High Density Lipoprotein-induced Signaling of the MAPK Pathway Involves Scavenger Receptor Type BI-mediated Activation of Ras*

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High density lipoprotein (HDL) stimulates multiple signaling pathways. HDL-induced activation of the mitogen-activated protein kinase (MAPK) pathway can be mediated by protein kinase C (PKC) and/or pertussis toxin-sensitive G-proteins. Although HDL-induced activation of MAPK involves Raf-1, Mek, and Erk1/2, the upstream contribution of p21ras (Ras) on the activation of Raf-1 and MAPK remains elusive. Here we examine the effect of HDL on Ras activity and demonstrate that HDL induces PKC-independent activation of Ras that is completely blocked by pertussis toxin, thus implicating heterotrimeric G-proteins. In addition, the HDL-induced activation of Ras is inhibited by a neutralizing antibody against scavenger receptor type BI. We conclude that the binding of HDL to scavenger receptor type BI activates Ras in a PKC-independent manner with subsequent induction of the MAPK signaling cascade.

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The abbreviations used are: HDL, high density lipoprotein; MAPK, mitogen-activated protein kinase; SR-BI, scavenger receptor type BI; elicited by signal transduction pathways in which HDL receptors at the cell surface are believed to transmit the signal to intracellular signaling proteins (1, 2). This concept of HDL receptor-mediated signaling was recently supported by the identification of a PDZ-containing adaptor protein (3) interacting with the cytoplasmic domain of the intensively studied HDL receptor scavenger receptor type BI (SR-BI). More importantly the SR-BI-dependent and HDL-mediated activation of endothelial nitric-oxide synthase has been demonstrated (4, 5).

Several laboratories have examined the plethora of signaling responses generated by the interaction of HDL with cells. The diversity of HDL-mediated cellular responses can in part be explained by the heterogeneity in the content of the particles (lipids, apolipoproteins, and enzymes) as well as by the different HDL receptors possibly involved. HDL triggers a variety of intracellular signaling events, including activation of phosphatidylinositol- and phosphatidylycholine-specific phospholipases C and D (PI-PLC, PC-PLC, and PC-PLD), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), tyrosine kinase, and heterotrimeric G-proteins (6, 7) but also production of cyclic AMP (cAMP), nitric oxide (NO), and ceramide (4) and intracellular Ca2+ release. Both lipid and protein components of HDL have been implicated in the activation of different classes of cellular phospholipases and the mobilization of intracellular calcium but also in the stimulation of mitogenesis in vascular smooth muscle cells. In respect to the HDL-induced activation of the MAPK pathway it was demonstrated that G-protein-dependent signaling proceeds phosphorylation of Raf-1 and Mek-1 (8–10). Indeed it was recently shown that sphingosylphosphorylcholine and lysosulfatide in HDL3 particles interact with receptors of the endothelial differentiation gene family. This leads to dual activation of signaling through heterotrimeric G-proteins that in turn activates PI-PLC. This G-protein-dependent activation of PI-PLC is inhibited by pertussis toxin (PTX). Activation of PI-PLC results in the immediate production of inositol 1,4,5-trisphosphate and diacylglycerol with the subsequent activation of PKC.

It is believed that PKC plays a pivotal role regulating the signaling cascade for the HDL-induced phosphorylation of the Raf-1/Mek/MAPK pathway (8, 11). However, a number of observations have demonstrated that HDL-induced activation of the MAPK pathway does not completely depend on PKC signaling. First, in fibroblasts down-regulation or inhibition of PKC only partially (40–50%) blocks HDL-induced MAPK activation (8). Second, in smooth muscle cells HDL-mediated activation of MAPK requires a PKC-independent but PTX-sensitive pathway, indicating the involvement of G-proteins (9). However, the potential contribution of p21ras (Ras), which is one of the best studied activators of the Raf-1/MAPK pathway, remains unclear, and to date there is no evidence that the

