# Parte II

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# 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 p21$^{ras}$ (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.**

In peripheral cells many beneficial effects of high density lipoproteins (HDL)[1] on the removal of cellular cholesterol are 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 phosphatidylcholine-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 $Ca^{2+}$ 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 $HDL_3$ particles interact with receptors of the endothelial differentiation gene family. This leads to dual activation of signaling through heterotrimeric $G_i$-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 p21$^{ras}$ (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

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[1] The abbreviations used are: HDL, high density lipoprotein; MAPK, mitogen-activated protein kinase; SR-BI, scavenger receptor type BI; 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, phosphatidylcholine; PL, phospholipase; Mek, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Erk, extracellular signal-regulated kinase; PDGF, platelet-derived growth factor; P-, phospho-.

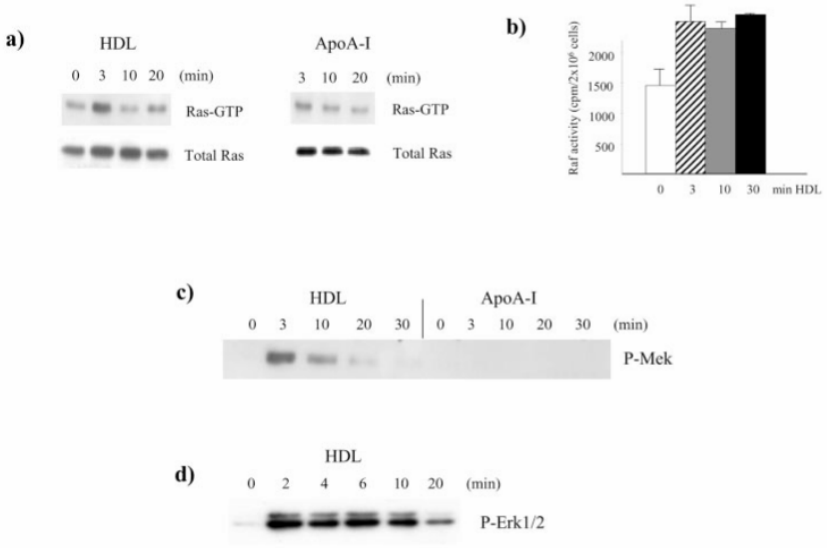


FIG. 1. **Effect of HDL on Ras and the MAPK signaling pathway.** *a*, CHO cells were incubated with $HDL_3$ (40 $\mu$g/ml) for the indicated time periods. Lysates were subjected to RBD pull-down to analyze the amount of Ras-GTP. *b*, Histogram showing the mean ± S.D. of Raf-1 activity (18) from lysates of cells incubated with $HDL_3$ for 0–30 min. *c* and *d*, HDL-induced activation of Mek and Erk1/2 by Western blot analysis with phosphospecific antibodies for Mek (*P-Mek*) and Erk (*P-Erk1/2*). Ras activity and P-Mek were also analyzed in CHO cells incubated with free apoA-I (15 $\mu$g/ml) (*a* and *c*). An anti-pan-Ras antibody was used to control for similar amounts of total Ras in the lysates.

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-MARCKS were from Cell Signaling, and PKC$\alpha$ 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 $\mu$g/ml) at 37 °C in 5% $CO_2$. For the measurement of Ras, Raf, and MAPK activity (see below) cells were preincubated overnight in the presence or absence of TPA (500 nM), PTX (0.1 $\mu$g/ml), or both.

*Preparation of HDL and ApoA-I*—High density lipoproteins ($HDL_3$, density 1.125–1.21 g/ml) were isolated from the plasma of normolipidemic volunteers by sequential density gradient ultracentrifugation as described previously (12, 13). After preparation HDL was stored in KBr at 4 °C and dialyzed extensively against phosphate-buffered saline before use. ApoA-I was isolated from HDL preparations as described previously (12).

