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Research Article

The nucleotide receptor $P2Y_{13}$ is a key regulator of hepatic High-Density Lipoprotein (HDL) endocytosis

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Abstract. Cell surface receptors for high-density lipoprotein (HDL) on hepatocytes are major partners in the regulation of cholesterol homeostasis. We recently identified a cell surface ATP synthase as a high-affinity receptor for HDL apolipoprotein A-I (apoA-I) on human hepatocytes. Stimulation of this ectopic ATP synthase by apoA-I triggered a low-affinity-receptor-dependent HDL endocytosis by a mechanism strictly related to the generation of ADP. This suggests that nucleotide G-protein-coupled receptors of the P2Y family are molecular components in this pathway. Only $P2Y_1$ and $P2Y_{13}$ are present on the membrane of hepatocytes. Using both a pharmacological approach and small interference RNA, we identified $P2Y_{13}$ as the main partner in hepatic HDL endocytosis, in cultured cells as well as in situ in perfused mouse livers. We also found a new important action of the antithrombotic agent AR-C69931MX as a strong activator of $P2Y_{13}$-mediated HDL endocytosis.

Key words. HDL; lipoprotein receptors; nucleotide receptors; cholesterol; endocytosis.

The risk of developing atherosclerosis, the leading cause of mortality in industrialized countries, is inversely related to the plasma concentrations of high-density lipoprotein (HDL)-cholesterol. This protective effect of HDL against atherosclerosis is classically attributed to its role in reverse cholesterol transport [1]. This process relies on specific interactions between HDL particles and cells, both peripherals (cholesterol efflux) and hepatics (cholesterol disposal), and on the maturation of HDL particles within the vascular compartment. Indeed, plasma HDL undergoes complex remodeling upon action of enzymatic and lipid transfer proteins, which enable the handling and esterification of large amounts of cell-derived cholesterol, resulting in the formation of large HDL particles further cleared by the liver. The plasma level of HDL-cholesterol will thus result from the complex interplay of those molecular partners. Among them, some contribute to HDL formation, e. g., lipoprotein lipase or the ATP-binding cassette A1 protein (ABCA1), while others are mostly involved in HDL catabolism, like hepatic lipase [2] or the endothelial lipase [3]. The physiological importance of reverse cholesterol transport has prompted numerous studies focused on the identification of cell surface receptors for HDL, which might regulate this pathway.

We have shown the presence of high-affinity receptors for HDL at the hepatocyte cell surface [4–5]. We have

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further demonstrated that this receptor is identical to the β-chain of ATP synthase, a major protein complex of the mitochondrial inner membrane [6]. Different experimental approaches confirmed this ectopic localization of components of the ATP synthase complex, as well as the corresponding ATP hydrolase activity at the hepatocyte cell surface. Stimulation by apolipoprotein A-I triggered endocytosis of holo-HDL particles (proteins and lipids) and the mechanism of stimulation was strictly dependent on ADP generation. Thus, we proposed a new role for the membrane-bound ATP synthase/hydrolase, modulating the levels of extracellular ADP and regulated by a major plasma apolipoprotein, apoA-I.

This suggests that nucleotide G-protein-coupled receptors of the P2Y family are molecular components in this pathway. To gain access to the molecular mechanisms linking the activation of cell surface ATP synthase and HDL endocytosis, we have focused our work on the three P2Y receptors preferentially activated by ADP: $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ [7]. Key roles for $P2Y_1$ and $P2Y_{12}$ receptors in platelet aggregation are well characterized [8], whereas the role of the recently cloned $P2Y_{13}$ receptors [9] is still unknown. Here, we show that $P2Y_{13}$ receptors are key components of the HDL endocytosis pathway regulated by ATP synthase/hydrolase.

Materials and methods

Materials. ADP, A2P5P, MRS2179 and Rolipram were from Sigma-Aldrich. Forskolin was purchased from Calbiochem. [^{3}H]cholesteryl-oleyl ether was from Amersham. AR-C69931MX used in perfused liver experiments was synthesized as already published [10]. The AR-C69931MX used in experiments on recombinant P2Y receptors was a gift from AstraZeneca.

Cell culture and siRNA preparation. $HepG_2$ cells were obtained from the American Type Culture Collection (HB-8065). The small interference RNA (siRNA) sequences targeting the human $P2Y_{13}$ receptor (GenBank/EMBL/DDBJ accession no. AF295368) were from position 406 and 359, respectively, in reference to the start codon. The siRNA duplex (21-nt RNAs) in deprotected and desalted form, the siRNA targeting the human $P2Y_1$ receptor, (mix of four siRNAs, M-005689), and the non-specific control siRNA (D-001206-01) were purchased from Dharmacon. Subconfluent $HepG_2$ cells were transiently transfected with siRNAs (final concentration, 100 nM) using Oligofectamine according to the manufacturer's protocol (Invitrogen). Cells were usually assayed 48 h after transfection. A specific silencing effect was confirmed in at least three independent experiments by Western blot analysis of $P2Y_1$ receptors. Briefly, 40 µg of $HepG_2$ homogenate proteins were separated by 8% SDS-PAGE under reducing conditions and were transferred to nitrocellulose membrane (30 min, 5.5 mA/cm^2). $P2Y_1$ receptor was immunodetected with antipeptide antibodies (0.6 µg/ml – AB5814, Chemicon) and revealed with a horseradish-peroxidase-conjugated second antibody. The proteins were visualized by enhanced chemiluminescence (ECL) detection (Perkin Elmer). In the case of $P2Y_{13}$ receptors, a real-time quantitative PCR was performed from total RNA isolated from control and siRNA-transfected $HepG_2$ cells using Trizol reagent (Invitrogen). Total RNA (5 µg) was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and treated with Dnase I (Invitrogen) according to the manufacturer's instructions. A real-time quantitative PCR assay was performed to amplify $P2Y_{13}$ and hypoxanthine phosphoribosyltransferase 1 (HPRT1) as control. The primers were designed using the PrimerExpress software developed by Applied Biosystems. Two sets of PCR primers were used: AGCTCCTCCACCTTCATCATCTAC and CAGGGTGCCAGGTGTGAGT, TGT GTT TTG CTC CAT TTC ATT TTG and TCT ACA GTC AGT CTT ATT GTT GGT TTG A for $P2Y_{13}$; TGG GAG GCC ATC ACA TTG T and TCC AGC AGG TCA GCA AAG AA for HPRT1. The first set of primers for $P2Y_{13}$ was designed to flank both sides of the siRNA-targeting positions. cDNA (250 ng) was amplified by real-time PCR using 2x SYBR Green Jumpstart Taq ready mix and primers (Sigma-Aldrich). The reactions were performed in duplicate in 25 µl of reaction volume with the following PCR conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. PCR was performed with an ABI PRISM 7000 sequence detection system (PE Applied Biosystems). Statistical analysis of the real-time quantitative PCR was performed using the ($2^{-\triangle\triangle Ct}$) method [11, 12], which calculates relative changes in gene expression of the target ($P2Y_{13}$) normalized to an endogenous reference gene (HPRT1).

