Phosphatidylinositol-3-Kinase Regulates Scavenger Receptor Class B Type I Subcellular Localization and Selective Lipid Uptake in Hepatocytes

Shoba Shetty, Erik R.M. Eckhardt, Steven R. Post, Deneys R. van der Westhuyzen

Objective—The high-density lipoprotein (HDL) receptor scavenger receptor Class B type I (SR-BI) plays a key role in mediating the final step of reverse cholesterol transport. This study examined the possible regulation of hepatic SR-BI by phosphatidylinositol-3-kinase (PI3K), a well known regulator of endocytosis and membrane protein trafficking.

Methods and Results—SR-BI–dependent HDL selective cholesterol ester uptake in human HepG2 hepatoma cells was decreased (≈50%) by the PI3K inhibitors wortmannin and LY294002. Insulin increased selective uptake (≈30%), and this increase was blocked by PI3K inhibitors. Changes in SR-BI activity could be accounted for by pronounced changes in the subcellular localization and cell surface expression of SR-BI as determined by HDL cell surface binding, receptor biotinylation studies, and confocal fluorescence microscopy of HepG2 cells expressing green fluorescent protein–tagged SR-BI. Thus, under conditions of PI3K activation by insulin, and to a lesser extent by the SR-BI ligand HDL, cell surface expression of SR-BI was promoted, resulting in increased SR-BI–mediated HDL selective lipid uptake.

Conclusion—Our data indicate that PI3K activation stimulates hepatic SR-BI function post-translationally by regulating the subcellular localization of SR-BI in a P13K-dependent manner. Decreased hepatocyte PI3K activity in insulin-resistant states, such as type 2 diabetes, obesity, or metabolic syndrome, may impair reverse cholesterol transport by reducing cell surface expression of SR-BI. (Arterioscler Thromb Vasc Biol. 2006;26:2125-2131.)

Key Words: SR-BI ■ HDL ■ PI3-kinase ■ reverse cholesterol transport ■ insulin resistance

A major atheroprotective function of high-density lipoprotein (HDL) is to promote the reverse cholesterol transport pathway.1,2 This pathway enables the removal of excess cholesterol from peripheral tissues and its delivery to the liver. Other cardioprotective effects of HDL may result from its anti-inflammatory and antioxidant properties and its role in endothelial nitric oxide synthase activation. The HDL receptor, scavenger receptor class B, type I (SR-BI), plays a key role in the terminal step of the reverse cholesterol transport pathway by promoting cholesterol ester uptake from plasma HDL into the liver and increasing biliary secretion of cholesterol and bile acids.3 Selective cholesterol ester uptake involves the preferential transfer of cholesterol ester from HDL into cells without accumulation or degradation of HDL apolipoproteins. The reverse cholesterol transport hypothesis is strongly supported by mice studies recently published by Zhang et al.4 These studies clearly demonstrated reverse cholesterol transport from macrophages to the liver and the key role of hepatic SR-BI in this pathway.

SR-BI is an 82-kDa glycosylated transmembrane protein expressed in various tissues, most prominently at sites of highest HDL selective lipid uptake, namely liver and steroi- 

dogenic tissues.5,6 In polarized cells, SR-BI is known to be localized largely at the plasma membrane, either apically (eg, enterocytes7) or basolaterally (eg, hepatocytes8 and Madin-Darby canine kidney [MDCK] cells9). Several reports have indicated that SR-BI functions as an endocytic receptor, particularly in polarized cells such as hepatocytes8 and kidney (MDCK)10 cells, and it has been proposed that selective lipid uptake from HDL occurs during SR-BI–mediated HDL recycling within cells.8 Other studies, however, have provided strong evidence that selective lipid uptake does not depend on HDL internalization by SR-BI and that the bulk of selective lipid uptake occurs at the plasma membrane.11,12 The importance of SR-BI in the regulation of HDL levels, as well as in biliary cholesterol secretion, has been well demonstrated in studies of SR-BI genetically altered mice.3,13 In addition to functioning in the liver as an HDL receptor, SR-BI also contributes to the metabolism of chylomicron remnants.14

Several reports have demonstrated an SR-BI–dependent activation of phosphatidylinositol-3-kinase (PI3K), by HDL.15,16 PI3K is an extensively studied signaling molecule which is known to play a key role in a wide range of biological processes.17,18 A major role of PI3K is its involve-
ment in endosomal membrane trafficking, which includes effects on the recruitment of regulatory proteins to the plasma membrane and effects on the endocytic uptake and recycling of receptors. Whether the activation of PI3K by HDL influences SR-BI function or trafficking is unknown. The regulation of SR-BI subcellular distribution in adipocytes by insulin and angiotensin II acting through PI3K was recently reported.

