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Inhibition of Low Density Lipoprotein Receptor Expression by Long-Term Exposure to Phorbol Ester via p38 Mitogen-Activated Protein Kinase Pathway

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Abstract The proximal region −234 to (+58 bp) of low-density lipoprotein receptor (LDLR) is responsible for its up-regulation by sterol regulatory element binding protein (SREBP). However, the mechanism of sterol-independent repression of LDLR has not been determined yet. In this study, we observed that there was an early induction and a later repression of LDLR by phorbol ester (PMA) in SK-Hep1 hepatocarcinoma cells and investigated the mechanisms through which PMA repressed LDLR transcription. SK-Hep1 cells were exposed to PMA and LDLR mRNA was evaluated by RT-PCR and Northern blot analysis. The effect of phorbol ester on LDLR transcriptional activity was studied using transient transfection of LDLR promoter-luciferase constructs. Overexpression of N-SREBP-2, a dominant positive SREBP2, did not reverse the PMA-repressed LDLR promoter activity. Serial deletion of LDLR promoter revealed that the region between −1,563 and −1,326 was responsible for the repression. The pretreatment with SB202190, an inhibitor for p38 mitogen-activated protein kinase pathway (p38-MAPK), but not other signaling inhibitors, reversed the PMA-induced repression. The 24 h-treatment with PMA efficiently arrested the SK-Hep1 cell cycle at G_0/G_1 as demonstrated by FACS analysis and decreased the ^{3}H-thymidine incorporation. The PMA-induced repression of LDLR transcription may be exerted by the factor(s), not SREBP2, induced or modified by p38-MAPK-mediated signaling pathway and associated with cell cycle blockage. J. Cell. Biochem. 96: 786–794, 2005.

Key words: atherosclerosis; cholesterol; gene expression; lipoproteins

The plasma level of low-density lipoprotein (LDL) cholesterol, a known cardiovascular risk factor, is regulated in part by clearing LDL by hepatic LDL receptor (LDLR) [Goldstein and Brown, 1977a; Goldstein et al., 1977b]. The more LDLR present in the liver, the greater is the rate of hepatic LDL clearance, leading to a concomitant reduction in plasma LDL cholesterol. The proximal region (from +58 to −234 bp) of LDLR promoter contains two Sp1 binding sites and a sterol-regulatory element-1 (SRE-1) which is a binding site for SRE binding protein (SREBP). SREBP is a 125 kDa membrane-bound transcription factor that regulates the synthesis and uptake of cholesterol and fatty acids in animal cells [Hua et al., 1993]. When cells are depleted of sterols, a proteolytic process releases the active portions of SREBP, which enter the nucleus and stimulate the transcription of genes in three pathways of lipid

Abbreviations used: LDLR, low-density lipoprotein receptor; SRE-1, sterol regulatory element-1; SREBP, sterol regulatory element binding protein; N-SREBP2, N-terminus of SREBP2; PMA, phorbol 12-myristate 13-acetate; p38-MAPK, p38-mitogen activated protein kinase; RT-PCR, reverse transcriptase polymerase chain reaction; PKC, protein kinase C; DMSO, dimethyl sulfoxide; PGC-1α, peroxisome proliferator activated receptor γ co-activator-1α; MEM, minimal essential medium.

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metabolism: (1) cholesterol biosynthesis; (2) uptake of cholesterol and fatty acids from plasma; (3) fatty acid biosynthesis.

Differently from the cholesterol-mediated regulatory mechanism, it is poorly understood whether the transcriptional regulation of LDLR gene could be modulated by factor(s) other than sterols and what the underlying mechanism for sterol-independent regulation of LDLR expression is. There are a few studies showing that the hormones including insulin, estrogen, and growth hormone transiently increase the expression of LDLR via SREBP [Streicher et al., 1996; Croston et al., 1997]. There are, however, also some evidences that the distal region, not SRE-1, of the LDLR promoter is responsible for the transient induction of LDLR [Pak, 1996]. Without demonstrable changes on cellular sterol metabolism, growth factors and mitogens increased LDLR gene expression in sterol-loaded human lymphocytes [Cuthbert and Lipsky, 1990], in HepG2 cells [Pak et al., 1996], or in mouse 3T3 cells [Mazzone et al., 1989]. In addition, second messengers such as calcium, cAMP, and protein kinase C (PKC) activators transiently induced LDLR mRNA expression, and then returned it to the low constitutive levels observed before induction in 24 h.

