Galactose-specific asialoglycoprotein receptor is involved in lipoprotein (a) catabolism

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Lp(a) [lipoprotein (a)] is a highly atherogenic plasma lipoprotein assembled from low-density lipoprotein and the glycoprotein apolipoprotein (a). The rate of Lp(a) biosynthesis correlates significantly with plasma Lp(a) concentrations, whereas the fractional catabolic rate does not have much influence. So far, little is known about Lp(a) catabolism. To study the site and mode of Lp(a) catabolism, native or sialidase-treated Lp(a) was injected into hedgehogs or ASGPR (asialoglycoprotein receptor)-knockout (ASGPR −) mice or wild-type (ASGPR +) mice, and the decay of the plasma Lp(a) concentration was followed. COS-7 cells were transfected with high- (HL-1) and low-molecular-mass ASGPR subunits (HL-2), and binding and degradation of intact or desialylated Lp(a) were measured. In hedgehogs, one of the few species that synthesize Lp(a), most of the Lp(a) was taken up by the liver, followed by kidney and spleen. Lp(a) and asialo-Lp(a) were catabolized with apparent half-lives of 13.8 and 0.55 h respectively. Asialo-orosomucoide increased both half-lives significantly. In mice, the apparent half-life of Lp(a) was 4–6 h. Catabolism of native Lp(a) by wild-type mice was significantly faster compared with ASGPR − mice and there was a significantly greater accumulation of Lp(a) in the liver of ASGPR + mice compared with ASGPR − mice. The catabolism of asialo-Lp(a) in ASGPR − mice was 8-fold faster when compared with native Lp(a) in wild-type mice. Transfected COS-7 cells expressing functional ASGPR showed approx. 5-fold greater binding and 2-fold faster degradation of native Lp(a) compared with control cells. Our results for the first time demonstrate a physiological function of ASGPR in the catabolism of Lp(a).

Key words: adenoviral vector, atherosclerosis, glycoprotein, hedgehog, lipoprotein (a), sialic acid.

INTRODUCTION

The physiological role and metabolism of Lp(a) [lipoprotein (a)] remain enigmatic [1]. On the other hand, there is compelling evidence from numerous studies that individuals with high plasma Lp(a) levels (> 30 mg/dl) are at a significantly increased risk of atherosclerosis, coronary heart disease and ischaemic stroke [2–5]. Lp(a) is a complex lipoprotein found in the plasma of primates. It consists of apo(a) [apolipoprotein (a)], which is covalently linked via a single disulphide bridge to apoB-100 of LDL (low-density lipoprotein). Apo(a) is a high-molecular-mass glycoprotein with repetitive domains, the so-called kringles (K), which show a high degree of sequence identity with plasminogen K-IV (kringle-4) [6]. K-IV T1 (K-IV type 1) and K-IV T3–T10 are present in a single copy, whereas the number of the 'repetitive kringles' K-IV T2 in apo(a) varies between individuals from 3 to 43; this variation is the basis of the genetically determined size polymorphism [7,8].

The latest developments in research have shown that there are still numerous gaps in the knowledge of metabolism of Lp(a). As far as Lp(a) biosynthesis is concerned, there are strong indications for an assembly of apo(a) with LDL in a two-step mechanism outside the liver cell or at its surface [9–11]. Since some apo(a) kringles have high affinity for lysine, apo(a) associates in the first step specifically with apoB and, in the second step, a disulphide bridge is formed that stabilizes the mature Lp(a). Another less probable possibility is the intracellular assembly of Lp(a) [12]. We were the first to demonstrate that the plasma Lp(a) concentration correlates significantly with the synthesis rate, but not with the fractional catabolic rate [13].

The site and mode of Lp(a) catabolism is unknown. Despite the fact that an integral part of Lp(a) consists of apoB, the LDL receptor does not appear to be significantly involved in Lp(a) catabolism [14–16]. Apo(a) is a glycoprotein with 25–30 % carbohydrates, consisting of N- and O-linked sugars rich in sialic acid [17]. Studies performed in mice, rabbits and hedgehogs, the latter being the only species, in addition to primates, to synthesize an Lp(a)-like lipoprotein, consistently showed that more than 50 % of human Lp(a) is catabolized by the liver [16,18,19]. This prompted us to search for specific mechanisms that may account for the uptake of Lp(a) by the liver. In the present study, we show that the galactose-specific ASGPR (asialoglycoprotein receptor) binds and internalizes Lp(a) and contributes to its *in vivo* catabolism.

EXPERIMENTAL

Isolation and radiolabelling of Lp(a)

Lp(a) was isolated from the fasting plasma of healthy individuals who were homozygous for the 15, 18, 21 or 25 K-IV repeat isoforms as described previously [20]. Immediately after drawing blood, the following preservatives were added to it: EDTA, butylated hydroxytoluene and Thiomersal at the final concentrations of 0.1, 0.005 and 0.05 % respectively, in addition to 1 mM PMSF and *p*-bromophenacyl bromide. This procedure, which

Abbreviations used: apo(a), apolipoprotein (a); ASGPR, asialoglycoprotein receptor; DELFIA, dissociation-enhanced fluorescence immunoassay; FCS, foetal calf serum; HL-1, HL-2, high- and low-molecular-mass ASGPR subunits; K-IV, kringle-4; LDL, low-density lipoprotein; Lp(a), lipoprotein (a).