In the present study, we investigated the role of PI3K in SR-BI function and subcellular localization in hepatocytes. Our results clearly demonstrate that SR-BI localization at the cell surface of hepatocytes is dependent on PI3K activation. Membrane recruitment of SR-BI and its ability to mediate selective uptake is markedly enhanced by insulin, most likely through its ability to activate PI3K, whereas PI3K inhibition results in a largely cytoplasmic distribution of SR-BI and reduced selective lipid uptake. These findings suggest that SR-BI function may be altered in insulin-resistant states in a manner that contributes to the dyslipidemia and cardiovascular risk associated with insulin resistance.

Materials and Methods

Cells and Treatments

Human hepatocellular liver carcinoma (HepG2) cells (American Type Culture Collection) were cultured in minimum essential α medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin G, and 50 μg/ml streptomycin (all from Invitrogen). For SR-BI overexpression, cells were plated in 12-well plates and once 70% to 80% confluent, were transduced with adenovirus containing SR-BI vector (Ad-SRBI) or empty vector (Ad-Null) at a multiplicity of infection of 20 in full medium for 18 hours. After 18 hours, medium was replaced with fresh medium and cells were used for experiments.

HDL Isolation and Radiolabeling

HDL and HDL₃ were isolated by sequential ultracentrifugation from human plasma as described previously. HDL₃ was double-labeled by radioiodination of the protein component using [%₁₂⁵I] and by tracing the cholesterol ester component with nonhydrolyzable [%₁⁻³H] cholesterol oleyl ether, all described previously. The integrity and purity of radio-labeled HDL preparations was verified by SDS-PAGE and nondenaturing gradient gel electrophoresis.

HDL Binding, Cell Association, and Selective Lipid Uptake

HDL cell association assays were performed in cell medium containing 0.2% BSA instead of serum as previously reported. Where indicated, cells were pretreated for 45 minutes and then assayed in the presence of insulin (Sigma, 200 nM) or the PI3K inhibitors wortmannin (200 nM) or LY294002 (50 μM/L; both purchased from BIOMOL). For 4°C binding experiments, the activator or inhibitors were present only during the 37°C preincubations, and media was buffered with 20 mmol/L HEPES instead of bicarbonate. After incubation, cells were washed, solubilized in 0.1 N NaOH, and lysate protein and radioactivity were then measured.

Statistical Analysis

Data are expressed as mean±SD. Significance was determined by paired t test or 1 way-ANOVA followed by Bonferroni’s post test as appropriate. P<0.05 was regarded as significant.

Results

PI3K Regulates SR-BI–Mediated Selective Lipid Uptake

Because PI3K has been implicated in endosomal trafficking, including the endocytic uptake and recycling of receptors, we assessed the possible effect of PI3K on SR-BI–dependent hepatic selective lipid uptake from HDL. HepG2, a human hepatoma cell line, was used as a hepatocyte model system. HepG2 cells express some endogenous SR-BI, and SR-BI–specific function was addressed by increasing SR-BI protein levels using adenovirus-mediated gene transfer as shown in Figure 1A. First, we determined whether PI3K activation affects selective lipid uptake from HDL. To this end, we analyzed the effects of wortmannin, a known PI3K inhibitor.
HDL2 (not shown), and insulin-induced Akt phosphorylation

