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Secretion of Atherogenic Risk Factor Apolipoprotein B-100 is Increased by a Potential Mechanism of JNK/PKC-Mediated Insulin Resistance in Liver Cells

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Abstract Apolipoprotein B-100 (ApoB) is the main protein of the atherogenic lipoproteins and plasma ApoB levels reflect the total numbers of atherogenic lipoproteins. Induction of insulin resistance was accompanied by a considerable rise in the production of hepatic very low density lipoprotein (VLDL) containing ApoB and triglyceride. Increased plasma levels of ApoB and triglyceride in VLDL are common characteristics of the dyslipidemia associated with insulin resistance and type 2 diabetes mellitus. Thus, we investigate whether phorbol 12-myristate-13-acetate (PMA)-induced insulin resistance affects the increase of ApoB secretion. PMA increased ApoB secretion and transcriptional level of microsomal triglyceride transfer protein (MTP). PMA treatment also resulted in increase of insulin receptor substrate 1 (IRS1) serine312 (Ser312) and serine1101 (Ser1101) phosphorylation and induction of IRS1 degradation. Additionally, PMA induced activation of c-jun N-terminal kinase (JNK) and protein kinase C (PKC) isoforms (α, βI, δ, ζ, θ), and reduced AKT8 virus oncogene cellular homolog (AKT) activation in a time dependent manner. PMA-induced ApoB secretion, MTP promoter activities, and IRS1 degradation was significantly decreased by treatment of JNK and PKCs inhibitors. Orthovanadate, a potent tyrosine phosphatase inhibitor, increased tyrosine phosphorylation of IRS1 and decreased ApoB secretion of Chang liver cells although PMA was co-treated. From the results, it was concluded that PMA-induced insulin resistance, through induction of serine phosphorylation of IRS1 mediated by activated JNK and PKCs, increases ApoB secretion in Chang liver cells. J. Cell. Biochem. 103: 908–919, 2008.

Key words: apolipoprotein B-100; insulin resistance; phorbol 12-myristate-13-acetate; dyslipidemia

Apolipoprotein B-100 (ApoB) is the structural protein required for the assembly and secretion of lipoproteins which transport hydrophobic lipids, cholesteryl ester, and triglycerides in their cores. ApoB is embedded in the phospholipids monolayer on the surface of triglyceride-rich lipoproteins including very low density lipoprotein (VLDL) and its metabolites intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) [Segrest et al., 2001; Olofsson and Borèn, 2005]. Human ApoB is a large amphipathic protein of 4,536 amino acids and a molecular mass of approximately 520 kDa that is synthesized in the liver [Cladaras et al., 1986; Knott et al., 1986]. There is also a truncated form of ApoB called ApoB-48 that is synthesized in human small intestine. ApoB-48 is also synthesized in livers of several lower species, but not in human liver and present on chylomicrones and their remnants [Dixon and Ginsberg, 1993; Olofsson and Borèn, 2005]. Except where noted, the term ApoB will refer to ApoB-100 at this paper. The assembly of

Abbreviations used: ApoB, apolipoprotein B-100; MTP, microsomal triglyceride transfer protein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; PMA, phorbol 12-myristate-13-acetate; JNK, c-jun N-terminal kinase; PKC, protein kinase C; AKT, AKT8 virus oncogene cellular homolog; ERK, extracellular signal-regulated kinase; IR, insulin receptor; IRS1, insulin receptor substrate-1; RT-PCR, Reverse transcription-polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

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ApoB into VLDL involves transfer of lipid by the microsomal triglyceride transfer protein (MTP). MTP functions by transferring lipid to ApoB during its translation and by transporting triglycerides into the endoplasmic reticulum to form ApoB-free lipid droplets. These droplets fuse with nascent ApoB-contained particles to form mature VLDL [Davidson and Shelness, 2000; Shelness and Ledford, 2005]. Recently, ApoB is regarded as the main protein of the atherogenic lipoproteins (VLDL, IDL, and LDL) and each lipoprotein has one ApoB molecule [Dixon and Ginsberg, 1993; Olofsson and Borèn, 2005]. Therefore, plasma ApoB levels reflect the total numbers of atherogenic lipoproteins. Moreover, lipid and lipoprotein indexes used in identifying cardiovascular risk and in judging the adequacy of therapy for hyperlipidemia as common lipid disorder include LDL cholesterol, non-high density lipoprotein (HDL) cholesterol, and ApoB [Stein et al., 2005].

The action of insulin is mediated via binding to cell surface insulin receptor (IR), which activates the protein kinase associated with IRβ subunit. Stimulation of protein kinase activity induces tyrosine phosphorylation of endogenous substrates, including IR substrates (IRSs) such as IRS1. This tyrosine phosphorylation of IRS1 allows them to interact with and recruit Src homology 2 (SH2)-domain-containing protein, including phosphatidyl inositol 3-kinase (PI3K), Grb2/mSos, protein tyrosine phosphatase with SH2 domains (SH-PTP2) for further downstream signaling molecules. These events lead to activation of multiple signaling pathways including glucose uptake, glycogen synthesis, mitogenesis, or gene expression [Virkamaki et al., 1999; Pederson et al., 2001; Jiang et al., 2004]. In addition to tyrosine phosphorylation, the IR and IRS proteins undergo serine phosphorylation that attenuates insulin signaling [Aguirre et al., 2001; Rui et al., 2001]. The inhibitory phosphorylation of IRS1 is known to occur on multiple serine residues, including serine312 (Ser312) (equivalent to Ser307 of rodent IRS1) and serine1101 (Ser1101) of human IRS1 [Kanety et al., 1995; Mothe and Van Obberghen, 1996; Griffin et al., 1999; Aguirre et al., 2001]. To date, several serine/threonine kinases have been implicated in this process; these include protein kinase C (PKC) isoforms (i.e., PKCθ) [Griffin et al., 1999; Ravichandran et al., 2001] and c-Jun N-terminal kinase (JNK) [Aguirre et al., 2000, 2001; Hirosumi et al., 2002]. Especially, serine phosphorylation of IRS1 results in ubiquitination and degradation of IRS1 and has been strongly implicated in a mechanism of insulin resistance [Pederson et al., 2001].