Cell lines expressing recombinant P2Y receptors. The CHO-K1 cell line stably expressing the human $P2Y_{13}$ receptor has been previously described [9]. The plasmid of human $P2Y_{12}$ receptor (hP2Y12-pEFIN5) was obtained from Euroscreen (Belgium) and was stably transfected into CHO-K1 cells using the FuGENETM6 transfection reagent (Roche Applied Science). Transfected cells were selected using G418 (400 µg/ml). The $hP2Y_{13}$-CHO-K1 and $hP2YhP2Y_{12}$-CHO-K1 cells were grown in Ham's F12 cell culture medium (Invitrogen) supplemented with fetal bovine serum (10%, v/v), penicillin (100 units/ml), streptomycin (100 µg/ml) and G418 (400 µg/ml).

Cyclic AMP assay. Stably transfected CHO-K1 cells (200,000 cells/dish) were seeded on 35-mm diameter cell culture dishes in complete medium 1 day before the experiment. Cells were incubated for 2 h in Krebs-

Ringer-Hepes (KRH) buffer (124 mM NaCl, 5 mM KCl, 1.25 mM MgSO4, 1.45 mM $CaCl_2$, 1.25 mM KH_2PO_4, 25 mM HEPES, pH 7.4, and 8 mM D-glucose) containing 25 µM Rolipram. Nucleotides were then added in the presence of 3 µM forskolin, usually for a 12-min incubation (37 °C). The reactions were stopped by aspiration of the incubation medium and addition of 1 ml of 0.1 M HCl. The lysates were then vacuum dried and resuspended in an appropriate volume of water. Cyclic AMP was quantified by ^{125}I radioimmunoassay, as previously described [9].

Internalization assays. Internalization assays were performed as described elsewhere [2] by incubating cells for 10 min at 37 °C with 75 µg/ml of either ^{125}I-TG-HDL_2 (triglyceride-rich HDL_2), ^{125}I-HDL_3 or ^{125}I-low-density lipoprotein (LDL). After washing and dissociation at 4 °C in PBS, cell radioactivity was determined. Results are expressed as the percentage of internalization with respect to the control (set at 0); the control correspond to a value of 400 ng, 300 ng, and 200 ng of TG-HDL_2, HDL_3 and LDL per milligram of cell protein, respectively. Data are the mean ± SE of seven, five and six independent experiments (for TG-HDL_2, HDL_3 and LDL respectively).

Perfused mouse liver experiments. C57BL/6J male mice (8 weeks old; Charles River) or $P2Y_1^{-/-}$ mice (25 weeks old) were anesthetized by intraperitoneal injections of ketamine hydrochloride and xylazine hydrochloride. The liver was perfused *in situ* for 10 min at 37 °C with HBSS medium (Invitrogen) containing 75 µg proteins/ml of ^{125}I-TG-HDL_2 (5000 cpm/ng of protein) or ^{3}H-cholesteryl-ether-TG-HDL_2 (15,000 dpm/µg of cholesteryl ester), with either PBS, apoA-I (10 µg/ml) or AR-C69931MX (10 µM). Livers were extensively washed at 4 °C in PBS and radioactivity associated with the liver was counted (n = 7 per group). Values represent means ± SE, with $p < 0.05$ (unpaired t test).

