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Research Article

Cell surface adenylate kinase activity regulates the F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis pathway on human hepatocytes

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Abstract. We have previously demonstrated on human hepatocytes that apolipoprotein A-I binding to an ecto-F_1-ATPase stimulates the production of extracellular ADP that activates a $P2Y_{13}$-mediated high-density lipoprotein (HDL) endocytosis pathway. Therefore, we investigated the mechanisms controlling the extracellular ATP/ADP level in hepatic cell lines and primary cultures to determine their impact on HDL endocytosis. Here we show that addition of ADP to the cell culture medium induced extracellular ATP production that was due to adenylate kinase (AK; $2ADP \leftrightarrows ATP + AMP$) and nucleoside diphosphokinase (NDPK; $ADP + NTP \leftrightarrows ATP + NDP$) activities, but not to ATP synthase activity. We further observed that *in vitro* modulation of both ecto-NDPK and AK activities could regulate the ADP-dependent HDL endocytosis. But interestingly, only AK appeared to naturally participate in the pathway by consuming the ADP generated by the ecto-F_1-ATPase. Thus controlling the extracellular ADP level is a potential target for reverse cholesterol transport regulation.

Keywords. High-density lipoprotein (HDL), apolipoprotein A-I, cholesterol, hepatocyte, nucleotide metabolism, adenylate kinase, nucleoside diphosphokinase.

Cell surface receptors for high-density lipoprotein (HDL) on hepatocytes are major partners in the regulation of cholesterol homeostasis [1]. We have previously identified on the cell surface of human hepatocytes a complex related to the mitochondrial ATP synthase, as a high-affinity receptor for HDL apolipoprotein A-I (apoA-I) [2]. In an attempt to characterize the enzymatic activity of this cell surface ATP synthase, we discovered that the complex could display an ATP hydrolase activity but could not synthesize ATP. We further elucidated a cell surface signal pathway for HDL endocytosis in which apoA-I binding to the ATP synthase (namely ecto-F_1-ATPase) stimulates extracellular ATP hydrolysis, and the ADP generated selectively activates the nucleotide receptor $P2Y_{13}$ and subsequent low-affinity-receptor-dependent HDL endocytosis [2, 3]. Strikingly, the ecto-F_1-ATPase activity ($ATP \rightarrow ADP + Pi$) provides a potent $P2Y_{13}$ receptor agonist (i.e. ADP) and confers on the ecto-F_1-ATPase its ability to stimulate HDL endocytosis. On the other hand, through degradation of extracellular ATP to adenosine, ecto-nucleotidases remove the potential to activate the P2Y receptor [4]. This characteristic of ecto-nucleotidases can be revealed using the ATP/ADPase apyrase, which mimics ecto-nucleotidase activity and inhibits HDL endocytosis by hepatocytes [2]. Therefore, adenine nucleotide metabolism in the extracellular environment of hepatocytes is likely to be important for the F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis. Besides ecto-nucleo-

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tidases, nucleotide-converting enzymes such as adenylate kinase (AK; 2ADP ⇆ ATP + AMP) and nucleoside diphosphokinase (NDPK; ADP + NTP ⇆ ATP + NDP) are important ADP-consuming/generating enzymes and may therefore potentially regulate the F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis. Although the presence and activities of such nucleotide-converting enzymes have previously been identified on the surface of various cell types [5–8], they have never been studied at the cell surface of hepatocytes.

In this study, we identified both AK and NDPK activities on the surface of $HepG_2$ hepatocarcinoma cells and primary human hepatocytes. These activities participate, in concert with the ecto-F_1-ATPase, in the interconversion of extracellular adenine nucleotides. In this context, we observed that both ecto-NDPK and AK activities could act on HDL endocytosis by modulating the extracellular ADP level. Interestingly, NDPK is unlikely to be constitutively active on human hepatocytes but the endogenous ADP-consuming activity of AK (2ADP → ATP + AMP) appears to downregulate the ecto-F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis pathway.

Materials and methods

Materials. Radiolabelled nucleotides were obtained from PerkinElmer and ^{125}I-Na from GE-Healthcare. The human hepatoblastoma-derived cell line $HepG_2$ was obtained from the American Type Culture Collection (HB-8065) and was cultured on collagen-I-coated plates (BD Biosciences) in high-glucose DMEM supplemented with 10% fetal bovine serum and antibiotics as previously described [9]. All cell culture reagents were from Invitrogen. Anti-NDPK (Nm23-H1) antibody was purchased from Insight Biotechnology. Nucleotides, diadenosine pentaphosphate (Ap_5A), apyrase type VII and all other reagents (analytical grade) were from Sigma. Ap_5A was treated with 20 units/ml grade VII apyrase for 2 h to hydrolyse contaminating ADP and ATP and apyrase was then inactivated by heat treatment at 100 °C for 10 min. ATP was then added to Ap_5A-treated samples and a luciferin-luciferase bioluminescence assay was performed to ensure that apyrase activity had been inactivated. Primary cultures of adult normal human hepatocytes were kindly provided by the group of P. Maurel (INSERM U128, Montpellier, France) and prepared as described previously [10].

