Studies on the pathophysiological aspects of the metabolic syndrome in transgenic mice

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Chapter 3

Plasma PAI-1 level is not regulated by the hepatic low-density lipoprotein receptor-related protein

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Abstract

Increased plasma levels of plasminogen activator inhibitor-1 (PAI-1) are associated with increased obesity, insulin resistance and cardiovascular diseases. While research has been directed towards the production of PAI-1, the clearance of PAI-1 remains poorly understood. *In vitro* studies have demonstrated that PAI-1 is bound, internalised and degraded by the low-density lipoprotein receptor (LDLR)-related protein (LRP). In the present study, we have investigated the role of hepatic LRP in the clearance of plasma PAI-1 *in vivo,* employing mice conditionally lacking hepatic LRP (LRP-). Plasma PAI-1 levels were similar between LRP- and control LRP+ littermates. LRP status also did not affect the clearance of both exogenously infused purified murine PAI-1 and endogenously endotoxin-stimulated PAI-1. Remarkably, adenovirus-mediated gene transduction of the LDLR gene family antagonist receptor-associated protein (RAP) resulted in a significant increase of plasma PAI-1 in both LRP+ and LRP- mice. In addition, the plasma PAI-1 decay was prolonged 2-fold in mice overexpressing RAP in the circulation. The plasma levels of PAI-1 in LDLR-/-, VLDR-/-, double deficient LRP-LDLR-/- and LRP-VLDLR-/- were not different from plasma PAI-1 levels in LRP+ mice. Therefore, we conclude that in contrast to the *in vitro* data, hepatic LRP does not contribute to the clearance of plasma PAI-1 to a significant extent. In addition, we propose that RAP-sensitive mechanisms other than hepatic LRP, LDLR and VLDLR are involved in the clearance of PAI-1 *in vivo*.

Keywords: PAI-1, LRP, clearance, mice

Introduction

Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of tissue-type and urokinase-type plasminogen activator (tPA, uPA). Increased plasma PAI-1 levels are strongly associated with obesity, diabetes and cardiovascular diseases.[1,2] Furthermore, increased plasma PAI-1 levels are associated with decreased fibrinolysis.[3] This increase is associated with enhanced PAI-1 expression in vascular endothelium, adipose tissue and liver.[1] Alternatively, decreased plasma PAI-1 clearance might contribute to the increased plasma PAI-1 levels. However, it remains unknown how PAI-1 is cleared from the circulation and to what extent decreased plasma PAI-1 removal contributes to increased plasma PAI-1 levels.

PAI-1 interacts with the low-density lipoprotein receptor (LDLR)-related protein (LRP) in vitro.[4] LRP is a multi-ligand endocytic receptor of the LDLR gene family, which also includes LDLR and very low-density lipoprotein receptor (VLDR). All ligand binding to LDLR gene family members is antagonised by the receptor-associated protein (RAP). LRP is a multi-ligand multifunctional receptor. It recognizes >30 structurally and functionally different ligands in vitro, including PAI-1.[4,5] PAI-1 contains binding sites for the low-density lipoprotein receptor (LDLR)-related protein (LRP).[6] *In vitro* studies have demonstrated that PAI-1 is bound, internalised and degraded by LRP.[7] Multiple in vitro studies have shown that PAI-1 in complex with its target proteins is a better ligand for LRP than PAI-1 alone. However, PAI-1 binds to LRP with similar affinity as factor VII (FVII), which is demonstrated to be regulated by LRP *in vivo*.[6,8]

In the present study, we studied the role of hepatic LRP in the regulation of plasma PAI-1 levels in vivo. To this end, we used the unique mouse model that allows Cre/loxP-mediated deletion of hepatic LRP.[9] In addition, we have addressed whether other RAP-sensitive mechanisms are involved in the clearance of plasma PAI-1 levels using adenovirus-mediated gene transfer of RAP. We propose that RAP-sensitive pathways other than hepatic LRP, LDLR and VLDLR are involved in the clearance of plasma PAI-1 in mice.