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involves column chromatography over Biogel A-15 M in 1 % proline, yields almost 100 % pure Lp(a) that is free from LDL. Protein concentrations were measured by the method of Lowry et al. [20a] using BSA as a standard. Lp(a) preparations were iodinated by the tyramine cellobiose method described previously [21]. Synthesis and subsequent radio-iodination of tyramine cellobiose was performed as described previously [22]. Na-^{125}I (1 mCi; Amersham Biosciences, Little Chalfont, Bucks., U.K.) was used for labelling 5 mg of protein. After exhaustive dialysis and chromatography over Sephadex G25M, < 3 % unbound (trichloroacetic acid-precipitable) radioactivity was found. Labelled lipoproteins were stored at 4 °C for less than 1 week.

Quantification of apo(a) by DELFIA (dissociation-enhanced fluorescence immunoassay)

Lp(a) concentrations in plasma were determined by DELFIA as described previously [20]. Briefly, a polyclonal antibody raised against apo(a) from rabbit was used for coating 96-well dishes, and a Europium-labelled polyclonal anti-apo(a) from sheep served as a detection antibody. Measurements were performed in the DELFIA reader (Wallac, Turku, Finland). The antibodies were affinity-purified and showed no cross-reactivity with plasminogen. Purified Lp(a) served as the primary standard. The DELFIA was sensitive to approx. 5 ng of apolipoprotein. The between-run precision of the assay was ± 5 %.

Desialylation of Lp(a) or orosomucoide by sialidase

Purified protein (5 mg) was dissolved in 2 ml of PBS and mixed with 1 ml of beaded agarose, to which sialidase was attached (51 units/g agarose; Sigma). Before use, the agarose was washed ten times with 5 ml of PBS (pH 7.4). The mixture was incubated for 48 h at room temperature (22 °C) under gentle shaking. Thereafter, it was filled into a small column and the protein was eluted with 3 ml of PBS. The degree of sialylation was monitored by the thiobarbituric acid assay [23]. Desialylated fractions were found to contain only insignificant amounts of sialic acid.

Construction of recombinant adenoviruses

In mice, ASGPR consists of two subunits, the high- (HL-1) and low-molecular-mass (HL-2) ASGPR subunits. Full-length cDNAs for the two subunits (ASGPR-HL-1 and ASGPR-HL-2) were separately inserted, under the control of the CMV promoter, into the adenoviral plasmid shuttle vector (pAvCvSv) kindly provided by L. Chan (Baylor College of Medicine, Houston, TX, U.S.A.). HL-1 and HL-2 cDNAs as well as monoclonal antibodies raised against receptor protein were kindly provided by M. Spiess (University of Basel, Basel, Switzerland). Recombinant vectors (pAvCvSv/HL-1 and pAvCvSv/HL-2) were used to transform *Escherichia coli* DH5-α competent cells to amplify recombinant plasmids. Positive clones were confirmed by restriction analysis and DNA sequencing. The recombinant shuttle plasmids (5 μg) were co-transfected with 5 μg of supercoiled pJM17 into HEK-293 (human embryonic kidney 293) cells by the calcium phosphate co-precipitation method. Infectious adenoviral plaques were picked 2 weeks after transfection, propagated and screened by PCR for ASGPR-HL-1 and ASGPR-HL-2 sequences respectively. Adenoviral vectors containing ASGPR subunits were further amplified in HEK-293 cells and the expression was determined by Western-blot analysis. Large-scale production of high-titre recombinant adenoviruses was performed as described previously [24]. Recombinant viruses were purified twice by caesium chloride density-gradient centrifugation and dialysed for 14 h at 4 °C against a buffer containing 10 mM Tris/HCl (pH 7.5), 1 mM $MgCl_2$ and 10 % (w/v) glycerol and stored at -70 °C. Virus titres, as measured by the plaque assay on HEK-293 cells for ASGPR-HL-1 and ASGPR-HL-2, were 4×10^{10} and 3×10^{10} pfu (plaque-forming units)/ml respectively. The recombinant viruses were termed HL-1-Ad and HL-2-Ad. As a negative control, lacZ-Ad was used.