ATP measurement by bioluminescence assay. $HepG_2$ cells were seeded on 24-well plates at 75,000 cells/well (~60% confluence) and allowed to adhere and grow for 48 h. Plates were then washed and equilibrated in either 10% fetal bovine serum or serum-free DMEM without red phenol for 1 h. Cells were then treated with different nucleotides or nucleotide analogues for 1 min. Supernatants were removed and centrifuged. For the experiments performed in 10% fetal bovine serum, serum proteins were removed from the cellular supernatants by chloroform extraction (1/1, v/v) before assaying ATP production on the upper phase of the extract. Aliquots (100 µl) of cellular supernatants were analysed using the ATP bioluminescence assay kit CLS II (Roche Diagnostics). Aliquots were added to the ATP assay mixture and luminescence was measured in a microplate luminometer Orion (Berthold detection systems) for 15 s. The ATP standard curve was performed in the same medium as the samples and in the 10^{-5} to 10^{-10} M concentration range. To monitor the number of cells in each well, adherent viable cells were lysed in NaOH 0.1 M and pH-equilibrated to 7.4 with HCl 1 M. Cellular protein concentration was then determined using the Bio-Rad protein assay dye and BSA as a standard. Data are expressed as picomoles of ATP produced per milligram of cell protein and the corresponding ATP molar concentrations have been specified in the results section when required.

Dual-label HPLC analysis. Nucleotides were separated by HPLC through PartiSphere SAX column (Whatman) and quantified on a continuous-flow, in-line scintillation detector (Parkard 500TR) as previously described [11]. The elution positions were determined using appropriate standards. No ATP was detectable in the [^{3}H]ADP preparation used in all experiments.

HDL_3, apoA-I preparations and ^{125}I-labelling. HDL_3 was isolated from the plasma of normolipidemic donors as previously described [12]. ApoA-I was isolated from HDL_3 by ion exchange chromatography as described in Mezdour et al. [13]. ^{125}I-labelling of HDL_3 was performed by the N-bromosuccinimide method [14]. Specific radioactivity of ^{125}I-ApoA-1 ranged from 600 to 1000 cpm/ng of protein.

HDL internalization assays. HDL internalization assays were performed as previously described [3]. Briefly, $HepG_2$ cells were seeded on 24-well plates at 75,000 cells/well (~60% confluence) and allowed to adhere and grow for 48 h. Primary human hepatocytes were seeded at confluence and used no longer than a week after preparation. Hepatocytes were washed and equilibrated in serum-free DMEM for 1 h at 37 °C, and were then incubated for 5 min at 37 °C with 75 µg/ml of ^{125}I-HDL_3. Cells were washed three times in serum-free DMEM, then dissociation of extracellular-membrane-bound HDL was performed at 4 °C in serum-free DMEM for 90 min. Cells were washed again three times in serum-free DMEM, then lysed in 0.5 ml NaOH 1 M for cell radioactivity counting and protein concentration measurement. Results are expressed as the percent variation of inter-

nalization compared to the control (set at 0); the control corresponds to a mean value of ~200 ng of internalized HDL_3 per milligram of cell protein.

Results

$HepG_2$ cells release ATP into the culture medium. We first determined whether $HepG_2$ cells were able to release ATP into the culture medium. After washing, $HepG_2$ cells were incubated in serum-free DMEM to assay extracellular ATP levels over time. $HepG_2$ cells released ATP immediately after medium change (Fig. 1a). The extracellular ATP concentration then decreased and stabilized over 1 h of incubation to a value of 76.5 ± 6.5 pmol ATP/mg cell protein (~12 nM). The same results were obtained when the experiment was performed in DMEM with 10% fetal bovine serum (data not shown). No lactate dehydrogenase activity could be detected in the medium sample (data not shown), indicating that no cell lysis occurred during the time of the experiment.

To determine whether ATP release was dependent on cell number, $HepG_2$ cells were seeded at various densities, allowed to grow for 48 h before incubation in serum-free medium for 1 h and ATP levels were measured. We observed a strong correlation between ATP concentration in the culture medium and the cell number (Fig. 1b).

Thus $HepG_2$ cells released ATP constitutively but the stabilization of extracellular ATP level over a short time (Fig. 1a) suggests a sharp regulation.

ADP increases the ATP concentration in cell culture medium. To clarify the mechanism of ATP release, we determined whether $HepG_2$ cells were able to convert nucleotides to ATP. $HepG_2$ cells were incubated with various nucleotides and the extracellular ATP concentration was measured. Figure 2a shows that addition of ADP (10 µM) to $HepG_2$ cells resulted in an increase of extracellular ATP content to 5871 ± 476 pmol ATP/mg cell protein (~1 µM), which corresponded to an ~100-fold increase of extracellular ATP content compared to the control without ADP added (vehicle). The same concentration (10 µM) of UDP, UTP, GDP, GTP or 2MeSADP (non-hydrolysable analogue of ADP) had no effect on the culture medium ATP concentration. Furthermore, ADP-induced ATP production correlated strictly with the added ADP concentration (Fig. 2b) and we could observe an ADP-induced ATP production (up to ~0.1 µM) with as little as 1 µM ADP added. Thus ADP to ATP conversion may participate significantly in extracellular ATP generation.

