Recycling of Apolipoprotein(a) After PlgRKT-Mediated Endocytosis of Lipoprotein(a)

Monika Sharma, Gregory M. Redpath, Michael J.A. Williams, Sally P.A. McCormick

Rationale: Lipoprotein(a) [Lp(a)] is a low-density lipoprotein–like lipoprotein and important cardiovascular risk factor whose cognate receptor and intracellular fate remains unknown.

Objective: Our study aimed to determine the intracellular trafficking pathway for Lp(a) and the receptor responsible for its uptake in liver cells.

Methods and Results: Human hepatoma cells were treated with Lp(a) purified from human plasma and Lp(a) uptake studied using Western blot analysis and intracellular localization of Lp(a) by confocal microscopy. Lp(a) was maximally internalized by 2 hours and was detected at an antiapop(a) antibody to be localized to Rab5-positive early endosomes, the trans-Golgi network, and subsequently Rab11-positive recycling endosomes. In human hepatoma cells, the apo(a) component from the internalized Lp(a) was resecreted back into the cellular media, whereas the low-density lipoprotein component was localized to the lysosomal compartment. Lp(a) internalization was reduced 0.35-fold in HAP1 and 0.33-fold in human hepatoma cells in which the plasminogen receptor (KT) was knocked out. Conversely, Lp(a) internalization was enhanced 2-fold in HAP1 and 1.6-fold in human hepatoma cells in which plasminogen receptor (KT) was overexpressed, showing for the first time the role of a specific plasminogen receptor in Lp(a) uptake.

Conclusions: The novel findings that Lp(a) is internalized by the plasminogen receptor, plasminogen receptor (KT), and the apo(a) component is recycled may have important implications for the catabolism and function of Lp(a).

Key Words: lipoprotein(a) • liver • plasminogen receptor • recycling endosomes

Lipoprotein(a) [Lp(a)] is an independent risk factor for developing cardiovascular disease.1 Lp(a) comprises a low-density lipoprotein (LDL) with its apolipoprotein B (apoB) moiety linked to apolipoprotein(a) [apo(a)].2 Apo(a) has its density lipoprotein (LDL) with its apolipoprotein B (apoB) moiety linked to apolipoprotein(a) [apo(a)]. Apo(a) has its evolutionary origin from plasminogen3 and shows homology to other plasminogen-related growth factors.4 Lp(a) is assembled after the secretion of apo(a) from the liver with assembly occurring extracellularly or on the hepatocyte surface5 or possibly intracellularly before secretion.6,7 Factors regulating apo(a) synthesis and secretion are reasonably well understood with the secretion rate of apo(a) being a major determinant of plasma Lp(a) levels.8 Whereas the anabolic fate of Lp(a) is well established, the catabolic fate of Lp(a) remains uncertain.

Editorial, see p 1050
In This Issue, see p 1045

Lp(a) uptake from plasma is primarily via the liver and kidney with liver clearance presumed to predominate.5 The receptor(s) and mechanism responsible for Lp(a) clearance have yet to be unambiguously identified. Recent studies have identified a role for the scavenger receptor B1 in the uptake of lipids from Lp(a)10,11; however, the receptor involved in whole Lp(a) particle remains elusive. Multiple receptors have been implicated, but evidence for each is conflicting. This is particularly the case with respect to the LDL receptor (LDLr). Early studies showed that Lp(a) bound to the same high affinity sites as LDL in skin fibroblasts,12 and its clearance was reduced in fibroblasts from familial hypercholesterolemia homozygotes.13 This was supported by studies showing that familial hypercholesterolemia subjects had higher Lp(a) levels than nonaffected individuals.13 Interestingly, recent trials with PCSK9 (proprotein convertase subtilisin/kexin type 9)–specific monoclonal antibodies, which block PCSK9-mediated degradation of the LDLr, show significant reductions in Lp(a) levels.14,15 A recent article showed that the LDLr is involved in Lp(a) metabolism in liver and fibroblast cells via its regulation by PCSK9,16 suggesting that the mechanism responsible for the reduction in Lp(a) levels may be indirectly through an enhanced hepatic LDL uptake. Contrary to these studies implicating a role for LDLr in Lp(a) uptake, many studies have indicated that the LDLr is not involved. Kinetic
Novelty and Significance

What Is Known?

- Lipoprotein(a) [Lp(a)] is taken up mostly by the liver.
- The role of various receptors in Lp(a) uptake, including the low-density lipoprotein (LDL) receptor, is unclear with disparate findings in different model systems.
- Although after uptake LDL has been shown to be degraded by lysosomes, the intracellular fate of Lp(a) is unknown.

What New Information Does This Article Contribute?

- Lp(a) uptake is mediated by the PlgRKT plasminogen receptor in liver cells.
- The LDL component of Lp(a) is degraded by lysosomes, but the apo(a) component traffics via the trans-Golgi network to recycling endosomes.
- The recycled apo(a) is resecreted from liver cells.

Elevated Lp(a) levels are an independent risk factor for cardiovascular disease. There are no current therapies to specifically lower plasma Lp(a) levels. The catabolism of Lp(a) occurs in the liver, but the receptor involved and intracellular fate of the Lp(a) is unknown. We found that Lp(a) is maximally internalized by liver cells within 2 hours where its LDL and apo(a) components have different catabolic rates. Although the LDL component is degraded in the lysosome, apo(a) follows a recycling fate which culminates in apo(a) resecretion. Using gain- and loss-of-function approaches, we show that the receptor mediating the majority of Lp(a) uptake in liver cells is the plasminogen receptor, PlgRKT. These findings provide important new information about Lp(a) catabolism and have implications for the development of therapies to modulate Lp(a) levels.

Nonstandard Abbreviations and Acronyms

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<tr>
<td>ASGPR: asialoglycoprotein receptor</td>
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<tr>
<td>HDFa: adult human dermal fibroblasts</td>
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<td>HepG2: human hepatoma cells</td>
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<td>LDL: low-density lipoprotein</td>
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<td>LDLR: low-density lipoprotein receptor</td>
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<td>Lp(a): lipoprotein(a)</td>
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<td>PCSK9: proprotein convertase subtilisin/kexin type 9</td>
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<td>PlgRKT: plasminogen receptor (KT)</td>
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studies have shown no difference in the catabolism of Lp(a) in familial hypercholesterolemia compared with nonaffected individuals. Furthermore, family studies showed no difference in Lp(a) levels between familial hypercholesterolemia and nonaffected family members with the same apo(a) isoforms. Some cell culture studies have also shown an insignificant role of the LDLR in Lp(a) uptake. Finally, statin treatment, which increases LDLR expression and effectively reduces plasma LDL levels, has no effect on plasma Lp(a) levels in humans.