Induction of insulin resistance was known to be accompanied with a considerable rise in the production of hepatic VLDL containing ApoB and triglyceride in vivo [Taghibiglou et al., 2000; Avramoglu et al., 2006]. Increased plasma levels of ApoB and triglyceride in VLDL are not only common characteristics of the dyslipidemia associated with insulin resistance and type 2 diabetes mellitus but also are the central pathophysiologic feature of the abnormal lipid profile [Ginsberg et al., 2005]. The dyslipidemia accompanying insulin resistance is characterized by distinct changes from a normal plasma lipid and lipoprotein profile. These changes include decrease of HDL levels in plasma, increase of VLDL production, a concomitant increase in ApoB, an essential structural component of these atherogenic lipoproteins, and increase of plasma LDL levels. Although the link between insulin resistance and dysregulation of lipoprotein metabolism is well established, a significant gap of linkage still exists regarding the underlying cellular and molecular mechanisms [Avramoglu et al., 2006; Reaven et al., 1993; Taskinen, 1995].

In this study, we investigated whether phorbol 12-myristate-13-acetate (PMA)-induced insulin resistance affects the increase of ApoB secretion in liver cells. We demonstrate that PMA increases ApoB secretion through the potential mechanism of JNK/PKCs-mediated insulin resistance in Chang liver cells.

MATERALS AND METHODS

Materials

PMA (Sigma), Gö6976 (Calbiochem), and SP600125 (Calbiochem) were solubilized in dimethyl sulfoxide (DMSO). Sodium orthovanadate (Sigma) was solubilized in water and cycloheximide (Sigma) was solubilized in ethanol.

Cell Culture and Treatment of Chemicals

Chang liver cells (ATCC, CCL-13) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; JBI Co., Daegu, Korea), 100 U/ml penicillin and

100 μg/ml streptomycin at under 5% CO_2 at 37°C. Chang cells were cultured under serum-free condition for 6 h, and these cells were cultured with or without 30 nM PMA for different times and with different concentrations of PMA for 24 h. In some cases, 5 μM Gö6976 (PKC inhibitor), 10 μM SP600125 (JNK inhibitor), and 0.2, 1, and 2 mM sodium orthovanadate (tyrosine phosphatase inhibitor) was added for 1 h before the addition of PMA. Fifty micromolars cycloheximide (protein synthesis inhibitor) was also added after the treatment of PMA for 2 h.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA from each cell was isolated using the Trizol reagent (JBI Co.), and the cDNAs were synthesized by reverse transcriptase with an oligo dT-adaptor prime from a Takara RNA PCR kit (Takara Shuzo, Shiba, Japan) according to the manufacturer's recommended protocol. The cDNA was amplified by PCR with the following primers: ApoB (607 bp), 5′-GAACTTGAAGAAAAGATAGTCAGTC-3′ (sense) and 5′-TGTATATCAGAAATGTGTGGTAGTT-3′ (antisense); MTP (500 bp), 5′-ATCCTCTTT-GATGTTCAGCT- 3′ (sense) and 5′-ATTCA-CATCCTGCTAATACG -3′ (antisense); β-actin (247 bp), 5′-CAAGAGATGGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (antisense). The use of equal amounts of mRNA in the RT-PCR assays was confirmed by analyzing the expression levels of β-actin. The PCR products were separated by gel electrophoresis on 1.5% agarose containing ethidium bromide with 1× TAE buffer.

Western Blot Analysis

Cells were homogenized in a buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_3, 100 μg/ml PMSF, and 1 μg/ml aprotinin. Cell lysates were centrifuged at 12,000 rpm and pellets were washed at three times. Membrane protein was extracted in a buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% NaN_3, 100 μg/ml PMSF, 1 μg/ml aprotinin, and 1% Triton X-100. For preparation of total protein, cells were also homogenized in the membrane protein extraction buffer. Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad). Twenty-microgram samples of cell lysates were size fractionated by SDS–PAGE and electrophoretically transferred to nitrocellulose membranes using the Hoefer electrotransfer system (Amersham Biosciences, UK). To confirm secreted ApoB in the cultured media, media were collected and aliquots of samples, based on cell protein, were used at SDS–PAGE. To detect target proteins, the membranes were incubated with the p-SAPK/JNK (Cell Signal), SAPK/JNK (Cell Signal), p-AKT (Santa Cruz Biotechnology), AKT (Santa Cruz Biotechnology), PKCs (α, βI, γ, δ, ε, ζ, η, θ) (Santa Cruz Biotechnology), ApoB (CN Co.), ISR1 (Santa Cruz Biotechnology), p-IRS1 (Ser1101) (Cell Signal), p-IRS1 (Ser312) (Abcam, Inc.), or GAPDH (Chemicon) antibodies. Detection was performed using a secondary horseradish peroxidase-linked anti-mouse antibody and an anti-rabbit antibody and the ECL chemiluminescence system (Amersham).

