Inactivation of the LRP1 Intracellular NPxYxxL Motif in LDLR-Deficient Mice Enhances Postprandial Dyslipidemia and Atherosclerosis

Philip L.S.M. Gordts, Sara Reekmans, Annick Lauwers, Amber Van Dongen, Leen Verbeek, Anton J.M. Roebroek

Objective—The purpose of this study was to determine the significance of the intracellular NPxYxxL motif of LRP1 for the atheroprotective role of this multifunctional receptor.

Methods and Results—LRP1 knock-in mice carrying an inactivating mutation in the NPxYxxL motif were crossed with LDLR-deficient mice, a model for atherosclerosis. In this LDLR−/− background the mutated mice showed a more atherogenic lipoprotein profile, which was associated with a decreased clearance of postprandial lipids because of a compromised endocytosis rate and reduced lipase activity. On an atherogenic diet LRP1 mutant mice revealed a 50% increased development of atherosclerosis. This aggravation was accompanied by an increase in smooth muscle cell (SMC) and collagen content and apoptotic cells in the lesions. The mutation showed, however, a limited impact on basal PDGFR-β expression and signaling and the antimigratory property of apoE on PDGF-BB–stimulated SMCs. Additionally, levels of LRP1 atherogenic ligands, like MMP2, t-PA, FVIII, and the inflammatory ligand TNF-α showed to be significantly elevated.

Conclusion—These findings demonstrate that the NPxYxxL motif is essential for the atheroprotective role of LRP1. This motif is relevant for normal control of lipid metabolism and of atherogenic and inflammatory ligands, but has no pronounced effect on regulating PDGF-BB/PDGFR-β signaling in SMCs. (Arterioscler Thromb Vasc Biol. 2009;29:1258-1264.)

Key Words: atherosclerosis • growth factors • immune system • lipases • lipids

The large endocytic receptor low-density lipoprotein (LDL) receptor–related protein 1 (LRP1) is a multifunctional protein that binds multiple extracellular ligands including apolipoprotein E (apoE) containing lipoproteins, lipoprotein lipase, complexes of proteinases-proteinase-inhibitors, hormones, matrix proteins, and growth factors like platelet-derived growth factor (PDGF; reviewed by Herz). This 600 kDa receptor is proteolytically cleaved by a furin-like endoprotease into 2 subunits of 515 kDa and 85 kDa. The very large extracellular α-subunit contains the ligand-binding domains and is noncovalent associated with the smaller β-subunit, containing an extracellular part, the membrane spanning domain and the cytoplasmic or intracellular domain (LRP1-ICD).

In several studies using conditional inactivation of LRP1 in mice, the receptor has been associated with a clear role in the pathogenesis of atherosclerosis. In the liver it has been shown that the receptor is important for the removal of atherogenic lipoproteins and other proatherogenic ligands from the circulation. Boucher et al, moreover, showed that LRP1 has a cholesterol-independent role in atherosclerosis by modulating the activity and cellular localization of the PDGF receptor-β (PDGFR-β) in vascular smooth muscle cells (SMCs). Finally, 2 studies argue that LRP1 in macrophages has an effect on atherosclerosis through the modulation of the extracellular matrix and inflammatory responses.

In all these studies, the overall function of LRP1 in a specific cell type was disrupted. These approaches, however, meet their limits when they are used for studies of large multidomain proteins where different individual domains are involved in diverse functions, as in the case of LRP1. It has been demonstrated that many motifs in the complex LRP1-ICD, being the 2 NPxY motifs, a YxxL motif, 2 di-leucine motifs, and a protein kinase A (PKA) consensus motif, are potentially involved in directing LRP1 in a cargo transporting or signaling function. The YxxL motif has been recognized as being the dominant endocytosis signal next to the distal NPxY and distal di-leucine motifs. The distal NPxY motif overlapping with the YxxL motif, forming the NPxYxxL double motif, is also capable of interacting with many cytoplas-
matic adaptor and scaffold proteins, which in addition can be modulated by the phosphorylation of the tyrosine residue.9,10

In the present study we investigated the role of the NPxYxxL motif in the LRP1-ICD in the development of atherosclerosis in vivo. To tackle this question we made use of mice carrying a knock-in mutation of the NPxYxxL motif into the endogenous \( \text{Lrp1} \) gene, which were generated by a recombinase-mediated cassette exchange (RMCE) strategy.11 The LRP1 knock-in mice were crossed into the LDLR\(^{-/-}\) background to study the effect of the LRP1-ICD mutation on the pathology in this atherosclerosis model. Our data demonstrate that the NPxYxxL motif is essential for the atheroprotective role of LRP1, not only via control of the lipid metabolism but also through regulation of levels of atherogenic factors like MMP2, tissue-type plasminogen activator (t-PA), and coagulation factor VIII (FVIII) and secretion of the proapoptotic cytokine, TNF-\( \alpha \). Furthermore, there was no significant effect of the NPxYxxL inactivation on LRP1 regulated PDGFR expression and signaling.