To assess whether PI3K activity affects the subcellular localization of SR-BI and thereby selective lipid uptake as shown in Figure 3A, we assessed the levels of cell surface SR-BI by 125I-labeled HDL binding to cells at 4°C. Binding was carried out after a preincubation of cells with or without inhibitors. The extent of binding at 4°C closely correlated with the relative rates of selective lipid uptake (Figure 3B). Thus, insulin increased 4°C binding of 125I-HDL in both the Ad-Null cells and Ad-SRBI cells (Figure 3B), which was inhibited by wortmannin. However the differences in the Ad-Null cells were not significant. The presence of wortmannin during the 4°C binding period only did not affect HDL binding, indicating that the observed effects of the inhibitor were not cause by a direct effect on the interaction of HDL with SR-BI (data not shown). In addition, wortmannin pretreatment significantly decreased the binding of fluorescently labeled Alexa-HDL to control cells and SR-BI overexpressing cells at 4°C, as assessed by fluorescence microscopy (Figure 3C). Taken together, these results indicate that PI3K inhibition decreases HDL binding to cell surface receptors, whereas insulin promotes cell surface binding to SR-BI. Such changes in cell surface expression of SR-BI correlated with changes in selective lipid uptake.

To assess whether altered HDL binding results from changes in the number of cell surface SR-BI receptors, cell surface receptor levels were measured directly by cell surface biotinylation. Biotinylation was performed after PI3K activation (insulin) or inhibition (wortmannin and LY294002). In agreement with our 4°C binding data, PI3K activation increased cell surface expression of SR-BI compared with untreated controls, both in control cells and SR-BI overexpressing cells (Figure 4). In contrast, PI3K inhibitors, both in the absence and presence of insulin, markedly decreased the number of receptors at the cell surface. Total SR-BI levels in cells were unchanged by the different treatments, indicating that the observed changes in SR-BI at the cell surface were not caused by changes in total cellular SR-BI. These data

### PI3K Regulates SR-BI Cell Surface Expression

To assess whether PI3K activity affects the subcellular localization of SR-BI and thereby selective lipid uptake as shown in Figure 1, we found that wortmannin significantly decreased selective lipid uptake from HDL (≈50%) in Ad-Null cells (Figure 1B) as well as in Ad-SRBI cells overexpressing SR-BI (Figure 1C). LY294002, a less specific PI3K inhibitor, was also shown to inhibit selective lipid uptake (data not shown). PI3K inactivation by wortmannin in both Ad-Null and Ad-SRBI cells, as well as its activation by HDL, was confirmed by an analysis of Akt phosphorylation (Figure 2). As shown previously13,16 we found that HDL3, HDL2 (not shown), and insulin-induced Akt phosphorylation at position Ser473. Wortmannin blocked the phosphorylation of Akt in each case indicating PI3K-dependent phosphorylation. In addition, LDL, a known ligand for SR-BI, also activated Akt in both cell types.

We next determined whether PI3K activation regulates SR-BI-dependent selective lipid uptake. PI3K activation after insulin treatment was found to stimulate selective uptake (≈30%) (Figure 3A). This increase was seen both for selective uptake by Ad-Null cells and Ad-SRBI cells, although the increase in Ad-Null cells was not statistically significant in repeated experiments. The increase in SR-BI-dependent selective lipid uptake by insulin suggests that the efficiency of hepatic selective lipid uptake may be significantly dependent on insulin and its downstream signaling components. Taken together, these results show that PI3K activity correlates with selective lipid uptake and that insulin, a potent activator of PI3K, stimulates this process.

### PI3K Regulates SR-BI Cell Surface Expression

To assess whether PI3K activity affects the subcellular localization of SR-BI and thereby selective lipid uptake as
demonstrate that the subcellular distribution of SR-BI is regulated by PI3K and that PI3K activation results in increased expression of SR-BI at the cell surface.

Insulin and HDL Induce Redistribution of SR-BI in a PI3K-Dependent Manner

To further study PI3K-induced redistribution of SR-BI, the subcellular distribution of EGFP-SRBI was analyzed by confocal microscopy. EGFP-SRBI, containing an N-terminal GFP tag, was previously shown to exhibit normal SR-BI-type ligand binding, selective lipid uptake, and subcellular distribution in Chinese hamster ovary cells. EGFP-SRBI or the EGFP vector alone were transfected into HepG2 cells by electroporation. Forty-eight hours after transfection, cells were serum starved for 3 hours to minimize PI3K activation. Cells were then incubated in serum-free media containing insulin, HDL, or both in the presence or absence of wortmannin. As shown in Figure 5A, EGFP-SRBI was found widely distributed throughout the cytoplasm in serum-starved cells. Strikingly, incubation for 60 minutes with either insulin or HDL resulted in a marked redistribution of the bulk of EGFP-SRBI to the cell surface. Wortmannin blocked this redistribution. In contrast, cellular localization of EGFP alone, which was distributed throughout the cytoplasm in serum-free medium, was unaffected by insulin (Figure 5A) or HDL (not shown), thereby excluding artificial effects such as oligomerization of the EGFP moiety with the various treatments. Translocation to the membrane by insulin was rapid and could be observed by 15 minutes and was almost complete by 30 minutes (Figure 5B). Translocation by HDL was slower and approached completion only after 60 minutes. When added together, insulin and HDL caused almost complete membrane recruitment of SR-BI after 15 minutes, suggesting an additive effect. To rule out possible artifactual receptor behavior caused by the EGFP tag, we also studied the subcellular localization of untagged SR-BI by immunostaining after the same experimental treatments. Immunostaining of SR-BI yielded similar findings as those obtained with EGFP-SRBI (data not shown).