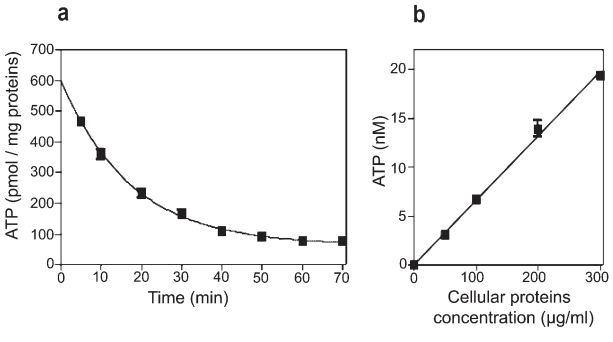

Figure 1. ATP release from $HepG_2$ cells as a function of medium change (*a*) and cell protein concentration (*b*). (*a*) $HepG_2$ cells were seeded on 24-well plates at 75,000 cells/well (~60% confluence) and allowed to adhere and grow for 48 h. Complete medium was then removed, replaced with serum-free DMEM; the extracellular medium was then collected at each indicated time point, and ATP was measured by luminometry. (*b*) $HepG_2$ cells were seeded at different densities from 25,000 to 150,000 cells/well then treated as in *a*, except that cells were incubated for 1 h with serum-free medium for stabilization before the ATP content was measured. Data shown are the mean ± SE (n = 3) and are representative of three separate experiments.

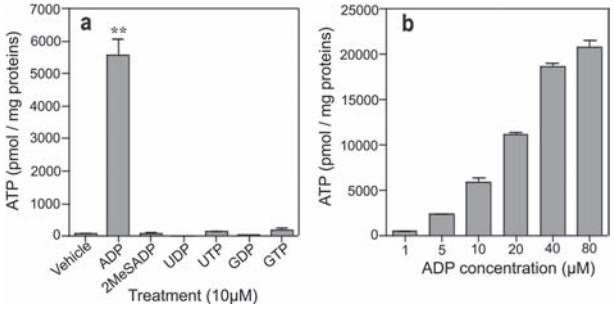

Figure 2. Effect of nucleotides on the ATP concentration in the culture medium. $HepG_2$ cells were seeded on 24-well plates at 75,000 cells/well (~60% confluence) and allowed to adhere and grow to confluence for 48 h. (*a*) Cells were incubated in serum-free DMEM for 1 h then nucleotides or nucleotide analogues (10 µM final concentration in DMEM) were added to $HepG_2$ cells for 1 min before samples of culture medium were taken and the ATP content measured by luciferin-luciferase assay. **$p < 0.05$ in comparison with vehicle. (*b*) Cells were incubated in serum-free DMEM for 1 h, then increasing concentrations of ADP (1–80 µM) were added to $HepG_2$ cells for 1 min before samples of culture medium were collected and the ATP content was measured by luciferin-luciferase assay. Data shown are the mean ± SE (n = 4) and are representative of three separate experiments.

Contribution of nucleotide conversion enzymes for extracellular ADP to ATP conversion on hepatocytes. Two important reactions by which ADP can be converted to ATP have been identified at the cell surface of various cell types: AK and ecto-NDPK [8, 15, 16]. AK catalyses the reaction 2ADP ⇆ ATP + AMP and is inhibited by Ap_5A [17]. NDPK catalyses a transphosphorylation reaction, transferring the γ-phosphate from a nucleoside triphosphate to a nucleoside diphosphate (e.g. ADP + NTP ⇆ ATP + NDP). However, these different enzymatic activities have never been clearly identified at the hepatocyte cell surface. Contrasting with these ADP-to-ATP conversion enzymes, the classical mitochondrial ATP synthase complex catalyses the synthesis of ATP from ADP and inorganic phosphate using a proton-motive force (ADP