*Measurement of Ras Activation*—The capacity of Ras-GTP to bind to RBD (Ras-binding domain of Raf-1) was used to analyze the amount of active Ras (15). Cells ($2 \times 10^6$) were incubated for 3 min with HDL (40 $\mu$g/ml), purified apoA-I (15 $\mu$g/ml), PDGF (10 ng/ml), or TPA (500 nM). In some experiments binding of HDL to SR-BI was inhibited by preincubation of cells with anti-SR-BI as described previously (14). Cells were harvested in lysis buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 1% (v/v) Triton X-100, 5 mM NaF, 10% (v/v) glycerol, 0.5% (v/v) 2-mercaptoethanol, 0.1 mM $Na_3VO_4$, and protease inhibitors). After centrifugation at 10,000 × *g* the protein concentration of the cleared cell lysate was determined (16). Then cellular proteins (600 $\mu$g) were incubated for 2 h at 4 °C with glutathione-Sepharose 4B beads precoupled with GST-RBD. Beads were washed four times in lysis buffer, and bound proteins were solubilized with Laemmli loading buffer and electrophoresed on 12.5% SDS-polyacrylamide gels (17). Proteins were transferred and immunoblotted using the anti-pan-Ras antibody.

*Raf-1 Kinase Activity Assays*—To measure Raf-1 activity, kinase assays following immunoprecipitation were performed essentially as described previously (18). Cells ($2 \times 10^6$) were harvested on ice in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1% (v/v) Triton X-100, 5 mM NaF, 0.1 mM $Na_3VO_4$, and protease inhibitors and cleared as described above. Supernatants (equalized for protein concentration) were immunoprecipitated for 2 h at 4 °C with 2 $\mu$g of anti-Raf precoupled with 20 $\mu$l of protein G-Sepharose (Pierce). Immunoprecipitates were washed three times in buffer A (30 mM Tris, 0.1 mM EDTA, 0.3% $\beta$-mercaptoethanol, 10% glycerol, 0.1% (v/v) Triton X-100, 5 mM NaF, 0.2 mM $Na_3VO_4$) with decreasing amounts of NaCl (1 M, 0.1 M, and salt-free) and incubated for 30 min at 30 °C in 20 $\mu$l of Mek buffer (salt-free buffer A plus 10 mM $MgCl_2$, 0.8 mM ATP, 6.5 $\mu$g/ml GST-Mek, and 100 $\mu$g/ml GST-Erk2). The reaction was terminated by the addition of 20 $\mu$l of ice-cold stop buffer (salt- and glycerol-free buffer A containing 6 mM EDTA). Following centrifugation, 6 $\mu$l of the supernatant was incubated for 15 min at 30 °C with 24 $\mu$l of MBP buffer (salt- and glycerol-free buffer A containing 10 mM $MgCl_2$, 0.1 mM ATP, 2.5 $\mu$l [$^{32}$P]ATP, 0.5 $\mu$g/$\mu$l myelin basic protein, and 0.16 $\mu$g/$\mu$l bovine serum albumin), and then aliquots of 24 $\mu$l 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 $HDL_3$ (40 $\mu$g/ml). Similar to the PDGF-induced activation of Ras (data not shown), incubation with $HDL_3$ 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. 1*a*). 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. 1*b*) and the HDL-induced phosphorylation of Mek and Erk1/2 (Fig. 1, *c* and *d*).

To determine whether the major apolipoprotein of $HDL_3$, apoA-I, is responsible for Ras activation, we compared $HDL_3$ and purified lipid-free apoA-I (21). In contrast to the immediate increase of Ras-GTP levels in $HDL_3$-incubated cells, addition of purified apoA-I to the culture medium had no effect on Ras-

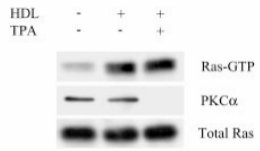


FIG. 2. **HDL activation of Ras is PKC-independent.** To down-regulate PKC, CHO cells were incubated overnight with or without TPA (500 nM) and then stimulated with $HDL_3$ (40 μg/ml) for 3 min as indicated. The inhibitory effect of TPA on PKCα is shown. The total amount of Ras in the cell lysates was determined as described above (Fig. 1).

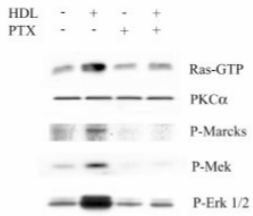


FIG. 3. **Effect of PTX on the HDL-induced activation of Ras.** CHO cells were preincubated with or without PTX (0.1 μg/ml for 6 h), and then $HDL_3$ (40 μg/ml) was added for 3 min as indicated. Then lysates were analyzed for Ras activity (RBD pull-down) as described above (Fig. 1*a*). Activation of MARCKS (*P-MARCKS*), Mek (*P-Mek*), and Erk1/2 (*P-Erk1/2*) was analyzed by Western blotting with phosphospecific antibodies as described above (Fig. 1, *c* and *d*). In addition, the expression of PKCα ± PTX was confirmed.

