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ScienceDirect
Metabolism Clinical and Experimental 57 (2008) 1677–1684
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Phosphatidylinositol acts through mitogen-activated protein kinase to stimulate hepatic apolipoprotein A-I secretion

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Received 14 April 2008; accepted 3 July 2008

Abstract

Phosphatidylinositol (PI) has been shown to stimulate reverse cholesterol transport in animal models and to increase plasma apolipoprotein (apo) A-I levels and high-density lipoprotein cholesterol in human subjects. The objective of this study was to determine the molecular mechanism through which PI stimulates apo A-I secretion in hepatic cells. PI (12 μmol/L) significantly stimulates apo A-I secretion from HepG2 cells over 24 hours. The stimulation in apo A-I secretion is completely blocked by phospholipase C inhibitors (D609 and U73122) and the Ras inhibitor sulindac sulfide. Apolipoprotein A-I secretion is augmented with a protein kinase C agonist (dioctanoyl glycerol) and inhibited by a protein kinase C inhibitor (dioleoyl ethylene glycol). The PI-induced apo A-I secretion is unaffected by PI-3-kinase inhibitors but is sensitive to mitogen-activated protein kinase (MAPK) inhibitors. Whereas the p38MAPK inhibitor SB203580 has no effect on PI-induced apo A-I secretion, the MAPK kinase 1/2 inhibitor U0126 and the *c*-Jun-*N*-terminal kinase/stress-activated protein kinase inhibitor SP600125 block PI-induced apo A-I secretion. PI also increased extracellular-regulated protein kinase 1 and 2 phosphorylation in HepG2 cells in a time-dependent manner. PI does not appear to stimulate apo A-I gene transcription, as cellular apo A-I messenger RNA levels remained unchanged over the 24-hour incubation. However, PI significantly decreases apo A-I binding and degradation in HepG2 cells. Collectively, the data suggest that PI acts through MAPK pathways to increase plasma apo A-I levels by protecting it from reuptake and degradation.

1. Introduction

The major protein component of HDL, apolipoprotein (apo) A-I, is expressed and secreted by the intestine and the liver [1]. Poorly lipidated apo A-I forms a high-density lipoprotein (HDL) building block that can acquire phospholipids and cholesterol from peripheral cells, and increase in size and form the more mature HDL complexes [2]. Low circulating apo A-I levels are considered to be an independent risk factor for cardiovascular disease and are closely associated with low HDL cholesterol levels [3]. Niacin has been shown to effectively raise plasma apo A-I and HDL cholesterol levels and to concomitantly reduce the risk of developing cardiovascular disease [4,5]. Niacin does not appear to raise HDL levels by stimulating apo A-I synthesis, but instead blocks the binding and reuptake of apo A-I and inhibits hepatic HDL uptake and catabolic pathways [6].

We have shown that anionic lipids have the ability to uniquely regulate intravascular lipid metabolism [7-10]. Phosphatidylinositol (PI) is a well-known intracellular signaling molecule and has been shown to have a profound impact on lipoprotein and lipid metabolism [8-10]. In phase 1 human trials, oral administration of PI resulted in significant increases in HDL and apo A-I and reductions in triglyceride levels [9]. High-density lipoprotein secretion has been implicated to involve Ras and mitogen-activated protein kinase (MAPK) pathways [11]. Whether this is causal or a consequence of apo A-I secretion is unclear, as it has also been shown that overexpression of apo A-I can increase extracellular-regulated protein kinase 1 and 2

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doi:10.1016/j.metabol.2008.07.023

(ERK1/2) phosphorylation [11,12]. Thus, the main objective for the present study was to evaluate if PI-induced apo A-I synthesis and secretion are mediated by MAPK in model hepatic cells.

We show that PI stimulates apo A-I secretion in a manner that is sensitive to inhibitors of phospholipase C (PLC), protein kinase C (PKC), Ras, and MAPK. PI promotes a rapid ERK1/2 phosphorylation, similar to that observed with insulin. PI, however, does not appear to increase apo A-I gene transcription, but instead blocks the reuptake and degradation of apo A-I.

2. Materials and methods

2.1. Chemicals

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC); 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA); and L-α-phosphatidylinositol (PI) were purchased from Avanti Polar Lipids (Alabaster, AL). U73122 (PI-PLC inhibitor), D609 (PC-PLC inhibitor), SP600125 (*c*-Jun-*N*-terminal kinase/stress-activated protein kinase [JNK] inhibitor), sulindac sulfide (RAS inhibitor), insulin, and wortmannin (PI-3-kinase [PI3K] inhibitor) were purchased from Sigma Chemical (St Louis, MO). The MAPK kinase (MEK) 1/2 and p38MAPK inhibitors and their inactive isoforms U0126, U0124, SB203580, and SB202474, respectively, were purchased from Calbiochem (La Jolla, CA). Unless otherwise stated, drugs and inhibitors were of analytical grade and solubilized in dimethyl sulfoxide . All chemical inhibitors were used at reported IC_{50} concentrations to block various signaling pathways.