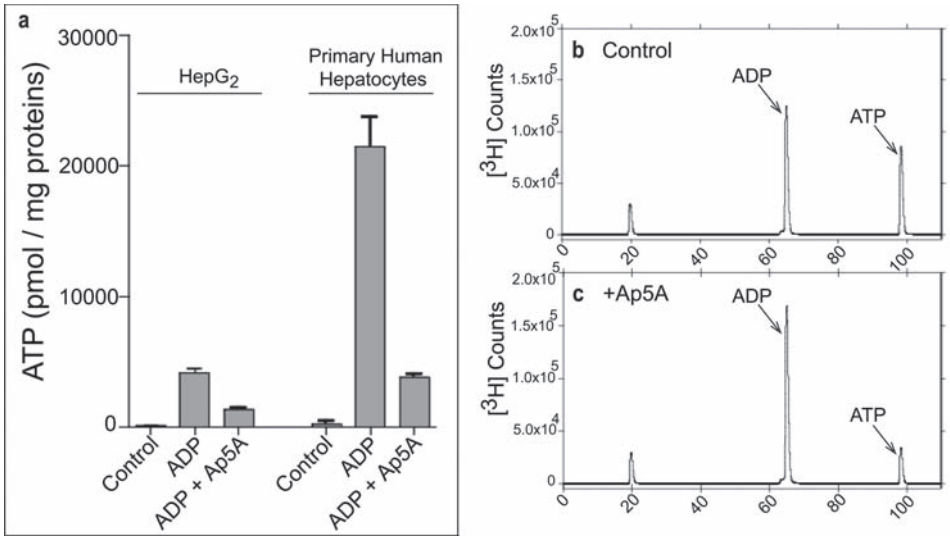

Figure 3. The conversion of ADP to ATP in culture medium of HepG2 cells and primary human hepatocytes is dependent on AK activity. (*a*) Confluent $HepG_2$ cells or primary human hepatocytes were washed and preincubated for 1 h in 0.5 ml of serum-free medium. The AK inhibitor Ap_5A (10 μM) was then added for 10 min. ADP (10 μM) was introduced for 1 min and cell culture medium samples were collected for ATP measurement by luciferin-luciferase assay. Data shown are the mean ± SE (n = 4) and are representative of three separate experiments. (*b*, *c*) HPLC analysis of metabolic products of ADP at the $HepG_2$ cell surface. Cells were washed and preincubated for 1 h in 0.5 ml of serum-free medium and subsequently incubated for 5 min in the presence of [^{3}H]ADP (1 μCi) alone (*b*) or simultaneously with Ap_5A (10 μM) (*c*). Samples of cell medium were then taken and nucleotides were separated by HPLC and [^{3}H] species detection was performed. The retention times for various standards are indicated with arrows. Data are representative of two separate experiments.

+ Pi ⇆ ATP). We have previously shown that the ecto-F_1-ATPase on hepatocytes could only hydrolyse ATP [2] whereas an ecto-F_1-ATPase present on the endothelial cell surface could both synthesise and hydrolyse ATP [18].

Using the ATP bioluminescence assay and HPLC analysis of radiolabelled nucleotides, we investigated the potential roles of cell surface AK, NDPK and F_1-ATPase activities in the extracellular conversion of ADP to ATP on $HepG_2$ cells and primary human hepatocytes.

By ATP bioluminescence assay, we first observed that the extracellular conversion of ADP to ATP was inhibited in the presence of Ap_5A (10 μM) by 65 ± 3% on $HepG_2$ cell and 82 ± 4% on primary human hepatocytes (Fig. 3a), suggesting that most of the ATP generated at the hepatocyte cell surface was produced by adenylate kinase activity (2ADP ⇆ ATP + AMP). Maximal inhibition of the ADP to ATP conversion occurred at 10 μM Ap_5A, as reported for other cell types [16], and higher concentrations of Ap_5A did not further inhibit ADP to ATP conversion (data not shown). To confirm that ADP was directly converted to ATP, HPLC analysis of extracellular [^{3}H]-ADP metabolic products was performed on $HepG_2$ cells. Incubation of $HepG_2$ cells with [^{3}H]-ADP (10 μM) for 5 min induced [^{3}H]-ATP production in the extracellular medium (Fig. 3b), and the generated [^{3}H]-ATP decreased by 68 ± 3% when 10 μM Ap_5A was added simultaneously with [^{3}H]-ADP (Fig. 3c), confirming the role of AK activity in the ADP-to-ATP conversion at the cell surface of hepatocytes.

Secondly, an ATP bioluminescence assay performed on $HepG_2$ cells and primary human hepatocytes indicated that the generation of extracellular ATP was higher upon incubation with both ADP (10 μM) and GTP (20 μM) compared to ADP or GTP alone (Fig. 4a) and ATP production correlated with the GTP concentration (data not shown). This is an indication of involvement of a transphosphorylation reaction using GTP as a phosphate donor. Moreover, the response to ADP + GTP was reduced to that of ADP alone in the presence of an anti-NDPK antibody (no effect was observed with a non-immune rabbit IgG; not shown). Finally, we tested the inhibition of NDPK activity by high concentration of UDP as reported previously [19]. Indeed, ATP generation was strongly reduced when a high concentration (1 mM) of UDP was added in combination with ADP + GTP (a higher concentration of UDP did not lead to further inhibition; not shown). Interestingly, ATP generation following ADP addition (without GTP) was decreased by ~50% with UDP (1 mM) whereas anti-NDPK antibody had no effect on the ATP level under the same conditions. Furthermore, HPLC analysis revealed rapid formation of [^{3}H]/[γ-^{32}P] double-labelled ATP when $HepG_2$ cells were incubated for 5 min with both [^{3}H]-ADP and [γ-^{32}P]-GTP (Fig. 4b, c), confirming that the γ-^{32}P of GTP had been transferred to the [^{3}H]-ADP through a cell surface transphosphorylation NDPK activity. The presence of NDPK at the cell surface was confirmed by flow cytometry performed on intact $HepG_2$ cells using the anti-NDPK antibody