**Methods**

An expanded Methods section can be found in supplemental material (available online at http://atvb.ahajournals.org).

**Statistical Analysis**

Statistical significance between groups was determined by Student \( t \), 1-way ANOVA, Mann–Whitney rank sum, and Kruskal–Wallis tests using STATISTICA version 6 software (StatSoft Inc). \( P<0.05 \) was regarded statistically significant.

**Results**

Inactivation of the LRP1 NPxYxxL Motif Causes an Increase of Remnant Particles

The previously described NPxYxxL mutant11 (LRP1\(^{n2/n2}\)) was crossed with LDLR\(^{-/-}\) mice to study the impact of the LRP1 knock-in mutation on the phenotype of this LDLR\(^{-/-}\) mouse model for atherosclerosis. Because LRP1 is involved in the clearance of lipoproteins from the circulation, serum was obtained. The LDLR\(^{-/-}\)LRP1\(^{n2/n2}\) mice show a significant 1.5-fold and 1.6-fold increase in their total serum cholesterol as well as triglyceride concentrations, respectively, compared with the LDLR\(^{-/-}\) mice (respectively 486.7\(\pm\)29 mg/dL versus 320\(\pm\)31 mg/dL cholesterol and 265.4\(\pm\)20 mg/dL versus 162.0\(\pm\)17 mg/dL triglycerides; \( P<0.005 \)). Analysis of serum samples by size exclusion chromatography revealed a higher cholesterol and triglyceride content in the CR, VLDL, and LDL fractions in LDLR\(^{-/-}\)LRP1\(^{n2/n2}\) mice (Figure 1A and 1B). This was confirmed by the significant rise in the apoB48 and apoE concentrations seen in plasma of the LDLR\(^{-/-}\)LRP1\(^{n2/n2}\) mice (supplemental Figure I). Altogether, the results suggest an accumulation of triglyceride rich lipoprotein particles (TRLs).

**LDLR\(^{-/-}\)LRP1\(^{n2/n2}\) Mice Have a Delayed Postprandial Lipid Clearance**

A possible contributing factor to the observed increase of TRLs in the LDLR\(^{-/-}\)LRP1\(^{n2/n2}\) mice is a difference in hepatic VLDL production rates. In contrast to the increase in
TRLs the production rate of hepatic VLDL was significantly reduced in the LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> mice to 320 mg/dL/h compared to 560 mg/dL/h for the LDLR<sup>−/−</sup> mice (Figure 1C). Because there was no increased production of hepatic apoB containing particles—on the contrary a decrease was observed—one can assume that an altered clearance of postprandial lipoproteins might be a possible explanation. Therefore, we compared the postprandial response of LDLR<sup>−/−</sup> and LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> mice. We found that the plasma triglyceride clearance in LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> mice after an oral fat load was significantly impaired and marked by an increased accumulation of triglycerides up to 4 hours after administration (Figure 1D). The rate of postprandial triglyceride accumulation is both determined by the rate of LpL-mediated triglyceride hydrolysis and the rate of receptor-mediated clearance of remnant lipoprotein particles. In postheparin plasma, however, there was no difference in lipase activity (supplemental Figure II). Therefore, lipase activity in total homogenates and heparin extracts (surface bound activity) from liver, white adipose tissue (WAT), and muscle were evaluated (Table). The results revealed no difference in lipase activity for liver and muscle tissue in both extracts. Contrary wise in WAT the amount of LpL activity was significantly reduced in both heparin releasable and total extracts indicating a decreased expression of LpL. To determine the impact on receptor-mediated clearance the endocytosis rate of the LRP1 ligand apoE was evaluated in peritoneal macrophages derived from LDLR<sup>−/−</sup> and LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> mice (Figure 1E). This analysis illustrated a significant 20% reduction in the clearance rate when the NPxYxxL motif was inactivated. Evaluation of the endocytosis rate of α<sub>M</sub>, a specific LRP1 ligand, in mouse embryonic fibroblasts (MEFs) revealed a significant 30% reduction in the uptake for LRP1<sup>n2/n2</sup> MEFs and almost no uptake for LRP1<sup>−/−</sup> MEFs (PEA1312) compared to their wild-type controls (Figure 1F). To substantiate this impact on clearance further, 2 atherogenic LRP1 ligands, t-PA and FVIII, levels were determined in plasma. In the LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> t-PA and FVIII plasma levels were, respectively, significantly 1.4-fold and 1.6-fold increased compared to the LDLR<sup>−/−</sup> controls (supplemental Figure III). The data indicate that inactivation of the NPxYxxL motif of LRP1 in vivo leads to a delayed clearance of postprandial TRLs, via both decreased LpL activity in adipose tissue and a reduced endocytosis rate of the receptor.