Material and Methods

Plasma PAI-1 clearance in transgenic mice

We employed LRP, LDLR and VLDR deficient mice and combination thereof.[9-11] Age-matched 8-12-weeks old mice homozygous for the "floxed" LRP allele, either with or without the MX1Cre transgene ($MX1Cre^{+}LRP^{flox/flox}$ or $LRP^{flox/flox}$, respectively) littermates were used. LRP deficiency was induced as described.[8,9] In clearance experiments, male mice received a bolus of 1 μg/mouse purified latent murine PAI-1 (Innovative Research, CA) via the tail vein. Values are expressed as percentage of PAI-1 remaining in the circulation, with the amount

of PAI-1 present at 1 minute after injection considered as 100%. Data were corrected for endogenous PAI-1 levels. A one phase exponential fit was used to calculate the half-lives. For endogenous PAI-1 turnover, female mice received 5 μg of endotoxin (LPS Re 595, Sigma, MO) intraperitoneally as described.[12] All animal experiments were approved by the institutional committees on animal welfare of TNO-Quality of Life.

Plasma analysis

Blood samples were obtained by tail bleeding and collected in tubes containing 1/10 volume of 3.2% (w/v) citrate. Plasma was prepared by centrifugation (8000x*g* for 10 minutes at 4°C), snap-frozen and stored at -80°C prior to analysis. Mouse plasma PAI-1 antigen (Innovative Research, CA) and serum amyloid A (SAA; Biosource Europe, Belgium) were measured by enzyme-linked immunosorbent assay according to manufacturers instructions. Mouse plasma FVIII activity was measured using an one-stage coagulation assay as described.[13] Pooled plasma of C57BL/6J mice was used as reference.

Recombinant adenovirus transduction

Recombinant adenovirus ($1x10^9$ plaque-forming units) containing RAP (Ad-RAP) or β-galactosidase cDNA (Ad-β-Gal) were used for *in vivo* transduction as described.[14] Ad-RAP gene transduction results in hepatic overexpression of secretable RAP in plasma. Blood samples were collected 8 days after adenovirus injection. The PAI-1 decay experiments were performed at 8 days after virus injection. Mice intravenously received a bolus of purified murine PAI-1 (1 μg per mouse) and plasma elimination of PAI-1 was followed in time. The functionality of Ad-RAP was evaluated by measuring plasma cholesterol levels in LDLR-/- mice as described.[14]

Statistical analysis

Data are represented as geometric means and 68% confidence intervals (CI), which represent one standard deviation from the geometric mean if a log-normal distribution is assumed. Data are analyzed by means of the Mann-Whitney *U* test. $P < 0.05$ was regarded as statistically significant.

Results and Discussion

Plasma PAI-1 levels and clearance in LRP deficient mice

In vitro studies have shown that LRP plays a major role in the catabolism PAI-1. To explore the physiological relevance of hepatic LRP in the regulation of plasma PAI-1 clearance, we measured plasma PAI-1 in induced $MX1Cre^{+}LRP^{flox/flox}$ (LRP-, n = 31) mice and control $LRP^{flox/flox}$ (LRP+, n = 33) littermates (Figure 1A). LRP- mice displayed similar plasma levels

as controls. Plasma PAI-1 levels were 1.3 (1.2-1.5) ng/mL and 1.6 (1.4-1.8) ng/mL for LRP- and control LRP+ littermates, respectively (p = 0.19). Significant increase in plasma FVIII activity was observed in these LRP- [5.4 (5.0-5.9) U/mL] and LRP+ [2.7 (2.1-3.4) U/mL] mice (P < 0.05), which is consistent with our previous findings, indicating adequate induction of LRP deficiency (Figure 1B).[8] To further investigate whether LRP contributes to the clearance