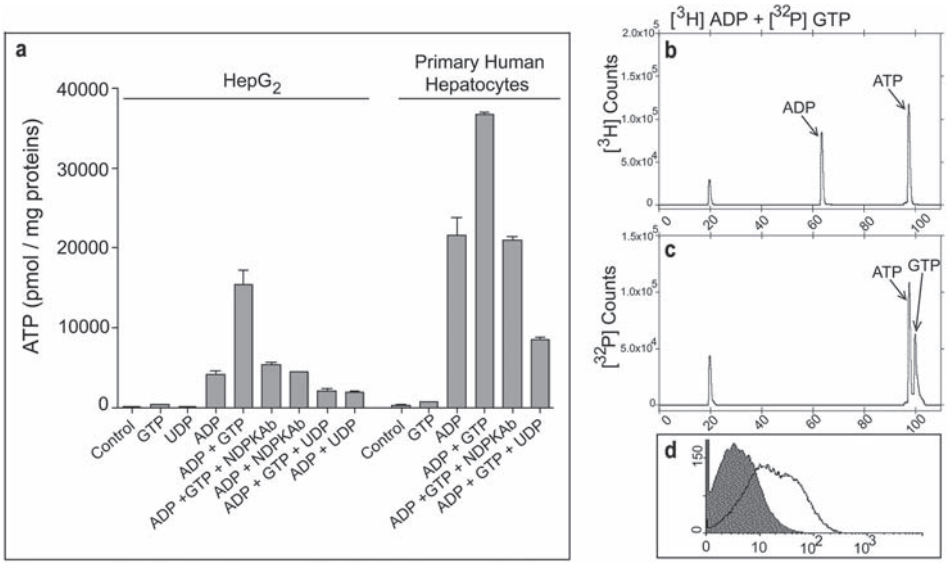

Figure 4. The conversion of ADP to ATP in culture medium of HepG2 cells and primary human hepatocytes is dependant on NDPK activity. (*a*) ADP and other nucleotides were introduced alone or simultaneously to confluent $HepG_2$ cells or primary human hepatocytes for 1 min. Cell culture medium samples were then collected and ATP was measured by luciferin-luciferase assay. Where not shown, ADP was used at 10 µM, GTP at 20µM and UDP at 1 mM. When used, NDPK antibody (100 µg/ml) was pre-incubated for 30 min prior to addition of nucleotides. Data shown are the mean ± SE (n = 4) and are representative of two separate experiments. (*b*, *c*) HPLC analysis of metabolic products of ADP at the $HepG_2$ cell surface. Cells were washed and pre-incubated for 1 h in 0.5 ml of serum-free medium and subsequently incubated for 5 min in the presence of [^{3}H]ADP (1 µCi) + γ[^{32}P]GTP (1 µCi). Samples of cell medium were then taken, nucleotides were separated by HPLC, and dual detection was performed (*b*: [^{3}H] species detection; *c*: [^{32}P] species detection). The retention times for various standards are indicated with arrows. (*d*) NDPK is present at the cell surface of hepatocytes. Flow cytometry on intact $HepG_2$ cells. Undashed histogram represents cells incubated with an antibody against the NDPK enzyme. Dashed histogram represents cells incubated with the isotypic control antibody.

(Fig. 4d). Altogether, these data strongly implicate NDPK in extracellular ATP generation, at least in the presence of extracellular triphosphate nucleotides, such as GTP.

ATP synthase uses Pi as a substrate for ATP production. However, an ATP bioluminescence assay performed on $HepG_2$ cells and primary human hepatocytes showed that addition of inorganic phosphate (50 mM) to the culture medium together with ADP did not further increase extracellular ATP production (Fig. 5a), as previously reported by Yegutkin et al. [16] on human endothelial cells (HUVECs). In addition, incubation of $HepG_2$ cells with [^{3}H]-ADP (10 µM), [^{32}Pi] and unlabelled phosphate (10 mM) induced [^{3}H]-ATP production in the extracellular medium (Fig. 5b) but the [^{3}H]-ATP generated was not ^{32}P-labelled (Fig. 5c), strongly indicating that extracellular ATP was not produced by the ecto-F_1-ATPase activity, as previously reported [2]. Similar results were obtained at 1 and 10 min incubation time (data not shown). As confirmation, oligomycin (an inhibitor of mitochondrial ATP synthase [20]) did not reduce the extracellular ADP-to-ATP conversion compared to the control (Fig. 5a). Altogether, these data indicate that the ecto-F_1-ATPase is not involved in the extracellular ADP-to-ATP conversion on hepatocytes, a process which more likely implicates AK and NDPK activities.

Contribution of NDPK and AK activities to HDL endocytosis by hepatocytes. The F_1-ATPase-mediated HDL endocytosis pathway is strictly dependent on the extracellular ADP level through the activation of the ADP-activated $P2Y_{13}$ receptor [2, 3]. Since the above results suggested that cell surface AK and NDPK activities were important in the extracellular metabolism of adenine nucleotides, we investigated the impact of these activities on HDL endocytosis by $HepG_2$ cells and primary human hepatocytes.

Addition of ATP + GDP to the $HepG_2$ cell medium to generate extracellular ADP through NDPK transphosphorylation activity (ATP + GDP → ADP + GTP) stimulated HDL endocytosis as efficiently as ADP addition (Fig. 6a). This stimulation was abolished when cells were pre-incubated with the anti-NDPK antibody, confirming that NDPK was specifically involved under ATP + GDP treatment. Importantly, anti-NDPK antibody did not significantly modulate HDL endocytosis in the absence of exogenous nucleotides or in the presence of ADP alone. This suggests that NDPK activity is unlikely to be involved in the basal level of HDL endocytosis. Similar results were obtained with primary human hepatocytes (data not shown).