Because evidence for the role of the LDLR in Lp(a) uptake is controversial, the roles of several other potential receptors for Lp(a) catabolism have been investigated. Various studies have implicated a role for the very low-density lipoprotein (VLDL) and megalin/gp330 receptors in Lp(a) uptake; however, neither receptor is expressed in the liver. Asialoglycoprotein receptor (ASGPR)−dependent Lp(a) uptake was observed in ASGPR−/− mice and COS-7 cells lacking ASGPR; however, these observations have not been investigated in relevant human cell lines. Previous studies have shown the binding of Lp(a) to plasminogen binding sites on various cell types. Both plasminogen and ε-aminocaproic acid, a lysine analogue that binds to the active site of plasminogen receptors, strongly reduce the binding of recombinant apo(a) to human hepatoma (HepG2) and primary fibroblast cells. However, it is unclear whether these results extend to the intact Lp(a) particle and, if so, which plasminogen receptor(s) are involved. Several plasminogen receptors exist, including α-enolase, H2B, p11/annexinA2, plasminogen receptor (KT; PlgRKT), and α5β1 integrin.

Although multiple receptors could be involved in Lp(a) uptake, a comparison of relevant receptors in the same cellular system has not been made. Furthermore, little is known about the intracellular fate of the Lp(a) particle once internalized. Our study aimed to investigate the internalization and intracellular trafficking route of Lp(a) in human liver and fibroblast cell lines. We show that internalized Lp(a) is trafficked to recycling endosomes via early endosomes and the trans-Golgi network, culminating in the resecretion of apo(a) into the media. Furthermore, we show that the plasminogen receptor, PlgRKT, is largely responsible for Lp(a) uptake.

Methods

Detailed descriptions of the experimental procedures are provided in the Online Data Supplement.

Results

Maximal Lp(a) Uptake Occurs at 2 Hours in HepG2 Cells

Immunostaining for apo(a) using the MAb-a5, apo(a)-specific monoclonal antibody, revealed a lack of endogenous apo(a) at 0 hour and maximum uptake of Lp(a) at 2 hours of Lp(a) treatment (green signal, Figure 1A) reducing to a minimal signal by 24 hours. Quantiﬁcation of Lp(a) uptake from Western blots of apo(a) in the cell lysates (Figure 1B) showed that Lp(a) uptake increased 2-fold between 1 and 2 hours (Figure 1C) then decreased down to 0.25-fold at 24 hours. A Western blot of apo(a) in the cell lysates at 6 hours (run under nonreducing conditions) showed the presence of both free apo(a) and Lp(a) (Figure 1D), suggesting that Lp(a) dissociates within the cell after internalization. Western blot analysis of apoB in Lp(a)-treated HepG2 cells from 0 to 24 hours (Online Figure IA and IB) and RT-PCR (reverse transcription polymerase chain reaction) analysis of apoB in Lp(a)-treated HepG2 cells at 6 hours (Online Figure IC) indicated continuous endogenous production of apoB not affected by the introduction of exogenous apoB from Lp(a). We also investigated Lp(a) uptake by tracer labeling using AlexaFluor-488–labeled Lp(a). Treatment of HepG2 cells with 5 µg/mL of AlexaFluor-488–labeled Lp(a) showed maximum fluorescence intensity from the internalized...
Lp(a) at 2 hours (100%) with a rapid decrease to 43% by 6 hours and 24% by 24 hours (Online Figure IIA and IIB). These results are in concordance with the time course of Lp(a) uptake as determined by Western blot and immunostaining.

To track the fate of the lipid and protein components of Lp(a) once internalized, the lipid component of Lp(a) was labeled using DiI stain (1,1’-dilinoleyl-3,3,3’,3’-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate), and the apo(a) component was detected as before using an anti-apo(a) antibody. Interestingly, the lipid and apo(a) component of Lp(a) were strongly colocalized at 1 and 2 hours (yellow signal, Figure 1E) but little colocalization was seen after 6 hours of Lp(a) treatment (Figure 1E), suggesting the separation of the lipid and apo(a) component of Lp(a) by 6 hours post-Lp(a) internalization.

**LDL of Lp(a) Is Degraded via Lysosomes**

Because we had observed the separation of the lipid and apo(a) component of Lp(a) (Figure 1E), we hypothesized that LDL would follow the well-established pathway of LDL degradation via lysosomes. Therefore, we investigated the fate of the LDL part of Lp(a) using Bodipy cholesterol labeling. Treatment of Bodipy-labeled Lp(a) onto HepG2 cells for 6 hours at 37°C revealed that the Bodipy-labeled Lp(a) (green signal, Figure 2A) was strongly colocalized (yellow signal, Figure 2A) with lysosomes (red signal, Figure 2A) similar to Bodipy-labeled LDL (Figure 2A).

As HepG2 cells synthesize their own apoB making it difficult to visualize the apoB component of Lp(a), we used haploid human fibroblast-like HAP1 cells (which do not express apoB) to determine whether the apoB component also tracked to the lysosomes. ApoB-specific immunostaining of Lp(a) using the 1D1, apoB-specific antibody, revealed that the apoB component of Lp(a) (green signal, Figure 2B) was strongly colocalized (yellow signal, Figure 2B) with lysosomes (red signal, Figure 2B) at 6 hours. Interestingly, the apo(a) component of Lp(a) (green signal, Figure 2C) showed little colocalization with lysosomes (red signal, Figure 2C), suggesting that the apoB component is preferentially trafficked to the lysosomes.

