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Activation of PKCβII and PKCθ is essential for LDL-induced cell proliferation of human aortic smooth muscle cells via Gi-mediated Erk1/2 activation and Egr-1 upregulation

Kyung-Sun Heo [a,d], Dong-Uk Kim [a], Lila Kim [a], Miyoung Nam [a], Seung-Tae Baek [a], Song-Kyu Park [b], Youngwoo Park [c], Chang-Seon Myung [d], Sung-Ook Hwang [e,*], Kwang-Lae Hoe [a,b,*]

[a] Functional Genomics Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yuseong, Daejeon, Republic of Korea
[b] Bio-Evaluation Center, KRIBB, Yuseong, Daejeon, Republic of Korea
[c] Therapeutic Antibody Research Center, KRIBB, Yuseong, Daejeon, Republic of Korea
[d] Department of Pharmacy, Chungnam National University, Yuseong, Daejeon, Republic of Korea
[e] Department of Obstetrics and Gynecology, Inha University Hospital, Incheon, Republic of Korea

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Abstract

Native LDL may be a mitogenic stimulus of VSMC proliferation in lesions where endothelial disruption occurs. Recent studies have demonstrated that the mitogenic effects of LDL are accompanied by Erk1/2 activation via an unknown G-protein-coupled receptor (GPCR). In this article, we report that LDL translocated PKCβII and PKCθ from cytosol to plasma membrane, and inhibition of PKCβII and PKCθ decreased LDL effects via the deactivation of Erk1/2. Moreover, pertussis toxin, but not cholera toxin or heparin, inhibited LDL-induced translocation of PKCβII and PKCθ, suggesting that Gi protein plays a role in LDL effects. Of LPA, S1P, and LDL, whose signaling is conveyed via Gi/o proteins, only LDL induced translocation of PKCβII and PKCθ. Inhibition of PKCβII or PKCθ, as well as of Erk1/2 and GPCR, decreases LDL-induced upregulation of Egr-1, which is critical for cell proliferation. This is the first report, to our knowledge, that the participation of PKCθ in VSMC proliferation is unique.

Keywords: Egr-1; Erk1/2 MAPK; GPCR; Low-density lipoprotein; PKC; Smooth muscle cell

Vascular smooth muscle cell (VSMC) proliferation and migration are important in physiological processes such as blood vessel development and in pathological conditions such as atherosclerosis, hypertension, and restenosis following angioplasty [1–3]. An increased level of low density lipoprotein (LDL) in blood plasma may develop into hypercholesteremia, hypertension, and atherosclerosis [4,5]. In particular, LDL could be a mitogenic and chemotactic regulator of VSMC in lesions where endothelial disruption occurs due to injury or angioplasty. LDL induces cell proliferation through multiple layers of signal transduction pathways, such as reactive oxygen species (ROS)-mediated extracellular signal-regulated kinase (Erk)1/2 activation and cell-cycle regulation [6,7]. The Erk1/2 cascade is one of the most important pathways for LDL-induced cell proliferation. Although the mechanisms of LDL-mediated Erk1/2 activation are not fully understood, it has been established that pertussis toxin (PTX)-sensitive G-protein activation is involved [6,8,9]. With stimulation, G-proteins dissociate to Gα and Gβγ subunits, both of which can initiate cellular signaling events [9]. Several mitogens that act via PTX-sensitive GPCR have been shown

* Corresponding authors. Fax: +82 2 860 4594.
E-mail addresses: sohwang@inha.ac.kr (S.-O. Hwang), kwanghoe@kribb.re.kr (K.-L. Hoe).

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to trigger specific signaling events, leading to proliferation through the activation of Ras and subsequent activation of Erk1/2 [8,9].

Protein kinase C (PKC) is a ubiquitously expressed family of proteins with 11 members subdivided into three groups based on their requirements for activation [10]. The conventional PKCs (α, β_{II}, and γ) require Ca^{2+}, lipid activators diacylglycerol (DAG), and phosphatidylserine (PS) as prerequisites for activation. The novel PKCs (δ, ε, η, θ, and μ) require only DAG and PS to catalyze their activation. The third subfamily of PKC is made up of atypical PKCs (ζ and ι/λ). This class of PKC requires neither Ca^{2+} nor DAG for activation. Various PKC isoforms participate in diverse stages of VSMC proliferation under pathophysiological conditions. PKC activation by mitogens, upstream from MAPK cascades, plays a pivotal role in transducing extracellular stimuli that modulate a number of cellular processes, including proliferation and angiogenesis [11,12]. It has also been implicated in the mitogenic response and growth of a number of different cell types such as VSMCs [13,14], airway smooth muscle cells [15], and fibroblasts [16].