MTP Promoter Assay

A 776 bp fragment from the 5′-promoter region of the MTP gene was cloned. Briefly, a 776 bp fragment at the 5′-flanking region of the human MTP gene was amplified by PCR using specific primers from the human MTP gene (accession no. S71339): 5′-GATATGGT-ACCCCCCTCTTAATCTCTTCCT (forward) and 5′-AGTCAAGCTTGTGGACTCTTTCAATGGCA (reverse). The pGL3-Basic vector containing the luciferase coding region was used to construct the expression vectors by subcloning PCR-amplified DNA of the MTP promoter into the *Kpn* I/*Hin*d III site of the pGL3-Basic vector. For the reporter analysis of the MTP promoter, the transient transfection of Chang cells was carried out using the Lipofactamine reagent. Briefly, cells were plated on six-well plates at a density of 10^5 cells/well and allowed to grow overnight. The cells were co-transfected with 1 μg of MTP promoter-luciferase reporter constructs and 1 μg of β-galactosidase reporter plasmid by the LipofecAMINE method (Invitrogen). These cells were cultured in DMEM/10% FBS medium for 24 h. The transfected cells were cultured under serum-free condition for 6 h and the various inhibitors were then added for 1 h followed by incubation with or without PMA. Luciferase activity and β-galactosidase activity were assayed using the luciferase and β-galactosidase enzyme assay system (Promega). Luciferase activity was normalized with the β-galactosidase activity in cell lysates and calculated as an average of three independent experiments.

Immunoprecipitation

Samples of the total protein (200 μg) were incubated with the anti-ApoB or anti-IRS1 polyclonal antibody (Santa Cruz Biotechnology) for 2 h at 4°C, followed by incubation with 10 μl of the protein A/G–agarose beads (Santa Cruz) for 4 h. The protein complexes were washed four times with an immunoprecipitation buffer [50 mM Tris-HCl, pH 7.4, 0.5% NP-40, 150 mM NaCl, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM dithiothreitol (DTT), 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride], and released from the beads by boiling in 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 2% glycerol, 0.02% bromophenol blue) for 5 min. the reaction mixture was then resolved by a 6% SDS–PAGE gel, transferred onto a nitrocellulose membrane by electroblotting and probed with the anti-ApoB or anti-phosphotyrosine antibody (Santa Cruz Biotechnology). The blot was developed using an ECL kit.

Statistical Analysis

Statistical analysis was performed using Student's t test. All determinations were confirmed using replication in three independent experiments. The intensity of the bands obtained from Western blot was estimated with Scion Image (Scion, MA). The values are calculated by percent or ratio of control and expressed as means ± SE of three independent experiments.

RESULTS

PMA Increases ApoB Secretion in Chang Liver Cells

The liver is the major organ for both the production of plasma lipoproteins and their uptake from plasma and catabolism. The production of

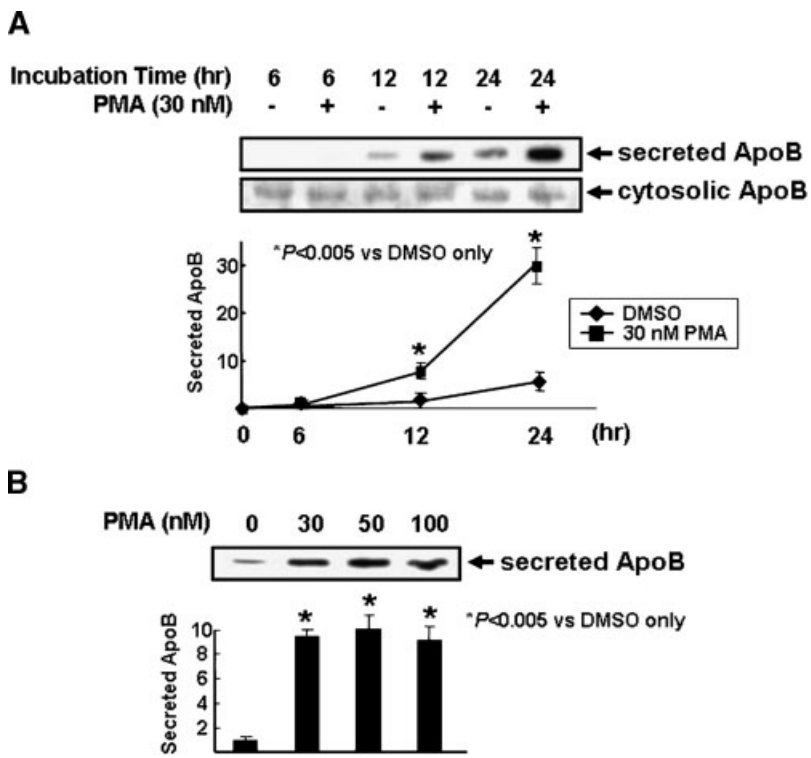

Fig. 1. Effects of PMA on ApoB expression and secretion of Chang cells. **A**: Chang cells were cultured under serum-free condition for 6 h to induce quiescence, and these cells were then incubated without or with 30 nM PMA for various times. For analysis of cytosolic ApoB, total proteins were immunoprecipitated with anti-ApoB polyclonal antibody and then ApoB was detected by Western blot analysis using same polyclonal antibody. The culture media was also used for detection of secreted ApoB levels using Western blot as described in Materials and methods. **B**: Chang cells were also incubated with different concentration of PMA for 24 h after starvation. The secreted ApoB was detected by immunobloting. The densitometric intensity of the bands was estimated as described in Material and methods and was represented in graphs.