**Figure 1. Lipoprotein(a) [Lp(a)] shows maximum internalization at 2 h in human hepatoma (HepG2) cells.** HepG2 cells were treated with 5 µg/mL Lp(a) for 0 to 24 h at 37°C. A, Confocal microscopy of Lp(a)-treated HepG2 cells stained with the apo(a)-specific monoclonal antibody, MAb-a5, detected by an anti-mouse AlexaFluor-488 antibody. A merged image of Lp(a) (green) with DAPI (blue) is shown. Scale bar=20 µm. At least 100 cells were analyzed per condition across 3 independent experiments and representative images shown. B, Western blot of cell lysates with the MAb-a5 and the apoB-specific monoclonal antibody, 1D1 using actin as a loading control. C, Apo(a) protein levels as detected by Western blotting were normalized against actin and expressed relative to that of Lp(a)-treated cells incubated for 1 h. Results are expressed as mean±SE for 3 independent experiments performed in triplicate. **P<0.01, ***P<0.001 compared with Lp(a) incubated for 1 h. D, Western blot analysis of Lp(a)-treated cell lysates (6-h incubation) under nonreducing conditions with the MAb-a5 and 1D1 antibodies. The purified Lp(a) in media before incubation with HepG2 cells is shown. E, HepG2 cells were treated with 5 µg/mL DiI-Lp(a) for 0 to 24 h at 37°C. Confocal microscopy of DiI-Lp(a) (red) treated HepG2 cells stained with an anti-mouse AlexaFluor-488 antibody. A merged image with DAPI is shown. Areas marked by white rectangles are zoomed. Scale bar=20 µm. At least 100 cells were analyzed per condition across 3 independent experiments, and representative images are shown.
suggesting that the apo(a) component had a different fate to that of the LDL.

**Apo(a) Is not Degraded via Lysosomes or Proteasomes**

To further investigate the fate of apo(a), we performed time course studies with organelle-specific immunostaining. As observed with HAP1 cells, there seemed to be little colocalization between apo(a) (green signal) and lysosomes (red signal) at any time point (Figure 2D). Furthermore, inhibition of lysosomal proteases by NH₄Cl treatment (which deacidifies the lysosomes) or pharmacologically (using the E64d lysosomal inhibitor) produced no increase in apo(a) levels in cell lysates after 5 µg/mL Lp(a) incubation (Online Figure IIIA and IIIC). In contrast, cells treated with 20 µg/mL LDL showed a distinct increase in intracellular apoB levels after both treatments (Online Figure IIIB and IIID).

We next investigated whether apo(a) was degraded via the proteasome. Treatment of HepG2 cells with Clasto-Lactacystin β-lactone, a widely used inhibitor of the 20S proteasome complex, had no effect on either apo(a) or apoB protein levels after Lp(a) or LDL incubation (Online Figure IIIE and IIIF), confirming that neither apo(a) nor LDL is degraded via the proteasome.

**Apo(a) Traffics Through Early Endosomes to the Trans-Golgi Network**

As apo(a) was not degraded via lysosomes or proteasomes, we further investigated the intracellular fate of apo(a) using organelle-specific markers. Localization of apo(a) (green) with Rab5-positive early endosomes (red) produced no increase in apo(a) levels in cell lysates after 5 µg/mL Lp(a) incubation (Online Figure IIIA and IIIC). In contrast, cells treated with 20 µg/mL LDL showed a distinct increase in intracellular apoB levels after both treatments (Online Figure IIIB and IIID). We next investigated whether apo(a) was degraded via the proteasome. Treatment of HepG2 cells with Clasto-Lactacystin β-lactone, a widely used inhibitor of the 20S proteasome complex, had no effect on either apo(a) or apoB protein levels after Lp(a) or LDL incubation (Online Figure IIIE and IIIF), confirming that neither apo(a) nor LDL is degraded via the proteasome.

**Figure 2.** The low-density lipoprotein (LDL) but not the apo(a) component of Lipoprotein(a) [Lp(a)] colocalizes with lysosomes. A, Purified Lp(a) and LDL were labeled with the Bodipy fluorophore (green), and human hepatoma (HepG2) cells were treated with 5 µg/mL Bodipy Lp(a) or 5 µg/mL Bodipy LDL for 6 h at 37°C. Confocal microscopy of Bodipy-labeled Lp(a) or LDL-treated HepG2 cells stained with LysoTracker Red DND-99. B and C, HAP1 cells were treated with 5 µg/mL Lp(a) for 6 h at 37°C. Confocal microscopy of Lp(a)-treated HepG2 cells stained with (B) LysoTracker Red DND-99 and 1D1 or (C) LysoTracker Red DND-99 and MAb-a5 detected by an anti-mouse AlexaFluor-488 antibody. D, HepG2 cells were treated with 5 µg/mL Lp(a) for 0 to 24 h at 37°C. Confocal microscopy of Lp(a)-treated HepG2 cells stained with LysoTracker Red DND-99 and Mab-a5 detected by an anti-mouse AlexaFluor-488 antibody. A merged image with DAPI is shown. Areas marked by white rectangles are zoomed. Scale bar=20 μm. At least 100 cells were analyzed per condition across 3 independent experiments, and representative images are shown.
Apo(a) Is Found Within Recycling Endosomes
We speculated that apo(a) might be found in a recycling endosomal compartment and investigated this after transfecting HepG2 cells with the recycling endosome marker plasmid, dsRed Rab11 wild type (WT). Apo(a) (green) colocalized with dsRed Rab11 WT (red) in the perinuclear region (merged, yellow signal, Figure 4A) at 6 hours. To confirm that apo(a) was following an intracellular recycling pathway, we investigated the colocalization of apo(a) with exogenously added transferrin to label endogenous intracellular recycling pathways. Coincubation of HepG2 cells with AlexaFluor-594–conjugated wheat germ agglutinin (WGA) after triton X-100 permeabilization (trans-Golgi network). Lp(a) was stained with MAb-a5 detected by an anti-mouse AlexaFluor-488 antibody. A merged image with DAPI is shown. Areas marked by white rectangle are zoomed. Scale bar=20 μm. At least 100 cells were analyzed per condition across 3 independent experiments, and representative images are shown.

Apo(a) Is Resecreted Into the Media
The presence of apo(a) in a recycling compartment begged the question: was apo(a) being resecreted into the media? To investigate this, HepG2 cells were incubated with Lp(a) for 6 hours, then Lp(a)-containing media removed, and cells extensively washed and placed in fresh media. Western blot analysis showed apo(a) to be present in the fresh media from 1 hour after Lp(a) removal (Figure 4C) with levels increasing 2.1-fold by 24 hours compared with 2 hours (Figure 4D). To exclude the possibility that the release of surface-bound Lp(a) was the source of apo(a) in the media, HepG2 cells were trypsinized after 6 hours of Lp(a) treatment and reseeded in fresh media for 24 hours to detect the rescreted apo(a) in media. Western blot analysis showed the presence of apo(a) in media from 1 hour post-Lp(a) removal in both washed and trypsinized cells (Figure 4C). Because our HepG2 cells are null for apo(a), our results indicate rescretion of the exogenously sourced apo(a).