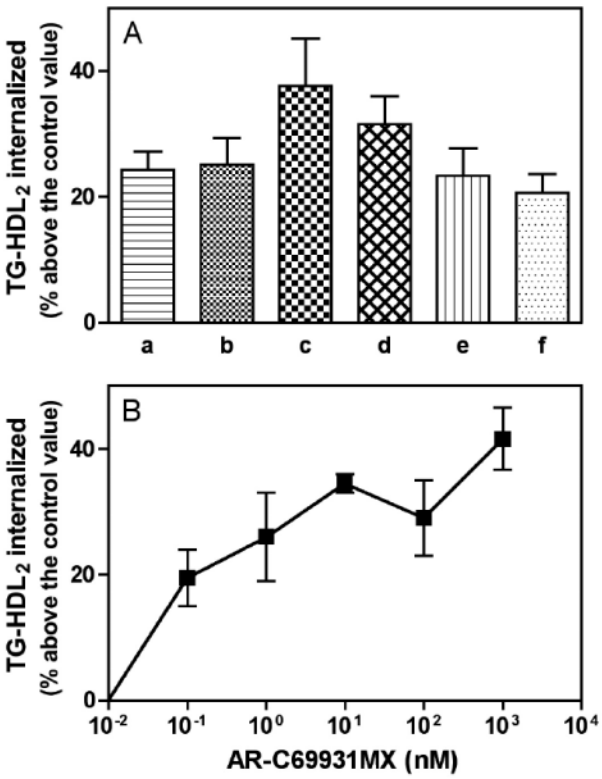

Figure 1. Effect of ADP and pharmacological modulators of P2Y receptors on TG-HDL_2 internalization by $HepG_2$ cells. (A) Cells were incubated for 10 min at 37 °C with 75 µg/ml ^{125}I-TG-HDL_2 without (control) or with lipid-free apoA-I 10 µg/ml (a), ADP 100 nM (b), AR-C69931MX 100 nM (c), ADP 100 nM + AR-C69931MX 100 nM (d), ADP 100 nM + A2P5P 100 µM (e), ADP 100 nM + MRS2179 10 µM (f). Data are expressed as the percentage of internalized radioactivity with respect to the control value (set as 0) (n > 5). (B) Dose-response curves of the effect of AR-C69931MX on TG-HDL2 internalization by $HepG_2$ cells were performed for 10 min at 37 °C as described in Materials and methods. Data are expressed as the percentage of internalized radioactivity with respect to the control value (set as 0) (n > 5).

Results

Despite the detection of $P2Y_1$ transcripts in $HepG_2$ cells, no increase of $[Ca^{2+}]_i$ was observed in response to ADP, suggesting the lack of functional $P2Y_1$ receptors in that cell line [13]. Furthermore, Northern blot analysis of $P2Y_1$ expression was negative in human liver [14]. $P2Y_{13}$ transcripts [9, 15] but not $P2Y_{12}$ transcripts [16–18] were detected in human liver, whereas both messengers were present in freshly isolated rat hepatocytes [19]. The discrepancy between the different observations concerning $P2Y_1$ receptor expressions, probably due to differential expression and/or sensitivity of the techniques used, led us to confirm by RT-PCR (not shown) that $P2Y_1$ and $P2Y_{13}$ but not $P2Y_{12}$ messengers are present in HepG2 and mouse hepatocytes.

In an attempt to measure modulations of HDL endocytosis, we used HDL_2 enriched in triglycerides (TG-HDL_2) as a substrate for internalization experiments [2]. This typical post-prandial HDL particle is able to bind and to be internalized only through low-affinity HDL binding sites on human hepatocytes, thus avoiding the contribution of the high-affinity apoA-I receptor, the cell surface ATP synthase [6]. In a pharmacological approach, we used A2P5P and MRS2179, specific antagonists of $P2Y_1$ receptors [20], and AR-C69931MX, a dual antagonist of $P2Y_{12}$ and $P2Y_{13}$ receptors [10; 21]. As already described [6], lipid-free apoA-I (fig. 1A) and ADP (fig. 1A) stimulated the internalization of ^{125}I-labelled-TG-HDL_2 by $HepG_2$ cells (20% above control cells). Addition of the $P2Y_1$ receptor antagonists, A2P5P (fig. 1A) and