Other researchers further confirmed the involvement of PKC in rapid increase of LDLR mRNA. They demonstrated that phorbol ester, a potent tumor promoter, which presumably binds to and activates PKC, enhanced the LDLR mRNA levels in 1–2 h in Jurkat leukemia T cells [Makar et al., 1998], HepG2 [Kumar et al., 1997], and THP-1 cells [Huang et al., 1997] in a cellular cholesterol-independent manner. Mehta et al. [2002] demonstrated that protein kinase Cε (PKCε) mediated induction of LDLR transcription by depletion of cholesterol and suggested that sterols might directly modulate its function by hindering the PKCε binding to its activators. On the other hand, it was reported that a chronic or long treatment of HepG2 hepatocarcinoma cells with phorbol 12-myristate 13-acetate (PMA) inhibited cell growth and PKC might be involved in the growth arrest and apoptosis [Duronio et al., 1990]. Similar results were also reported using SK-Hep1 cells [Bode et al., 1998]. These observations are difficult to reconcile with the fact that stimulation of PKC leads to sustained activation of several signaling pathways that are required for cell proliferation. The signaling pathways include mitogen activated protein kinase (MAPK) as well as ERK and JNK pathways.

In the present study, we observed that PMA showed a biphasic transcriptional regulation of LDLR in SK-Hep1 hepatocarcinoma cells; a rapid and transient induction in 2 h and a repression after 6 h. The mechanism for the sterol-independent repression of LDLR transcription has not been reported partly because the LDLR mRNA was too low to detect when repressed. We used the luciferase reporter constructs of LDLR promoter with different lengths to determine the effect of phorbol ester in SK-Hep1 cells. Evidence has been provided that a 24 h-treatment of PMA represses the LDLR promoter-driven transactivation via p38-mitogen activated protein kinase (p38-MAPK) pathway in a sterol- or SREBP-independent manner.

MATERIALS AND METHODS

Reagents

Minimal essential medium (MEM) and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY). Wortmannin, BAPTA/AM, Genistein, PD 98059, U-73122, SB 202190, bisindolylmaleimide 1 (GF109203X) were purchased from CalBiochem (San Diego, CA). PMA, dimethyl sulfoxide (DMSO), and the rest of chemicals were purchased from Sigma Co. (St. Louis, MO). [α-^{32}P]dCTP (3,000 Ci/mmol) was obtained from Amersham Biosciences (Buckinghamshire, UK). Propidium iodide was purchased from CalBiochem (San Diego, CA). All the other chemicals used were of the purchased grade available.

Plasmids

A HindIII fragment (1,563 bp) of human LDLR promoter from pLDLR-CAT 1563 [Pak et al., 1996] was subcloned into HindIII site of pGL2-Basic vector (Promega, Madison, WI), and named as pLR1563-luc. The serially truncated LDLR promoter-reporter vectors, pLR1326-luc, pLR974-luc, pLR650-luc, and pLR234-luc were also constructed by PCR using pLR1563-luc as a template and primers containing HindIII restriction sites. The expression vector of human N-terminus of SREBP-2 was a kind gift from Dr. Kim Kyung-Sup (Yonsei University, Korea).

Cell Culture and Transfection

The SK-Hep1 human hepatoma cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C/5% CO_2. SK-Hep1 cells in 6-well plate were transiently transfected with the designated reporter gene and expression vectors using calcium phosphate co-precipitation method [Pak et al., 1996; Han et al., 2000]. The cells were washed twice with PBS 4 h after transfection and cultured in DMEM containing 0.5% FBS. Sixteen hours later phorbol ester or DMSO vehicle (10 μl) was added into the medium for the indicated time periods. The cells were then harvested and assayed for luciferase activity using the luciferase assay kit (Promega) and luminometer (Berthold, Badwildbad, Germany) as previously described [Han et al., 2000]. The transfection efficiencies were normalized with regard to β-galactosidase activity co-transfected with the reporter.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA from rat liver or cultured cells was isolated using TRIzol Reagent followed by manufacturer's instruction (Invitrogen, Carlsbad, CA) [Pak et al., 1996]. Total cDNA synthesized from 1 μg of total RNA was amplified for 30 cycles at 94°C for 30 s, 48°C or 56°C for 30 s, and 72°C for 1 min. The primer sets for LDLR (5′-atg cat ctc cta caa gtg ggt-3′ and 5′-agt ttc cat cag agc act gga a-3′), and β-actin (5′-ttc tac aat cag ctg cgt gtg gct 3′ and 5′-gct tct cct taa tgt cac gca cga 3′) were used for amplification of 629 and 378 bp fragments of LDLR and or β-actin, respectively. The PCR products were examined by 1.2% agarose gel electrophoresis and normalized to the RT-PCR products for β-actin mRNA [Kim et al., 2001].

Northern Blot Analysis

Twenty micrograms of total RNA were separated by 1% formaldehyde-agarose gel electrophoresis and transferred to a Nytran membrane (Schleicher & Schuell, Inc., Dassel, Germany) using TurboBlotter (Schleicher & Schuell, Inc.). The membrane was hybridized with the ^{32}p-labeled specific cDNA probes for LDLR (629 bp PCR product) in Quickhyb hybridization solution (Stratagene, La Jolla, CA) for 12 h at 65°C, washed twice for 15 min at room temperature with a 2× SSC/0.1% SDS, then washed once for 30 min at 60°C with 0.1× SSC/0.1% SDS. The membrane was exposed to K-type imaging screen and visualized using Molecular Imaging System FX (Bio-Rad, Hercules, CA).