Further validation that the rescretion of apo(a) was from internalized Lp(a) and not from surface-bound Lp(a) was gained by immunostaining of Lp(a) on the cell surface. There was no evidence of surface-bound Lp(a) (green signal, Online Figure V) on the HepG2 cell membrane as stained with wheat germ agglutinin (red signal, Online Figure V), suggesting that the internalized Lp(a), and not cell surface-bound Lp(a), was the source of rescreted apo(a).
To establish whether the resecreted apo(a) was in the form of free apo(a) or Lp(a), the resecreted apo(a) was immunoprecipitated from the media and removed from the beads under nonreducing conditions. Western blot analysis showed the presence of both free apo(a) and Lp(a) in the media (Figure 4E). However, free apo(a) was first observed at 2 hours, whereas Lp(a) was not observed until 6 hours. Interestingly, the appearance of Lp(a) coincided with the appearance of apoB in the media at 6 hours (Figure 4C and 4E). On the basis of this observation, we speculate that free apo(a) may be reassembled into Lp(a) after the endogenous secretion of apoB. Both apo(a) and Lp(a) levels seemed to remain constant in the media from 6 until 24 hours (Figure 4E). With respect to how much of the initial apo(a) added to cells was being recycled, quantification of the recycled apo(a) in media by an apo(a)-specific ELISA showed that $\approx30\%$ was being recycled into the media after 24 hours (Figure 4F).

Finally, the presence of resecreted apo(a) in media was detected after the treatment of HepG2 cells with AlexaFluor-488--labeled Lp(a). Fluorescence in media was seen 6 hours post-Lp(a) removal increasing by 24 hours (Online Figure IIC). This was in conjunction with the appearance of apo(a) in the media at 6 hours which was increased at 24 hours, as observed by Western blotting of an apo(a) immunoprecipitation from the media (Online Figure IIC, inset). Overall, these results corroborate the novel recycling fate of apo(a).
Adult Human Dermal Fibroblasts Cells Also Internalize Lp(a) and Resecrete Apo(a) Into the Media

To validate our results of Lp(a) uptake, recycling and resecretion of apo(a) in a primary human cell line, adult human dermal fibroblasts (HDFa), were treated with 5 µg/mL Lp(a). HDFa cells displayed a similar uptake as HepG2 cells (Figure 5A); however, in contrast, the apo(a) signal (green) did not diminish over time. The localization of apo(a) within the cell and recycling endosomes (Figure 5B and 5D) was also seen in HDFa cells. Apo(a) was not present in lysosomes in HDFa cells (Figure 5C). Like in HepG2 cells, apo(a) was shown to be recycled into the media (Figure 5E) with ≈30% of the initial Lp(a) being rescreted (Figure 5F). Because HDFa cells do not secrete endogenous apoB, the rescreted apo(a) cannot be reassembled into Lp(a) as seems to occur in HepG2 cells.

Lp(a) Uptake Is Independent of the LDLR and ASGPR

Last, we wanted to determine the receptor responsible for Lp(a) internalization. We investigated whether the LDLR was responsible for Lp(a) uptake. Preincubation of HepG2 cells with an anti-LDLR antibody followed by Lp(a) treatment of HepG2 cells showed no change in Lp(a) uptake (Figure 6A). LDLR uptake, however, was markedly reduced (Figure 6A) by the LDLR antibody. Confocal microscopy performed on Lp(a)-treated HepG2 cells showed little colocalization between internalized apo(a) (green signal) and the LDLR (red) (Figure 6D).
We next investigated whether the ASGPR was involved in Lp(a) uptake. Coincubation of GalNAc, a natural ligand for the ASGPR, and Lp(a) in HepG2 cells did not alter Lp(a) uptake (Figure 6B). Blocking the ASGPR by preincubating HepG2 cells with an anti-ASGPR1 antibody did not alter Lp(a) uptake (Figure 6B). There was no colocalization observed between internalized apo(a) (green signal) and ASGPR (red) on confocal microscopy of HepG2 cells (Figure 6E). Together, these results suggest that neither the ASGPR nor LDLR is the receptor responsible for Lp(a) internalization.

Plasminogen Receptor, PlgRKT, Is Responsible for the Majority of Lp(a) Uptake

Because of the high homology of apo(a) with plasminogen, we hypothesized that Lp(a) uptake might be mediated via plasminogen receptors. PlgRKT is a recently identified plasminogen receptor that is hypothesized to be the predominant plasminogen receptor in humans. Coincubation of Lp(a) with ε-aminocaproic acid for 6 hours severely reduced apo(a) uptake (Figure 6C), as did preincubation of the cells with an anti-PlgRKT antibody before Lp(a) treatment (Figure 6C). Confocal microscopy of Lp(a)-treated HepG2 cells showed colocalization between the internalized apo(a) (green signal) and PlgRKT (red signal; merged, yellow signal; Figure 6F).

To investigate the role of the PlgRKT in mediating Lp(a) uptake, PlgRKT knockout (HAP1) cells were obtained. PlgRKT knockout HAP1 cells were shown to have no detectable PlgRKT as detected by Western blot analysis, whereas the receptor was detected in wild-type (WT) HAP1 cells (Online Figure VIA). Treatment of both cell lines with 5 μg/mL Lp(a) for 6 hours showed Lp(a) uptake to be markedly reduced in PlgRKT knockout cells (Figure 7A) compared with WT HAP1 cells (0.35-fold reduction). The lack of LDLR involvement in Lp(a) uptake was further confirmed when blocking of the LDLR with an anti-LDLR antibody in HAP1 PlgRKT knockout cells had no cumulative effects on Lp(a) uptake (Online Figure VIIA). Control experiments showed that neither LDL nor plasminogen uptake was affected in HAP1 PlgRKT knockout cells (Online Figure VIIIA and VIIIB). The lack of an effect of the PlgRKT on plasminogen uptake was confirmed by a plasminogen competition assay which showed that plasminogen did not compete with Lp(a) in HepG2 cells.