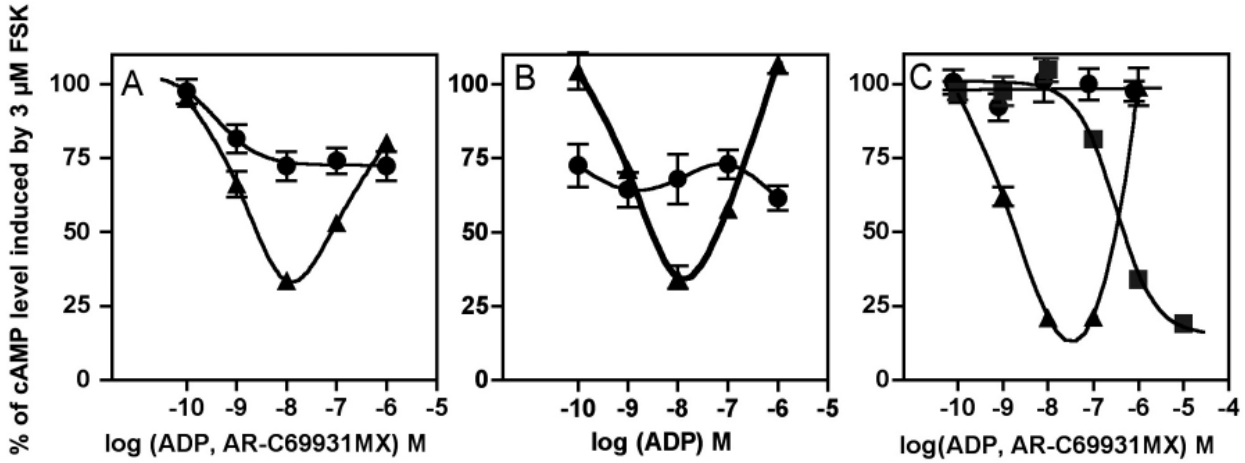

Figure 2. Effect of ADP and AR-C69931MX on recombinant $P2Y_{13}$ and $P2Y_{12}$ receptor activity. (A) Effect of ADP (▲) and AR-C69931MX (●) on forskolin (FSK; 3 µM)-induced cAMP formation in $hP2Y_{13}$-CHO-K1 cells. The cells were incubated with various concentrations of these agents for 12 min as described in Materials and methods. Data are expressed as a percentage of the value with forskolin alone and as the mean ± SD of triplicates in one representative experiment out of four. (B) Effect of AR-C69931MX (100 nM) on the modulation by ADP of FSK-induced cAMP formation. AR-C69931MX and ADP (at increasing concentrations) were added simultaneously with forskolin (3 µM) to $hP2Y_{13}$-CHO-K1 cells for a 12-min incubation period. Data are expressed as a percentage of the value with forskolin alone and presented as the mean ± SD of triplicates in one representative experiment out of two (▲) , ADP without AR-C69931MX; (●), ADP + AR-C69931MX (100 nM). (C) Effect of ADP (▲), AR-C69931MX (●) and ADP in the presence of AR-C69931MX (100 nM) (■) on (FSK)-induced cAMP formation in $hP2Y_{12}$-CHO-K1 cells. The cells were incubated with various concentrations of these agents for 12 min (FSK = 3 µM). Data are expressed in % of the value with forskolin alone and presented as the mean SE of three (ADP) or two (AR-C69931MX) independent experiments, each performed in triplicate.

MRS2179 (fig. 1A), had no effect on the stimulation by ADP of the TG-HDL_2 internalization. Surprisingly, AR-C69931MX (fig. 1A) per se increased TG-HDL2 internalization to a slightly greater extent than ADP itself and the effect of an equimolar combination of AR-C69931MX and ADP (100 nM each; fig. 1A) was equal to the effect of AR-C69931MX alone. Interestingly, the effect of AR-C29931MX on HDL endocytosis was observed at a concentration as low as 0.1 nM (fig. 1B) and with a time-course reaching a maximum between 5–10 min (not shown). This unexpected effect of AR-C69931MX might be explained by analyzing its behavior toward the $P2Y_{13}$ receptor as revealed by experiments on recombinant CHO-K1 cells. ADP has been previously shown to have a biphasic effect on the cAMP formation induced by forskolin in these cells [9, 21], i.e., an inhibition of cAMP accumulation at low nanomolar concentrations of ADP which is reversed at micromolar concentrations (a feature previously observed with other G_i-coupled receptors such as the α_2-adrenergic receptor). In the present experiments, the IC_{50} value for cAMP inhibition by ADP was 0.6 ± 0.2 nM and the maximal decrease was 70% (mean of five experiments, fig. 2A). In the same kind of experiments, AR-C69931MX also inhibited cAMP accumulation with a maximal inhibition of 25% and an IC_{50} of 0.5 ± 0.1 nM (mean of four experiments, fig 2A). However, by contrast to ADP, there was no reversal of that inhibition at higher concentrations of AR-C69931MX (fig 2A). As a control, AR-C69931MX had no effect on cAMP accumulation on plain CHO-K1 cells (data not shown). In fact, the behavior of AR-C69931MX was consistent with that of a partial agonist: indeed it mimicked the inhibitory effect of submaximal concentrations of ADP, but reduced the maximal inhibition produced at 10 nM ADP, as shown in figure 2B. In CHO-K1 cells stably expressing the human $P2Y_{12}$ receptor, AR-C69931MX had no effect per se on cAMP accumulation by forskolin, but shifted the ADP inhibition to higher concentrations, as expected for a pure competitive antagonist [10; 18] (fig. 2C). Thus, this partial agonist effect was specific for the $P2Y_{13}$ receptor.

The specific implication of $P2Y_{13}$ receptors in the TG-HDL_2 endocytosis by $HepG_2$ cells was then analyzed using siRNA [22]. First, silencing of $P2Y_1$ receptors by a pool of four specific $P2Y_1$ receptor siRNAs, was confirmed by Western blot experiments (fig. 3C), but had no effect on the stimulation of the TG-HDL_2 endocytosis by lipid-free apoA-I, ADP, AR-C69931MX (fig. 3A). By contrast, $P2Y_{13}$ receptor silencing by two different specific siRNAs (as confirmed by real-time PCR quantification; fig. 3D) induced a dramatic inhibition of the TG-HDL_2 endocytosis after stimulation by apoA-I, ADP, or AR-C69931MX (fig. 3B). A similar inhibition of internalization by silencing of $P2Y_{13}$ receptors was observed when using ^{125}I-labeled HDL_3, a major sub-class of HDL (fig. 4A). Indeed, $P2Y_{13}$ receptor siRNAs induced again a