^{3}H-Thymidine Incorporation Assay

SK-Hep1 cells (5×10^4) were grown to 80% confluence on 24-well plates, made quiescent by serum deprivation and were labeled with 0.5 μCi/ml [^{3}H]-thymidine (Amersham BioSciences) per well in the presence or absence of PMA (100 ng/ml) for different time periods before terminating the reaction. The media were removed and the cells were washed with PBS. Cellular DNA was precipitated with 0.5 ml sample of ice-cold 10% TCA at 4°C, washed with methanol, and redissolved in 0.5 ml of 0.5N NaOH. The incorporated radioactivity into DNA was counted in Beckman scintillation counter. The amount of [^{3}H]-thymidine incorporated into DNA (% incorporation) was normalized to the radioactivity per well.

Cell Cycle Analysis by FACS

SK-Hep1 cells (5×10^5) in 100 mm dishes were cultured in DMEM containing 0.5% FBS for 16 h. The cells were treated with either PMA (150 ng/ml) or DMSO for 24 h. The cells were harvested by trypsinization, resuspended in 1 ml PBS, and fixed in 1 ml of 70% cold ethanol for 2 h at 4°C. The fixed cells were stained in PBS containing 0.1% BSA, RNase A (50 μg/ml), and 50 μg/ml propidium iodide for 30 min at 37°C after PBS washing. The cells were analyzed on a fluorescence activated cell sorter (FACS Calibur, Becton Dickinson, San Jose, CA). The cell cycle distribution was evaluated on DNA plots by ModFit LT software (Verity Software House, Inc., Topsham, ME).

Statistical Analysis

Data are expressed as mean + SD and statistical differences ($P < 0.05$) between mean values were determined by a two-tailed Student's *t*-test. Experiments were performed in triplicate on three separate occasions.

RESULTS AND DISCUSSION

Biphasic Regulation of Endogenous LDLR Expression by Time-Dependent Exposures of PMA

To examine the time-dependent effects of PMA on LDLR expression, we treated SK-

Hep1 human hepatoma cell for 2 or 24 h with PMA (150 ng/ml), and determined the steady state levels of endogenous LDLR mRNA by RT-PCR and Northern blot analyses. The 2 h-treatment of PMA increased the levels of LDLR mRNA approximately 10-fold as determined by Northern blot as reported [Kumar et al., 1997] and 3-fold by RT-PCR. However, the 24 h-treatment of PMA repressed endogenous LDL receptor mRNA up to 50% of the vehicle-treated control without significantly affecting expression of a house-keeping β-actin gene (Fig. 1).

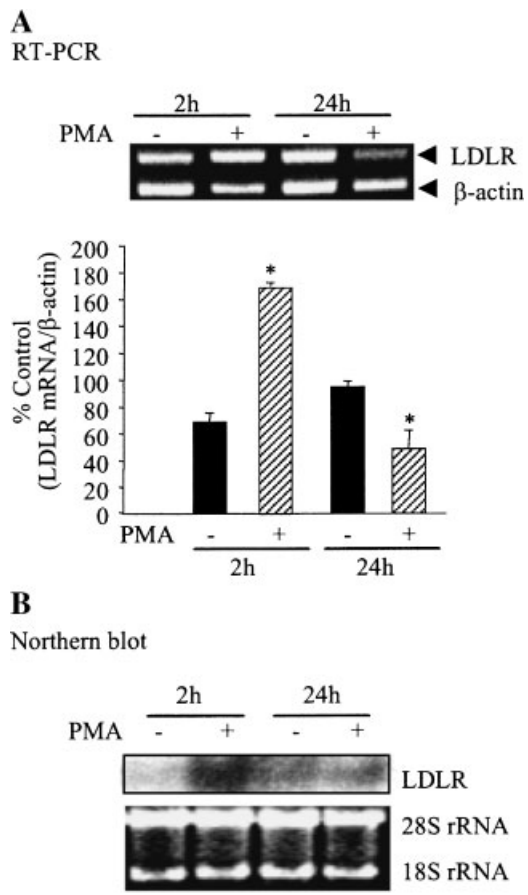

Fig. 1. Time-dependent regulation of endogenous low-density lipoprotein receptor (LDLR) expression by exposure to phorbol 12-myristate 13-acetate (PMA). Confluent SK-Hep1cells in 100 mm plates were cultured in DMEM containing 0.5% FBS for 16 h and treated with either PMA (150 ng/ml) or vehicle (DMSO) for 2 or 24 h. The cells were harvested for total RNA preparation using TRIzol. **A**: RT-PCR. The PCR fragments LDLR (629 bp) and β-actin (378 bp) are shown. The band intensities of LDLR on 1% agarose gel were determined by densitometry and normalized to β-actin mRNA. Values are mean ± SD of three independent experiments. *: $P < 0.005$ versus the vehicle-treated. **B**: Northern blot. The hybridized signals of LDLR are shown. Equivalent loading of RNA was verified by 28S and 18S rRNAs on the agarose gel stained with ethidium bromide.