ApoB-containing lipoproteins by the liver is required for the assembly and secretion of VLDL and LDL [Davis and Hui, 2001]. ApoB is the only constant component in all of these atherogenic lipoproteins and there is always just one ApoB per lipoprotein particle [Stein et al., 2005]. The human hepatic cells including HepG2 [Thrift et al., 1986], Chang [Kang et al., 2004], and Huh7 [Higashi et al., 2003] cells, when grown under serum-free conditions, secrete a majority of their ApoB in lipoproteins. Although the increasing information on the relationship between lipoprotein secretion and atherosclerotic risks, however, the exact molecular mechanism(s) responsible for atherogenic ApoB secretion has not been explained yet in liver cells. From the current information on the atherogenic ApoB secretion, first, we have examined whether the level of ApoB secretion is changed by PMA treatment in Chang cells. Incubation of Chang cells in serum-free medium with 30 nM PMA for various times (6, 12, and 24 h) significantly resulted in the increase of ApoB secretion in medium without change in the cytosolic ApoB in Chang cells (Fig. 1A). To determine the effects of different PMA concentrations on ApoB secretion, Chang cells were also treated with 30, 50, and 100 nM PMA. The level of secreted ApoB was highly increased by treatment of 30 nM PMA compared to a negative control (DMSO only). In treatments of 50 and 100 nM PMA, ApoB secretion was not changed as compared with 30 nM PMA (Fig. 1B). As shown in Figure 1, 30 nM PMA was enough to induce ApoB secretion in Chang cells.

PMA Increases Transcriptional Levels of MTP but not ApoB

From the above results, it was very interesting to examine the effect of PMA on transcriptional regulation of ApoB and MTP genes in Chang cells. Therefore, we studied whether PMA has an effect on changes in RNA levels of these genes and in MTP promoter activity by RT-PCR analysis and promoter-luciferase assay, respectively. For RT-PCR analysis, Chang cells were incubated with 30 nM PMA for different times (0.5–24 h) and the total RNAs were purified and used for RT-PCR as described in Materials and methods. The incubation of Chang cells increased the expression of MTP gene in a time-dependent manner. In contrast, the expression of ApoB gene was not changed (Fig. 2A). On the other hand, Chang cells were also transfected with a human MTP promoter-luciferase reporter construct and then treated with 30 nM PMA for 0.5–24 h. The MTP promoter activities were remarkably elevated by incubation with 30 nM PMA after 6 h and reached a maximum level at 12 h (Fig. 2B). These results are consistent with the PMA effect on MTP mRNA level, suggesting that PMA up-regulates MTP mRNA level mainly through its transcriptional regulation but not ApoB.

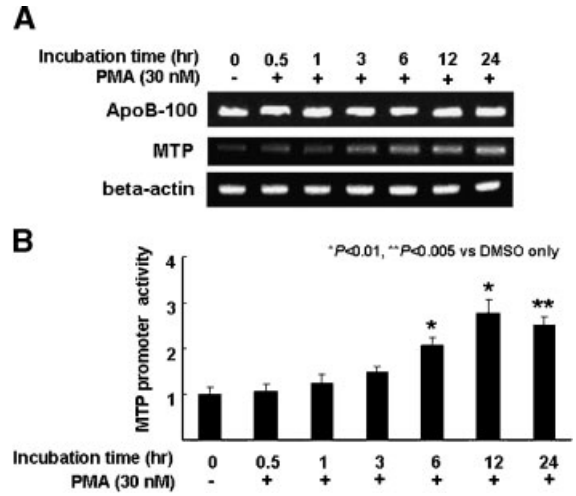

Fig. 2. Effects of PMA on transcription levels of ApoB and MTP. **A**: To examine the effect of PMA on transcription of ApoB and MTP genes, Chang cells were treated without or with 30 nM PMA for various times after starvation. Total RNAs were purified and used for RT-PCR as described in Materials and methods. Beta-actin was included as an internal control. **B**: For analysis of MTP promoter activity, Chang cells were also transfected with human MTP promoter-luciferase reporter construct and β-galactosidase reporter plasmid as an internal control. The transfected Chang cells were treated without or with 30 nM PMA for various times. Relative luciferase activity was normalized with β-galactosidase activity derived from β-galactosidase reporter plasmid. Data are the mean ± SD of three independent experiments. Statistical significance was determined by the nonpaired Student's *t*-test.

PMA Induces the Degradation of IRS1 Through Decreased Tyrosine Phosphorylation as well as Increased Seine Phosphorylation

From the previous studies [Taghibiglou et al., 2000; Ginsberg et al., 2005; Avramoglu et al., 2006], it was well known that insulin resistance increases hepatic ApoB-contained VLDL secretion causing hyperlipidemia. IRS1 protein is an important intracellular molecule that mediates insulin receptor tyrosine kinase signaling and serine-phosphorylated IRS1 may be one of the causes of insulin resistance via its degradation [Pederson et al., 2001; Jiang et al., 2004]. To

determine whether PMA affects on stability and phosphorylation of IRS1, we have examined the degradation and three different phosphorylations of IRS1. Chang cells were cultured under serum-free condition for 6 h. After starvation, Chang liver cells were treated with PMA for 2 h followed by incubation with cycloheximide to inhibit protein synthesis for 6, 12, and 24 h after PMA treatment. As shown in Figure 3A, following a 24 h treatment of cycloheximide, Chang cells treated with only PMA did not show any loss of IRS1 protein. However, cells treated together with PMA and cycloheximide had a significant decay of IRS1 protein in a time-dependent manner. Therefore, our data demonstrate that IRS1 is a stable protein, but degraded by PMA treatment.