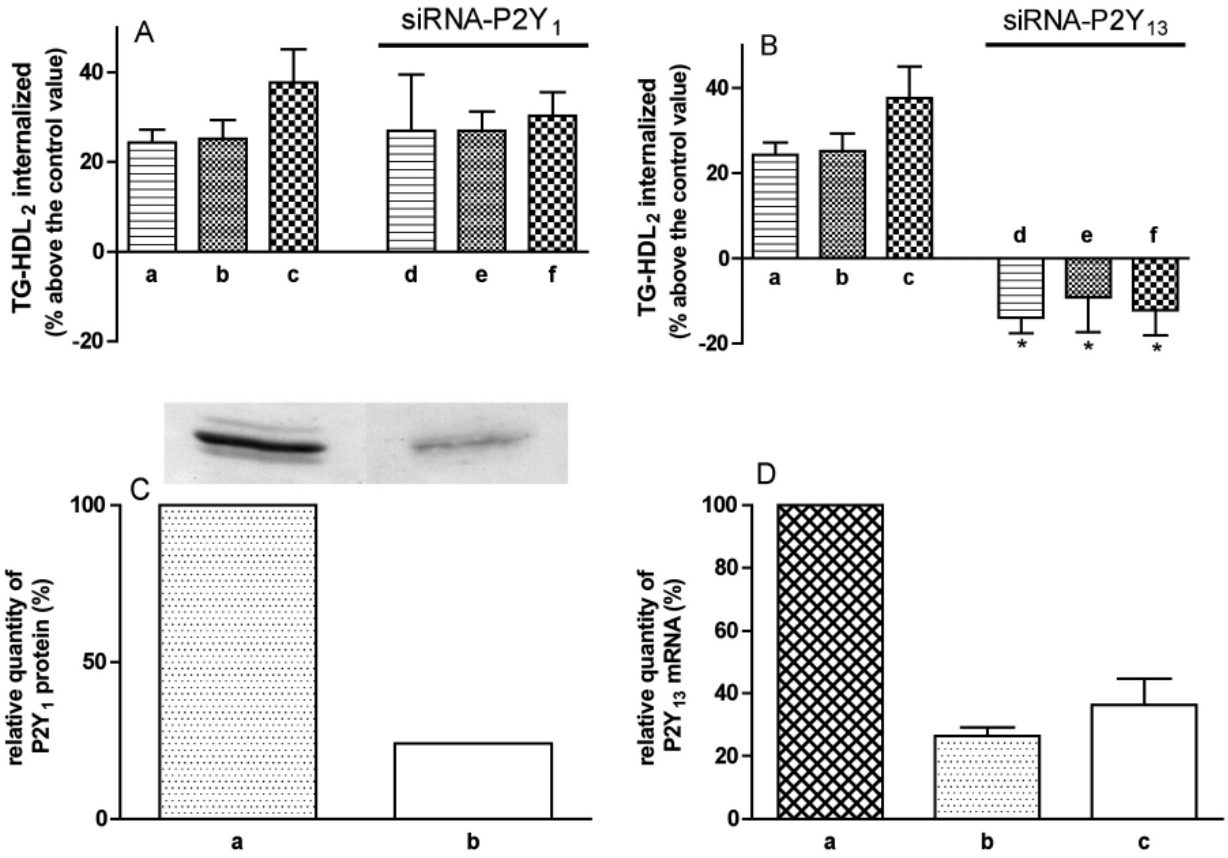

Figure 3. Involvement of human $P2Y_1$ and $P2Y_{13}$ receptors in the internalization of TG-HDL_2 by $HepG_2$ cells. (A) $P2Y_1$ receptor effect on TG-HDL_2 internalization. $HepG_2$ cells were transiently transfected with siRNA specific for the human $P2Y_1$ receptor (column d, e, f) or with non specific siRNA as control (a, b, c). 48 h later, cells were incubated for 10 min at 37 °C with 75 µg/ml ^{125}I-TG-HDL_2 without (control) or with lipid-free apoA-I 10 µg/ml (a, d), ADP 100 nM (b, e), AR-C69931MX 100 nM (c, f). Data are expressed as in figure 1A ($n > 5$). (B) $P2Y_{13}$ receptor effect on TG-HDL_2 internalization. Experiments were performed as in figure 3A with non-specific control siRNA (a, b, c) or with siRNA directed to the human $P2Y_{13}$ receptor (d, e, f) ($n > 7$). * $p < 0.05$, (unpaired t test). (C) The control of the efficacy of the siRNA designed to human $P2Y_1$ (b) on $HepG_2$ cells was performed for 48 h in comparaison to non-specific siRNA (a) using Western blot experiments as described in Materials and methods. Results are representative of three independent series of experiments. (D) Real-time PCR measurement of the $P2Y_{13}$ mRNA expression in $HepG_2$ cells after transient transfection for 48 h of two sets of siRNA specific for human $P2Y_{13}$ receptor (b, c) or with non-specific siRNA as control (a). Data are expressed as a percentage of the relative value obtained with non-specific siRNA and are representative of three independent series of experiments.

strong inhibition of basal HDL_3 internalization as well as after stimulation by ADP and AR-C69931MX (fig. 4A). Finally, as previously shown with lipid-free apoA-I [6], we confirmed the specificity of this endocytosis pathway toward HDL particles by the lack of stimulation of internalization of ^{125}I-labelled LDL by ADP (fig. 4B) or AR-C69931MX (fig. 4B), both without or with $P2Y_{13}$ receptor silencing.

To evaluate the physiological relevance of the $P2Y_{13}$ receptor as a partner in HDL endocytosis, we carried out in situ ^{125}I-labeled-TG-HDL_2 internalization experiments in perfused mouse liver. Indeed, the very short half-life of AR-C69931MX injected in animals does not allow long-term in vivo experiments [23]. Remarkably, lipid-free apoA-I (fig. 5A) and AR-C69931MX (fig. 5A) induced a rapid (10 min) and marked increase (up to 34% over control) of TG-HDL_2 internalization by the liver, indicating that $P2Y_{13}$ receptors were implicated in hepatic HDL endocytosis. Moreover, stimulated endocytosis was not restricted to the protein moiety of HDL (labeled with ^{125}I), since AR-C69931MX also stimulated the internalization of ^{3}H-cholesteryl-ether-labeled TG-HDL_2 (up to 27% over control, fig. 5B). Interestingly, the ratio of cholesteryl-ether to protein internalized by the liver was up to 20-fold more important (in 10 min) than in the original TG-HDL_2, indicating a selective uptake of cholesteryl-ether in the mouse liver, at variance with what we had previously observed in human hepatocytes [6]. Finally, as for human hepatocytes, HDL endocytosis in mouse liver was independent of $P2Y_1$ receptors as demonstrated by the marked increase (38% above the control) of TG-HDL_2 internalization by the liver of $P2Y_1^{-/-}$ mice [24], in