PKC stimulation activates Erk1/2 through different pathways, depending on the cell type. Activated Erk1/2 translocates to the nucleus, where it can phosphorylate downstream kinases that directly activate transcription factors. For example, arterial injury in vessel cells activates Erk1/2, which in turn induces rapid and transient upregulation of the early growth response gene (*Egr)-1* [17]. It has been suggested that Egr-1 plays a role in proliferation and differentiation in response to a variety of growth stimulants in vascular cell types [18,19]. Despite research over a long period of time, the signaling pathway of the LDL effect is still unclear, as the responsible receptor is not clearly defined. In this regard, little information exists about LDL-generated signal transduction pathways in hAoSMC proliferation. Given the importance of LDL signaling as it relates to the pathophysiology of vascular disease such as intimal hyperplasia, restenosis, and atherosclerosis, the elucidation of LDL signaling pathways is important.

Materials and methods

Chemicals and antibodies. U0126, SB253580, PTX, CTX, 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), $PKC\beta_{II}$ inhibitor, and myristoylated PKCθ pseudosubstrate inhibitor (MYR-PKCθ-PS) were purchased from Calbiochem. Radioisotope was purchased from Amersham. PCR primers and PCR premix were purchased from Bioneer. All reagents, such as heparin, lysophosphatic acid (LPA), sphingosine 1-phosphate (S1P), and Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma unless otherwise stated.

Isolation of LDL and cell cultures. LDL (density 1.019–1.063 g/ml) was isolated from plasma of normocholesterolemic subjects (serum cholesterol <6.2 mM) by differential ultracentrifugation as previously described [20]. No oxidation was observed in isolated LDL for periods of up to 3 weeks, as determined by the thiobarbiturate method. Human AoSMCs (Clonetics) were cultured in SmGM-2 Bullet kit medium (Clonetics) at 37 °C in 5% CO_2. Cells were used at passages 3 through 8. For all experiments, the cells were grown to 80–90% confluence and made quiescent by starvation in a maintenance medium (DMEM containing 0.1% FBS, Invitrogen) for at least 24 h.

Cell proliferation assay (BrdU incorporation method). Approximately 1×10^4 cells per well were incubated in 96-well plates overnight at 37 °C, starved for 24 h, and pretreated with various inhibitors in the presence or absence of LDL. After an additional 24-h incubation, cell proliferation was measured by BrdU incorporation assay (Roche). BrdU incorporation into DNA was evaluated by photometric analysis (A_{450}) using a microplate reader (Emax, Molecular devices).

Western blot analysis. Cells were lysed in SDS sample buffer. Each sample was resolved by 10% SDS–PAGE, transferred onto PVDF membranes (Pall), analyzed with antibodies according to the supplier's protocol, and visualized with peroxidase and an enhanced-chemiluminescence system (ECL kit, Amersham). Normalization was performed with the SMC-specific α-actin antibody (M0851, BD Sciences). To examine the translocation of PKC isoforms, cells were transferred to ice-cold lysis buffer (25 mM Tris, 250 mM NaCl, 3 mM EDTA, and 1% Triton X-100) and disrupted rapidly in a sonicator. The lysed samples were then separated into soluble and particulate fractions, which were immediately boiled in Laemmli SDS-stop buffer. Rabbit polyclonal antibodies specific for phospho-Erk1/2 (#9101), PKCα (#2056), PKCδ (#2058), PKCθ (#2059), and IgG (#7074) were obtained from Cell Signaling. Mouse monoclonal antibody specific for $PKC\beta_{II}$ (#sc-13149) was obtained from Santa Cruz biotechnology.

Northern blot analysis. Total RNA from hAoSMC was prepared with Trizol reagent according to the manufacturer's protocol (Gibco). Northern blotting was performed as described previously [20]. Northern probe was prepared by gel-extraction of PCR-products. Primer sequences for PCR amplication of *egr-1* were as follows; *egr-1* forward, 5′-CACCAT GGACAACTACCCTAAGCTGG-3′, reverse, 5′-CCGACAGCAGTCC CATTTACTCAGCGG-3′; size of PCR product = 536-bp.