Strikingly, the AK inhibitor Ap_5A stimulated HDL endocytosis by $HepG_2$ cells and primary human hepatocytes

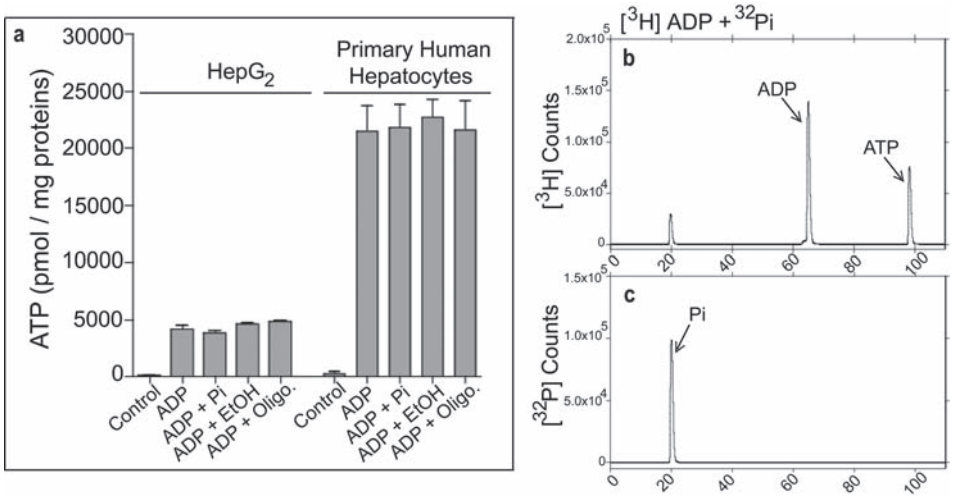

Figure 5. Ecto-F_1-ATPase activity is not involved in the conversion of ADP to ATP in culture medium of $HepG_2$ cells and primary human hepatocytes. (*a*) ADP (10 µM) was introduced alone or simultaneously with inorganic phosphate (50 mM) to confluent $HepG_2$ cells or primary human hepatocytes for 1 min. Cell culture medium samples were then collected and ATP was measured by luciferin-luciferase assay. When used, oligomycin (10 µg/ml) was pre-incubated for 10 min prior to addition of ADP (10 µM). Because oligomycin was dissolved in ethanol, an ethanol (1/100) control was performed. Data shown are the mean ± SE (n = 3) and are representative of three separate experiments. (*b*, *c*) HPLC analysis of metabolic products of ADP at the $HepG_2$ cell surface. Cells were washed and pre-incubated for 1 h in 0.5 ml of serum-free medium and subsequently incubated for 5 min in the presence of [^{3}H]ADP (1 µCi) + ^{32}Pi (0.5 µCi). Samples of cell medium were then taken and species were separated by HPLC and dual detection was performed (*b*: [^{3}H] species detection; *c*: [^{32}P] species detection). The retention times for various standards are indicated with arrows.

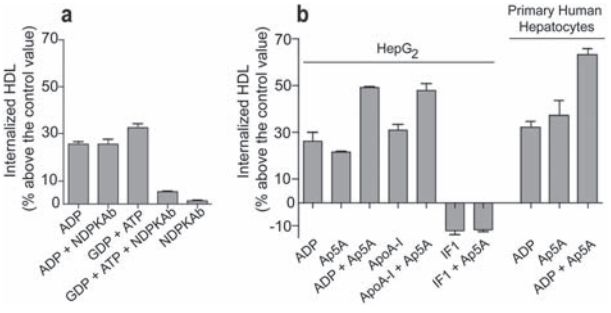

Figure 6. Effect of NDPK and AK activity on HDL_3 internalization by $HepG_2$ cells. Cells were incubated for 5 min at 37 °C with 75 µg/ml ^{125}I-HDL_3 and different modulators were then applied as described in Materials and methods. Where not shown, nucleotides and IF_1 were used at 100 nM, Ap_5A was used at 1 µM, apoA-I at 10 µg/ml and anti-NDPK antibody (NDPK Ab) at 100 µg/ml.

and this effect was additive to ADP stimulation (Fig. 6b). This reveals that unlike NDPK, AK is constitutively active and consumes extracellular ADP (2ADP → ATP + AMP), thus inhibiting the ADP-dependent $P2Y_{13}$-mediated HDL endocytosis pathway. As previously reported [2], apoA-I stimulated HDL endocytosis up to ~30% (Fig. 6b). Interestingly, the inhibition of AK activity by Ap_5A enhanced by ~50% the stimulation of HDL entocytosis by apoA-I (Fig. 6b), which suggests that AK activity counteracts the apoA-I effect by consuming the ADP generated through apoA-I interaction with the ecto-F_1-ATPase. Finally, we previously observed that the F_1-ATPase inhibitor IF_1 could inhibit both extracellular ADP generation and HDL endocytosis by $HepG_2$ cells [2]. Here, we observed that, in the presence of IF_1, the AK inhibitor Ap_5A could not upregulate HDL endocytosis (Fig. 6b). This is again a strong indication that, in basal conditions, AK mostly downregulates HDL internalization through consuming the ADP generated by the ecto-F_1-ATPase.