Cell culture and recombinant adenovirus infection

COS-7 cells were cultured under standard conditions (37 °C, 5 % CO_2 and 95 % humidity) and grown in Dulbecco's modified Eagle's medium containing 10 % FCS (foetal calf serum). Before experiments, cells were trypsinized, counted and seeded into 24-well trays (6×10^4 cells/well). After 5 h, the cells were co-infected with 15 MOI (multiplicity of infection, i.e. the number of viable virus particles applied per cell) of HL-1-Ad and HL-2-Ad respectively. Control cells were infected with 30 MOI of lacZ-Ad. The cells were infected in a culture medium containing 2 % FCS for 90 min at 37 °C. After infection, fresh medium with 10 % FCS was added to the cells. Cell-culture studies were performed 36 h after infection.

Immunoblot analysis

Samples were mixed with loading buffer [20 % glycerol, 5 % (w/v) SDS, 0.15 % (w/v) Bromophenol Blue and 63 mM Tris/HCl, pH 6.8] in the ratio 1:1, incubated for 10 min at 95 °C and analysed by SDS/PAGE (15 % gel) for 1 h at 150 V. After electrophoresis, proteins were transferred to nitrocellulose, and incubated with 1:2000 dilutions of a polyclonal antibody recognizing HL-1 and HL-2 or a monoclonal antibody reacting with HL-1 of ASGPR. A second incubation was performed with the corresponding horseradish peroxidase-labelled antibodies raised against rabbit or mouse IgG (Sigma) and protein bands were visualized by ECL® (Amersham Biosciences).

Determination of Lp(a) cell binding, internalization and degradation

These studies were performed in COS-7 cells overexpressing the HL-1 and HL-2 subunits of ASGPR (see below) or in control cells expressing β-galactosidase. Cells were incubated 36 h after transfection for 6 h at 37 °C with increasing amounts of either native or sialidase-treated [^{125}I]Lp(a). Some incubations were performed in the presence of 50-fold molar excess of asialo-orosomucoide relative to Lp(a) concentration. Degradation of ^{125}I-labelled apolipoproteins was determined by measuring the trichloroacetic acid-non-precipitable radioactivity in the medium after the removal of free iodine with $AgNO_3$ in a γ-counter (Cobra Quantum-Packard, VWR International, Vienna, Austria). To determine non-cell-mediated degradation, control experiments were performed without cells. Subsequently, cells were extensively washed with PBS and the ASGPR-specific binding of Lp(a) was measured in a γ-counter after incubation with a 50-fold excess of lactose (by weight) for 1 h at 4 °C. Lactose at this concentration proved to be as effective as asialo-orosomucoide, yet was much cheaper. Lp(a) internalization (internalization + unspecific association) was analysed in the cell lysate after extensive washing of the cells with PBS and solubilization for 4 h in 0.3 M NaOH at room temperature.

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Animals

Three- to five-year-old male or female hedgehogs (Asian species) from the Hangzhou area of China were studied during summertime. The weight of these animals ranged from 330 to 400 g.

Mice used in the present study were obtained from Jackson Laboratory (Bar Harbour, ME, U.S.A.). ASGPR-knockout mice (B6, 129-Asgr2[tm1Her]) lacking HL-2 were described previously [25]. Knockout mice lacking HL-1 or both subunits were not commercially available. B6129F2/J mice were used as a wild-type control, according to the suggestions of Jackson Laboratory. The animals were housed at 22 °C under a constant light/dark cycle and had free access to water and rodent chow (4.5 % fat and 21 % protein; Sniff, Soest, Germany). The animal experiments were approved by the Austrian Ministry of Education, Science and Culture according to Section 8 of the Regulations for Animal Experimentation.

In vivo metabolic studies

Hedgehogs

Animals were starved overnight and 0.1 mg of labelled lipoproteins (specific activity, 100–200 μCi/mg of protein) was injected intravenously. Blood was drawn at the indicated time points and trichloroacetic acid-precipitable plasma radioactivity was counted. Some animals were co-injected with a 50-fold excess of mannan from *Saccharomyces cerevisiae* (Sigma) or asialo-orosomucoide to block the corresponding ASGPRs. In separate experiments, similarly treated animals were anaesthetized 2 h after lipoprotein injection with Isofluran (Amersham Biosciences and Upjohn, Guyancourt, France) and perfused through the left ventricle with 500 ml of PBS. Different organs were harvested and 300–500 mg pieces were analysed in the γ-counter.

Mice

Six female ASGPR − and six ASGPR + mice were anaesthetized with Isofluran and injected in the morning via the tail vein with 0.25 mg of either native or sialidase-treated Lp(a) in 150 μl of PBS. Blood was collected at different time intervals during a period of 23 h from anaesthetized animals by retro-orbital puncture, and the decay of the Lp(a) plasma concentration was followed using the DELFIA described above. To study the uptake of Lp(a) by different tissues, five female ASGPR − and five ASGPR + mice were injected with 0.05 mg of [^{125}I]Lp(a) (180 c.p.m./ng of protein) in 100 μl of PBS. After 15 min, mice were perfused through the left ventricle with 20 ml of PBS. Different organs were harvested and 300 mg pieces were counted in the γ-counter.

Statistics

Results were expressed as means ± S.D. Significance of differences was examined using Student's *t* test.