2.2. Cell culture

HepG2 cells were cultured in Dulbecco modified Eagle medium growth medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Confluent cells were then subjected to treatment/stimulation with drugs and lipids for 24 hours in serum-deficient conditions, as indicated.

2.3. Preparation of phospholipids vesicles

PI, POPC, and DOPA vesicles in phosphate-buffered saline (PBS) (1 mg/mL) were prepared by sonication as previously described [1]. In brief, 1 mg of phospholipids was dried under nitrogen. One milliliter of PBS was added to the dry lipids and sonicated for 1 minute at 100% duty cycle. Vesicles were then incubated for 30 minutes at 37°C and sonicated for 5 minutes at 95% duty cycle.

2.4. Apo A-I enzyme-linked immunosorbent assay

Protein in conditioned medium, from each stimulation, was analyzed by enzyme-linked immunosorbent assay (ELISA) on a 96-well plate according to manufacturer's instructions, with minor modifications. Briefly, the Nunc-Immuno Maxisorp 96-well plates (Nalqe Nunc International, Rochester, NY) were coated overnight with a mouse anti–human apo A-I monoclonal antibody. Samples and standards were incubated in the wells for 2 hours followed by a 1-hour incubation with a horseradish peroxidase–linked goat anti–human antibody. Both antibodies were purchased from BioDesign (Saco, ME). K-Blue Max TMB substrate (Neogen Corp, Lexington, KY) was added to each well, and the reaction was stopped using a 1-mol/L HCl solution; the absorbance was recorded at 450 nm.

2.5. Western blot analysis

After incubation with drugs for indicated time and doses, cells were washed twice with ice-cold PBS-T on ice. Cells were lysed by adding buffer (NaF, 1 mmol/L; NaCl, 5 mmol/L; EDTA, 1 mmol/L; NP40, 1 mmol/L [Roche Diagnostics, Indianapolis, IN]; HEPES, 10 mmol/L; pepstatin A, 1 mg/mL; leupeptin, 1 mg/mL; aprotinin, 1 mg/mL; Na_3VO_4, 1 mmol/L; phenylmethylsulfonyl fluoride, 1 mmol/L), and total protein was extracted. Cell proteins were separated by 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and were analyzed by Western blot using specific phosphorylated ERK1/2 and total ERK1/2 antibodies. Band intensity was analyzed using spot densitometer by AlphaImager software (Alpha Innotech, San Leandro, CA), and obtained phosphorylated value was normalized to the total ERK1/2 values.

2.6. Quantification of messenger RNA levels by real-time polymerase chain reaction

Total RNA was extracted from HepG2 cells using TRI Reagent from Sigma according to the manufacturer's instructions. Complementary DNA (cDNA) was reverse-transcribed from the total RNA using a Superscript II RNAse H-RT Kit from Invitrogen (Burlington, Ontario, Canada). Complementary DNA was hydrolyzed using 50 mmol/L EDTA and 10 N NaOH and was then purified using the Promega Wizard PCR Preps DNA Purification System from Promega (Madison, WI). The purified cDNA was quantified using the OliGreen ssDNA Quantitation Kit (Invitrogen). All samples were incubated in the fluorescent dye, and the fluorescence was measured using the Fluorostar (BMG Labtechnologies, Durham, NC). Oligonucleotide primers were designed from the GenBank cDNA sequence of the human apo A-I (accession no. NM000039) gene using Lightcycler LC Probe Design Software (Roche). Real-time polymerase chain reaction (PCR) of apo A-I was performed using LightCycler Fast Start DNA Master SYBR Green I Kit in the Lightcycler (Roche), with the following set of primers: forward primer—5′-GATGAAAGCTGCGGTG-3′ and reverse primer—5′-CTGCCGCTGTCTTTGA-3′, and resulted in a PCR product of 153 base pairs. The amplification reaction was performed with thermal cycling conditions of 10 minutes at 95°C and 55 cycles of 15 seconds at 95°C, 15 seconds at 50°C, and 15 seconds at 72°C. A standard curve was used to quantify apo A-I messenger RNA

(mRNA) levels using a plasmid containing an insert of the full-length cDNA of apo A-I (pDNR-LIB vector). Cellular apo A-I mRNA levels were also quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

2.7. Northern blot analysis

HepG2 cells were grown to 90% confluency in 100-mm culture dishes and incubated with or without PI for 24 hours. Total RNA was isolated and was fractionated by electrophoresis through a 1.5% agarose gel containing 2.2 mol/L formaldehyde and transferred to a nylon membrane [13]. The apo A-I cDNA probe was labeled with [α-32P]-dCTP from PerkinElmer (Shelton, CT) by oligolabeling [14], hybridized to the RNA on the membrane, and washed 0.15 mol/L NaCl, 15 mol/L sodium citrate, 0.1% SDS, pH 7.0 for 20 minutes followed by 3 washes at 60°C. The membrane was exposed in an exposure cassette for 24 hours at 4°C. Autoradiographs were developed in a phosphoimager, and the amount of signal was quantified by densitometry using Quantity One software (BioRad Laboratories, Hercules, CA).

