. It is interesting to note in this regard that ERK-2 exhibits a preference for the bHLH-ZIP family of transcription factors (39), and SREBPs are members of this family (11, 12). Thus, SREBPs are potential and attractive targets for ERK-mediated transcriptional regulation of the LDL receptor gene. However, although preliminary observations have suggested that SREBP undergoes a mobility shift consistent with its phosphorylation (40), it remains to be established that SREBP is phosphorylated in vivo. An involvement of SREBP would be consistent with the in vivo footprinting results that have reported protection at SRE-1 in response to TPA-induction of LDL receptor expression (41). TPA-mediated gene activation even in the presence of sterols suggests that LDL receptor induction by SREBP may also occur in a sterol-independent manner (24). It is possible that signaling by TPA may override sterol-dependent transcriptional repression by modulating either the nuclear transport or binding of SREBP, or modifying SREBP-associated transcription activators, for example CREB-binding protein (42). The lack of activation by TPA of the sterol-responsive squalene synthase gene (Fig. 2), which contains a functional SRE-1 (43), suggests that activation of SREBP alone may not account for LDL receptor induction by TPA. This raises the possibility for the involvement of additional factor(s) in TPA induction of the LDL receptor gene, and studies are in progress to identify the relevant *cis*- and *trans*-acting elements.

In conclusion, this study has begun to link the molecular events generated in the cell membrane by TPA to transcriptional induction of the LDL receptor gene. A seqential cascade involving PKCα, Raf-1, MEK, and ERK appear to represent key events. Because many of the transcriptional modulators that induce LDL receptor expression rapidly activate PKC and the MAPK cascade after receptor activation (44–46), this pathway may mediate the actions of other inducers too.

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