RESULTS

Catabolism of Lp(a) in hedgehogs

The site of Lp(a) catabolism in humans is unknown. Hedgehogs and primates are the only animals that synthesize Lp(a) and primates are hardly accessible for such studies. Therefore the first experiments for elucidating Lp(a) catabolism were performed in hedgehogs.

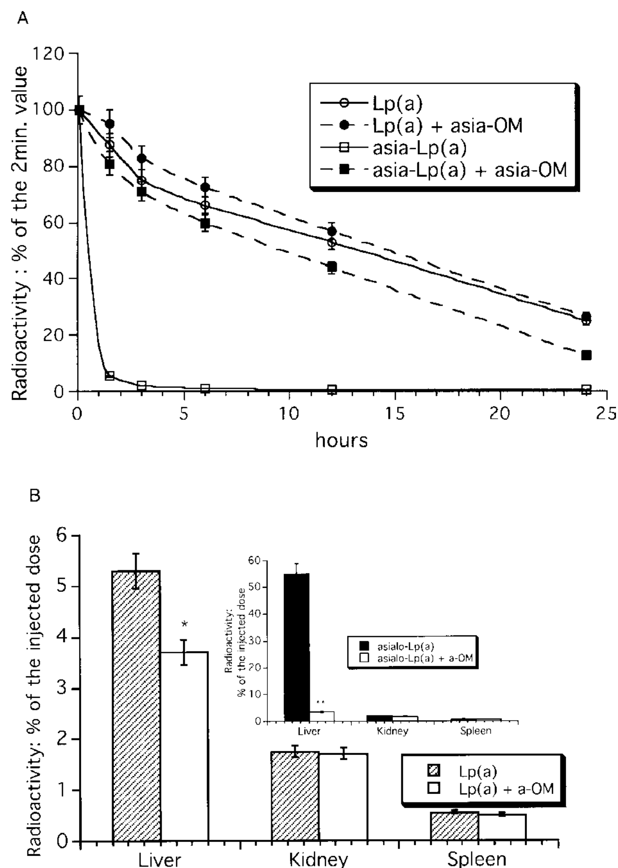

Figure 1 Catabolism of Lp(a) in hedgehogs

(**A**) Three hedgehogs were injected each with 0.1 mg of radiolabelled Lp(a) or asialo-Lp(a) [asia-Lp(a)] in the presence or absence of a 50-fold excess of asialo-orosomucoide (asia-OM). The trichloroacetic acid-precipitable plasma radioactivity was measured in a γ-counter and plotted as a percentage of the 2 min value against time. (**B**) Experimental details are same as in (**A**), except that animals were killed 2 h after injection, perfused with PBS and organs were counted. Values are means ± S.D. of the radioactivity found in whole organs of five animals each (*$P < 0.05$, **$P < 0.01$).

Figure 1(A) exhibits the decay of ^{125}I-tyramine-cellobiose-labelled Lp(a) and of asialo-Lp(a) in the presence and absence of asialo-orosomucoide in three hedgehogs each.

The apparent half-life of native Lp(a) in these animals was 13.8 h. Neuraminidase treatment of Lp(a) resulted in a very fast removal from plasma with an apparent half-life of 0.55 h, suggesting that asialo-Lp(a) is catabolized by ASGPR. There are basically two known ASGPRs, namely galactose- and mannose-specific ASGPRs. To test which of these two receptors might be involved, similar experiments as above were performed in the presence of the competitive inhibitors asialo-orosomucoide or mannan respectively. Whereas asialo-orosomucoide increased asialo-Lp(a) half-life from 0.55 to 9.9 h (Figure 1A), mannan had no effect at all (results not shown). Interestingly, asialo-orosomucoide also increased statistically significantly the half-life of native Lp(a) from 13.8 to 15.1 h ($P < 0.02$).

Subsequent experiments in hedgehogs were aimed at identifying the site of Lp(a) catabolism. Animals were injected with Lp(a) or asialo-Lp(a) as above, killed after 2 h, perfused and the distribution of radioactivity in organs was measured. Figure 1(B) demonstrates that native Lp(a) and, even more so, asialo-Lp(a) are predominantly taken up by the liver, followed by kidney and spleen. The uptake of Lp(a) by all other organs was only