2.8. Apo A-I binding and degradation

The binding and degradation of apo A-I were evaluated with the use of ^{125}I-labeled protein as previously described [6]. Apolipoprotein A-I was purified from normolipidemic HDL by anionic exchange chromatography [2]. IODO-GEN precoated iodination tubes purchased from Pierce (Rockford, IL) were rinsed with sodium phosphate buffer. An equal amount of apo A-I was added to each tube followed by the addition of 350 μCi of ^{125}I from Amersham Biosciences (Piscataway, NJ). Tubes were stirred constantly for 1 minute. This mixture was loaded onto PD-10 Sephadex columns (Amersham Biosciences) that had been preequilibrated with PBS. Fractions were collected, and the 4 most radioactive ^{125}I-apo A-I fractions were pooled and then incubated with 5 mg of cold HDL overnight at 37°C. [^{125}I-apo A-I]-HDL was isolated by density centrifugation and dialyzed extensively, and the protein concentration was quantified. ^{125}I-labeled HDL exhibited a similar charge and size to the freshly isolated HDL.

HepG2 cells were grown to near confluence in 20-mm plates and incubated in serum-free medium with or without 12 μmol/L PI for 24 hours. The medium was removed, the cells were washed with PBS, and fresh serum-free medium was put back on the cells. Cells were incubated with 50 μg [^{125}I-apo A-I]-HDL for 4 to 16 hours at 37°C. The medium and cell lysate from each well were collected, and radioactivity was measured. The medium was further analyzed after trichloroacetic acid (TCA) precipitation to determine the amount of free ^{125}I, which directly correlates with the amount of apo A-I degradation. For specific binding studies, cells were incubated for 4 hours with 50 μg labeled HDL and 50-fold excess cold HDL at 4°C.

2.9. Statistical analysis

Values are shown as mean ± SEM of at least 4 independent experiments. Differences between mean values were evaluated by 1-way analysis of variance on ranks by a pairwise multiple comparison using the Student-Newman-Keuls post hoc test (SigmaStat; Systat Software, San Jose, CA), and P less than .05 was considered significant.

3. Results

3.1. PI increases apo A-I secretion in HepG2 cells

The ability of PI, POPC, and DOPA (12 μmol/L) to stimulate apo A-I secretion was evaluated in HepG2 cells. Fig. 1 shows that PI significantly increased the secretion of apo A-I at 24 hours relative to controls ($P < .001$). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and DOPA had no effect on apo A-I secretion, and curves appeared similar to control incubations. Total cell protein and intracellular apo A-I levels in HepG2 cells were not significantly affected by treatments with PI and were similar for both controls and other phospholipid treatments (data not shown). Dose titration has shown that 12 μmol/L PI promotes a maximal apo A-I secretion from HepG2 cells over 24 hours [15]. PI has been shown to also significantly stimulate apo A-I secretion in primary human hepatocytes [15].

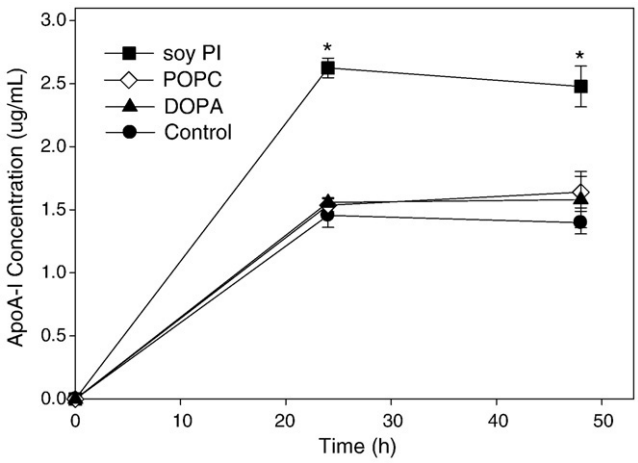

Fig. 1. Phosphatidylinositol. Phosphatidylinositol promotes apo A-I secretion from HepG2 cells. Confluent and quiescent HepG2 cells were incubated with various pure lipids, and apo A-I secretion was measured. Twelve micromoles per liter of aqueous vesicular mixtures of soy PI, POPC, and DOPA was added to the cells and incubated for 24 and 48 hours. Conditioned medium was collected, and apo A-I concentration was determined by ELISA. Apolipoprotein A-I secretion is presented relative to medium volume. Total cell protein values were similar for all incubations. Values are expressed as means ± SEM of at least 4 independent experiments. *$P < .001$ vs control.