Transient transfection and luciferase reporter assay. The promoter assay for Egr-1 was performed as described previously [20]. Briefly, 60% confluent hAoSMC (1×10^6) was transfected with 1 μg of the pFR-Luc-*egr-1* plasmid along with the normalization vector, pSV-β-galactosidase (0.5 μg, Promega). An empty luciferase plasmid, pFR-Luc, was used as a basal control. After transfection using the Superfect reagent (Qiagen), the cells were starved in serum-free maintenance medium for 16 h, and then pretreated with PTX, U0126, $PKC\beta_{II}$ inhibitor, and MYR-PKCθ-PS for 30 min in the presence or absence of LDL. The cells were then harvested and luciferase activity was measured using a Biolumat 9505 luminometer (Berthold Technology). The pFR-Luc-*egr-1* plasmid was constructed from the parent vector pFR-Luc (the PathDetect luciferase *cis*-reporter system, Stratagene) as follows. First, a 2.7-kb DNA fragment containing the promoter region of *egr-1* (GenBank Accession No. 1958) was amplified by PCR using a pair of specific primers: forward 5′-CGGAAGCTTAAT GAGAGCCGGGTTTCCC (with a HindIII extension underlined), and reverse 5′-CGGGGTACCGCGCATCACACAAAAGGCA (with a KpnI extension underlined). The PCR products were then confirmed by DNA sequencing. The 2.7-kb DNA fragment containing the *egr-1* promoter region was digested with restriction enzyme, and ligated into the HindIII/KpnI-digested pFR-Luc vector.

Statistical analysis. Experimental data were analyzed with the GraphPad Prism program, version 2.00 (GraphPad Software). All values are reported as mean ± SD of at least three independent experiments. The unpaired Student's *t*-test was used to assess the significance of the differences between the two groups. A value of $p < 0.05$ was accepted as significant.

Results and discussion

$PKC\beta_{II}$ and PKCθ translocate from cytosol to plasma membrane in response to LDL

PKC is a well-known regulator of cell proliferation in VSMC [13,14]. To determine which isoforms of PKC are related to LDL-induced hAoSMC proliferation, the effect

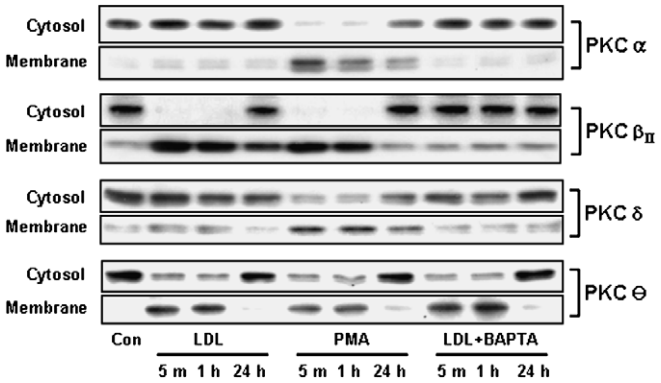

Fig. 1. LDL-induced translocation of PKCβII and PKCθ form cytosol to plasma membrane. AoSMCs were stimulated with LDL (100 μg/ml), PMA (100 nM), or BAPTA (10 μM) in the presence of LDL for the indicated times. Western blot analysis was then performed using antibodies of the indicated PKC isoforms. The pictures represent typical examples of each of three independent experiments.

of LDL on PKC translocation was examined using specific antibodies. Out of 11 PKC isoforms, six isoforms (α, βII, δ, ε, θ, and ζ) have been reported to coexist in vascular smooth muscle, although their expression patterns vary depending upon species and vascular bed [21]. However, PKCε and PKCζ were unrecognizable due to signals weaker than the detection range (data not shown). As shown in Fig. 1, α, βII, δ, and θ PKC isoforms located in the cytosol without stimulation and the positive control (PMA) translocated all the tested PKC isoforms to the membrane, as expected. However, PKCβII and PKCθ only translocated to the membrane in response to LDL. The translocation was at its maximum at 5 min after LDL treatment, which lasted for 1 h and decreased to the basal level around 24 h. When Ca^{2+} inhibitor (BAPTA) was pretreated before LDL, only the translocation of conventional PKCβII was completely abolished, due to its sensitivity to Ca^{2+}, without affecting the localization of novel PKCθ. Taken together, LDL treatment resulted in translocation of PKCβII and PKCθ in hAoSMC. In particular, changes in intracellar Ca^{2+} concentration were not related to LDL-induced translocation of novel PKCθ, but were related to translocation of conventional PKCβII, which is consistent with the previous report [8].