Figure 6. Lipoprotein(a) [Lp(a)] uptake is independent of the low-density lipoprotein receptor (LDLR) and asialoglycoprotein receptor (ASGPR) and is mediated by the PlgRKT plasminogen receptor. A, Human hepatoma (HepG2) cells were preincubated with 10 μg/mL anti-LDLR followed by incubation with 5 μg/mL Lp(a) or 20 μg/mL LDL at 37°C for 6 h. B, HepG2 cells were either treated with 5 μg/mL Lp(a) and 20 μmol/L GalNAc or preincubated with 5 μg/mL anti-ASGPR1 followed by 5 μg/mL Lp(a) at 37°C for 6 h. C, HepG2 cells were either treated with 5 μg/mL Lp(a) and 5 μmol/L ε-aminocaproic acid (ε-ACA) or preincubated with 10 μg/mL anti-PlgRKT antibody followed by 5 μg/mL Lp(a) at 37°C for 6 h. Cell lysates were subjected to Western blotting with MAb-a5 and 1D1 antibodies using actin as a loading control. Each experiment was done in triplicates of at least 2 independent experiments, and representative blots are shown. Confocal microscopy of Lp(a)-treated HepG2 cells stained with MAb-a5, detected by an anti-mouse AlexaFluor-488 antibody, LDLR staining with an anti-LDLR antibody detected by an anti-rabbit AlexaFluor-594 antibody, ASGPR staining with an anti-ASGPR1 antibody detected by an anti-rabbit AlexaFluor-647 antibody, and PlgRKT staining with an anti-PlgRKT antibody detected by an anti-rabbit AlexaFluor-594 antibody. Merged images with DAPI are shown. Areas marked by white rectangle are zoomed. Scale bar=20 μm. At least 100 cells were analyzed per condition across 3 independent experiments, and representative images are shown.
Overall, these results demonstrate that the PlgRKT receptor specifically mediates Lp(a) uptake independent of the LDLR, and its role in plasminogen uptake in these cells is minimal.

An siRNA-mediated knockdown of the human PlgRKT gene was also performed in HepG2 cells to strengthen the role of the PlgRKT-mediated Lp(a) uptake in HepG2 cells. Transfection of HepG2 cells with 30 nmol/L PlgRKT-specific siRNA showed reduced PlgRKT levels (Online Figure VIC). The PlgRKT-specific siRNA transfection followed by treatment with 5 µg/mL Lp(a) for 6 hours showed a significant reduction in Lp(a) internalization to 0.33-fold relative to untransfected HepG2 cells (Figure 7C).

To further corroborate the role of the PlgRKT in mediating Lp(a) uptake, the human PlgRKT gene was overexpressed in both WT HAP1 and HepG2 cells. An increase in the PlgRKT protein level was apparent in HAP1 and HepG2 cells (Online Figure VIIB and VID, respectively), which was associated with a 2-fold increase in Lp(a) uptake in HAP1 (Figure 7B) and a 1.6-fold increase in HepG2 cells (Figure 7D) relative to untransfected cells. Collectively, these results suggest that the plasminogen receptor, PlgRKT, plays a significant role in mediating Lp(a) uptake.

**Discussion**

The liver has been established as the predominant site for Lp(a) catabolism; however, the receptor involved in Lp(a) uptake has not been well established, and the intracellular fate of Lp(a) has never been reported. Here, we show the time-dependent uptake of Lp(a) in liver cells using immunostaining and tracer-labeling experiments. We also show that the plasminogen receptor, PlgRKT, plays a significant role in mediating Lp(a) uptake.
provide useful knowledge for the future design of therapies aimed at enhancing Lp(a) catabolism.

Because of the similarity of Lp(a) to LDL, we expected that Lp(a) catabolism might follow the LDLR pathway as established by Brown and Goldstein in which LDL is degraded by lysosomes after LDLR-facilitated internalization. Certainly, evidence for Lp(a) being catabolized by the LDLR has been presented, although for every article that suggested involvement there was one to suggest otherwise. Here, we present evidence that Lp(a) does not interact with the LDLR, including no interference of Lp(a) uptake by an LDLR antibody that blocks LDL uptake and no colocalization between apo(a) and LDLR in liver cells. Our results are in concordance with a recent study showing that Lp(a) uptake in both primary hepatocytes and fibroblasts cells are unaffected by PCSK9-specific monoclonal antibody treatment, indicating no role for the LDLR, but are in contrast with another recent study showing that Lp(a) uptake in hepatocytes and fibroblasts is affected by PCSK9-specific monoclonal antibody treatment implying the involvement of the LDLR.

Labeling of the lipid and protein components of Lp(a) showed that the internalized Lp(a) is dissociated into the LDL and apo(a) moieties within 6 hours. The 2 components then follow different fates. Labeling of the lipid and apoB component of Lp(a) showed that the LDL part of Lp(a) is found in lysosomes 6 hours after Lp(a) treatment. The apo(a) component, on the other hand, does not traffic to lysosomes as seen by a lack of colocalization between apo(a) and lysosomes in both liver and fibroblast cells and a lack of apo(a) accumulation after treatment with lysosomal inhibitors. Our experiments tracking the fate of apo(a) via apo(a)-specific immunohistochemistry led us to find that apo(a) was trafficked to the trans-Golgi network via Rab5-positive early endosomes and from there was placed into Rab11-positive recycling endosomes resulting in apo(a) being secreted back into the media (Figure 8). The apo(a) observed in the media was initially free but seemed to be reassembled into Lp(a) in association with the appearance of apoB particles secreted from HepG2 cells. This is in keeping with studies showing an extracellular assembly of Lp(a) in cell culture media. Resecretion of free apo(a) was further confirmed after the incubation of primary fibroblasts with Lp(a) showed free apo(a) appearing in the media on a similar time scale and concentration to that seen in HepG2 cells. A tracer experiment with AlexaFluor-488–labeled Lp(a) validated the resecretion of apo(a) into the media. Exactly at what point the apo(a) is released from the intact Lp(a) particle is not known, but free apo(a) appears in the cell lysate by 1 to 2 hours. A previous study using FRET imaging has shown that disulphide bond reduction activity is found in many endosomal compartments but not in lysosomes or the Golgi apparatus. This suggests that apo(a) may be released from LDL in either the early or recycling endosomal compartments. However, the location and mechanism for apo(a) release remain unknown.