PMA-treated Chang liver cells were also used to examine IRS1 phosphorylation. After 6 h-starvation, cells were treated with PMA and harvested at the different time points. Tyrosine phosphorylation of IRS1 was apparently decreased at 3–24 h after PMA treatment although temporally increased at 0.5–1 h. Two forms of serine phosphorylation, Ser1101 and Ser312 as factors causing the insulin resistance, were gradually increased by treatment of PMA (Fig. 3B). These results clearly indicate that PMA induces the degradation of IRS1 protein through increase of serine phosphorylation.

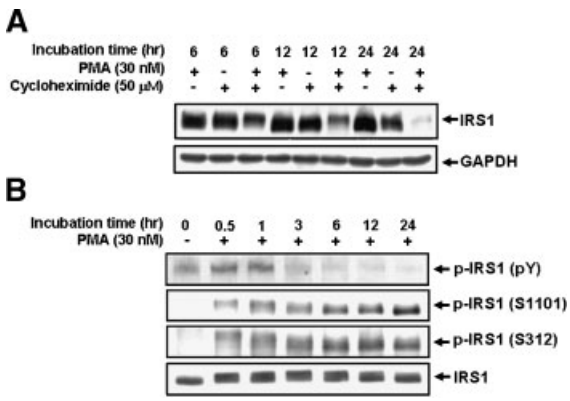

Fig. 3. Effects of PMA on degradation and phosphorylation of IRS1. **A**: Chang cells were cultured under serum-free condition for 6 h. After starvation, Chang cells were treated with PMA for 2 h followed by incubation with cycloheximide to inhibit protein synthesis for 6, 12, and 24 h after PMA treatment. Total proteins were used for Western blot analysis performed with antibodies recognizing IRS1. GAPDH was included as an internal control. **B**: PMA-treated Chang cells were also used to examine IRS1 phosphorylation. After 6 h-starvation, cells were treated with PMA and harvested at the different time points. For detection of tyrosine phosphorylated (pY) IRS1, total proteins were immunoprecipitated with anti-IRS1 antibody and then immunoprecipitated IRS1 proteins were probed with anti-phosphotyrosine antibody for Western blot. Western blot analysis was also performed for detection of serine phosphorylated (Ser1101 and Ser312) and total IRS1 (IRS1) using total proteins without immunoprecipitation.

The Effects of PMA on Activation of AKT, JNK, and PKC Isoforms

PMA has been previously shown to inhibit insulin signaling in cultured cells [Takayama et al., 1988; De Fea and Roth, 1997]. AKT phosphorylation (activation) is a key event in insulin signaling and has been commonly used as a surrogate marker for assessing the level of activation of the proximal insulin signaling pathway [Shepherd et al., 1998]. We, therefore, determined whether PMA inhibits AKT phosphorylation in the Chang liver cells. As shown in Figure 4, PMA treatment resulted in an apparent decrease in phosphorylated AKT in a time-dependent manner, suggesting that PMA negatively affect insulin signaling in Chang liver cells. JNK has also been reported to phosphorylate IRS1 at Ser307 and inhibits insulin signaling [Aguirre et al., 2000, 2001; Rui et al., 2001; Hirosumi et al., 2002]. Therefore, we have examined whether JNK was activated by PMA in the Chang liver cells. As shown in Figure 4, PMA treatment leads to phosphorylation (activation) of JNK in a time-dependent manner.

PMA is also known to bind to and activate multiple isoforms of PKCs [Liu and Heckman,

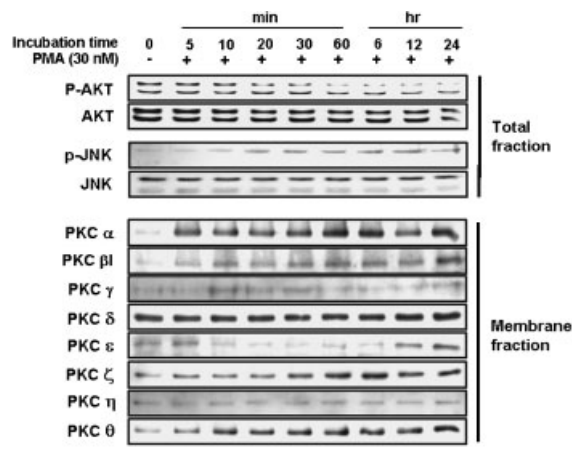

Fig. 4. Effects of PMA on activation of AKT, JNK, and PKC isoforms. To examine the effect of PMA on activation of AKT, JNK, and PKC isoforms, Chang cells were treated without or with 30 nM PMA for various times after starvation. The cytosolic and membrane proteins are prepared and used for Western blot as described in Materials and methods.

1998; Shepherd et al., 1998]. Especially, phosphorylation of IRS1 at site of Ser1101 is mediated by the PKC θ [Griffin et al., 1999; Ravichandran et al., 2001]. To identify which the PKC isoforms were activated by PMA in the Chang liver cells, Western blots were performed using eight different antibodies against PKC isoforms that were translocated to the membrane. Such translocation is known to correlate to the activation and catalytic activity of the PKC isoforms [Liu and Heckman, 1998]. Although PMA did not activate PKC γ, ε, and η, it activated PKC α, βI, δ, ζ, θ in a time-dependent manner (Fig. 4). Taken together, these results suggest that PMA induces JNK and PKC-mediated serine phosphorylation of IRS1.