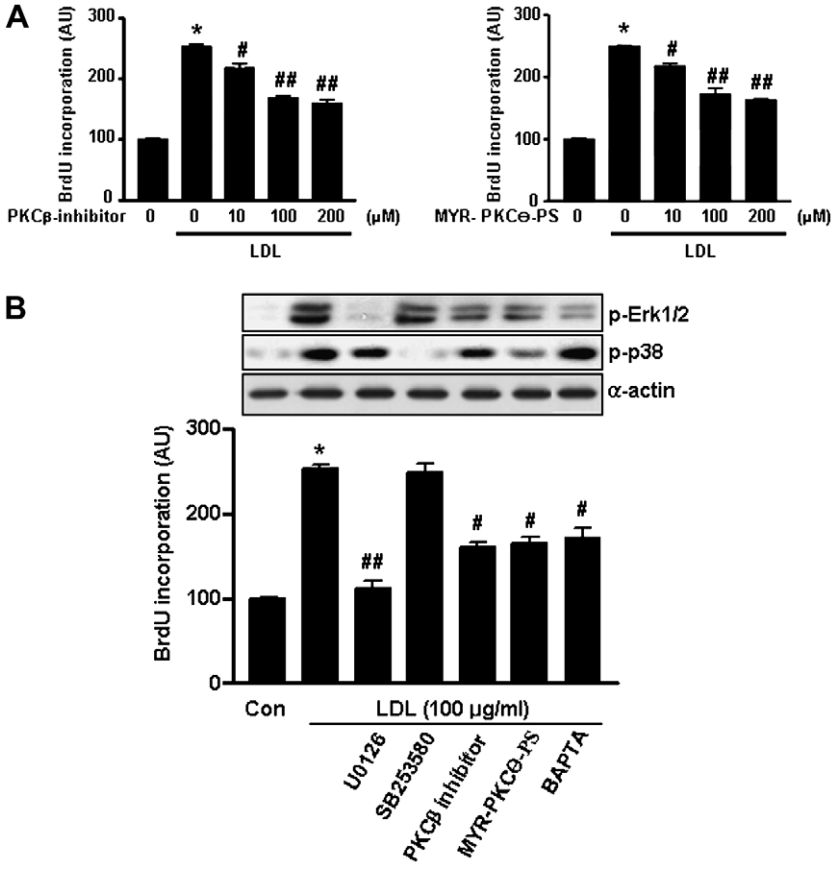

Fig. 2. Participation of PKCβII and PKCθ in the LDL effect via Erk1/2 activation. (A) Inhibition of PKCβII or PKCθ decreased LDL-induced DNA synthesis in a dose-dependent manner. PKCβII (left) or PKCθ inhibitors (right) were pretreated at the indicated concentrations for 30 min before LDL treatment and their effects on cell proliferation were analyzed by BrdU incorporation. $n = 7$, $^{*}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LDL. (B) Inhibition of PKCβII or PKCθ decreased the LDL effects via the deactivation of Erk1/2. The indicated inhibitors for Erk1/2 (U0126, 20 μM), p38 (SB253580, 10 μM), PKCβII (100 μM), PKCθ (MYR-PKCθ-PS, 100 μM), or Ca^{2+} signaling (BAPTA, 10 μM) were pretreated for 30 min and their effects on cell proliferation were analyzed by BrdU incorporation after an additional 24-h incubation in the presence of LDL (lower). $n = 7$, $^{*}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LDL. At the same time, their effects on Erk1/2 and p38 activation were analyzed by Western blot analysis after a 5-min exposure to LDL (upper). The α-actin was used as a normalization control.