Discussion

This work was designed to characterize the ecto-enzymatic activities that control the ADP/ATP level on human hepatocytes and to determine their impact on the F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis pathway [2, 3].

First, it is clear that the ecto-F_1-ATPase substrate, i.e. ATP, is present at the surface of hepatocytes independently of cell lysis (Fig. 1). Indeed, mechanical perturbations induced by changing cell culture medium [21] trigger the release of ATP (Fig. 1a) [22] that stabilizes after 1 h. This basal extracellular ATP level most likely reflects a dynamic equilibrium in which the ATP metabolism at the cell surface of resting cells is balanced by constitutive ATP release. This constitutive ATP release might depend on ATP-binding cassette (ABC) transporters [23], the cell surface voltage-dependent anion channel VDAC [24], vesicle secretion [21, 25] or a network of mitochondria-associated tubules [26].

Interestingly, ADP but not ATP can stimulate the ecto-F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis pathway [2, 3]. This indicates that the extracellular level of ATP is not limiting in this pathway, but the availability of ADP produced from ATP by the ecto-F_1-ATPase is the limiting step of $P2Y_{13}$ activation. The concentration of extracellular ADP may be regulated by ecto-kinases activities that modulate extracellular ATP/ADP levels, such as AK ($2ADP \leftrightarrows ATP + AMP$) or NDPK ($ADP + NTP \leftrightarrows ATP + NDP$), both of which have been reported at the cell surface of various cell types [6–8, 15, 16, 25]. Here, we clearly showed that both AK and NDPK activities are also present at the cell surface of hepatocytes. Indeed, we have observed that the conversion of exogenously added ADP to ATP occurs rapidly and increases in parallel with ADP concentration (Fig. 2a, b). The higher activity observed on primary hepatocytes compared to $HepG_2$ cells may be attributable to an increased metabolism already described in primary cultures [27, 28]. This extracellular ADP-to-ATP conversion is mainly mediated by AK, as shown by the 68% and 82% inhibition observed in the presence of the AK inhibitor Ap_5A for $HepG_2$ cells and primary human hepatocytes, respectively. However, because Ap_5A does not completely abolish the conversion of ADP to ATP, a small fraction of ATP may be produced by other ecto-nucleotide kinase enzymes. We also found that ecto-NDPK activity, which uses a γ-phosphate donor in the reaction $ADP + NTP \leftrightarrows ATP + NDP$, can take part in extracellular ATP production when hepatocytes are incubated with both ADP and GTP. Indeed, this extracellular ADP-to-ATP conversion increases in a dose-dependant manner with GTP concentration, can be reduced by NDPK antibody to the level obtained with ADP alone, and is strongly inhibited in the presence of a high concentration (1 mM) of UDP, as previously reported [19]. The last effect may be attributed to the ability of UDP to also counteract AK activity in an alternative reaction $ADP + UDP \leftrightarrows AMP + UTP$ [29]. This hypothesis is supported by the fact that the anti-NDPK antibody strictly inhibits the GTP-induced ADP-to-ATP conversion and has no effect on ATP production when ADP is added alone (Fig. 4a). However, we do not totally exclude the possibility that NDPK may be activated by adding ADP alone, as nucleoside triphosphate, particularly UTP, has been reported to be constitutively released by cells [30, 31].

Although we have previously reported that the ecto-ATP synthase (namely ecto-F_1-ATPase) had only an ATP hydrolysis activity on hepatocytes [2], this ecto-enzyme was also found to both hydrolyse and synthesise ATP on keratinocytes [8] and endothelial cells [18]. We therefore investigated again whether an ecto-ATP synthase activity ($ADP + Pi \leftrightarrows ATP$) could be involved in the extracellular ADP-to-ATP conversion observed on hepatocytes. Contrasting with other studies reporting that oligomycin (a potent inhibitor of the mitochondrial F_1F_o ATP synthase by interaction with the F_o subcomplex [20]) could partially inhibit the extracellular ADP-to-ATP conversion [8], we did not observe in our system any effect of oligomycin on extracellular ATP production by hepatocytes. Furthermore, following incubation of $HepG_2$ cells with [^{3}H]-ADP and [^{32}Pi], we unambiguously observe that the generated [^{3}H]-ATP is not ^{32}P-labelled (Fig. 6b, c), suggesting a reaction different from that of the conventional ATP synthase, as previously reported by Yegutkin et al. [16] on human endothelial cells. Consistent with our data on hepatocytes, Yegutkin et al. [16] attributed this extracellular ATP production on endothelial cells to AK and NDPK activities but not to ATP synthase activity. Therefore, cell surface ATP synthesis observed on endothelial cells by other groups is probably not only due to the ATP synthase activity and further investigations are needed regarding other ecto-nucleotide kinases activities. However, it remains to be elucidated whether we are dealing with similar entities of the ATP synthase on the different cell types.

Thus, our data rule out the ability of the ecto-F_1-ATPase to synthesize ATP on hepatocytes and show that the ADP-to-ATP conversion at the hepatocyte cell surface is mainly due to AK and ecto-NDPK activities, the later being fully active when a co-substrate (e.g. GTP) is added.