Together, these results demonstrate that the plasma membrane localization and activity of SR-BI in hepatocytes is dependent on PI3K activity and provide an explanation for our finding that insulin increased SR-BI selective lipid uptake from HDL.

Discussion

In this study, we describe the involvement of PI3K in the post-translational regulation of SR-BI activity in HepG2 cells. The major findings presented are: (1) PI3K activity strongly affects cell surface expression of SR-BI through the regulation of receptor subcellular localization; (2) insulin and the SR-BI ligand HDL both strongly enhance cell surface expression of SR-BI in a PI3K-dependent manner, whereas SR-BI is largely localized intracellularly under conditions of serum starvation; and (3) increased cell surface expression of SR-BI by insulin increases selective cholesterol ester uptake from HDL. The current findings suggest that PI3K-dependent post-translational regulation of SR-BI in hepatocytes may play an important physiological role in the liver by influencing a key final step in the reverse cholesterol transport pathway.

SR-BI is subject to transcriptional as well as post-transcriptional control. Transcriptional regulation includes hormonal regulation in steroidogenic tissues, such as by corticotropin (ACTH) in adrenal parenchymal cells, estrogen in ovarian cells, and gonadotropin in Leydig cells. Hepatic SR-BI is transcriptionally regulated in response to a number...
of signals, including dietary plant polyunsaturated fatty acids, estrogen, and proinflammatory stimuli including lipopolysaccharide, tumor necrosis factor α and interleukin-1. Post-transcriptional regulation of SR-BI has also been suggested by numerous findings showing a lack of correlation between receptor protein and mRNA levels in hepatocytes. SR-BI stability was shown to be dependent on cellular expression of PDZK1, a tissue specific adaptor protein with 4 PDZ domains. In cells lacking PDZK1, SR-BI fails to be transported to the cell surface and is subject to increased degradation. Whether this PDZK1-dependent mechanism of SR-BI trafficking is subject to physiological regulation is not known. Another interesting, albeit unresolved, regulatory mechanism is the induction of post-translational degradation of SR-BI by farnesyl-transferase. Although farnesyl-transferase decreases PDZK1, evidence indicates that the regulation of hepatic SR-BI by farnesyl-transferase is not through its effects on PDZK1. Our study highlights a post-translational regulatory mechanism whereby receptors are translocated to the cell surface after PI3K activation. Similar findings were recently reported for SR-BI localization in adipocytes, where SR-BI expression at the cell surface of 3T3-L1 mouse adipocytes was shown to increase in response to insulin and angiotensin II. Mobilization of SR-BI to the cell surface was shown to increase the uptake of 22-(N-nitrobenz-2-oxa-1,3-diazo-4-yl)-amino-23,24-bisnor-5-cholen-3-ol (NBD cholesterol) from HDL, but effects on selective lipid uptake were not studied.