3.2. Intracellular signaling inhibitors impact PI-mediated apo A-I secretion

Control experiments were undertaken with each inhibitor to confirm that no significant changes in cell growth and viability had occurred at the dose and time indicated. The effects of inhibiting PLC on apo A-I secretion from HepG2 cells were evaluated. The inhibitors U73122 (PI-PLC inhibitor) and D609 (PC-PLC inhibitor) were incubated with cells for 30 minutes, and then the cells were incubated with PI (12 μmol/L) for 23.5 hours. Both PLC inhibitors significantly blocked the PI-mediated apo A-I secretion (Fig. 2). Both PLC inhibitors reduced the PI effect from 1.7- to 1.1-fold relative to untreated control cells ($P < .001$). Inhibiting Ras with the inhibitor sulindac sulfide also blocked apo A-I secretion and resulted in about a 50% decrease in PI induction ($P < .05$) (Fig. 2).

Experiments were undertaken to evaluate the importance of PKC and PI3K in apo A-I secretory events. Incubation of HepG2 cells with the PKC agonist dioctanoyl glycerol (DOG) alone had no effect on apo A-I secretion, but the agonist stimulated apo A-I secretion when combined with PI (Fig. 3, left panel). Similarly, the PKC inhibitor dioleoyl ethylene glycol (DOEG) had no effect on basal apo A-I secretion, but completely blocked the PI-induced apo A-I secretion (Fig. 3). In contrast, the PI3K inhibitor wortmannin had no effect on apo A-I secretion (Fig. 3, right panel).

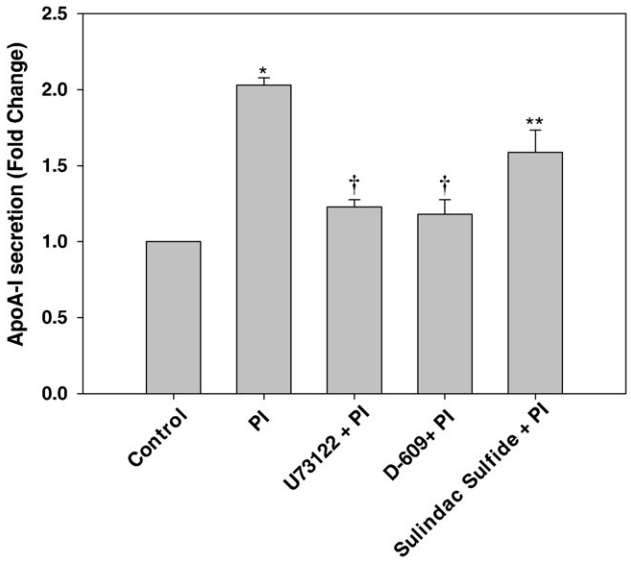

Fig. 2. Effect of PLC and Ras on PI-induced hepatic apo A-I secretion. Confluent and quiescent HepG2 cells were pretreated with or without U73122 (PI-PLC inhibitor, 10 μmol/L), D609 (PC-PLC inhibitor, 10 μmol/L), or sulindac sulfide (Ras inhibitor, 10 μmol/L) for 30 minutes and then with PI (12 μmol/L) for 24 hours. Conditioned medium was collected, and apo A-I concentration was determined by ELISA. Apolipoprotein A-I secretion was corrected to total cell protein and is presented as a fold-change relative to control values. Values are expressed as means ± SEM of at least 4 independent experiments. $^{*}P < .001$ vs control; $^{**}P < .05$ and $^{\dagger}P < .001$ vs PI.