PTX, pertussis toxin; CHO, Chinese hamster ovary; TPA, 12-O-tetradecanoylphorbol-13-acetate; GST, glutathione S-transferase; RBD, Ras-binding domain; MARCKS, myristoylated alanine-rich C kinase substrate; PI, phosphatidylinositol; PC, phosphatidylycholine; PL, phospholipase; Mek, mitogen-activated protein kinase/extracellular signal-regulated kinase; Erk, extracellular signal-regulated kinase; PDGF, platelet-derived growth factor; P- phospho-.
interaction of HDL with cell surface receptors increases Ras activity leading to MAPK phosphorylation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Nutrient mixture Ham’s F-12, glutathione, TPA, and PDGF were from Sigma (Madrid, Spain). Pertussis toxin was from List Biological Laboratories Inc. Fetal calf serum was purchased from Biological Industries. Peroxidase-labeled antibodies and SDS-PAGE molecular weight markers were from Bio-Rad. Monoclonal anti-pan-Ras was purchased from Oncogene Sciences. Polyclonal anti-P-Mek and anti-MAPK (P-MAPK) were purchased from Cell Signaling. Antibodies against phospho-MARKS were from Cell Signaling, and PKCs and c-Raf-1 were from BD Biosciences. Rabbit antiserum against the extracellular domain of mSR-BI (KKB-1) was kindly provided by Karen Kosarzky (14).

Cell Culture—CHO cells were grown in Ham’s F-12 supplemented with 10% fetal calf serum, l-glutamine (2 mm), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in 5% CO2. To measure the presence or absence of TPA (500 nM), PTX (0.1 μM Na3VO4, and protease inhibitors and cleared as described above. Supernatants (equalized for protein concentration) were immunoprecipitated for 2 h at 4 °C with 2 μg of anti-Raf precoupled with 20 μl of protein G-Sepharose (Pierce). Immunoprecipitates were washed three times in buffer A (30 mM Tris, 0.1 mM EDTA, 0.3% β-mercaptoethanol, 10% glycerol, 0.1% [v/v] Triton X-100, 5 mM NaF, 0.2 mM Na3VO4, and decreasing amounts of NaCl (1 M, 0.1 M, and salt-free) and incubated for 30 min at 30 °C in 20 μl of Mek buffer (salt-free buffer A plus 10 mM MgCl2, 0.8 mM ATP, 6.5 μg/ml GST-Mek, and 100 μM/ml GST-Erk2). The reaction was terminated by the addition of 20 μl of ice-cold stop buffer (salt- and glycerol-free buffer A containing 6 mM EDTA). Following centrifugation, 6 μl of the supernatant was incubated for 15 min at 30 °C with 24 μl of MPR buffer (salt- and glycerol-free buffer A containing 10 mM MgCl2, 0.1 mM ATP, 2.5 μl [32P]ATP, 0.5 μg/μl myelin basic protein, and 0.16 μg/μl bovine serum albumin), and then aliquots of 24 μl were loaded onto P81 sheets, washed three times (20 min each) in 75 mM orthophosphoric acid, and counted.

RESULTS AND DISCUSSION

To identify a possible role of Ras in HDL signaling we analyzed the HDL-induced activation of the MAPK pathway in CHO cells. Ras activity was measured by GST-RBD pull-down experiments with cell lysates from CHO cells incubated from 3 to 20 min with HDL3 (40 μg/ml). Similar to the PDGF-induced activation of Ras (data not shown), incubation with HDL3 resulted in a strong induction of Ras activity in CHO cells. Production of Ras-GTP peaked after 3 min of exposure to HDL and returned to basal levels at later time points (Fig. 1a). Kinetics of growth factor- and TPA-induced Ras-GTP production are characterized by a peak of Ras activity immediately after addition of activating agents (19). These findings demonstrate that HDL-induced activation of Ras follows kinetics similar to those for receptor tyrosine kinase (epidermal growth factor receptor)- or PKC (TPA)-mediated stimulation of Ras in CHO cells (20). Similar to results described in vascular smooth muscle cells (9), incubation of CHO cells with HDL resulted in the downstream activation of the Raf-1/MAPK pathway as shown by the immediate increase (70 ± 10%) of Raf-1 activity after 3 min of HDL exposure (Fig. 1b) and the HDL-induced phosphorylation of Mek and Erk1/2 (Fig. 1, c and d).