Biphasic Regulation of LDL Receptor Transcription by PMA

We studied whether the time-dependent biphasic regulation of LDLR expression by PMA occurred at transcriptional level using pLR1563-luc, a luciferase reporter ligated to LDLR promoter (−1,563 to +58). Interestingly, time-dependent PMA treatment resulted in a biphasic transcriptional regulation of LDLR; a rapid and transient induction in early time points (2–3 h) and a repression after 6 h (Fig. 2A). Since most of known regulatory *cis*-acting elements including SRE-1 are present between −234 and +58 bp of the LDLR promoter, we transfected SK-Hep1 cells with two different LDLR promoter-luciferase constructs, pLR234-luc (containing −234 to +58 bp) and pLR1563-luc to localize the LDLR promoter region which is responsible for PMA-induced repression. Both 2 h- and 24 h-treatment of PMA induced the transcription of pLR234-luc. In pLR1563-luc-transfected cells, however, PMA induced the reporter activity at 2 h while it repressed the activity at 24 h, suggesting the *cis*-acting silencing element(s) which might be responsible for a long-term treatment of PMA might be present above −234 bp of the promoter (Fig. 2B).

N-SREBP2 Overexpression Does Not Reverse the PMA-Repressed LDLR Transcription

Next, we examined whether the PMA-induced repression of LDLR was influenced by SREBP2 in the context of LDLR promoter length. SK-Hep1 cells were co-transfected with the pLR234-luc or pLR1563-luc and the expression vector of N-SREBP2, constitutively active SREBP2. Figure 3 showed pLR1563-luc displayed higher basal transcription activity than pLR234-luc. The pLR1563-luc was suppressed by 24 h-treatment with PMA (150 ng/ml) as compared to the vehicle-treated. The overexpression of N-SREBP2 induced both pLR234-luc and pLR1563-luc activities, but did not reverse the PMA-induced pLR1563-luc suppression. The same PMA treatment increased the pLR234-luc activity regardless the presence of N-SREBP. Since phorbol ester is a strong AP1(c-Jun/c-Fos) complex activator [Angel and Karin, 1991], Ap1 may bind to putative AP1 binding site(s) on the region between −234 and +58 bp to synergistically enhance the LDLR promoter activity in the presence of N-SREBP2.

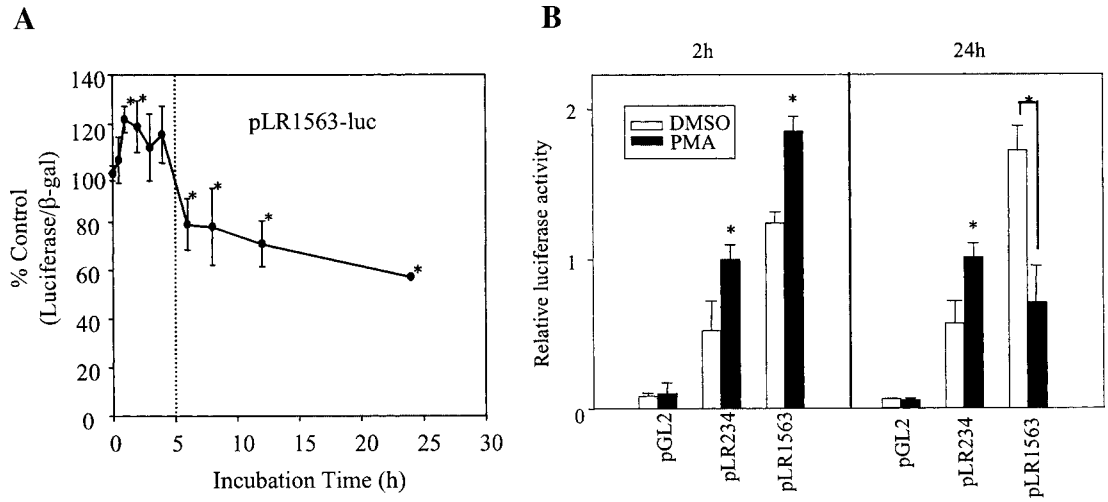

Fig. 2. **A**: Time-dependent regulation of LDLR transcription by PMA. The pLR1563-luc-transfected cells were treated with PMA (150 ng/ml) for various time periods and harvested for luciferase assay. **B**: Repression of LDLR promoter activity by PMA treatment for 24 h. The SK-Hep1 cells were transfected transiently with pLR234-luc or pLR1563-luc and cultured in DMEM containing 0.5% FBS for 16 h. The cells were treated with PMA (150 ng/ml) or DMSO for 2 or 24 h and harvested for luciferase assay. Luciferase activity was normalized to β-galactosidase activity and expressed as a relative value to activity of pLR234 treated with PMA. Data are mean ± SD of three independent experiments performed in triplicates. *: $P < 0.05$ versus DMSO control.