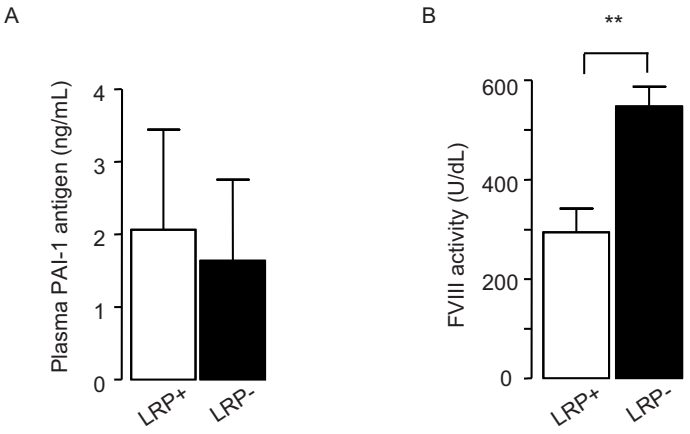

Figure 1 **Plasma PAI-1 antigen and FVII activity in hepatic LRP deficient mice LRP.**
Plasma PAI-1 antigen levels (A) and FVII activity (B) in LRP deficient (LRP-, n = 31) and control littermates (LRP+, n = 33). **p < 0.01, significantly different from control LRP+ littermates.

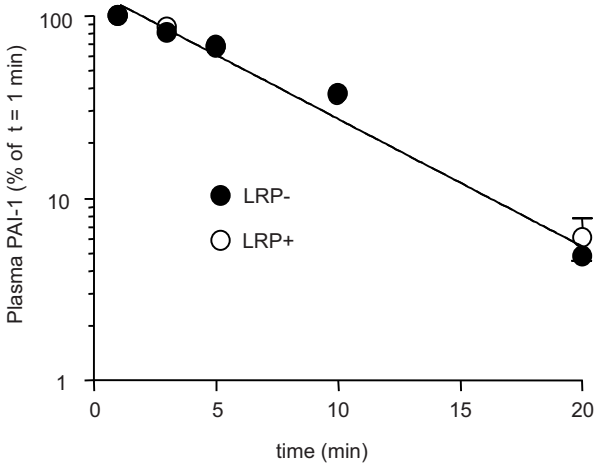

Figure 2 **Plasma PAI-1 clearance in hepatic LRP deficient mice**
LRP- and control LRP+ littermates (n = 6) intravenously received a bolus of purified mice PAI-1 (1 μg/mouse) and the plasma elimination of PAI-1 was followed in time. A one-exponential fit was used to calculate the half-lives, considering the amount of PAI-1 present at 1 minute after injection as 100%.

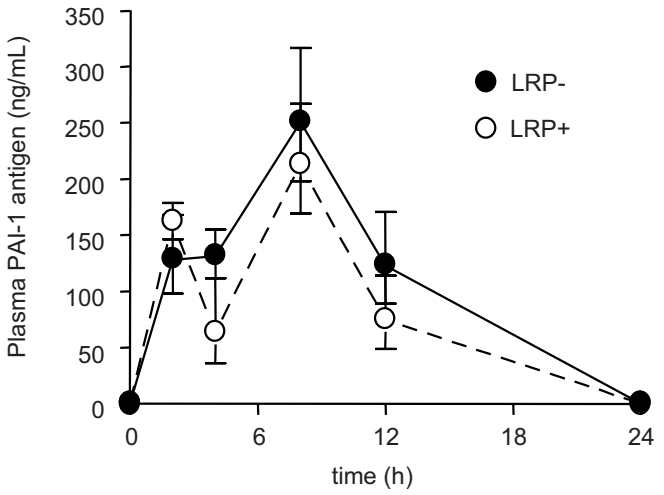

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Figure 3 **Plasma PAI-1 antigen and clearance of plasma PAI-1 upon LPS challenge**
LRP- (n = 8) and control LRP+ (n = 7) littermates intraperitoneally received 5 μg/mouse endotoxin LPS. Subsequently, plasma PAI-1 was measured during 24-hours.