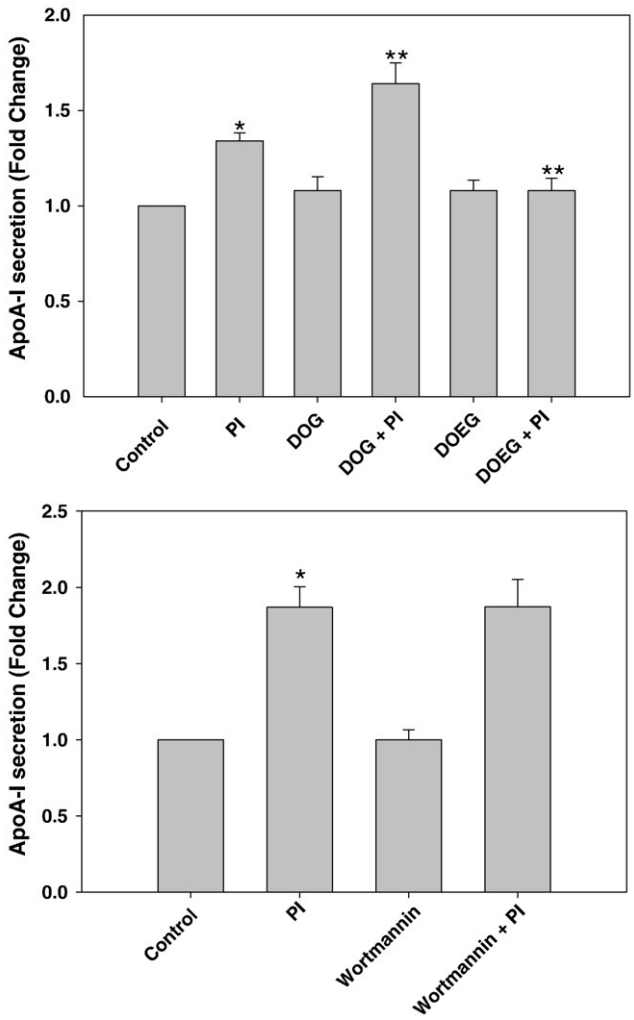

Fig. 3. Effect of PKC and PI3K on PI-induced hepatic apo A-I secretion. Confluent and quiescent HepG2 cells were pretreated with or without DOG (PKC agonist, 10 μmol/L) and DOEG (PKC inhibitor, 10 μmol/L) for 30 minutes and then with or without PI (12 μmol/L) (left panel), or pretreated with or without wortmannin (PI3K inhibitor, 1 μmol/L) and then with or without PI (12 μmol/L) (right panel) as indicated for 24 hours. Conditioned medium was collected, and apo A-I concentration was determined by ELISA. Apolipoprotein A-I secretion was corrected to total cell protein and is presented as a fold-change relative to control values. Values are expressed as means ± SEM of at least 4 independent experiments. $^{*}P < .05$ vs control and $^{**}P < .05$ vs PI.

Similar results were seen with the PI3K inhibitor LY94002 (data not shown).

To determine whether PI may be acting through MAPK pathways, various MAPK inhibitors were used. Inhibition of MEK1/2 with the inhibitor U0126 almost completely blocked the PI-induced apo A-I secretion, whereas the inactive MEK1/2 inhibitor U0124 exerted no effect on PI stimulation of apo A-I (Fig. 4). The inhibitor for p38MAPK (SB203580) and its inactive homologue (SB202474) had no effect on apo A-I secretion. Preincubation with the JNK inhibitor SP600125 also significantly decreased the stimulatory effect of PI (Fig. 4).

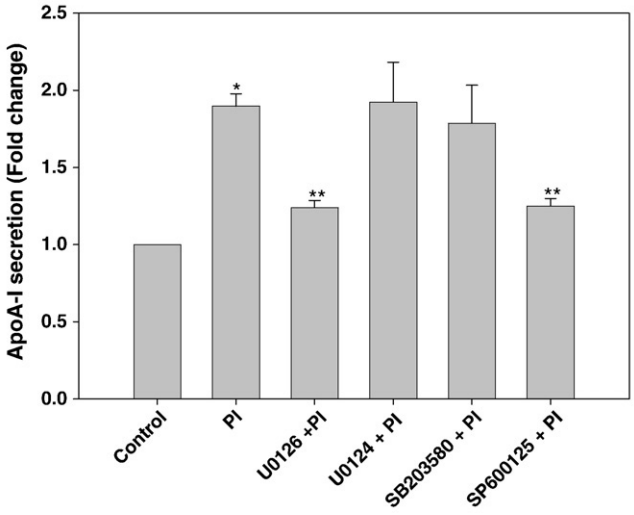

Fig. 4. Effect of MAP kinase inhibitors on PI-induced hepatic apo A-I secretion. Confluent and quiescent HepG2 cells were pretreated with or without U0126 (MEK1/2 inhibitor, 10 μmol/L), U0124 (inactive form of U0126, 10 μmol/L), SB203580 (p38MAPK inhibitor, 10 μmol/L), or SP600125 (JNK inhibitor, 10 μmol/L), and then with PI (12 μmol/L) for 24 hours. Conditioned medium was collected, and apo A-I concentration was determined by ELISA. Apolipoprotein A-I secretion was corrected to total cell protein and is presented as a fold-change relative to control values. Values are expressed as means ± SEM of at least 4 independent experiments. $^{*}P < .001$ vs control and $^{**}P < .001$ vs PI.

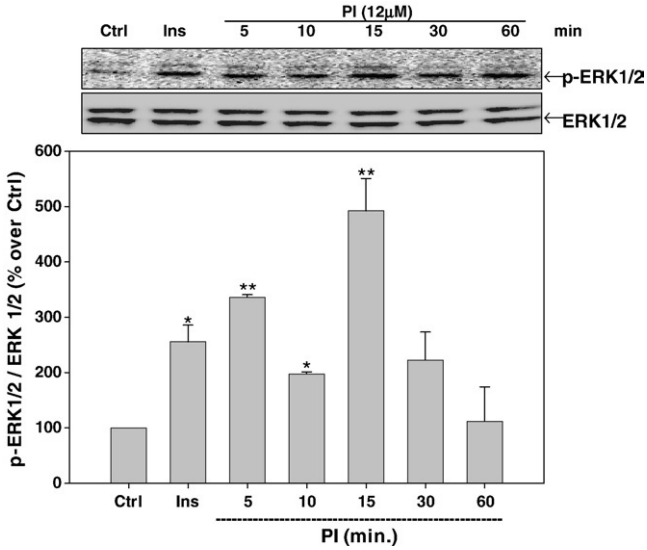

Fig. 5. Phosphatidylinositol. Phosphatidylinositol promotes ERK1/2 phosphorylation in HepG2 cells. HepG2 cells were incubated with or without insulin (100 nmol/L) for 5 minutes or with PI (12 μmol/L) for indicated time points, and phosphorylated ERK1/2 (upper panel) and total ERK1/2 (middle panel) were analyzed by Western blot using specific antibodies. Lower panel is a histogram of analyzed values. Values are expressed as means ± SEM of at least 4 independent experiments. $^{*}P < .05$, $^{**}P < .001$ vs control.