### Increased Atherosclerotic Lesion Area and Smooth Muscle Cell and Collagen Content

To investigate the impact of the NPxYxxL mutation on the development of atherosclerosis, 12-week-old LDLR<sup>−/−</sup> (n=23) and LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> (n=15) mice were fed an atherogenic diet for 12 weeks. On the atherogenic diet, LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> mice had increased triglyceride levels compared to the levels in LDLR<sup>−/−</sup> mice, however cholesterol levels were not different (Figure 2A and 2B). Similar to the chow diet, an accumulation of TRLs was also reflected in their lipoprotein and apolipoprotein profile (supplemental Figure IV). En face analysis of these mice showed a significant 1.5-fold increase for the development of atherosclerosis in LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> mice compared to the LDLR<sup>−/−</sup> control (respectively 18.8±1.9% versus 12.6±0.6%; P<0.01, Figure 2C). Thus, inactivation of the NPxYxxL motif in LRP1 leads to an increased atherosclerosis development, indicating that this motif is relevant for the atheroprotective properties of the receptor. To evaluate the impact of the LRP1 knock-in mutations on the composition of atherosclerotic lesions, we determined the percentage of SMC, macrophage and collagen content in lesions of LDLR<sup>−/−</sup> (n=6) and LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> (n=6) mice. As shown, the percentage of SMC lesion content at the level of the individual lesions for the LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> mice was significantly 2-fold higher compared to the control LDLR<sup>−/−</sup> mice (Figure 2D through 2F). Furthermore, the mean percentages of collagen content in individual lesions of LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> mice were also significantly increased compared to the LDLR<sup>−/−</sup> mice (Figure 2D, 2G, and 2H). In contrast, results obtained with the antimouse macrophage antibody indicated no differences for macrophages (Figure 2D). Lesion classification indicated no significant differences in the low amount of early lesions but revealed a significant shift from less moderate to more advanced lesions in LDLR<sup>−/−</sup>LRP<sub>1</sub><sup>n2/n2</sup> mice compared to the LDLR<sup>−/−</sup> controls (supplemental Figure V). These results suggest that inactivation of the NPxYxxL motif is essential for an increased SMC content in the lesion area, which is associated concomitantly with an increased collagen content in the atherosclerotic lesions, likely reflecting a phenotypic shift of the lesions.