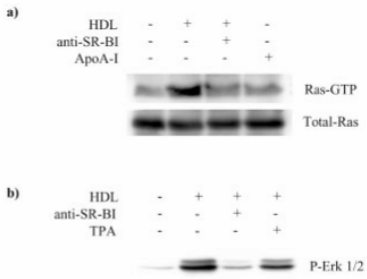


FIG. 4. **HDL activation of Ras and P-Erk1/2 is SR-BI-dependent.** *a*, CHO cells preincubated with the neutralizing anti-SR-BI antibody (KKB.1, 10 μg/ml) were incubated with $HDL_3$ (40 μg/ml) or apoA-I (15 μg/ml) for 3 min as indicated. Lysates were subjected to RBD pull-down, and the amount of Ras-GTP was determined. The total amount of Ras in the cell lysates was determined as described above (Fig. 1). *b*, CHO cells were preincubated with the neutralizing anti-SR-BI antibody (KKB.1, 10 μg/ml) or TPA (500 nM) and then incubated with $HDL_3$ (40 μg/ml) for 3 min as indicated. Then the HDL-induced activation of Erk was analyzed by Western blotting with a phosphospecific antibody (*P-Erk1/2*) as described above (Fig. 1*d*).

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 substate, 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 $Ca^{2+}$ 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 $HDL_3$, 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. 4*a*). 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. 4*b*). 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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## RESUMEN PARTE II:

## HIGH DENSITY LIPOPROTEIN-INDUCED SIGNALING OF THE MAPK PATHWAY INVOLVES SCAVENGER RECEPTOR TYPE BI-MEDIATED ACTIVATION OF RAS

### Antecedentes

Las lipoproteinas de alta densidad (HDL) ha sido demostrado que son potentes señalizadores, a través de los múltiples receptores celulares descritos. Entre estas señalizaciones destaca por su importancia fisiológica la activación mitogénica, y en concreto la vía de Raf-1 / MEK / MAPK. El enzima PKC es asimismo activado por las HDL y parece necesario para la activación de esta vía, si bien algunos trabajos contradicen esta contribución. Del mismo modo, la activación de proteínas G heterotriméricas acopladas a receptores endoteliales es necesaria para la activación de Raf-1. No había sido descrita, no obstante, la activación de la GTPasa Ras, uno de cuyos efectores más importantes es Raf-1. Asimismo, no existían evidencias directas de la participación del receptor SR-BI en ninguna de estas señalizaciones, a pesar de ser uno de los receptores de mayor relevancia fisiológica en el metabolismo *in-vivo* del colesterol.

### Objetivos

1. Determinar si las HDL son capaces de activar Ras en células CHO. Si es así, discriminar que componente de la partícula es el encargado de activar la señalización.
2. Analizar el resto de la vía de las MAPKs así como la contribución exacta del enzima PKC.
3. Describir el receptor implicado en dicha señalización.

Resultados

Por primera vez se describe la activación de Ras inducida por el tratamiento con HDL. La activación de Ras acontece de forma rápida (1-3 minutos), al igual que ocurre al estimular el mismo sistema celular con factores de crecimiento. Al cabo de 15 minutos, la activación de Ras retorna a niveles basales.

La generación de Ras-GTP se acompaña de un incremento subsiguiente de Raf-1, MEK y MAPK, completando la vía prototípica de las MAPKs. En esta señalización, al contrario de lo que ocurre en otros sistemas, no es necesaria la presencia del enzima PKC, de tal forma que en células pretratadas con ésteres de forbol para deplecionar al enzima la activación de Ras, Raf, MEK y MAPK acontece con normalidad. No obstante, al analizar uno de los sustratos de la PKC activa, P-MARCKS, se observa que el enzima resulta en efecto activado por las HDL.

Por último, describimos como la incubación de las células, de forma previa al tratamiento con las HDL, con un anticuerpo capaz de bloquear la unión del receptor SR-BI a la partícula, conlleva la inhibición de la señalización mitogénica descrita. Este hecho indica, en células CHO, que la asociación de alta afinidad de la partícula al receptor SR-BI, presente en las caveolas, es necesaria para la activación mitogénica. En este proceso, además, se requiere la existencia de proteínas $G_i$ heterotriméricas.

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