3.3. PI increases the phosphorylation of ERK1/2

To further examine MAPK involvement, the effect of PI on ERK1/2 phosphorylation was measured. HepG2 cells were incubated with PI (12 μmol/L) for various times; and the phosphorylation of ERK1/2 relative to total ERK1/2 protein levels was compared with controls. Each control had a basal level of ERK1/2 phosphorylation. Phosphatidylinositol had an immediate impact on ERK1/2 phosphorylation at 5 minutes, much like insulin, which also increased ERK1/2 phosphorylation at 5 minutes (Fig. 5). PI then promoted a second pulse in ERK1/2 phosphorylation at 15 minutes.

3.4. Effect of PI on apo A-I mRNA levels

To determine if increased apo A-I secretion is related to an increase in apo A-I transcription and mRNA levels, HepG2 cells were treated with 12 μmol/L PI for 24 hours; and apo A-I mRNA levels were quantified. Both Northern blot and real-time PCR were used to measure the levels of apo A-I mRNA. Quantitative real-time PCR showed no significant differences in apo A-I mRNA levels between control, PI-treated, and POPC-treated cells at 24 hours (Fig. 6). Relative PCR measurements showed similar results relative to the control protein GAPDH (Fig. 6, inset A). Time course real-time PCR measurements showed no changes in mRNA at the early time points, up to 8 hours (Fig. 6, inset B). Northern blots also showed no changes in hybridized RNA levels at 24 hours (data not shown).

3.5. Effect of PI on apo A-I cellular association and degradation

Apolipoprotein A-I binding and degradation were measured by tracking the cellular association and degradation of ^{125}I-labeled apo A-I. HepG2 cells were treated for

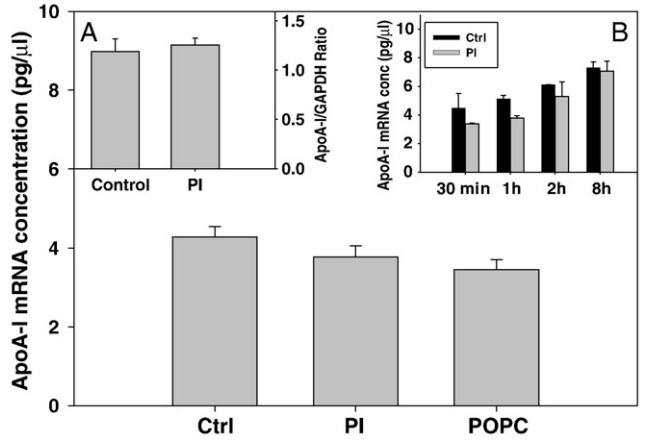

Fig. 6. Effect of PI on apo A-I mRNA levels. Confluent and quiescent HepG2 cells were incubated with or without 12 μmol/L of PI for 24 hours. Cells were collected using TRI Reagent, and total RNA was isolated. Total RNA was isolated and converted to cDNA, and apo A-I mRNA was quantified using specific primers and real-time PCR. Values indicated are means ± SEM for each group of 3 independent experiments. Relative apo A-I/GAPDH quantification is shown in inset A, and time course RNA quantification up to 8 hours is shown in inset B.

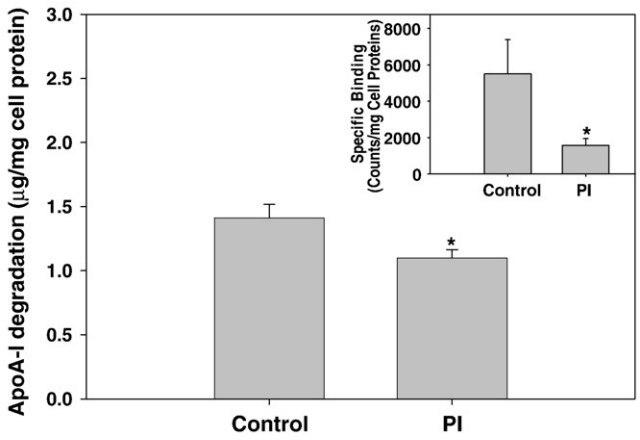

Fig. 7. Effect of PI on apo A-I degradation. Confluent and quiescent HepG2 cells were incubated with or without 12 μmol/L of PI for 24 hours, followed by a 4-hour incubation with [^{125}I-apo A-I]-HDL (50 μg). Cell medium was collected, and proteins were TCA precipitated. Supernatant from the precipitation was counted for ^{125}I as a measure of apo A-I degradation. High-density lipoprotein specific binding was measured at 4°C with addition of excess cold HDL (inset). Data are presented as means ± SEM for each group of 3 independent experiments. $^{*}P < .05$ vs PI.