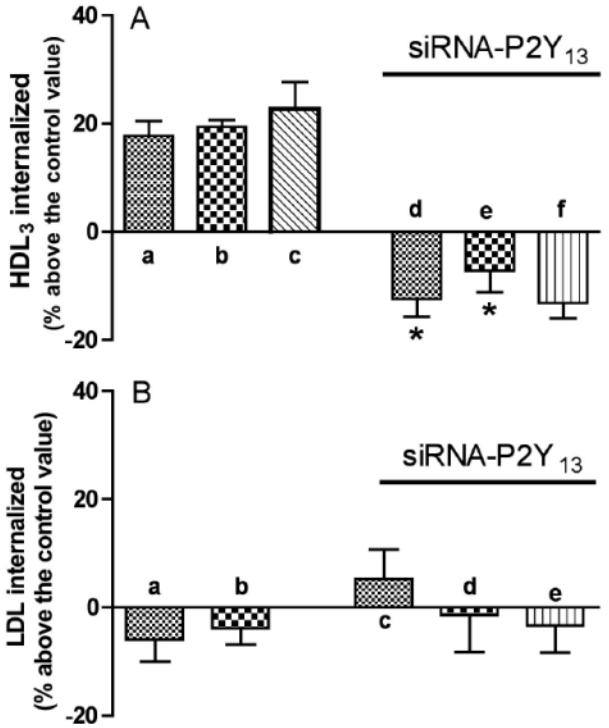

Figure 4. Involvement of human $P2Y_{13}$ receptors in the internalization of HDL_3 and LDL by $HepG_2$ cells. (A) $P2Y_{13}$ receptor effect on HDL_3 internalization. Cells were transiently transfected with siRNA specific for the human $P2Y_{13}$ receptor (columns d, e, f). Forty-eight hours later, cells were incubated for 10 min at 37 °C with 75 µg/ml ^{125}I-HDL_3 without (control) or with ADP 100 nM (a, d), AR-C69931MX 100 nM (b, e), lipid-free apoA-I 1 µg/ml (c) or PBS (f) ($n > 5$). * $p < 0.05$ (unpaired t test). (B) $P2Y_{13}$ receptor effect on LDL internalization. The experiment was performed as in figure 4A except that $HepG_2$ cells were incubated for 10 min at 37 °C with 75 µg/ml ^{125}I-LDL ($n > 6$).

response to AR-C69931MX (fig. 5C). Likewise, plasma cholesterol concentrations were not significantly different between wild-type mice (1.84 ± 0.18 mM, n = 6) and $P2Y_1^{-/-}$ mice (1.86 ± 0.11 mM, n = 6), confirming the absence of impact of $P2Y_1$ on HDL liver endocytosis.

Discussion

We have previously demonstrated that apoA-I binds with high affinity to the β chain of ATP synthase expressed on the surface of hepatocytes and triggers the endocytosis of HDL particles by a mechanism involving ATP hydrolysis into ADP [6]. ADP acts on three distinct P2Y receptors: $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ [7]. Which of these receptors are expressed in hepatocytes remains controversial. Using pharmacological inhibitors and siRNA we have shown both in vitro (HepG2 cells) and in situ (perfused mouse liver) that the effect of ADP is mediated by the $P2Y_{13}$ receptor and not by the $P2Y_1$ subtype.

Surprisingly, we have also observed that AR-C69931MX (currently in clinical development as an antithrombotic agent under the name Cangrelor) stimulates HDL endocytosis by HepG2 cells and by the perfused mouse liver. AR-C69931MX was first known as an inhibitor of platelet aggregation induced by ADP [10], and a competitive antagonist of the $P2Y_{12}$ receptor devoid of activity on the $P2Y_1$ receptor [18]. More recently, AR-C69931MX was shown to inhibit the stimulatory effect of ADP on the formation of inositol phosphates in 1321N1 cells stably expressing the human $P2Y_{13}$ receptor and the $G\alpha_{16}$ subunit [21]. Here, we have shown that in CHO-K1 cells it behaves as a partial agonist of the $P2Y_{13}$ receptor rather than as a pure antagonist. This partial agonist action might explain why it reproduces the stimulatory effect of ADP on HDL endocytosis by hepatocytes instead of inhibiting it. The question to be addressed is that this effect is actually greater than that of the full agonist ADP. One speculative explanation is that AR-C69931MX and ADP activate different signaling pathways associated with the $P2Y_{13}$ receptor. Indeed, AR-C69931MX mimicked the inhibitory effect of ADP on cAMP accumulation, mediated by G_i, but not the stimulation of inositol phosphate formation that involves $G\alpha_{16}$, nor the potentiation of cAMP accumulation that occurs at high ADP concentrations and is due to a promiscuous coupling to G_s.