Inhibition of PKCβ_{II} or PKCθ decreases LDL-induced cell proliferation via the deactivation of Erk1/2

The results reported above prompted us to investigate whether LDL-induced cell proliferation is related to translocation of PKCβ_{II} and PKCθ. As shown in Fig. 2A, inhibition of either PKCβ_{II} or PKCθ affected DNA synthesis in a dose-dependent manner. However, their inhibition did not lead to a complete abolition of cell proliferation evoked by LDL, suggesting that PKCβ_{II} and PKCθ are not fully responsible for Erk1/2 activation. Future study will be required to elucidate which is responsible for the full activation of Erk1/2 in response to LDL. Next, the effects of PKC inhibitors on DNA synthesis and Erk1/2 activation were investigated (Fig. 2B). Compared with the control, LDL treatment resulted in a 175% increase in DNA synthesis and Erk1/2 activation, which are essential for cell proliferation. MEK inhibitors (U0126) abolished the LDL effect, while p38 inhibitors (SB253580) had no effect at all. Inhibitors of PKCβ_{II}, PKCθ, or Ca^{2+} influenced cell proliferation by 56.3%, 52%, and 48.5% along with a concomitant decrease in Erk1/2 activation. An intriguing finding was that MYR-PKCθ-PS inhibited p38 activation as well as Erk1/2 activation, but to a much lesser extent than SB253580. These results suggest that PKCθ is likely to affect not only LDL-induced Erk1/2 activation but also p38 activation, which is critical for LDL-induced IL-8 production via activation of activating protein (AP)-1 and participation of nuclear factor (NF)-κB [20]. There is a consistent line of evidence that a novel type of PKCθ is related to signaling for cytokine upregulation and cell proliferation in T cells via the activation of transcription factors, such as AP-1, NF-κB, and nuclear factor of activated T cells (NFAT) [20,22,23].

Among mitogenic stimulants via Gi/o proteins, such as LPA, S1P, and LDL, only LDL induces translocation of both PKCβ_{II} and PKCθ, which is affected by PTX, but not by CTX and heparin

An accumulating body of evidence suggests that GPCR may be a receptor responsible for LDL effects [8]. Therefore, the effects of GPCR inhibitors on DNA synthesis and Erk1/2 activation were examined using heparin