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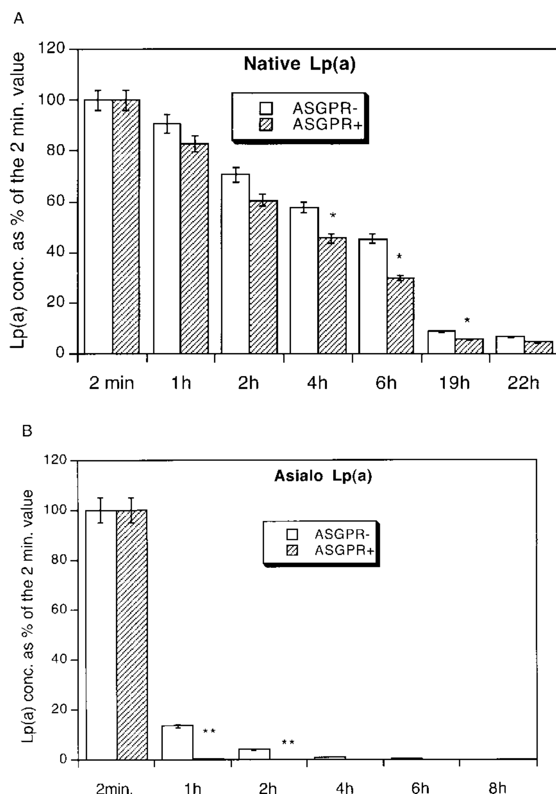

Figure 2 Catabolism of Lp(a) in wild-type mice and in knockout mice lacking HL-2

Female ASGPR+ and ASGPR− mice ($n = 6$ each) were injected with (**A**) native Lp(a) or (**B**) asialo-Lp(a) isolated from a donor with the 18 K-IV isoform. The decay of the plasma Lp(a) concentrations was measured by DELFIA. Plasma Lp(a) levels at the 2 min time point was set to 100 %. Values are means ± S.D. (*$P < 0.05$, **$P < 0.01$).

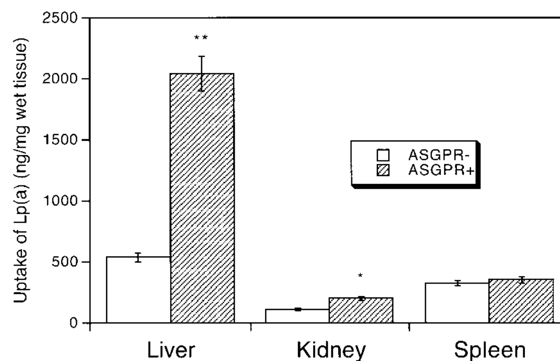

Figure 3 Accumulation of ^{125}I-labelled native Lp(a) in various organs of ASGPR − and ASGPR + mice

^{125}I-tyramine-cellobiose-labelled Lp(a) (18 K-IV isoform) was injected into ASGPR− and ASGPR+ mice ($n = 3$ for each group). The mice were perfused 15 min after injection through the left ventricle with PBS (20 ml); liver, kidney and spleen were harvested and the amount of Lp(a) accumulated was determined in a γ-counter (*$P < 0.05$, **$P < 0.01$).

slightly above background. In the presence of a 50-fold excess of asialo-orosomucoide, the uptake of asialo-Lp(a) by the liver was decreased to the degree of native Lp(a). Most importantly, however, asialo-orosomucoide also significantly decreased the uptake of native Lp(a) by the liver, but not by other organs. These experiments strengthened our view that native Lp(a) is catabolized by ASGPR. Further *in vivo* and *in vitro* experiments aimed to verify this assumption.

Catabolism of Lp(a) is faster in ASGPR + mice compared with ASGPR − mice

We tested the potential role of ASGPR in Lp(a) catabolism by injecting 0.25 mg of freshly isolated Lp(a) into six female ASGPR − and six ASGPR + mice. The dose applied resulted in an initial plasma concentration of 22 ± 4 mg/dl, which is comparable with physiological levels in humans. We collected blood samples between 2 min and 23 h after injection. Plasma Lp(a), as measured by DELFIA, decayed with an apparent half-life of 4–6 h. After 23 h, more than 90 % of the injected dose of Lp(a) was catabolized (Figure 2A). Catabolism of Lp(a) was faster in ASGPR + mice compared with ASGPR − mice; the difference was statistically significant 4 h after injection of Lp(a) ($P < 0.05$). At the 6 h time point, 45 ± 2.1 and 29.0 ± 1.4 % of Lp(a) remained in circulation in ASGPR − and ASGPR + mice respectively.

To test whether full desialylation should convert Lp(a) into a more avidly removed substrate by the ASGPR, we injected 0.15 mg of sialidase-treated Lp(a) into control and ASGPR − mice. As expected, desialylated Lp(a) disappeared from the circulation at a very high rate (Figure 2B): after 1 h, 99.5 % of asialo-Lp(a) was catabolized in wild-type mice and 86.6 % in ASGPR − mice. Interestingly, asialo-Lp(a) was also significantly faster catabolized in ASGPR − mice compared with native Lp(a). At 2 h after injection, only 4.7 % of asialo-Lp(a) remained in the blood circulation in ASGPR − mice.

Wild-type mice accumulate a high amount of Lp(a) in the liver

To study the role of ASGPR in the uptake of Lp(a) by various organs, we injected 0.05 mg of tyramine-cellobiose-labelled Lp(a) into ASGPR − or ASGPR + mice ($n = 5$ for each group). We perfused these mice through the left ventricle 15 min after injection with 20 ml of PBS, harvested liver, kidney and spleen and determined the degree of Lp(a) accumulation. As shown in Figure 3, significantly higher amounts of native Lp(a) accumulate in the liver of ASGPR + mice compared with ASGPR − mice (2040 ± 146 and 508 ± 35 ng/g of wet tissue respectively). Much smaller amounts of radioactivity were also found in spleen and kidney, yet there was no difference between ASGPR + and ASGPR − animals.