The Effects of Chemical Inhibitors on PMA-Induced ApoB, IRS1 Degradation, and MTP Promoter Activity

The above results indicate that PMA increases the ApoB secretion and triggers the degradation of IRS1 through its serine-phosphorylation mediated by activation of JNK and PKC isoforms. To determine if any chemical inhibitors of these protein kinases influence PMA-induced ApoB secretion and IRS1 degradation, we measured ApoB secreted by Chang liver cells in the media and intracellular IRS1 after treatment of signal inhibitors. Chang liver cells were treated with Gö6976 (PKC inhibitor) and SP600125 (JNK inhibitor) and then PMA was added as described in Materials and methods. As shown in Figure 5A,B, Gö6976 and SP600125 strongly inhibited the PMA-induced ApoB secretion and IRS1 degradation.

On the other hand, the effects of these chemical inhibitors on the MTP promoter activity induced by treatment of PMA were measured by luciferase assay. As shown in Figure 5C, Gö6976 and SP600125 inhibited PMA-induced MTP promoter activity (Fig. 5C). However, when the inhibitors were treated without PMA, Gö6976 and SP600125 did not change it (Fig. 5C). These results strongly support that PMA-induced ApoB secretion and transcriptional activity of

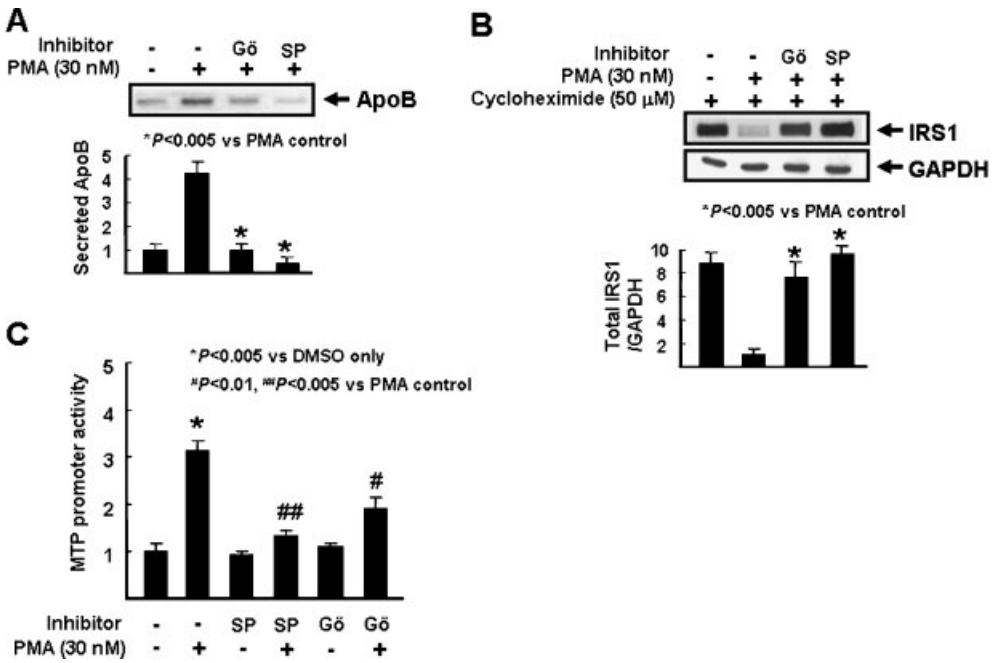

Fig. 5. Effects of chemical inhibitors on PMA-induced ApoB secretion, IRS1 degradation, and MTP promoter activity. To examine the effects of chemical inhibitor, Chang cells were treated with 5 μM Gö6976 (Gö) and 10 μM SP600125 (SP) after starvation and then Chang cells were treated with PMA for 2 h followed by incubation with cycloheximide to inhibit protein synthesis for 24 h after PMA treatment. **A**: ApoB in the medium was detected by Western blot. The densitometric intensity of the bands was estimated as described in Materials and methods and was represented in bar graphs. **B**: Total proteins were used for Western blot analysis performed with antibodies recognizing IRS1. GAPDH was included as an internal control. The densitometric intensity of the bands was represented in gar graphs. **C**: Chang cells were transfected with human MTP promoter-luciferase reporter construct and β-galactosidase reporter plasmid as an internal control. The transfected Chang cells were treated with these chemical inhibitors followed by additional incubation for 12 h in the absence or presence of 30 nM PMA. Relative luciferase activity was normalized with β-galactosidase activity derived from β-galactosidase reporter plasmid. Data are the mean ± SD of three independent experiments. Statistical significance was determined by the nonpaired Student's *t*-test.

MTP are mediated with activated PKCs and JNK.

The Effects of Inhibitors of Tyrosine Phosphatase on ApoB Secretion and Phosphorylation of IRS1

Orthovanadate, a potent tyrosine phosphatase inhibitor that is known to increase IRS1 tyrosine phosphorylation, decreases IRS1 serine phosphorylation, and prevent its degradation [Pandey et al., 1998; Pederson et al., 2001; Avramoglu et al., 2006]. To investigate the effect of orthovanadate on ApoB secretion and IRS1 phosphorylation, Chang liver cells were pretreated with various concentrations of orthovanadate before treatment with PMA. As shown in Figure 6, orthovanadate inhibited the secretion of ApoB induced by PMA in a dose-dependent manner. On the other hand, Orthovanadate also dose-dependently increased the IRS1 tyrosine phosphorylation and decreased IRS1 serine phosphorylation, inducing the degradation of it. Therefore, these results indicate that inhibitory effect of orthovanadate on IRS1 degradation is involved with decrease of ApoB secretion induced by PMA in Chang liver cells.