This ADP-to-ATP conversion may be essential in the regulation of the F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis pathway. Indeed, our results reveal that ecto-NDPK activity can stimulate HDL endocytosis only when the enzyme is activated with exogenous substrates (e.g ATP + GDP) to generate extracellular ADP. By contrast, the AK inhibitor Ap_5A stimulates HDL endocytosis, suggesting that ecto-AK is constitutively active in consuming extracellular ADP. Moreover, because Ap_5A does not further stimulate HDL endocytosis when the F_1-ATPase activity is inhibited by IF_1, it appears that most of the ADP constitutively generated by the ecto-F_1-ATPase ($ATP \rightarrow ADP + Pi$) is consumed by AK activity. Therefore, in the $P2Y_{13}$-mediated pathway of HDL endocytosis, the availability of ADP to stimulate $P2Y_{13}$ and finally HDL uptake is a crucial point of regulation. It may depend on the balance between the constitutive AK activity that removes extracellular ADP and the ecto-F_1-ATPase that synthesizes ADP. Activation of ADP synthesis by apoA-I unbalances the pathway and increases HDL endocytosis when required (Fig. 7).

The present work provides a better understanding of a cell surface nucleotide signal which is the initial step of the F_1-ATPase/$P2Y_{13}$ pathway and will eventually lead to the endocytosis of the holo-HDL particles (protein plus lipid) through a low-affinity HDL-binding site as previously described [2, 32, 33]. The identity of the low-affinity receptor is still unknown and needs further investigations. Although Cla-1 (the human orthologue of SR-BI) may be a good candidate [34], neither SR-

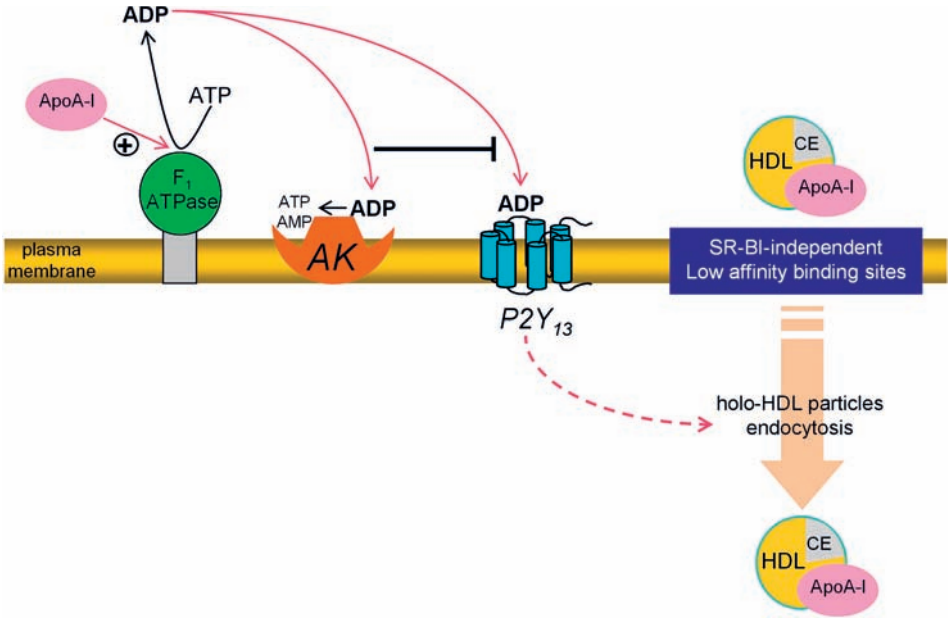

Figure 7. Model of F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis by hepatocytes. Cell surface AK activity downregulates an SR-BI-independent holo-HDL particle endocytosis pathway by consuming the ADP generated by the ecto-F_1-ATPase. This process depends on the balance between the AK activity that removes extracellular ADP and the ecto-F_1-ATPase that synthesizes ADP. Activation of ADP synthesis by apoA-I unbalances the pathway and increases HDL endocytosis when required. CE, cholesterol ester.

BI/Cla-1-blocking antibody nor SR-BI/Cla-1 siRNA on $HepG_2$ cells could modify the basal or ADP-induced HDL uptake by $HepG_2$ cells [1, and our unpublished data]. Thus, it is more likely that the final step of this pathway, i.e. uptake of the holo-HDL particles, occurs through an HDL low-affinity receptor other than SR-BI/Cla-1 (Fig. 7). This is consistent with other data suggesting that the selective lipid uptake, which is one of the main features displayed by SR-BI [34], does not require HDL endocytosis [35, 36] and that the amount of HDL trafficking through the SR-BI-dependent retroendocytic pool is too small to support the SR-BI-mediated selective lipid uptake [37].

In conclusion, this study provides new insights into the F_1-ATPase/$P2Y_{13}$-mediated HDL endocytosis pathway by showing that modulation of the extracellular ADP level through ecto-AK and ecto-F_1-ATPase activities is a potential target for reverse cholesterol transport regulation. This opens up new strategies for the design of drugs able to increase HDL-cholesterol clearance for enhanced atheroprotective effects.

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