Lp(a) is bound and degraded by adenovirus-transformed COS-7 cells expressing ASGPR

We also performed *in vitro* experiments with ASGPR-transfected cells in culture. COS-7 cells were infected at 15 MOI each with recombinant adenoviruses coding for HL-1 and HL-2 receptor subunits. As shown in Figure 4, both HL-1 (molecular mass, 46 kDa) and HL-2 (molecular mass, 48 kDa) subunits were expressed, as verified by Western-blot analysis using specific antibodies. To verify the functionality of the expressed receptors, binding and internalization of desialylated Lp(a), in comparison with Lac-Z-transfected cells used as a control, was investigated. Indeed, COS-7 cells expressing ASGPR bound 4–10-fold higher amounts of asialo-Lp(a) compared with Lac-Z-transfected cells (Figures 5A and 5B). Binding in Figure 5 refers to 'specific binding' as the values were obtained as lactose-releasable counts (50-fold excess of lactose, by wt). In addition, we show that the binding of asialo-Lp(a) is almost completely decreased to values

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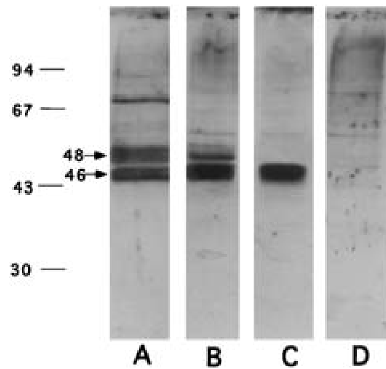

Figure 4 Expression of HL-1 and HL-2 subunits of ASGPR in COS-7 cells

The cells were co-infected with HL-1-Ad and HL-2-Ad at 15 MOI each, as described in the Experimental section. The cells were lysed 36 h after infection in SDS sample buffer, boiled for 10 min and proteins were separated by SDS/PAGE (12 % gel) under reducing conditions. After transferring the expressed proteins on to a nitrocellulose membrane, they were visualized using antibodies reacting with HL-1 + HL-2 (lanes A, B and D) or antibodies raised against HL-1 (lane C). Lane A, positive control using an extract from Hep-G2 cells; lanes B and C, COS-7 cells transfected with HL-1 and HL-2 (at 15 MOI each); lane D, negative control from Lac-Z-transfected COS-7 cells. Arrows indicate the molecular mass of the corresponding marker proteins.

seen in negative control, i.e. Lac-Z-transfected cells, by a 20-fold excess of asialo-orosomucoide; asialo-orosomucoide is the standard ligand for ASGPR. Lac-Z-transfected cells exhibited only background binding of asialo-Lp(a).

Next, we examined the role of ASGPR in cellular catabolism of native Lp(a) in a similar way as outlined above. COS-7 cells expressing both HL-1 and HL-2 subunits were able to bind and degrade significantly higher amounts of native Lp(a) when compared with control Lac-Z-transfected cells. The addition of a 20-fold excess of asialo-orosomucoide competitively inhibited binding and degradation to the amount observed with control cells. Asialo-orosomucoide had no influence on background binding of native Lp(a) to Lac-Z-transfected cells (Figures 6A and 6B).

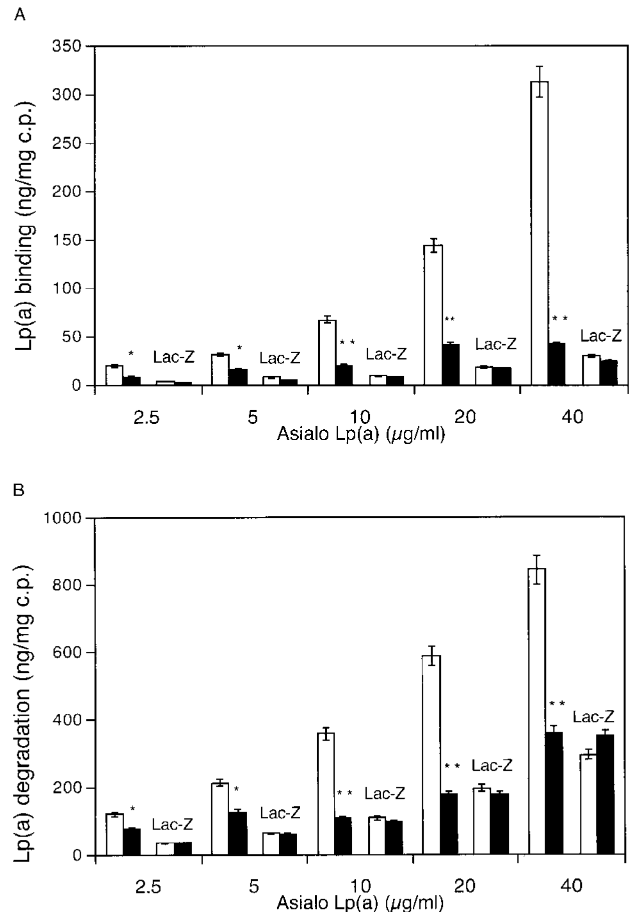

Figure 5 Binding (A) and degradation (B) of sialidase-treated Lp(a) using ASGPR-transfected COS-7 cells