HDL endocytosis can occur in hepatocytes through two different pathways. The first one might be dependent on scavenger receptor class B type I (SR-BI) [25], or its human ortholog CLA-1. SR-BI triggers internalization of the holo-HDL particle followed by selective transcytosis of lipoprotein cholesterol and recycling of the protein moiety, the so-called selective cholesterol uptake. A second mechanism might be independent of SR-BI and involves the uptake and degradation of the holo-HDL particle by an unknown receptor [26]. Interestingly, the ratio of cholesteryl ester to protein internalized by the mouse liver in our experiments was up to 20-fold more important than in the original TG-HDL_2, indicating a selective uptake of cholesteryl ester in the mouse liver. This suggests that SR-BI might be involved in the HDL endocytosis stimulated by ADP or AR-C69931MX in the mouse liver. In keeping with the strong differences in lipoprotein metabolism between humans and rodents, another mechanism of HDL endocytosis might operate in human hepatocytes. The involvement of CLA1 and SR-BI could also be different regarding hepatic HDL endocytosis in humans and rodents, respectively. Indeed, we already published results using blocking SR-BI antibodies that show that in human hepatocytes the involvement of SR-BI/CLA1 in HDL endocytosis was negligible [6]. We confirmed this observation using specific CLA-1/SR-BI siRNA on HepG2 cells (data not shown). Furthermore, although the importance of SR-BI in HDL metabolism is clearly demonstrated in mice, this

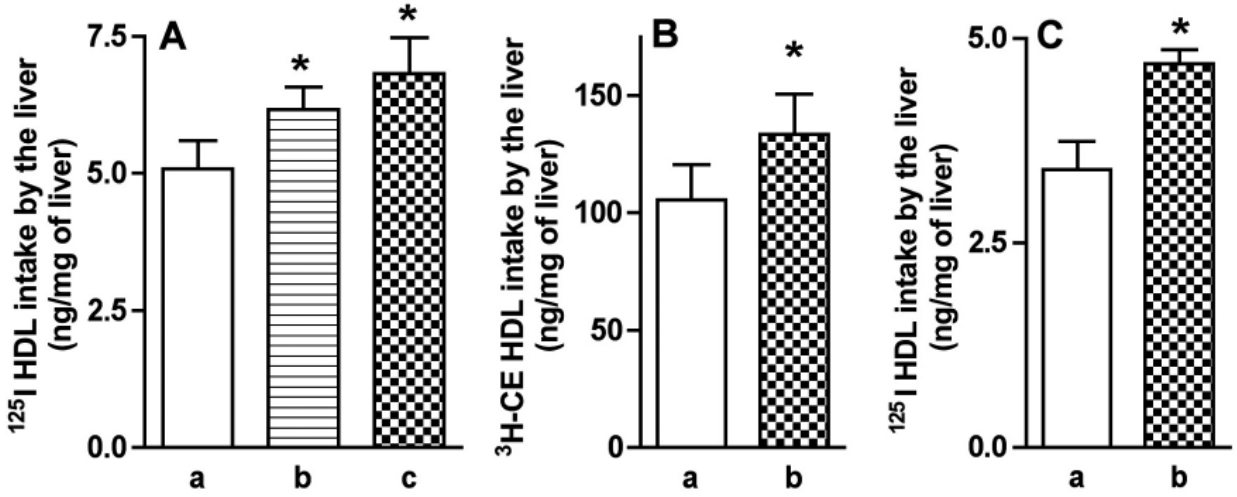

Figure 5. In situ TG-HDL_2 endocytosis by perfused mouse liver. (A) The liver from C57BL/6J male mice (8 weeks old) was perfused in situ for 10 min at 37 °C in HBSS medium with 75 µg/ml of ^{125}I-TG-HDL_2 in the presence of PBS (a), lipid-free apoA-I 10 µg/ml (b) or AR-C69931MX 10 µM (c). ^{125}I radioactivity associated with the livers was determined ($n > 4$). * $p < 0.05$ (unpaired t test). (B) Experiment as in A except that 75 µg/ml of ^{3}H-cholesteryl-ether-TG-HDL_2 was used in the presence of PBS (a) or AR-C69931MX 10 µM (b). ^{3}H radioactivity associated with the livers was determined ($n > 4$). * $p < 0.05$ (unpaired t test). (C) The liver from $P2Y_1^{-/-}$ C57BL/6J male mice (25 weeks old) was perfused as in A with 75 µg/ml of ^{125}I-TG-HDL_2 in the presence of PBS (a) or AR-C69931MX 10 µM (b). ^{125}I radioactivity associated with the livers was determined ($n > 3$). * $p < 0.05$ (unpaired t test)

point is still discussed in humans, especially because all of the CLA1/SR-BI mutations described so far in humans have shown no correlation with plasma HDL levels, suggesting a minor role for CLA1 in this metabolism [27]. Finally, whatever the differences observed in cholesterol metabolism between humans and rodents might be, we clearly found that $P2Y_{13}$ receptors have a key role in specifically regulating HDL endocytosis both in human hepatocytes and in mouse liver.

The physiological relevance of our observations is an important point for the validation of this pathway. Indeed, in situ experiments on perfused mouse liver are largely in favor of the specificity of $P2Y_{13}$ and not of $P2Y_1$ receptors. This needs to be further documented when $P2Y_{13}$ receptor knockout mice become available. However, we have previously published that when using IF1, a specific natural antagonist of the ectopic ATP synthase, in perfused rat liver, we decreased HDL endocytosis by about 50%, strongly indicating that the pathway we describe in the present paper could represent 50% of the total HDL endocytosis in rodents.

This unique role of $P2Y_{13}$ receptors in regulating reverse cholesterol transport raises possibilities for the control of cholesterolemia, a fundamental issue in the therapy of cardiovascular diseases. Indeed, we have demonstrated that the $P2Y_{13}$ receptor is a new pharmacological target able to control cholesterol catabolism by the liver, the only pathway of cholesterol elimination by the body. Moreover, the identification of AR-C69931MX, currently in clinical development, as a stimulator of this pathway opens up the way for the design of new drugs able to increase HDL-cholesterol clearance, thus increasing the atheroprotective effect of the HDL.