DISCUSSION

Many investigators have used HepG2 cells for studying ApoB secretion [Thrift et al., 1986; Au et al., 2003; Borradaile et al., 2003; Allister et al., 2005]. However, since the HepG2 cell line is already a hepatocellular carcinoma cell, the cells are not suggested to be the best source for study of ApoB secretion. In this study, therefore, we have chosen a liver Chang cell line that is originally derived from normal liver tissue but is subsequently established via HeLa cell contamination [Kang et al., 2004]. Moreover, human hepatic Chang cells secrete a majority of their ApoB in lipoproteins [Kang et al., 2004]. Previous studies in hepatoma cells have shown that insulin and naringenin decrease the secretion of hepatic ApoB and lipoproteins via down-regulation of MTP transcription and up-regulation of LDL receptor [Au et al., 2003; Borradaile et al., 2003; Allister et al., 2005]. Recent studies reported that there was an early induction and a later repression of LDL receptor by PMA in SK-Hep1 hepatoma cells [Oh et al., 2005] and PMA enhanced the LDL receptor mRNA levels in 1–2 h Jurkat leukemia T cells [Makar et al., 1998]. However, there is no any report that shows the effect of PMA on ApoB secretion in liver cells. Here, we demonstrate, for the first time, that PMA increases the ApoB secretion in Chang liver cells.

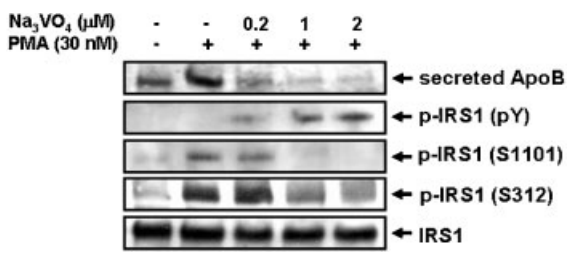

Fig. 6. Effects of orthovanadate on PMA-induced ApoB secretion, IRS1 phosphorylation. After 6 h-starvation, Chang cells were treated without or with 0.2, 1, and 2 mM orthovanadate for 1 h followed by additional incubation for 23 h in the absence or presence of 30 nM PMA. ApoB in the medium was detected by Western blot. Detection of phosphorylated IRS1 was performed as described in Figure 3B.

Previous studies showed that ApoB mRNA levels in HepG2 cells were unaffected by treatment of oleate or insulin, although former stimulated and latter inhibited secretion of ApoB and the regulation of hepatic ApoB secretion is not modulated transcriptionally, but rather translationally or post-translationally [Pullinger et al., 1989]. Furthermore, our data have clearly shown that treatment of PMA affected to the level of the secreted ApoB only, but not to the protein level in Chang cells (Fig. 1). In addition, as shown in Figure 2, treatment of PMA also increased transcriptional level of MTP without any change in ApoB mRNA level. On the other hand, MTP is known to be rate limiting for the assembly and secretion of ApoB-contained lipoproteins [Au et al., 2003]. Although oleate treatment was reported to stimulate secretion of ApoB-contained lipoprotein by preventing the intracellular degradation of ApoB, not by altering MTP mRNA levels [Lin et al., 1995; Mitchell et al., 1998], insulin decreases MTP mRNA levels and ApoB secretion in HepG2 cells [Au et al., 2003]. Furthermore, several researchers demonstrated that insulin regulated ApoB-contained lipoprotein metabolism through insulin-mediated signal transductions [Phung et al., 1997; Wetterau et al., 1997]. Our results are consistent with the previous reports and support the regulation pattern of intracellular ApoB expression.Insulin resistance, defined as the decreased ability of cells or tissues to respond to physiological levels of insulin, is

thought to be the primary defect in the pathophysiology of type 2 diabetes [Jiang et al., 2004]. IRS1 protein is an important intracellular molecule that mediates insulin receptor tyrosine kinase signaling, and IRS1-related defect may be one of the causes of insulin resistance [Pederson et al., 2001]. Serine phosphorylation of IRS1 has been strongly implicated as a mechanism of insulin resistance. Serine-phosphorylated IRS1 proteins have a reduced ability to interact with the insulin receptor and to be tyrosine phosphorylated by the insulin receptor [Pederson et al., 2001; Rui et al., 2001]. Such negative phosphorylation of IRS1 is known to occur on multiple serine residues, including Ser312 and Ser1101 of human IRS1 [Kanety et al., 1995; Mothe and Van Obberghen, 1996; Griffin et al., 1999; Aguirre et al., 2001]. JNK and PKC isoforms as several serine/threonine kinases associate with Ser312 and Ser1101 in IRS1 and phosphorylate it, respectively [Griffin et al., 1999; Aguirre et al., 2000; Ravichandran et al., 2001]. Several observations showed that PMA and TNF-α inhibit insulin signaling by promoting IRS1 phosphorylation at multiple serine residues [Kanety et al., 1995; Aguirre et al., 2000; Rui et al., 2001; Jiang et al., 2003]. Furthermore, it was reported that activation of JNK and PKC isoforms mediates the inhibitory effects of PMA and TNF-α on insulin signaling [Aguirre et al., 2000; Jiang et al., 2003]. We therefore investigated whether PMA induces IRS Ser312 and Ser1101 phosphorylation in the Chang cells. As shown in Figure 3, PMA treatment resulted in increase of IRS1 Ser312 and Ser1101 phosphorylation and induction of IRS1 degradation. Interestingly, tyrosine phosphorylation of IRS1 was temporally increased by PMA, although it was significantly decreased after 3 h. This result indicates that insulin signaling may be enhanced for a short time by PMA treatment. However, an early induction of tyrosine phosphorylation did not attenuate the degradation of IRS1 by PMA (Fig. 3). Additionally, PMA induced activation of JNK and PKC isoforms (α, βI, δ, ζ, θ) and reduced AKT activation in a time dependent manner (Fig. 4). These results suggest that PMA induce Ser312 and Ser1101 phosphorylation of IRS1 through activation of JNK and PKC and lead to insulin resistance as reflected in reduced AKT activation.