To further confirm the recycling of apo(a), we looked whether apo(a) colocalizes with a classic recycled protein, transferrin. Indeed, colocalization of apo(a) and transferrin was apparent. None of the plasminogen family members have previously been reported as being recycled so the finding that apo(a) is recycled is novel and raises many questions most notably why is it recycled? Recycling proteins often have functional roles in carrying extracellular ligands into the cell. In the case of transferrin, the ligand is extracellular iron. Apo(a) is known to bind many ligands, including oxidized phospholipids and fibrin. Recycled apo(a) may be involved in the removal of oxidized phospholipids from cell surfaces with the apo(a)/oxidized phospholipids complex being delivered via the scavenger receptor B1 receptor after reformation of Lp(a). Alternatively, recycled apo(a) could be involved in the clearance of fibrin degradation products from circulation because proteomic studies have revealed that all 3 fibrin chains peptides are present on the apo(a) component of Lp(a). Interestingly, recycling of apolipoproteins is not unprecedented. Previous studies have shown that a significant portion of apolipoprotein E (apoE) escapes degradation by lysosomes and is rerouted via the Golgi for resecretion. The recycled apoE has been proposed to play a role in lipoprotein assembly, cholesterol efflux, and chylomicron clearance.
Likewise, recycled apo(a) might have some physiological relevance in Lp(a) metabolism.

The finding that apo(a) is recycled could have implications for Lp(a) kinetic studies because this would suggest that there may be 2 pools of Lp(a) in circulation, one initially assembled from newly secreted apo(a) and another formed later from reassembly of Lp(a) after apo(a) resecretion. Interestingly, previous kinetic studies have identified that apo(a) has a significantly reduced fractional catabolic rate compared with the apoB within Lp(a), suggesting that the apo(a) component has a longer plasma resident time than the apoB.4,45 The authors from both studies concluded that this was likely because of the dissociation of apo(a) from Lp(a) and its reassociation with other apoB-containing particles in plasma. We propose that the extended plasma resident time of apo(a) might be in part because of the recycling of apo(a) from the liver effectively creating 2 pools of Lp(a).

Nonetheless, some kinetic studies have shown similar fractional catabolic rates for the apo(a) and apoB components of Lp(a) arguing against the presence of 2 pools of Lp(a) in circulation.7,46,47 Clearly, further studies are needed to elucidate whether recycled apo(a) makes a significant contribution to the plasma pool of Lp(a).

Our findings might also bear some light on previous studies reporting the excretion of free apo(a) in urine.48 Our study showing the resecretion of free apo(a) may suggest that free apo(a) is available for degradation via the kidney if it is not reassembled back into Lp(a). It is possible that the recycled apo(a) is further modified from its original form which may make it a better substrate for metabolism by the kidney. Further studies are required to determine this.

We showed that Lp(a) internalization is regulated by the plasminogen receptor, PlgRKT, after knockout of this receptor in a haploid fibroblast-like HAP1 cell line, and a PlgRKT-specific siRNA knockdown of this receptor in HepG2 cells significantly reduced Lp(a) uptake. Similarly, the overexpression of the PlgRKT significantly enhanced Lp(a) uptake in both cell lines. Because of the high homology of apo(a) with plasminogen, it is perhaps not surprising that apo(a) would interact with plasminogen receptors. Indeed, earlier studies had shown Lp(a) to interact with plasminogen binding sites on the surface of U937 and endothelial cells,44 and lysine analogues and plasminogen disrupted binding of apo(a) to cell surface receptors.23 Interestingly, PlgRKT contains an exposed C-terminal lysine residue which is important for its interaction with plasminogen34 and presumably apo(a). However, because complete knockout of PlgRKT only reduced 65% of apo(a) uptake, it remains possible that other plasminogen receptors such as p11/annexinA2 may be responsible for further Lp(a) uptake. Furthermore, our study used a limited range of apo(a) size isoforms, and it would be of interest to evaluate the effect of different sizes of apo(a) on PlgRKT-mediated uptake and recycling of apo(a) in future studies.

In conclusion, we show 2 novel findings for Lp(a) metabolism namely that Lp(a) internalization is mediated by the plasminogen receptor, PlgRKT, and that after internalization, the apo(a) protein is resecreted to the extracellular space via recycling endosomes (Figure 8). The identification of a receptor for Lp(a) catabolism has implications for manipulations of Lp(a) levels. At present, there is only 1 therapy, apo(a) antisense, that can specifically lower Lp(a), and this is targeted at reducing apo(a) synthesis.49 Drugs targeted at increasing catabolism will add to the repertoire of possible Lp(a)-lowering therapies so that in the future, like LDL, we may have multiple agents with which to intervene and lower Lp(a) and associated cardiovascular disease risk.

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Disclosures

None.

References

Supplemental Material

Detailed Methods

Lipoprotein isolations

Lipoproteins (LDL and Lp(a)) were isolated from pooled plasma samples from healthy individuals with Lp(a) levels >50 mg/dL as previously described. Individuals in the pool had apo(a) isoform sizes ranging from 17-20 with a predominant apo(a) isoform size of 19. Ethical approvals for the samples used in this study was granted by the Lower Regional South Ethics Committee (LRS/12/01/005). Purified LDL and Lp(a) protein levels were quantified using the Qubit protein assay kit (ThermoFisher Scientific, Waltham, MA).

Cell culture

Hepatocellular carcinoma (HepG2) cells (American Type Culture Collection, Manassas, VA) and adult human dermal fibroblast (HDFa) cells (Life Technologies, Carlsbad, CA) were maintained in Advanced Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (Bio International, Auckland, New Zealand), 2 mM L-glutamine, 0.25 µg/mL amphotericin B, 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies) at 37°C in a humidified environment with 5% CO₂. Haploid human fibroblast-like cells (HAP1) containing a PlgRKT knockout (PlgRKT<sup>-/-</sup>) and wild type HAP1 cells (WT) were purchased from Horizon Discovery (Waterbeach, Cambridge, United Kingdom) and maintained in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified environment with 5% CO₂. Western blot analysis on cell lysates showed the absence of apo(a) in all cell lines and the absence of PlgRKT in the HAP1 PlgRKT<sup>-/-</sup> cells.

Cell treatments

Cells were seeded at a density of 5 x 10⁴ in a 24-well plate and treated with purified 5 µg/mL Lp(a) or 20 µg/mL LDL for the indicated times in serum free media. HepG2 cells were transfected with 500 ng of the dsRed Rab11 WT plasmid (Addgene, Cambridge, MA), for labelling of recycling endosomes, using Lipofectamine® 3000 (Life Technologies) for 24 hours at 37°C before Lp(a) treatment. HAP1 and HepG2 cells were transfected with NKP33 GFP (1032) plasmid containing the human PlgRKT gene (Addgene) using Lipofectamine® 3000 (Life Technologies) for 72 hours at 37°C prior to Lp(a) treatment. siRNA-mediated PlgRKT knockdown in HepG2 cells was performed using Silencer® Select siRNAs from Ambion® specific to the human PlgRKT gene (ThermoFisher Scientific) using Lipofectamine® 3000 for 72 hours at 37°C prior to Lp(a) treatment.