Cells were co-transfected with both ASGPR subunits (HL-1 + HL-2) or with Lac-Z, as described in the Experimental section. Increasing amounts of ^{125}I-labelled sialidase-treated Lp(a) were added in the absence (open bars) or presence (black bars) of a 20-fold excess of sialidase-treated orosomucoide (asialo-orosomucoide) used as a competitive inhibitor, and cells were incubated for 6 h at 37 °C. The medium was harvested for measuring Lp(a) degradation. (**A**) Cells were washed and incubated for 2 h at 4 °C with a 50-fold excess of lactose to measure specific asialo-Lp(a) binding. (**B**) Asialo-Lp(a) degradation, calculated from the trichloroacetic acid-non-precipitable radioactivity in the medium. Open and black bars, HL-1 + HL-2-transfected cells; controls, Lac-Z-transfected cells. Values are means ± S.D. from three separate experiments performed in triplicate with Lp(a) isoforms containing 15 or 18 K-IV repeats (*$P < 0.05$, **$P < 0.01$).

DISCUSSION

The pathways responsible for the catabolism of Lp(a) in humans are mostly unexplored. Several groups, including our own, have shown that the apo(a) antigen of Lp(a) is cleaved in the circulation by metalloproteinases, yielding fragments of variable sizes [26,27]. Only a small fraction of these fragments is secreted into urine, yet most of it is rapidly cleared from the circulation by various tissues and organs in transgenic mice [26]. In humans, we calculated that less than 1 % of the catabolic rate of Lp(a) is accounted for by the urinary secretion of apo(a) fragments [28]. Most of the Lp(a) in animal experiments is taken up by the liver [16,18,19], but the role of various lipoprotein-specific receptors in Lp(a) catabolism is controversial and has not been fully explored for the *in vivo* situation [14,15,29–32]. The reason for this is that Lp(a) is found only in primates, which are hardly accessible for *in vivo* studies. Therefore first experiments were performed in hedgehogs, an animal species which synthesizes Lp(a) [33].

Apo(a), the specific antigen of Lp(a), is a highly glycosylated protein of variable size, which is determined by the number of K-IV T2 repeats [34]. The most complete characterization of the sugars in apo(a) was recently published by Garner et al. [35], who demonstrated that 17 % of the oligosaccharide structures consist of two major arsine-linked N-oligosaccharides [35]. N-glycans are complex biantennary structures in either a mono- or disialylated state. Approx. 80 % of the Ser/Thr O-linked oligosaccharides present in all apo(a) isoforms are represented by the monosialylated core type 1 structure, NeuNAcα2-3Galβ1-3GalNAc and the remaining 20 % consist of disialylated and non-sialylated O-glycans. The latter finding prompted us to elucidate the possibility that Lp(a) may bind to the ASGPR.

In the present study, we particularly took care that antimicrobial agents, among other preservatives, were added immediately after drawing blood and also throughout the different isolation steps, and the freshest Lp(a) preparation possible was used for the experiments.

In vivo experiments in hedgehogs revealed that desialylated Lp(a) is catabolized 25 times faster than native Lp(a) and is almost exclusively taken up by the liver. When desialylated Lp(a) was injected concomitantly with asialo-orosomucoide, the half-life and the catabolism by the liver were decreased to a value comparable with native Lp(a) (Figures 1A and 1B). Mannan, the competitive inhibitor for the mannose-specific ASGPR, had no

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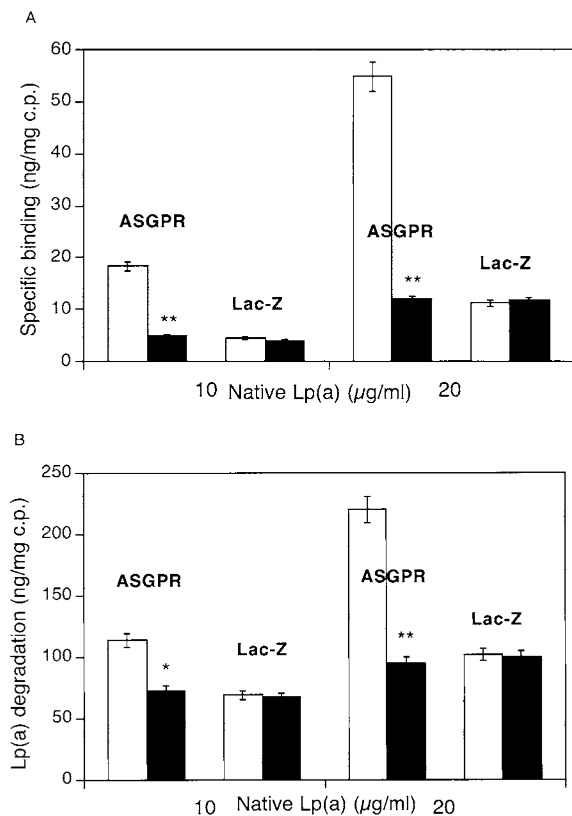