These results suggested that the long-term treatment of PMA might suppress the LDLR promoter activity without interacting SRE-1 or SREBP2, and this suppression might be strong enough to make the proximal promoter-mediated LDLR activation vanish.

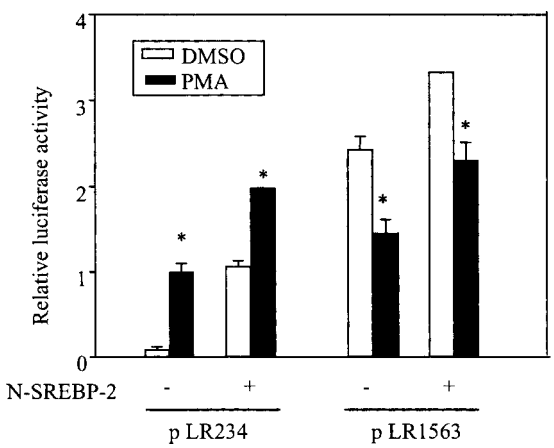

Fig. 3. Repression of both basal and N-SREBP-2 induced LDLR promoter activity by 24 h-treatment with PMA. SK-Hep 1 cells were transiently transfected with pLR234-luc or pLR1563-luc in the presence or absence of pCMV-N-SREBP-2. The transfected cells were treated with either PMA (150 ng/ml) or DMSO for 24 h after incubating in DMEM containing 0.5% FBS for 16 h. Luciferase activity of the cell lysates was normalized to β-galactosidase activity and expressed as a relative value to activity of pLR234 treated with PMA. Data are mean ± SD of three independent experiments performed in triplicate. *: $P < 0.05$ versus DMSO control.

LDLR Promoter Region Between −1,563 and −1,326 bp Is Necessary for LDLR Repression by PMA

To identify the LDLR promoter region which was responsible for repression by 24 h-treatment of PMA, we constructed the LDLR promoter-luciferase reporter series (pLR1563-luc, pLR1326-luc, pLR974-luc, pLR650-luc, and pLR234-luc) by sequential deletion as shown in Figure 4A. The basal activities of these pLR-luc constructs demonstrated that pLR1326-luc activity was lower than pLR974-luc, and pLR1563-luc was much higher than pLR974-luc (Fig. 4B). Therefore, we would assume that a putative activator(s) might bind between −1,563 and −1,326 and between −650 and −234 and repressor(s) between −1,326 and −650 as depicted in Figure 4C. Furthermore, only pLR1563-luc showed a strong inhibition of luciferase activity by PMA, but the other pLR-luc reporters were not repressed by PMA treatment (Fig 4B). The induction by PMA was repeated with the pLR234-luc and pLR650-luc. However, pLR974-luc and pLR1326-luc activities after PMA incubation were comparable to the basal activity of pLR1326-luc. The results implied that the PMA-mediated activation via the region from −234 to +58 was cancelled out by repression through the region above −650 to −1,326. PMA actively induced an inhibition through the element present between −1,563