The LDLR, ASGPR and PlgRKT on HepG2 cells were blocked using anti-LDLR (Abcam), anti-ASGPR1 (Sigma, St. Louis, MO), and anti-PlgRKT (HPA011144, Sigma) antibodies respectively. Cells were preincubated with 10 µg/mL of anti-LDLR antibody or 5 µg/mL of anti-ASGPR1 or 10 µg/mL of anti-PlgRKT antibody for an hour prior to the 5 µg/mL Lp(a) or 20 µg/mL LDL treatment for 6 hours at 37°C. The ASGPR was also blocked using N-Acetyl-D-galactosamine (GalNAc) (Sigma). Briefly, cells were coincubated with 20 µM GalNAc and 5 µg/mL Lp(a) for 6 hours at 37°C. Cells were lysed for western blotting after treatments. The plasminogen receptors were also blocked using 5 µM ε-aminocaproic acid (ε-ACA) (Sigma). Briefly, cells were coincubated with 5 µM ε-ACA and 5 µg/mL Lp(a) for 6 hours at 37°C. Cells were lysed for western blotting after treatments.

Western blot

Cell lysates from Lp(a)-treated HepG2, HDFa and HAP1 cells were harvested in RIPA buffer supplemented with complete mini protease inhibitors (Roche, Basel, Switzerland). Cell lysates (40 µg protein) were resolved on 4%, 10% or 4-12% gradient (ThermoFisher Scientific) polyacrylamide gels dependent on the protein being analysed. Proteins were transferred to nitrocellulose membrane and the membrane blocked before incubation with primary antibody. Primary antibodies included an anti-apo(a) monoclonal antibody, MAb-a5, an anti-apoB monoclonal antibody, 1D13 and an anti-PlgRKT antibody (Sigma). An anti-actin antibody (Sigma) was use to normalise all western blots for
quantification. After incubation with the appropriate secondary antibody (either an anti-rabbit IgG-hrp conjugate or an anti-mouse IgG-hrp conjugate (Life Technologies), membranes were developed and imaged with a Fujifilm LAS3000 (R&D systems, Minneapolis, MN). Quantification was performed using the ImageQuant TL software (Amersham Biosciences, Piscataway, NJ) with the protein of interest normalized against actin.

Immunocytochemistry

Immunocytochemistry was performed on HepG2, HDFa and HAP1 cells as previously described. Briefly, cells were treated with 5 μg/mL Lp(a) or LDL for the indicated times then fixed with 4% paraformaldehyde and blocked and permeabilized in 3% (v/v) goat serum with 0.1% triton X-100. Cells were incubated with MAb-a5 or 1D1 followed by anti-mouse IgG AlexaFluor 488 (Life Technologies) for Lp(a) visualisation, LysoTracker® Red DND-99 (Life Technologies) for lysosomes, Wheat Germ Agglutinin AlexaFluor®594 Conjugate (Life Technologies) for the trans-Golgi network and an anti-Rab5 antibody followed by an anti-rabbit IgG AlexaFluor 594 Conjugate (Life Technologies) for early endosomes. Transferrin AlexaFluor® 594 Conjugate (Life Technologies) was used as a recycling marker. In HDFa cells, Wheat Germ Agglutinin AlexaFluor®594 Conjugate (Life Technologies) was used as a membrane stain prior to fixation and permeabilisation of the cells. Mounting was performed with ProLong® Gold Antifade with DAPI (Life Technologies) and an Olympus FluoView™ FV1000 confocal microscope was used for imaging. Image analysis was done using the Olympus FluoView™ FV1000 Image Examiner software and Fiji image software (http://fiji.sc/).

Immunoprecipitation of cellular media

To detect the resecretion of Lp(a) into the media, cells were incubated with 5 μg/mL Lp(a) for 6 hours at 37°C. Lp(a)-containing media was removed and cells were either extensively washed with PBS or trypsinised and reseeded and then fresh media added. Media was harvested at 1, 2, 6, 12 and 24 hours. An anti-Lp(a) antibody raised in goat (DAKO, Glostrup, Denmark) was immobilized on PureProteome™ Protein A/G magnetic beads (EMD Millipore, Billerica, MA) and Lp(a) was immunoprecipitated from the media after an overnight incubation at 4°C. Lp(a) was eluted from the beads under reducing or non-reducing conditions. Samples were then subjected to western blot using MAb-a5 and 1D1 antibodies.

Lp(a) ELISA

Quantification of recycled Lp(a) into the media was performed by an enzyme-linked immunosorbent assays (ELISA) using the apo(a)-specific monoclonal antibody, MAb a-6, as the coating antibody and MAb a1-1-hrp as the detection antibody.

Labelling of purified Lp(a)

For cholesterol labelling of Lp(a) and LDL, 0.5 μM Bodipy cholesterol (Avanti Polar Lipids) was incubated with 100 μg of purified Lp(a) or LDL overnight at 37°C from was used. Purified Lp(a) was also labelled with Fast Dil-C18 (Invitrogen) to visualise the surface lipids of Lp(a) by incubating 0.3 mg of Dil with 100 μg of Lp(a) overnight at 37°C. Free Bodipy cholesterol or Dil label was removed on a Sephadex G-25 column. HepG2 cells were then incubated with 5 μg/mL of Bodipy labelled-Lp(a) or LDL or 5 μg/mL of Dil labelled-Lp(a) for 6 hours or 0-24 hours respectively at 37°C and confocal microscopy was performed.