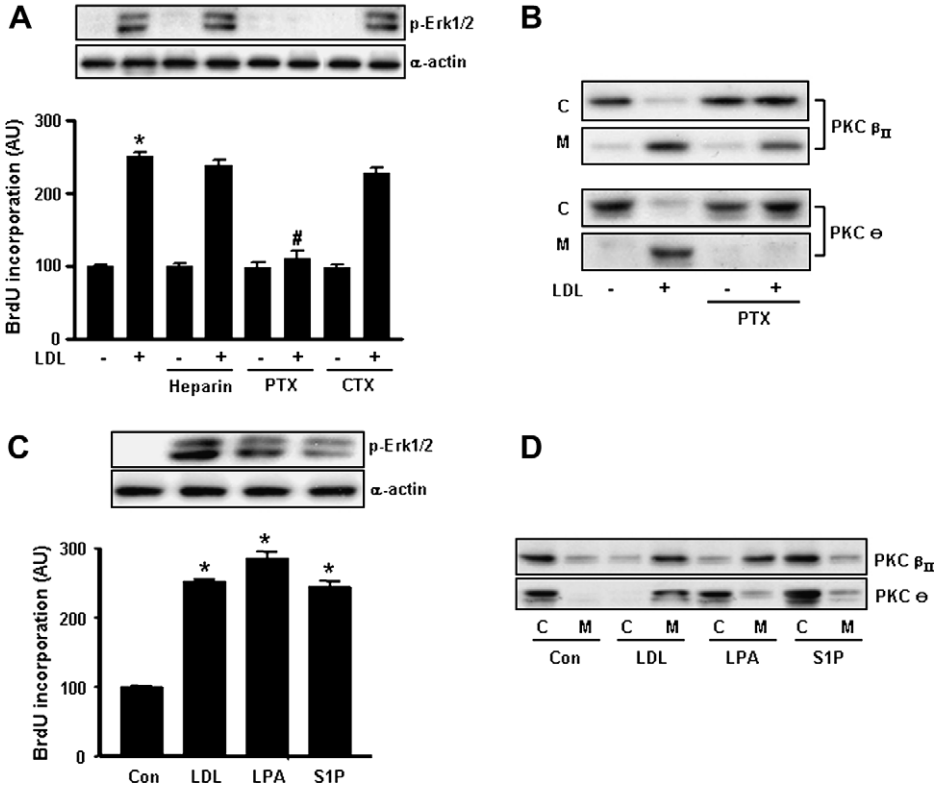

Fig. 3. Participation of PKCβ_{II} and PKCθ in the LDL effects via a novel GPCR. (A) PTX abolished the LDL effect via the deactivation of Erk1/2. Cells were pretreated with heparin (100 μg/ml), PTX (100 ng/ml), or CTX (100 ng/ml) for 1 h. Cell proliferation was then assayed by BrdU incorporation after an additional 24-h incubation in the presence or absence of LDL (100 μg/ml) (lower). At the same time, Erk1/2 phosphorylation was measured by Western blot analysis after a 5-min incubation in the presence or absence of LDL (upper). Results are represented as mean ± SEM from three separate experiments. $n = 8$, $^{*}P < 0.01$ versus control, $^{\#}P < 0.01$ versus LDL. (B) PTX abolished LDL-induced translocation of PKCβ_{II} and PKCθ. Cells were pretreated with PTX for 1 h. Translocation of either PKCβ_{II} or PKCθ was then measured by Western blot analysis after a 5-min incubation in the presence or absence of LDL. (C) LPA, S1P, and LDL, which are well-known mitogenic stimulants via Gi/o protein, induced cell proliferation via Erk1/2 activation. Cells were treated with LDL (100 μg/ml), LPA (20 μM), or S1P (100 nM). Cell proliferation (lower) or Erk1/2 phosphorylation (upper) was then measured after a 24-h or 5-min incubation. $n = 4$, $^{*}P < 0.01$ versus control. (D) LDL translocated both PKCβ_{II} and PKCθ. Cells were treated with LDL, LPA, or S1P and translocation of PKCβ_{II} or PKCθ was then measured by Western blot analysis after a 5-min exposure to LDL.

(classical LDL receptor inhibitor), PTX (Gαi/o-protein coupled receptor inhibitor), and CTX (Gαs-protein coupled receptor inhibitor). As shown in Fig. 3A, LDL increased DNA synthesis by 175% with Erk1/2 activation. The LDL effect was not affected by heparin, suggesting that the classical LDL receptor is not related to LDL-induced cell proliferation. Moreover, PTX, but not CTX, completely abolished LDL-induced DNA synthesis and Erk1/2 activation. These results support previous reports that LDL is believed to exert its proliferation effects via a GPCR (not yet identified) instead of the conventional LDL receptor, LDLR [6,8,24]. Next, it was determined whether PTX affects LDL-induced translocation of $PKC\beta_{II}$ and PKCθ (Fig. 3B). The LDL-induced translocation of PKCθ to the membrane was completely abolished by PTX, while $PKC\beta_{II}$ translocation was mostly abolished. The results suggest that Gαi/o-protein, not Gαs-protein, is responsible for the LDL effect.

In addition to LDL, a few lysophospholipids, such as LPA and S1P, have been identified as stimulants for cell proliferation via Gi/o-protein-coupled receptors [25,26]. Therefore, LDL was compared with LPA and S1P in terms of cell proliferation and PKC isoforms. As shown in Fig. 3C, LPA and S1P increased DNA synthesis as much as LDL and activated Erk1/2 to a much greater extent than LDL. These results led us to investigate their effects on translocation of $PKC\beta_{II}$ and PKCθ, compared with LDL. As expected, LDL induced translocation of both $PKC\beta_{II}$ and PKCθ to the membrane fraction. In contrast, LPA induced translocation of $PKC\beta_{II}$ to the membrane fraction, but not PKCθ, whereas S1P had no effect on translocation of either $PKC\beta_{II}$ or PKCθ (Fig. 3D). These results are consistent with a previous report that LPA enhances DNA synthesis of rat AoSMCs via the activation of $PKC\beta_{II}$ in a PTX-sensitive manner [27]. Taken together, these results suggest that although LDL and LPA induce cell proliferation via a common $PKC\beta_{II}$ signaling pathway, LDL has an extra PKCθ signaling pathway, which is unique compared to other mitogens such as LPA or S1P. This is the first finding, as far as we know, that PKCθ is involved in cell proliferation of VSMCs.