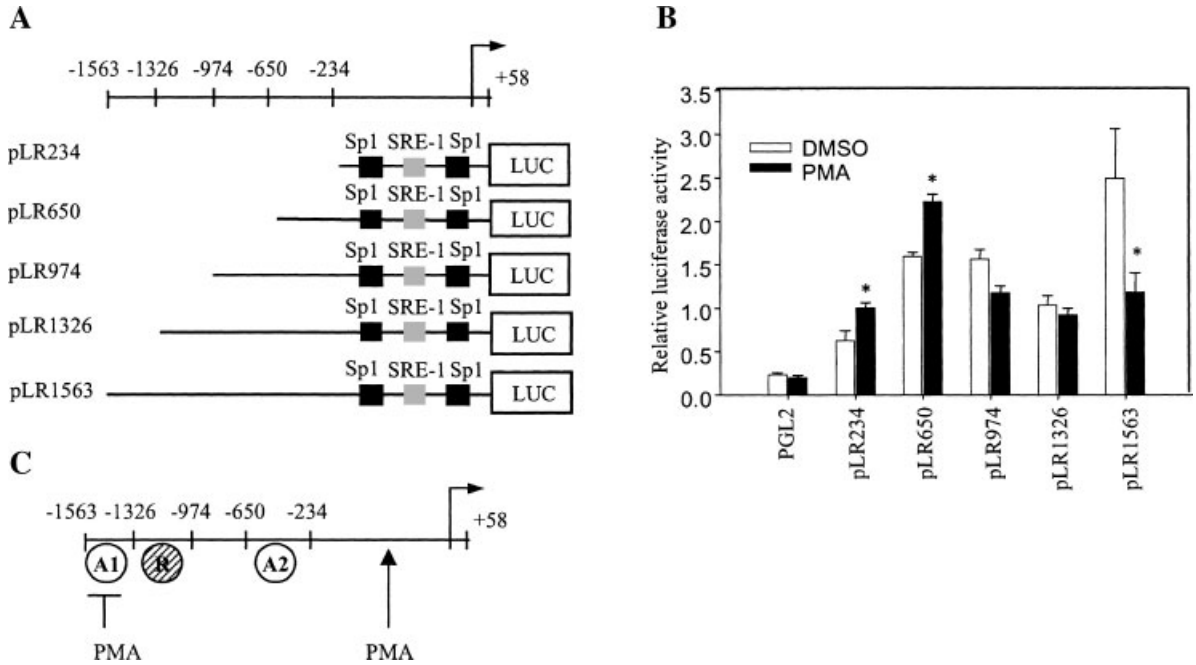

Fig. 4. The distal region of LDLR promoter is essential for the repression by PMA. **A**: Schematic presentation of plasmids used in this study. The serially truncated LDLR promoter-reporter vectors, pLR1563-luc, pLR1326-luc, pLR974-luc, pLR650-luc, and pLR234-luc, were constructed as described in ''Materials and Methods.'' **B**: PMA effects on the serially deleted pLR-luc activities. SK-Hep1 cells transfected with the designated reporter construct were cultured in DMEM containing 0.5% FBS for 16 h. The quiescent cells were treated with PMA (150 ng/ml) or DMSO for 24 h. Luciferase activity of cell lysates was normalized to β-galactosidase activity and expressed as a relative value to activity of pLR234 treated with PMA. Data are mean ± SD of three independent experiments performed in triplicates. *: $P < 0.05$ versus DMSO control. **C**: Binding scheme of activators or repressors as predicted from (B) A and R are putative activator and repressor, respectively. PMA not only enhanced proximal promoter-mediated LDLR transactivation but also repressed the transcription via the region between −1,563 and −1,326 bp.

and −1,326. Therefore, we suggest that a putative activator which might interact with the LDLR promoter region between −1,563 and −1,326, might be inactivated by the long-term treatment of PMA. The *cis*-acting PMA responsive element for LDLR induction is located between −234 and +58 but not for the LDLR repression (Fig. 4C).

The Specific p38 MAPK Inhibitor, SB202190, Reversed the PMA-Induced Repression of LDLR Transcription Activity

To investigate if protein kinase activity was involved in the repression of LDLR expression elicited by PMA, we studied the effect of GF109203X (a general PKC inhibitor), PD98059 (a MEK inhibitor), wortmannin (a PI3 kinase inhibitor), BAPTA/AM (an intracellular calcium chelator), genistein (a tyrosine kinase inhibitor), U73122 (a phospholipase C inhibitor), and SB202190 (a p38-MAP kinase inhibitor). Figure 5 shows that p38-MAPK inhibitor abolished the PMA-mediated repression and further increased the pLR1563-luc activity. However, inhibition of other protein kinases had no influence on the repressive effect of PMA. The results lead us to conclude that p38-MAPK pathway may be involved in PMA-mediated repression of LDLR, possibly by inactivation of the putative activator which binds to the region from −1,563 to −1,326. Confusingly, inhibitors of MEK and tyrosine kinase increased the basal LDLR promoter activity as compared to the vehicle-treated control. MEK pathway has been proved to be essential for LDLR induction by a variety of transcriptional modulators [Singh et al., 1999] and tyrosine kinase has been reported as a potential activator of LDLR expression [Kanuck and Ellsworth, 1995]. Discrepancy of our results with these reports may be explained by the differences of LDLR expression measurements. We monitored the LDLR promoter activity while the others measured endogenous LDLR messages. It has been reported LDLR mRNA is stabilized as a post-transcriptional modification

by phorbol ester [Wilson et al., 1998] or hepatocyte growth factor [Pak et al., 1996], which is not possible to monitor by reporter assay. In our study, however, we focused to search for modulator(s) or *cis*-acting promoter elements to regulate the LDLR transcription, thus the pathway for mRNA stabilization would be excluded. The regulatory loop of LDLR transcription by PMA is summarized on Figure 5B. Identification of putative activator(s) which is inactivated by p38-MAPK would be useful in development of novel LDLR transcription activator in order to reduce the serum cholesterol.