The protein component of purified Lp(a) was labelled using Alexa Fluor® 488 Microscale Protein Labelling Kit (ThermoFisher) as per the manual’s instructions. Briefly, 100 μg of purified Lp(a) was mixed with 2 μl of AlexaFluor488 protein dye and sodium bicarbonate for 15 minutes at room temperature. The unbound dye was removed using BioGel-6 resin (supplied with the kit). The AlexaFluor488 labelled Lp(a) was used for uptake and recycling studies of Lp(a).
Supplemental References


Supplementary figures and figure legends

Online Figure I

Online Figure I: No effect of Lp(a) treatment on apoB protein and mRNA expression levels in HepG2 cells. (A) HepG2 cells were treated with 5 µg/mL Lp(a) for 0 to 24 hours at 37°C. Western blot of cell lysates with 1D1 using actin as a loading control. (B) ApoB protein levels as detected by western blotting were normalized against actin and expressed relative to that of untreated cells. Results are expressed as mean ± S.E for triplicate blots. (C) HepG2 cells were treated with 5 µg/mL Lp(a) for 6 hours at 37°C. ApoB mRNA expression was quantified by RT-PCR using apoB specific primers and apoB expression levels were normalised using RPL27 and β2m reference genes. Results are expressed as mean ± SEM for at least two experiments performed in triplicate.
Online Figure II: Time dependent uptake and recycling of AlexaFluor labelled-Lp(a) in HepG2 cells. (A) Purified Lp(a) was labelled with AlexaFluor488 protein dye and HepG2 cells were treated with 5 µg/mL AlexaFluor488 labelled-Lp(a) from 0-24 hours at 37ºC and the fluorescence intensity of internalised Lp(a) in cell lysates was measured. (B) The percentage uptake and recycling of AlexaFluor488 labelled Lp(a) relative to Lp(a) uptake at 2 hours in HepG2 cells was quantified. (C) To investigate recycling, HepG2 cells were treated with 5 µg/mL AlexaFluor488 labelled-Lp(a) for 6 hours at 37ºC, cells were washed extensively and the fluorescence intensity of the media measured at 0, 6 and 24 hours post-Lp(a) treatment. Lp(a) IP was performed on media collected at 0, 6 and 24 hours post-Lp(a) treatment and the IP subject to western blot analysis under reducing conditions with the MAb-a5 antibody. Two independent experiments were performed.
Online Figure III: Lp(a) is not degraded via lysosomes or proteasomes. HepG2 cells were treated with (A) 5 µg/mL Lp(a) or 5 µg/mL Lp(a) and 20 mM NH₄Cl, (B) 20 µg/mL LDL or 20 µg/mL LDL and 20 mM NH₄Cl (C) 5 µg/mL Lp(a) or 5 µg/mL Lp(a) and 40 µM E64d, (D) 20 µg/mL LDL or 20 µg/mL LDL and 40 µM E64d, (E) 5 µg/mL Lp(a) or 5 µg/mL Lp(a) and 10 µM Clasto-Lactacystin β-lactone, (F) 20 µg/mL LDL or 20 µg/mL LDL and 10 µM Clasto-Lactacystin β-lactone for 6 hours at 37°C. Cell lysates were subjected to western blotting with MAb-a5 and 1D1 antibodies. Apo(a) and apoB protein levels as detected by western blotting were normalized against actin and expressed relative to that of Lp(a) and LDL treated cells respectively. Results are expressed as mean ± S.E for at least two independent experiments performed in triplicate. *, p<0.05 **, p< 0.01 compared with Lp(a) or LDL treated cells.
Online Figure IV: Lp(a) does not traffic to the cis-medial Golgi and brefeldin A redistributes Lp(a) into trans-Golgi tubular processes. (A) HepG2 cells were treated with 5 µg/mL Lp(a) for 2 hours at 37°C. Confocal microscopy was performed of Lp(a)-treated HepG2 cells stained with an anti-giantin antibody detected with an anti-rabbit AlexaFluor 594 antibody and MAb-a5 detected by an anti-mouse AlexaFluor 488 antibody. (B) HepG2 cells were treated with 5 µg/mL Lp(a) and 10 µg/mL of Brefeldin A (BFA) for 6 hours at 37°C. Confocal microscopy of Lp(a) and BFA treated HepG2 cells stained with AlexaFluor 594 conjugated WGA (trans-Golgi network) and MAb-a5 detected by an anti-mouse AlexaFluor 488 antibody. A merged image with DAPI is shown. Areas marked by white rectangles are zoomed. Scale bar = 20 µm. At least 100 cells were analysed per condition across three independent experiments and representative images are shown.
Online Figure V: Lack of surface bound Lp(a) after Lp(a) treatment. HepG2 cells were treated with 5 µg/mL Lp(a) for 6 hours at 37°C and cells washed extensively with PBS. Confocal microscopy was performed on Lp(a)-treated and washed HepG2 unpermeabilised cells stained with AlexaFluor 594 conjugated WGA (membrane stain) and the MAb-a5 anti-mouse AlexaFluor 488 antibody. A merged image with DAPI is shown. Scale bar = 20 μm. At least 100 cells were analysed per condition across three independent experiments and representative images are shown.
Online Figure VI: PlgRKT plasminogen receptor expression in HAP1 and HepG2 cells. (A) Western blot of cell lysates with a PlgRKT-specific antibody in HAP1 wild type (WT) cells and HAP1 PlgRKT<sup>−/−</sup> cells. Actin was used as a loading control. (B) Western blot of cell lysates with a PlgRKT-specific antibody in HAP1 WT cells transfected with a plasmid containing the human PlgRKT gene. Actin was used as a loading control. (C) Western blot of cell lysates with a PlgRKT-specific antibody in HepG2 cells transfected with 30 nM of a PlgRKT-specific siRNA. Actin was used as a loading control. (D) Western blot of cell lysates with a PlgRKT-specific antibody in HepG2 cells transfected with a plasmid containing the human PlgRKT gene. Actin was used as a loading control.
Online Figure VII: Lp(a) uptake is independent of the LDLR in HAP1 cells and plasminogen does not compete with Lp(a) uptake in HepG2 cells. (A) HAP1 WT cells and HAP1 PlgRKT−/− cells were preincubated with 10 µg/mL anti-LDLR antibody and treated with 5 µg/mL Lp(a) for 6 hours at 37°C. (B) HepG2 cells were treated with 5 µg/mL Lp(a) or 5 µg/mL Lp(a) and either 5, 25 nM or 50 nM plasminogen. Cell lysates were subjected to western blot with MAb-a5 using actin as a loading control. For each case, triplicate blots were run and a representative blot is shown.
Online Figure VIII: LDL and plasminogen uptake is not mediated by PlgRKT in HAP1 cells. (A) HAP1 WT cells and HAP1 PlgRKT−/− cells were treated with 5 µg/mL LDL for 6 hours at 37°C. Cell lysates were subjected to western blot with 1D1 using actin as a loading control. (C) HAP1 WT cells and HAP1 PlgRKT−/− cells were treated with 50 µM plasminogen for 6 hours at 37°C. Cell lysates were subjected to western blot with anti-plasminogen antibody using actin as a loading control. For each case, triplicate blots were run and a representative blot is shown.