24 hours with PI and then incubated with 50 μg [^{125}I-apo A-I]-HDL for 4 hours at 37°C or at 4°C. Whereas total cell association of HDL-apo A-I was unaffected by treatments with PI, HDL specific binding was reduced by approximately 75% (Fig. 7, inset). Cellular degradation of the protein was also reduced. The amount of free ^{125}I was measured in the medium after TCA precipitation and used as a measure of apo A-I degradation. Results showed that PI significantly reduced that amount of apo A-I degradation by approximately 22% after 4 hours (Fig. 7). PI also reduced apo A-I degradation after 16 hours by 27% (data not shown). These results suggest that the increase in apo A-I protein secretion observed in PI-treated HepG2 cell medium may be partly due to a decrease in apo A-I reuptake and degradation.

4. Discussion

Plasma HDL and apo A-I levels are inversely correlated to risk of developing cardiovascular disease [16-19]; and as such, there has been significant interest to develop novel therapeutic compounds to increase HDL production. Two fundamental classes of compounds have been developed: drugs that increase HDL secretion (niacin, fibrates) and compounds that block HDL catabolism (cholesterol ester transfer protein [CETP] inhibitors). PI is a safe and potent HDL-raising agent [9] and appears to act at a hepatic level to increase HDL secretion. PI is a potent intracellular signaling precursor molecule; and therefore, experiments were undertaken to determine whether PI might be acting through MAPK, PI3K, and/or associated signaling cascades.

Niacin (nicotinic acid) is the most effective agent used in the treatment of hypoalphalipoproteinemia and significantly raises HDL cholesterol and apo A-I levels in the blood [20]. Niacin has been shown to impact MAPK through the activation of a G-protein–coupled receptor [21] and activation of PKC [22]. Insulin is also known to stimulate apo A-I secretion through activation of PKC [23], and data from the present study suggest that PI acts similarly. Activation of PKC by DOG does not impact apo A-I secretion directly; however, DOG did augment PI-induced hepatic apo A-I secretion. On the other hand, PI-induced hepatic apo A-I secretion was attenuated by PKC inhibition by DOEG. Collectively, this suggests that PI may act through PKC to increase hepatic apo A-I secretion. PI-induced hepatic apo A-I secretion was also blocked by inhibition of PI-PLC and PC-PLC, which are upstream regulators of PKC [24]. Many receptor protein tyrosine kinase–stimulated responses leading to the activation of PKC are known to be mediated by PLC, which often acts through Ras-Raf-MAPK signaling [25]. PI-induced apo A-I secretion appears to involve a G-protein activation, as activity was blocked by Ras inhibition with sulindac sulfide (Fig. 2).

Although chemical inhibitors are often not specific for target proteins, the data cumulatively show that PI is acting through PLC and PKC to activate Ras-MAPK signaling pathways (Fig. 8). Phosphatidylinositol may act through a cell surface G-protein–coupled receptor directly or through a receptor protein tyrosine kinase. Signaling proteins that bind to the intracellular domain of receptor tyrosine kinases include PLC and the adaptor proteins such as Shc and Grb2. PI may therefore act through a PLC pathway to generate a diglyceride cascade stimulation of PKC. PLC production of diglyceride activates PKC together with other adaptor proteins (Shc, Grb2), which are known to activate the Ras GTPase signaling cascade and the ERK and JNK MAPK pathways. Both proteomic arrays (data not shown) and Western blot studies suggest that the expression and the phosphorylation status of several components of the ERK and JNK MAP kinase pathways are affected by treatment of HepG2 cells with PI.

Inhibition of MEK1/2 and JNK blocked the PI-induced apo A-I secretion, which suggests that the PI induction is through MEK-ERK1/2 and JNK MAPK pathways. This may be a direct effect of PI or a secondary effect of the increased amount of apo A-I/HDL in the medium. HDL and apo A-I have been shown to impact the ERK1/2-MAPK [10] and PI3K/protein kinase B (PKB)/Akt pathways [26]. However, the very rapid impact (at 5 minutes) of PI on the phosphorylation of ERK1/2 (Fig. 5) in HepG2 cells suggests that PI is the primary stimulant of the ERK1/2 MAPK pathway. We have also shown that PI can increase PKB phosphorylation in a biphasic manner [15]. This work therefore appears to corroborate findings that PKB and ERK1/2 phosphorylation can have biphasic responses in various cell lines [27-29]. Early-phase activation of phosphorylation is believed to impact rapid signaling events, whereas later phases are associated with