The mechanism involved in PI3K-dependent cell surface localization of SR-BI is not yet clear. PI3K activity is associated with a variety of cellular events, dependent on the particular activation signal and on the cellular context. A major role of PI3K is its involvement in endosomal membrane traffic. For example, PI3K inhibition slowed degradation of the PDGF receptor after ligand stimulation and also slowed recycling of the transferrin receptor to the plasma membrane. Wortmannin inhibition of PI3K in polarized cells was also found to inhibit transcytosis. Evidence suggests that PI3K inhibition affects trafficking early in the endocytic pathway as well as the process of inward vesiculization that is responsible for the formation of multivesicular bodies present in later stages of the endocytic pathway. PI3K functions in these processes by promoting the generation of 3-phosphoinositides that serve as binding sites for proteins containing structural motifs, including PH, FYVE, and PHOX domains, which recognize 3-phosphoinositides. A classic example of a protein where trafficking to the cell surface is highly regulated by PI3K is the insulin responsive glucose transporter-4 (GLUT-4). Insulin activates PI3K through auto-phosphorylation of the insulin receptor that in turn activates a series of downstream proteins, including insulin receptor substrates (IRS proteins 1 and 2) that activate PI3K. This leads to the activation of certain intracellular signaling molecules such as PKD and Akt at the plasma membrane, which in turn promote GLUT-4 trafficking to the cell surface. In insulin resistance, one of the reasons for the inability of GLUT-4 to traffic to the cell surface is decreased PI3K activation because of the failure of IRS proteins to activate PI3K. Similarly, our studies indicate that insulin regulates cell surface expression of SR-BI and consequently selective lipid uptake. Although insulin activates signaling cascades in addition to PI3K, our finding that wortmannin blocked insulin stimulation of SR-BI cell surface expression indicates the dependency of this effect on PI3K.

Our finding that SR-BI function is dependent on PI3K activity suggests that under conditions of reduced PI3K activity, such as found in insulin-resistant states, SR-BI-dependent clearance of HDL cholesterol may be significantly compromised because of decreased cell surface receptor expression. Although this requires more thorough investigation, there is suggestive evidence supporting such a hypothesis. The unusual dyslipidemia characterized by increased, large cholesterol ester-rich HDL particles (HDLI) in the severely insulin-resistant mouse models of obesity, leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice, might be explained by decreased SR-BI-dependent HDL lipid clearance attributable altered subcellular distribution of SR-BI. Defective hepatic HDL selective lipid uptake has, in fact, been shown in ob/ob mice. HDL binding to hepatocytes from ob/ob mice was reduced when compared with control mice despite, interestingly, similar hepatic SR-BI protein levels in the 2 mouse strains. In another study, SR-BI levels were reported to be altered in ob/ob mice in comparison to control mice. The explanation for the difference between these 2 studies with respect to SR-BI levels is not known. Our results suggest that an altered SR-BI subcellular localization resulting from insulin resistance may
account for the reduced activity of SR-BI in ob/ob mice. Leptin administration reversed the abnormal plasma lipid profile and defective HDL catabolism in ob/ob mice, and we propose that this may be because of the known ability of leptin to activate PI3K and improve overall insulin sensitivity. Clearly, a reduction in SR-BI-mediated selective uptake in response to reduced PI3K activation may potentially lead to a reduction in reverse cholesterol transport and consequently increase the risk for atherosclerosis.

In conclusion, our findings demonstrate that PI3K plays a key role in the trafficking and plasma membrane localization of SR-BI in hepatocytes that is necessary to facilitate SR-BI-mediated selective cholesterol ester uptake from HDL. Such post-translational regulation of SR-BI, for example by insulin, may influence the rate of reverse cholesterol transport to the liver as well as other SR-BI-mediated functions, such as its role in chylomicron clearance, lipopolysaccharide uptake, and cholesterol efflux. The importance of PI3K activation for SR-BI function demonstrates the need to understand how other signaling molecules may contribute to this regulatory process. Activation of SR-BI function by insulin suggests possible cross-talk between signaling pathways regulating lipoprotein and carbohydrate metabolism.

Acknowledgments
We thank Dr Nancy Webb and Dr Lei Cai for valuable discussions and for providing the adenoviral expression vectors used in this study. We also thank John Cranfill and Susan Bridges for excellent technical assistance.

Sources of Funding
This work was supported by an American Heart Association Established Investigator Award and National Institutes of Health (NIH)
References


Disclosures

None.
Phosphatidylinositol-3-Kinase Regulates Scavenger Receptor Class B Type I Subcellular Localization and Selective Lipid Uptake in Hepatocytes

Shoba Shetty, Erik R.M. Eckhardt, Steven R. Post and Denys R. van der Westhuyzen

Arterioscler Thromb Vasc Biol. 2006;26:2125-2131; originally published online June 22, 2006; doi: 10.1161/01.ATV.0000233335.26362.37

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/26/9/2125

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/