To determine whether the major apolipoprotein of HDL3, apoA-I, is responsible for Ras activation, we compared HDL3 and purified lipid-free apoA-I (21). In contrast to the immediate increase of Ras-GTP levels in HDL3-incubated cells, addition of purified apoA-I to the culture medium had no effect on Ras-
indicated. The inhibitory effect of TPA on PKC activity (Fig. 1). The total amount of Ras in the cell lysates was determined as described above (Fig. 1).

GTP or the P-Mek levels as compared with the control at any time point analyzed (Fig. 1, a and c). These findings indicate that lipid-free apoA-I does not activate Ras-GTP production.

Since HDL-induced activation of PKC could be responsible not only for the downstream induction of the Raf-1/Mek/MAPK pathway but also the activation of Ras, we studied the involvement of PKC in the activation of Ras after HDL stimulation. Cells were preincubated overnight in the presence or absence of TPA, a potent PKC inhibitor (8, 12). Inhibition of PKC was confirmed by Western blot analysis of PKC (Fig. 2) and reduced phosphorylation of a PKC substrate, P-MARCKS (22) (data not shown). HDL-induced production of Ras-GTP was clearly detectable even after down-regulation of PKC, indicating that HDL-induced activation of Ras in CHO cells occurs mainly via a PKC-independent pathway (Fig. 2).

Nofer and co-workers (9) recently reported that HDL-induced activation of the MAPK pathway involves PTX-sensitive G-proteins. To test the possible involvement of G-proteins in HDL-mediated activation of Ras and Erk1/2, RBD assays were performed with lysates from PTX-preincubated CHO cells. Strong inhibition of HDL-induced activation of Ras-GTP production and the MAPK pathway as shown by the reduced phosphorylation of Mek and Erk1/2 proteins was observed upon pretreatment with PTX (Fig. 3). Taken together these findings indicate that HDL-mediated activation of Ras requires G-protein-mediated signaling in CHO cells.

SR-BI, which binds HDL particles with higher affinity than lipid-free apoA-I (23, 28), could be a candidate for HDL signaling. First, SR-BI is located in caveolae (24–27) and is responsible for HDL-mediated activation of endothelial nitric-oxide synthase (4, 5). Second and similar to the results described above, Yuhanna et al. (5) reported that only binding of native HDL to SR-BI is associated with induction of NO production, whereas binding of apoA-I had no effect, indicating that binding of HDL particles to SR-BI can promote signaling events. In addition, in CHO cells the incubation of HDL (50 μg/ml) does not lead to changes in intracellular Ca²⁺ levels in agreement with Smart and co-workers (4) (data not shown).

Thus to identify a possible role for SR-BI in HDL-mediated Ras activation, CHO cells were preincubated with an antibody against the extracellular domain of SR-BI that inhibits HDL binding to SR-BI (14). Cells were then incubated with HDL₃, and lysates were prepared to determine Ras-GTP levels in RBD assays (Fig. 4). In these experiments we observed a strong reduction of HDL-induced activation of Ras and reduced amounts of phosphorylated Erk1/2 in CHO cells preincubated with the inhibitory SR-BI antibody (Fig. 4a). These findings suggest that interaction of HDL and SR-BI plays a crucial role in the activation of Ras in CHO cells.

It was previously shown that, in vascular smooth muscle cells treated with HDL, the inhibition of PKC did not interfere with the Raf-1 or the MAPK activity (9). However, it has been shown in fibroblasts that PKC may contribute in some extent in the HDL-mediated stimulation of MAPK (8). The demonstration in the present study that HDL activates Ras and the complexity and cross-talk of signal transmission prompted us to determine the possible involvement of PKC in the overall stimulation of Ras. Here we clearly demonstrate that when PKC was completely depleted, by the action of TPA, no changes in the amount of Ras-GTP (Ras activity) (Fig. 2) or in the P-Erk1/2 could be observed (Fig. 4b). Therefore, as a major step in this direction, we report here for the first time that HDL activates Ras through SR-BI in a PKC-independent manner.

To understand the HDL-mediated activation of the MAPK pathway, it is essential to identify the key signal-transducing proteins at the cell surface and/or the endosomal compartment that are activated by the interaction of HDL with receptors. Mapping the specific location where HDL-mediated activation of downstream molecules occurs will have important implications in the understanding of the development of cardiovascular disease.

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