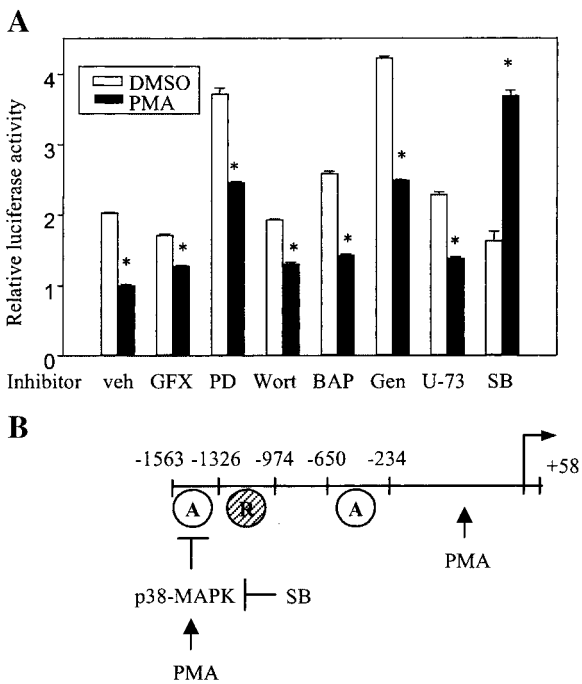

Fig. 5. **A**: Repression of the LDLR promoter activity by PMA via p38 MAPK pathway. The pLR1563-luc-transfected cells were pretreated with various inhibitor as indicated (GFX, 50 nM GF109203X PKC inhibitor; PD, 10 μM PD98059 MEK inhibitor; Wort, 50 nM Wortmannin PI3-kinase inhibitor; BAP, 1.25 μM BAPTA-AM a intracellular calcium chelator; Gen, 12.5 μM Genistein tyrosine kinase (TK) inhibitor; U-73, 10 μM U-73122 PLC inhibitor; SB, 20 μM SB202190 p38 MAP kinase inhibitor) for 30 min before PMA(150 ng/ml PMA) incubation for 24 h. The cells were harvested for luciferase assay. Luciferase activity was normalized to β-galactosidase activity and expressed as a relative value to activity of pLR1563 treated with PMA without inhibitor. Data are mean ± SD of three independent experiments performed in triplicate. *: $P < 0.05$ versus DMSO control. **B**: Schematic presentation of protein kinase action point on the scheme of Figure 4C. The long-term effect of PMA might be mediated by p38-MAPK. Neither MEK nor TK is involved in the PMA effect, whereas MEK or TK suppresses the basal LDLR transcription.

PKC-Activated Signaling Pathway Is Not Involved in the Long-Term Effect of PMA on LDLR Expression

PMA and other phorbol esters are potent activators of PKC. It is well recognized that PMA promotes cell proliferation, differentiation, and apoptosis [Hsu et al., 1998]. Moreover, there were several reports showing that PKC isoforms played a role in LDLR regulation [Mehta et al., 2002; Kapoor et al., 2003]. In contrast, our results shown in Figure 5 imply that PKC-mediated pathways may be not linked to PMA-repressed LDLR expression. Therefore, we determined the expression levels of representative PKC isoforms by Western blot. As expected, a 24 h-treatment of PMA resulted in depletion of PMA-sensitive PKC isoforms (α, δ, and ε) and no influence on the PMA-insensitive PKC isoforms (ζ, λ, and ι) in SK-Hep1 cells (Fig. 6). The PKCε, which was suggested to be critical in up-regulation of LDLR expression, was clearly decreased even in 2 h-treatment of PMA. In SK-Hep1 cells, PMA treatment for 2 h increased LDLR expression and pLR1563-luc activity under PKCε depletion, thus the induction of LDLR might be elicited by either PKCε-independent signaling pathway or activation of PKCε occurred earlier than 2 h. Furthermore,

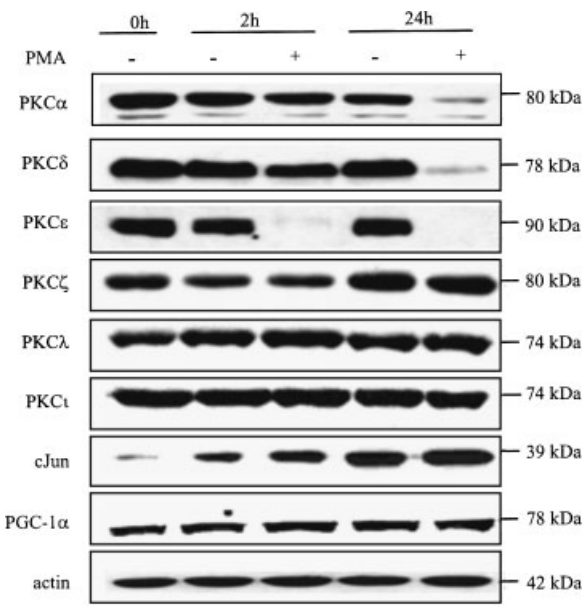

Fig. 6. Time-dependent changes of different PKC isoforms by PMA treatment. SK-Hep1 cells were cultured and treated with PMA as described in Figure 1. The cells were harvested and analyzed by Western blot using antibodies against various PKC isoforms, cJun, PGC-1α, or actin as indicated.

general inhibition of PKC by GF109203X did not influence the PMA-mediated repression, thus it was not likely that any isoforms of PKC played a role in PMA-repressed LDLR.