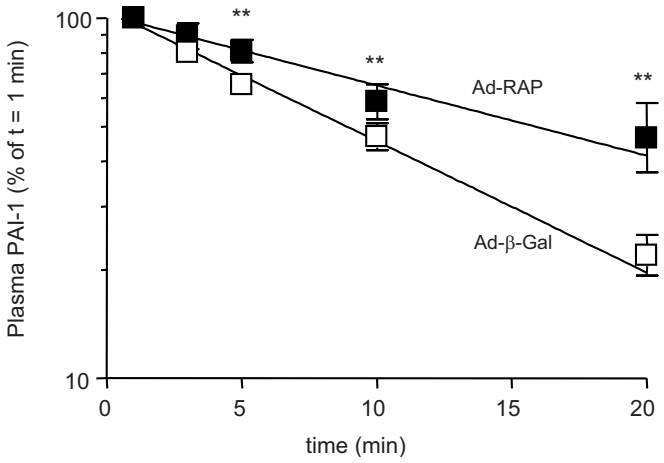

Figure 4 **Plasma PAI-1 clearance in mice overexpressing RAP**
Mice intravenously received 1 x 109 plaque forming units of recombinant Ad-β-Gal or Ad-RAP (n = 6). Eight days after adenovirus administration, mice intravenously received a bolus of purified murine PAI-1 (1 μg/mouse) and the plasma elimination of PAI-1 was followed in time as in figure 2. **p < 0.01, significantly different from Ad-β-Gal treated mice.

of PAI-1, we studied the plasma elimination of intravenously administered purified murine PAI-1. PAI-1 half-lives were identical between LRP- and LRP+ littermates (Figure 2). The half-lives were calculated to be 4.3 (3.7-4.3) minutes and 4.3 (3.7-4.3) minutes for LRP- and LRP+, respectively.

It has been established that lipopolysaccharide (LPS) can induce increased plasma PAI-1 levels in mice.[12] Therefore, we challenged LRP- and LRP+ mice with endotoxin to induce a transient rise in endogenous plasma PAI-1 levels. Indeed, upon LPS challenge a transient rise in endogenous plasma PAI-1 levels was observed (Figure 3). More importantly, the subsequent plasma PAI-1 elimination in LRP- and LRP+ littermates was similar (Figure 3). The areas under the curve were 2.8 (2.3-3.5) μg/mL.h and 2.1 (1.6-2.8) μg/mL.h for LRP- and LRP+ mice, respectively (p = 0.61). These data indicated that hepatic LRP is not involved in the regulation of plasma PAI-1 levels to a significant extent *in vivo*.

Adenovirus-mediated overexpression of RAP in hepatic LRP deficient mice

In vitro studies have previously demonstrated that the LDLR gene family antagonist receptor-associated protein (RAP) inhibits the endocytosis and degradation of PAI-1.[15] Hence, we investigated whether RAP-dependent mechanisms other that hepatic LRP are involved in the regulation of plasma PAI-1 levels. Administration of adenovirus containing RAP cDNA (Ad-RAP) evoked a significant increase in plasma PAI-1 levels in both LRP+ and LRP- mice, as compared to mice that received control adenovirus containing β-galactosidase cDNA (Ad-β-Gal, Table 1). However, no difference in plasma PAI-1 levels was observed between LRP+ and LRP- mice following Ad-RAP administration ($P = 0.49$). As plasma PAI-1 levels also increased after Ad-β-Gal administration, we considered the possibility that the plasma PAI-1 increase is due to an acute phase reaction upon adenovirus administration. Therefore, we measured the acute phase protein SAA. Indeed, adenoviral administration resulted in a significantly increased SAA. However, the increased SAA was more pronounced in mice that received Ad-β-Gal as compared to Ad-RAP. Plasma SAA levels were 2.1 (0.8-5.1) μg/mL and 99.7 (82.3-120.8) μg/mL for Ad-RAP and Ad-β-Gal, respectively ($P < 0.05$). This strongly suggests that the increased plasma PAI-1 in mice overexpressing RAP is independent of the systemic inflammatory response to adenovirus. Of note, although injection were standardized according to the plaque forming units, the absence of a SAA elevation following Ad-RAP likely reflects difference in Ad-β-Gal and Ad-RAP batches with regard to the content of non-infectious viral particles. However, we cannot fully exclude the possibility that RAP itself modulates SAA levels.