Inhibition of either $PKC\beta_{II}$ or PKCθ, as well as Erk1/2 and GPCR, decreases LDL-induced upregulation of Egr-1, which is critical for cell proliferation

It has been suggested that Egr-1 plays a role in the proliferation and differentiation of vascular cell types [28]. The promoter regions of many genes that encode mitogenic and migratory factors expressed by VSMCs contain recognition elements for the transcriptional regulator Egr-1 [29]. As shown in Fig. 4A, LDL induced upregulation of Egr-1 expression in a time-dependent manner. LDL-induced upregulation of Egr-1 reached its maximum by 20 min and decreased to basal levels through 120 min. The effects of inhibitors for GPCR, Erk1/2, $PKC\beta_{II}$, or PKCθ on Egr-1 transcriptional expression were investigated next

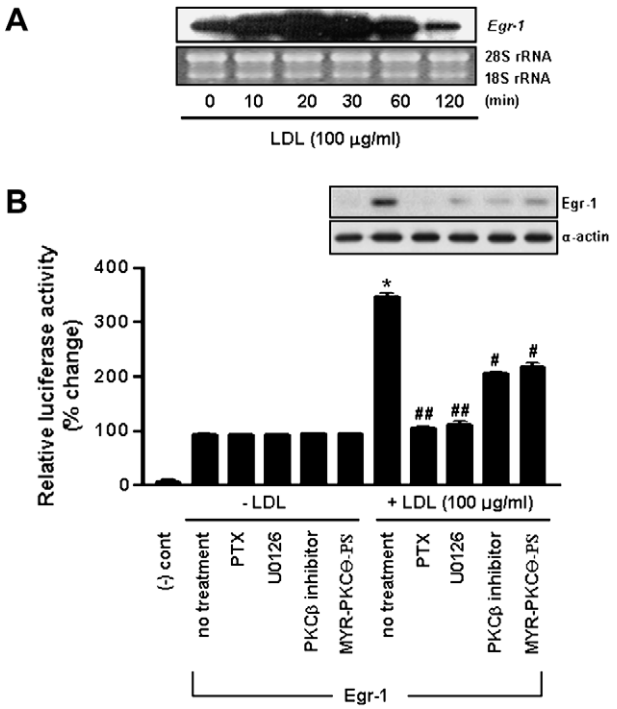

Fig. 4. Participation of $PKC\beta_{II}$ and PKCθ in LDL effect via the upregulation of *Egr-1*. (A) LDL induces upregulation of *Egr-1* in a time-dependent manner. Cells were treated with LDL (100 μg/ml) for the indicated time and the mRNA levels of Egr-1 were determined by Northern blot analysis. Ribosomal RNA (rRNA) was used as a normalization control. (B) Inhibition of $PKC\beta_{II}$ or PKCθ decreased the LDL effect via the down-regulation of Egr-1. Cells harboring the pFR-Luc-*egr-1* plasmid were pretreated with the indicated inhibitors (PTX, U0126, $PKC\beta_{II}$ inhibitor, or MYR-PKCθ-PS) for 30 min in the presence or absence of LDL, and levels of Egr-1 were measured by luciferase assay (upper) and Western blot assay (upper) after an additional 30-min incubation. $n = 11$, $^{*}P < 0.01$ versus control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LDL.

(Fig. 4B). According to the results of a Luciferase reporter assay, pretreatment with PTX and U0126 completely abolished LDL-induced transcriptional activation of Egr-1, whereas pretreatment with $PKC\beta_{II}$ or PKCθ inhibitors decreased the LDL effects by 54% or 56% (Fig. 4B, lower). These results point to the importance of PTX-sensitive G-protein-mediated Erk1/2 activation and subsequent Egr-1 upregulation for LDL-stimulated hAoSMC proliferation. Consistent with these results, Western blot analysis also showed that pretreatment with PTX, U0126, $PKC\beta_{II}$ or PKCθ inhibitors almost abolished LDL-induced Egr-1 upregulation (Fig. 4B, upper). It has been previously reported that the activation of $PKC\beta_{II}$ is a critical upstream regulator of Egr-1 [30]. However, the participation of PKCθ in LDL-induced upregulation of Egr-1 is a new finding from the present study.

We have found, for the first time, which $PKC\beta_{II}$ and PKCθ play key roles in LDL-induced cell proliferation via the activation of Erk1/2 and the upregulation of Egr-1 expression through PTX-sensitive Gi/o-protein-coupled receptors. The results suggest that the participation of novel PKCθ in the LDL signaling pathways may constitute

a novel therapeutic target for the treatment of proliferative cardiovascular diseases. Furthermore, we have provided a line of evidence that implies another role of PKCθ as a cytokine mediator via p38 activation.

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