We have observed that over-expression of peroxisome proliferator activated receptor γ co-activator-1α (PGC-1α) suppressed LDLR expression at transcriptional level. We also determined the protein level of PGC-1α to examine if PGC-1α was a mediator for PMA effect. But the protein levels of PGC-1α were not altered by PMA (Fig. 6). Similarly, c-Jun protein, of which overexpression increased LDLR expression, was neither influenced by both short- and long-term treatment of PMA.

Decrease of Cell Proliferation by PMA

There are abundant evidences that cholesterol uptake via LDLR is required for concomitant cell proliferation. To conform whether the PMA-mediated repression resulted in blockade of cell proliferation, we measured time-dependent ^{3}H-thymidine incorporation in SK-Hep1 cells. A biphasic time-dependent effect of PMA on cell proliferation was observed; an increase of cell proliferation at 0.5 h post PMA treatment and a repression started at 3 h and completely achieved after 8 h (Fig. 7A). The time course of cell proliferation by PMA was similar to that of LDLR expression. Whether the LDLR repression and cell proliferation inhibition might be linked each other needs to be studied further.

To examine the effect of PMA on cell cycle progression, we analyzed the SK-Hep1 cells with and without 150 ng/ml PMA incubation for 24 h by FACS. As expected, PMA decreased both S and M phase populations from 25.6% and 11.6% to 13.2% and 7.4%, respectively, and increased G_0/G_1 phase population from 62.8% to 79.4% after 24 h-treatment of SK-Hep1 cells (Fig. 7B).

Cholesterol homeostasis is finely controlled by several factors. LDLR regulations are one of the most important steps to control the level of serum cholesterol. However, only sterol-mediated regulations are intensively investigated in the field. Since the long LDLR promoter constructs showed different regulatory patterns as shown in this study, uncovering of unidentified regulators which functioned on the distal region of LDLR promoter and/or a better understanding of the signaling pathway affecting these regulators would make us visualize the in vivo effects of therapeutics as well as cholesterol. Future study to identify the unknown activator responding to p38-MAPK would provide us a novel target for developing therapeutics.

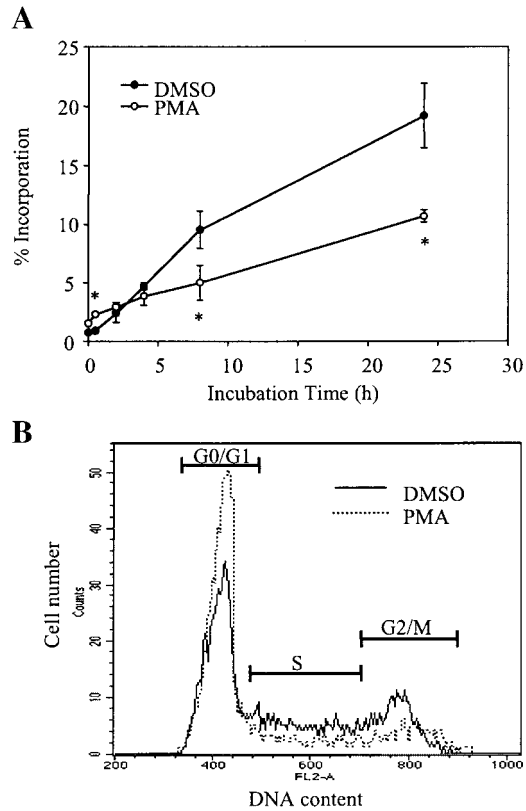

Fig. 7. Repression of cell proliferation by the 24 h-treatment of PMA. **A**: ^{3}H-thymidine incorporation assay. SK-Hep1 cells were grown to 80% confluence on 24-well plates, made quiescent by serum deprivation and were labeled with 0.5 μCi/ml [^{3}H]-thymidine per well in the presence or absence of PMA (150 ng/ml) for different time periods before terminating the reaction. After the indicated time period of incubation, the cells were washed, and [^{3}H]-thymidine incorporation into cellular DNA was measured as described in "Materials and Methods." Each point represents the mean ± SD for triplicate dishes of cells. *: $P < 0.05$ versus DMSO control. **B**: Cell cycle analysis by FACS. SK-Hep1 cells (5×10^5) in 100 mm dishes were cultured in DMEM containing 0.5% FBS for 16 h. The cells were treated with either PMA (150 ng/ml) or DMSO. After 24 h, the attached cells were harvested by trypsinization, resuspended in 1 ml PBS, and fixed in 1 ml of cold ethanol for 2 h at 4°C. The fixed cells were resuspended in PBS containing 0.1% BSA, 50 μg/ml RNase A, and 50 μg/ml propidium iodide after PBS washing. The harvested cells were analyzed by FACS.

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