Figure 6 Binding (A) and degradation (B) of native Lp(a) using ASGPR-transfected cells or Lac-Z-transfected control cells

Experiments were performed as described in Figure 5, but native Lp(a) was used. Values are means ± S.D. from three separate experiments performed in triplicate with Lp(a) isoforms containing 15 or 18 K-IV repeats. The experiments were performed in the absence (open bars) or presence (black bars) of a 20-fold excess of sialidase-treated orosomucoide (asialo-orosomucoide) used as a competitive inhibitor (*$P < 0.05$, **$P < 0.01$).

effect (results not shown). Similar results were observed in wild-type mice where desialylated Lp(a) is catabolized 50–100 times faster than sialylated Lp(a). Here also, only asialo-orosomucoide, but not mannan, acted as a competitive inhibitor. Thus any Lp(a) preparation isolated from human plasma may contain only a small fraction of the unsialylated Lp(a), because of its very short half-life in the blood of the donor. Whether such an unsialylated Lp(a) is directly secreted from the liver or is generated during circulation in blood or an artifact is generated during Lp(a) preparation cannot be answered from the present study. Any difference in clearance of Lp(a) between ASGPR − and ASGPR + mice was expected to be small, if measurable at all. Indeed, we observed that knocking out the HL-2 subunit of ASGPR led to a measurable retardation of Lp(a) catabolism. The amount of Lp(a) labelled with the non-degradable isotope [^{125}I]tyramine cellobiose accumulating in the liver of knockout mice was significantly lower compared with wild-type mice, which is a strong indication that ASGPR is indeed involved in Lp(a) catabolism.

In ASGPR − mice, Lp(a) was still catabolized at a reasonable rate, indicating that, in addition to ASGPR, other mechanisms contribute to Lp(a) clearance, such as LDL receptor-related protein, glycoprotein 330 or the LDL receptor. Earlier studies indicated that Lp(a) shows a less efficient interaction *in vivo* with the LDL receptor compared with LDL [36]. This possibly leads to prolonged circulation times and to partial desialylation of Lp(a), which in turn is then recognized by ASGPR. We also show that asialo-Lp(a) is still readily cleared in ASGPR − mice (i.e. 86.6 % at 1 h after injection). Previous studies by Roos et al. [37] demonstrated two galactose-specific receptors in the liver: one of them, the Kupffer cell-specific glycoprotein receptor [38], does interact readily with galactose-exposing particles of the size of LDL [39]. It is probable that this receptor is responsible for the clearance of asialo-Lp(a) in ASGPR − mice. However, the Kupffer cell-specific glycoprotein receptor is not expressed in humans, as human genome analysis reveals a pseudogene that is not translated to a protein [38]. Therefore we assume that ASGPR plays an even more significant role in Lp(a) clearance in humans when compared with mice and hedgehogs.

For ethical reasons, similar *in vivo* experiments as outlined above cannot be performed in humans; however, we believe that mice are a valid compromise, since this animal species was used extensively in the past in studies of plasma lipoprotein metabolism in transgenic and knockout experiments. Our *in vivo* studies were further supported by additional experiments with COS-7 cells transfected with both subunits of ASGPR. In these experiments, we demonstrated that adenovirus-mediated gene transfer results in the functional expression of the two ASGPR subunits. When these cells were incubated with increasing amounts of freshly isolated native Lp(a), a significantly higher amount of Lp(a) was bound and degraded by these cells. A 50-fold excess of lactose (by wt) dissociated most of this binding, confirming a role for galactose in Lp(a) as a recognition marker.

Altogether, we demonstrate for the first time that freshly isolated native Lp(a) binds in hedgehogs, one of the few animals that synthesize Lp(a), and in mice to ASGPR and, subsequently, can be internalized and degraded. This also sheds new light on the significance of ASGPR, for which a physiological substrate has not been fully explored until now. We realize that Lp(a) is not a naturally occurring lipoprotein in mice, but there are many other glycoproteins present in mice with comparable N- and O-glycosylations that may follow the pathway proposed here for Lp(a). In fact, experiments are currently performed that demonstrate that β-glycoprotein is such a candidate. Finally, we suggest that our findings may lead to treatment strategies for individuals who are at an increased risk of coronary heart disease and stroke owing to high levels of plasma Lp(a).

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