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1 Sviridov D. and Nestel P. (2002) Dynamics of reverse cholesterol transport: protection against atherosclerosis. Atherosclerosis, **161:** 245–254
2 Guendouzi K., Collet X., Perret B., Chap H. and Barbaras R. (1998) Remnant high density lipoprotein2 particles produced by hepatic lipase display high-affinity binding and increased endocytosis into a human hepatoma cell line (HEPG2). Biochemistry **37:** 14974–14980
3 Badellino K. O. and Rader D. J. (2004) The role of endothelial lipase in high-density lipoprotein metabolism. Curr. Opin. Cardiol. **19:** 392–395
4 Barbaras R., Collet X., Chap H. and Perret B. (1994) Specific binding of free apolipoprotein A-I to a high-affinity binding site on HepG2 cells: characterization of two high-density lipoprotein sites. Biochemistry **33:** 2335–2340
5 Martinez L. O., Georgeaud V., Rolland C., Collet X., Tercé F., Perret B. et al. (2000) Characterization of two high-density lipoprotein binding sites on porcine hepatocyte plasma membranes: contribution of scavenger receptor class B type I (SR-BI) to the low-affinity component. Biochemistry **39:** 1076–1082
6 Martinez L. O., Jacquet S., Esteve J. P., Rolland C., Cabezon E., Champagne E. et al. (2003) Ectopic beta-chain of ATP synthase is an apolipoprotein A-I receptor in hepatic HDL endocytosis. Nature **421:** 75–79
7 Abbracchio M. P., Boeynaems J. M., Barnard E. A., Boyer J. L., Kennedy C., Miras-Portugal M. T. et al. (2003) Characterization

of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. Trends Pharmacol. Sci. **24:** 52–55
8 Gachet C. (2001) Identification, characterization, and inhibition of the platelet ADP receptors. ADP receptors of platelets and their inhibition. Int. J. Hematol. **74:** 375–381
9 Communi D., Gonzalez N. S., Detheux M., Brezillon S., Lannoy V., Parmentier M. et al. (2001) Identification of a novel human ADP receptor coupled to G(i). J. Biol. Chem. **276:** 41479–41485
10 Ingall A. H., Dixon J., Bailey A., Coombs M. E., Cox D., McInally J. I. et al. (1999) Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J. Med. Chem. **42:** 213–220
11 Livak K. J. and Schmittgen T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **25:** 402–408
12 Schmittgen T. D. (2001) Real-time quantitative PCR. Methods **25:** 383–385
13 Schofl C., Ponczek M., Mader T., Waring M., Benecke H., Muhlen A. von zur Muhlen et al. (1999) Regulation of cytosolic free calcium concentration by extracellular nucleotides in human hepatocytes. Am. J. Physiol. **276:** G164–G172
14 Janssens R., Communi D., Pirotton S., Samson M., Parmentier M. and Boeynaems J. M. (1996) Cloning and tissue distribution of the human P2Y1 receptor. Biochem. Biophys. Res. Commun. **221:** 588–593
15 Zhang F. L., Luo L., Gustafson E., Palmer K., Qiao X., Fan X. et al. (2002) P2Y(13): identification and characterization of a novel Galphai-coupled ADP receptor from human and mouse. J. Pharmacol. Exp. Ther. **301:** 705–713
16 Zhang F. L., Luo L., Gustafson E., Lachowicz J., Smith M., Qiao X. et al. (2001) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. J. Biol. Chem. **276:** 8608–8615
17 Hollopeter G., Jantzen H. M., Vincent D., Li G., England L., Ramakrishnan V. et al. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature **409:** 202–207
18 Takasaki J., Kamohara M., Saito T., Matsumoto M., Matsumoto S., Ohishi T. et al. (2001) Molecular cloning of the platelet P2T(AC) ADP receptor: pharmacological comparison with another ADP receptor, the P2Y(1) receptor. Mol. Pharmacol. **60:** 432–439
19 Dixon J., Hall J. F., Webb T. E. and Boarder M. (2004) Regulation of that hepatocyte function by P2Y receptors: Focus on Control of glycogen phosphorylase and cyclic AMP by 2-Methylthioadenosine 5´-Diphosphate (2004). J. Pharmacol. Exp. Ther. **311:** 334–341
20 Ralevic V. and Burnstock G. (1998) Receptors for purines and pyrimidines. Pharmacol. Rev. **50:** 413–492
21 Marteau F., Le Poul E., Communi D., Communi D., Labouret C., Savi P. et al. (2003) Pharmacological characterization of the human P2Y13 receptor. Mol. Pharmacol. **64:** 104–112
22 Caplen N. J. and Mousses S. (2003) Short interfering RNA (siRNA)-mediated RNA interference (RNAi) in human cells. Ann. N. Y. Acad. Sci. **1002:** 56–62
23 Storey R. F., Oldroyd K. G. and Wilcox R. G. (2001) Open multicentre study of the P2T receptor antagonist AR-C69931MX assessing safety, tolerability and activity in patients with acute coronary syndromes. Thromb. Haemost. **85:** 401–407
24 Leon C., Hechler B., Freund M., Eckly A., Vial C., Ohlmann P. et al. (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. J. Clin. Invest. **104:** 1731–1737
25 Acton S., Rigotti A., Landschulz K. T., Xu S., Hobbs H. H. and Krieger M. (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science **271:** 518–520
26 Silver D. L., Nan W., Xiao X. and Tall A. R. (2001) HDL particle uptake mediated by SR-BI results in selective sorting of HDL cholesterol from protein and polarized cholesterol secretion. J. Biol. Chem. **276:** 25287–25293
27 Yu W., Nohara A., Higashikata T., Lu H., Inazu A. and Mabuchi H. (2002) Molecular genetic analysis of familial hypercholesterolemia: spectrum and regional difference of LDL receptor gene mutations in Japanese population. Atherosclerosis **165:** 335–342