In our results, insulin resistance is induced by PMA via activation of JNK and PKC isoforms. We hypothesized that induction of ApoB secretion by PMA in Chang cells is associated with triggered insulin resistance. The treatments of JNK and PKCs inhibitors revealed that activation of these serine/threonine kinases was strongly implicated in induction of ApoB secretion. When activation of JNK and PKCs was respectively inhibited by SP600125 and Gö6976, PMA-induced ApoB secretion and IRS1 degradation were remarkably decreased (Fig. 5A,B). Two inhibitors also decreased MTP promoter activities induced by PMA, respectively (Fig. 5C). These results demonstrate that activated JNK and PKCs play a central role in the effects of PMA on induced ApoB secretion and MTP promoter activity. Moreover, it is interesting to note that PMA-induced ERK activation was remaining in treatment of SP600125 and Gö6976 for 24 h (data not shown). It has been suggested that insulin regulates ApoB secretion through both PI3K and ERK signaling pathway [Lin et al., 1995; Phung et al., 1997; Wetterau et al., 1997]. Insulin-mediated activation of PI3K inhibits the secretion of ApoB-containing lipoprotein particles and thereby promotes the intracellular degradation of ApoB [Phung et al., 1997]. The ERK signaling involves insulin-mediated inhibition of MTP gene transcription [Lin et al., 1995; Au et al., 2003]. However, our results indicate that PMA-induced insulin resistance may increase ApoB secretion through induction of MTP transcription mediated by ERK signaling and involve with activated JNK and PKCs.

Previous evidence demonstrates that protein tyrosine phosphatase-1B (PTP-1B) plays an important role in regulation of insulin signal transduction and lipoprotein analysis of plasma from PTP-1B knockout mice reveals a significant reduction in ApoB-containing lipoproteins [Qiu et al., 2004; Avramoglu et al., 2006]. Moreover, orthovanadate, a potent tyrosine phosphatase inhibitor, increase or maintain IRS1 tyrosine phosphorylation and partially inhibit degradation of IRS1 [Pandey et al., 1998; Pederson et al., 2001]. In our results, orthovanadate increased tyrosine phosphorylation of IRS1 and decreased ApoB secretion of Chang cells although PMA was co-treated (Fig. 6). These results strongly support that insulin resistance triggered by PMA increase ApoB secretion of Chang cells. However, induction of insulin resistance was accompanied by a considerable rise in the production of hepatic VLDL

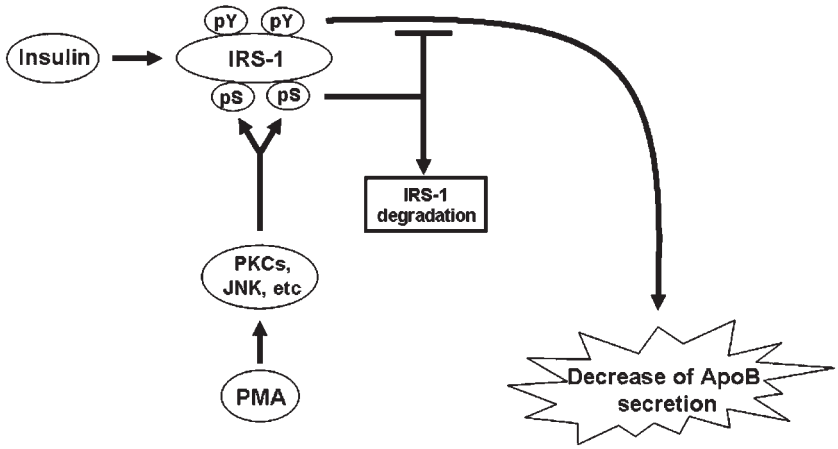

Fig. 7. Schematic diagram illustrating signaling pathway for PMA-mediated increase on ApoB secretion. A schematic summarizes the results from the current experiments and describes our proposed mechanism as PMA-induced ApoB secretion is mediated by insulin resistance via IRS1 degradation. pY, tyrosine phosphorylation. pS, serine phosphorylation.

containing ApoB [Taghibiglou et al., 2000; Avramoglu et al., 2006]. Insulin resistance is commonly associated with an atherogenic dyslipidemia that contributes to significantly higher risk of atherosclerosis and cardiovascular disease. Indeed, insulin insensitivity appears to cause increased hepatic VLDL secretion causing hypertriglyceridemia and reduced plasma levels of HDL cholesterol [Avramoglu et al., 2006].

In summary, Figure 7 summarizes the working hypothesis on the effects of PMA on ApoB secretion in Chang liver cells. In short, we can conclude that PMA-induced insulin resistance, through induction of serine phosphorylation of IRS1 mediated by activated JNK and PKCs, increase ApoB secretion of Chang liver cells.

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