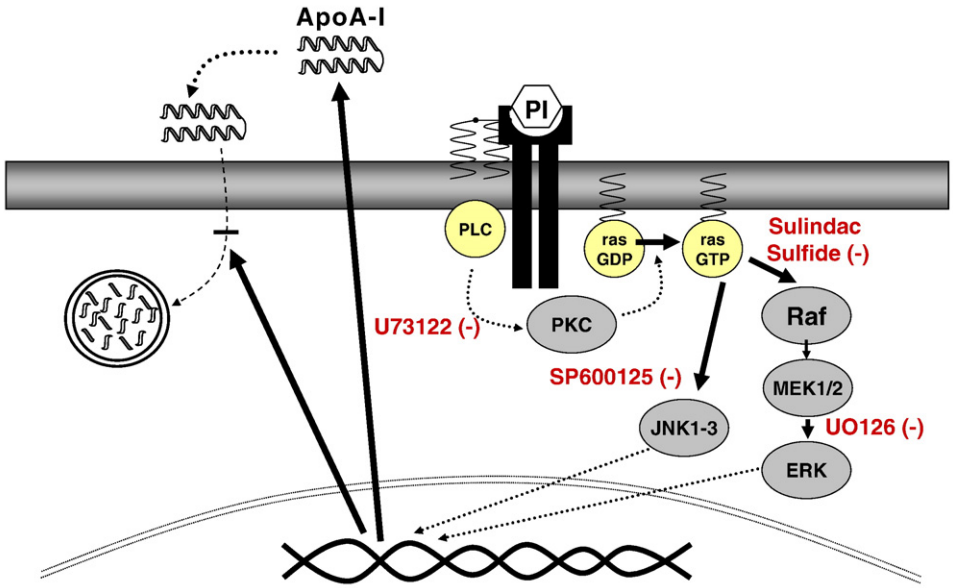

Fig. 8. Proposed signaling pathways of PI-mediated apo A-I secretion. Phosphatidylinositol appears to act through PLC to stimulate apo A-I secretion. Production of diglyceride activates PKC and initiates a Ras GTPase signaling cascade and the ERK and JNK MAP kinase pathways. Activation of the pathway does not appear to stimulate apo A-I gene transcription but instead promotes apo A-I secretion by blocking the reuptake and degradation of apo A-I.

transactivation of other protein targets to induce signaling. On the contrary, attenuation of PI3K did not alter the PI-induced apo A-I secretion in HepG2 cells. Phosphatidylinositol was, however, shown to rapidly induce PKB/Akt phosphorylation in HepG2 cells [15]. Although PI may affect PI3K signaling, it does not appear that the PI3K and PKB pathways are directly involved in hepatic apo A-/I secretion.

PI had no effect on cell growth and proliferation and did not impact cellular apoptotic pathways (data not shown). Both ERK and JNK pathways are known to impact cellular processes outside of their established mitogenic and apoptotic roles [30,31]. In many cases, ERK and JNK pathways have been shown to work in concert [32,33]. However, the involvement of JNK in apo A-I secretion differs and is opposite from that reported by Beers et al [33], wherein they showed that apo A-I promoter activity was increased in incubations with the JNK inhibitor SP600125. In contrast, we show that inhibition of JNK with the same compound completely blocks PI-mediated apo A-I secretion from HepG2 cells. Factors that affect apo A-I gene regulation may therefore differ from those that affect apo A-I secretion. The ERK1/2 MAPK pathway has been implicated in apo A-I gene regulation and protein secretion for many years. Insulin has been thought to impact apo A-I expression and secretion through the ERK1/2 MAPK cascade [34].

PI appears to increase the amount of apo A-I secreted by HepG2 cells, partly by blocking apo A-I binding and degradation. Although the activation of the MAPK pathway might be expected to impact the target gene transcription, PI did not appear to affect apo A-I transcription. PI stimulation of MAPK pathways did not coincide with an increase in cellular apo A-I mRNA levels. Niacin has been shown to increase HDL and apo A-I levels in humans [20]. The drug also stimulates apo A-I secretion from HepG2 cells but does not appear to affect transcription because niacin has no affect on cellular apo A-I mRNA levels [35]. Niacin has instead been shown to increase apo A-I secretion by inhibiting the cellular association and reuptake of apo A-I in HepG2 cells [6]. Although PI does not appear to modify the cellular association of apo A-I, our studies suggest that a PI stimulation in apo A-I secretion is related to a significant reduction in apo A-I uptake and degradation in PI-treated HepG2 cells. PI therefore appears to have a similar mechanism of action to that of niacin. PI increases hepatic apo A-I secretion through a G-protein activation of MAPK cascades, which act to decrease apo A-I protein recycling and degradation.

Acknowledgment

Studies were supported by a grant from the Heart and Stroke Foundation of Canada (DLS) and from Liponex (NRP). SH is a recipient of an Industrial Postgraduate Scholarship from the National Sciences and Engineering Research Council of Canada.

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