Table 1 **Plasma PAI-1 in control, LRP-, LDLR-/-, VLDLR-/-, LRP-LDLR-/- and LRP-VLDLR-/- mice with and without adenovirus-mediated overexpression of RAP.**

genotype	adenovirus	PAI-1 (ng/mL)	n
LRP+	-	1.6 (1.4-1.8)	33
LRP-	-	1.3 (1.2-1.5)	31
LRP+	ad-β-Gal	32.5 (24.9-42.3)	4
LRP+	ad-RAP	159.3 (98.9-256.6)*	4
LRP-	ad-β-Gal	19.6 (11.7-33.0)	4
LRP-	ad-RAP	261.9 (192.2-357.0)#	4
LDLR-/-	-	1.5 (1.4-1.6)	14
VLDLR-/-	-	2.1 (1.8-2.3)	5
LRP-LDLR-/-	-	1.6 (1.4-1.8)	16
LRP-VLDLR-/-	-	1.7 (1.5-2.0)	7

For the adenovirus-mediated overexpression of RAP, mice received 1x109 plaque forming units recombinant adenovirus containing RAP (Ad-RAP) or control β-galactosidase (Ad-β-Gal) cDNA. Blood samples were collected 5 days after adenovirus administration. Blood samples were then analysed for PAI-1. Data represent geometric mean with 68% CI. *p < 0.05, significantly different from ad-β-Gal injected LRP+ mice. #p < 0.05, significantly different from ad-β-Gal injected LRP- mice.

To study whether the increased plasma PAI-1 levels in Ad-RAP treated mice can be attributed to impaired clearance, we followed the clearance of exogenously injected PAI-1 in these mice. The clearance of PAI-1 was 2-fold slower in mice overexpressing RAP (Figure 4). The half-lives were calculated to be 15.4 (13.3-18.4) minutes in Ad-RAP treated mice and 8.3 (7.6-9.1) minutes in mice that received Ad-β-Gal (P < 0.01). These data indicate that RAP-dependent mechanisms other than hepatic LRP are involved in the regulation of PAI-1 *in vivo*.

RAP-sensitive receptors include LDLR and VLDLR. Therefore, we measured plasma PAI-1 levels in LDLR-/-, VLDLR-/-, and double deficient LRP–LDLR-/- and LRP– VLDLR-/- mice. Plasma PAI-1 levels in these mice were not different from plasma PAI-1 in LRP+ mice (Table 1), suggesting that neither LDLR nor VLDLR is critically involved in the regulation of plasma PAI-1 levels. Additional studies are required to establish which RAP-sensitive mechanisms are involved in the regulation of plasma PAI-1 levels. The question remains whether the LRP/PAI-1 interaction is of any physiological importance. It could be possible that the interaction between LRP and PAI-1 is of importance only when PAI-1 is in complex with its target proteases. The high affinity LRP binding site in PAI-1 is demonstrated to be exposed when PAI-1 is in complex with t-PA.[4] However, the similar plasma PAI-1 levels between LRP- and LRP+ (Figure 1) are a strong argument against a significant accumulation of plasma PAI-1 complexes in the present study. Alternatively, the PAI-1/LRP interaction might only be of importance in cellular signaling locally. It has been shown that PAI-1 is a potent chemoattractant molecule, an activity that depends on the interaction with LRP for cell signalling.[16] Identification of the molecular mechanisms that underlie the regulation of PAI-1 levels in the circulation may further advance our understanding of increased plasma PAI-1 levels in patients with obesity, diabetes and cardiovascular diseases.

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