### No Pronounced Impact on LRP1 Mediated PDGFR-β Regulation In Vivo

In the search for a potential mechanism contributing to the increased SMC content in the atherosclerotic lesions of mice bearing the knock-in mutation, we wanted to evaluate whether the NPxYxxL inactivation had an effect on the expression of the PDGFR-β and its signaling as seen for the vascular specific LRP1 knock-out mice. Therefore, protein extracts...
from aortas were checked for expression and phosphorylation of PDGFR-β and ERK by immunoblotting. Determination of the relative optical density of the bands revealed no significant increase in PDGFR-β expression and phosphorylation of PDGFR-β and ERK (supplemental Figure VI). Because these data did not suggest a strong effect on PDGFR-β expression and signaling, we wanted to assess whether the LRP1 mutation had an effect on the LRP1 regulated apoE-mediated inhibition of PDGF-BB–induced SMC migration. The role of the LRP1 knock-in mutation was assessed in vascular SMCs isolated from wild-type (LRP1+/+) and LRP1n2/n2 mice (Figure 3A). The results in the SMCs showed for the NPxYxxL mutation at low concentrations of apoE a loss of the antimigratory effect, whereas higher concentrations of apoE were reducing the migratory property of PDGF-BB as compared to the wild-type control cells. Similar results were obtained using the derived MEFs (supplemental Figure VII). Thus overall, the NPxYxxL mutation seems to have a comparable to the wild-type control cells. Similar results were obtained using the derived MEFs (supplemental Figure VII). Thus overall, the NPxYxxL mutation seems to have only a limited impact on the antimigratory property of apoE in vitro. Among ligands for LRP1 involved in migration of SMCs are matrix-metalloproteinases and t-PA, which was obtained using the derived MEFs (supplemental Figure VI). Because these data did not suggest a strong effect on PDGFR-β expression and signaling, we wanted to assess whether the LRP1 mutation had an effect on the LRP1 regulated apoE-mediated inhibition of PDGF-BB–induced SMC migration. The role of the LRP1 knock-in mutation was assessed in vascular SMCs isolated from wild-type (LRP1+/+) and LRP1n2/n2 mice (Figure 3A). The results in the SMCs showed for the NPxYxxL mutation at low concentrations of apoE a loss of the antimigratory effect, whereas higher concentrations of apoE were reducing the migratory property of PDGF-BB as compared to the wild-type control cells. Similar results were obtained using the derived MEFs (supplemental Figure VII). Thus overall, the NPxYxxL mutation seems to have only a limited impact on the antimigratory property of apoE in vitro. Among ligands for LRP1 involved in migration of SMCs are matrix-metalloproteinases and t-PA, which was already shown to be increased in the plasma of the LDLR−/−LRP1n2/n2 mice. Therefore, MMP2 and MMP9 activity in aortic lysates were evaluated in 12-week-old mice via zymography. The mice having the NPxYxxL inactivation had a significant 2.7-fold increase of MMP2 activity in the aortas, whereas no differences were seen for MMP9 activity (Figure 3B). Altogether, these results suggest that inactivation of the NPxYxxL motif leads to increased levels of aortic MMP2 and plasma t-PA, both LRP1 ligands with promigratory properties.

Proapoptotic Effects via Increased Secretion of TNF-α in Macrophages

Because it has been shown that LRP1 is involved in the regulation of apoptosis,13 the amount of apoptotic cells in the atherosclerotic lesions were determined via TUNEL staining (Figure 4A and 4B). As illustrated in Figure 4C, there was a significant 2-fold increase in the amount of apoptotic cells relative to the plaque size in LDLR−/−LRP1n2/n2 mice. The apoptosis rate in atherosclerotic plaques is largely on the account of the oxysterols present in oxidized LDL particles. Therefore we examined the impact of the NPxYxxL inactivation on apoptosis in mouse peritoneal macrophages after a 20-hour treatment with 0.5 μg/mL staurosporine (STS), 10 μg/mL 7-ketocholesterol or 20 μg/mL 25-hydroxycholesterol (supplemental Figure VIII). No significant difference in apoptosis was observed between LDLR−/−LRP1n2/n2 and LDLR−/−macrophages for STS and both oxysterols. In search for an alternative explanation, the secretion of the proapoptotic inflammatory molecule TNF-α was evaluated in the peritoneal macrophages. It has been shown that LRP1 influences TNF-α levels through its binding to αM and subsequent internalization of the complex.5 We quantified the amount of TNF-α accumulating in media from lipopolysaccharide (LPS)-stimulated macrophages over time. In comparison to LDLR−/−macrophages, the NPxYxxL inactivation increased the accumulation of TNF-α 1.8-fold and 2.2-fold after 4 and 20 hours incubation, respectively (Figure 4D). Because it has also been shown that macrophage LRP1 influences monocyte chemotactic protein 1 (MCP-1) secretion,4 we next investigated the effect of the NPxYxxL inactivation on its secretion. No effect was observed in LPS stimulated macrophages (supplemental Figure IX). Therefore
clearance of postprandial lipoproteins from the circulation,² has been described that LRP1 in the liver is involved in and triglyceride levels compared to the background control. It resulted in a significant increase in total serum cholesterol and LDL concentration in the circulation of LDLR mice, which are not linked to increased hepatic triglyceride production. This was substantiated further in vivo by the increased levels of LRPI atherogenic ligands like t-PA and FVIII and in vitro via evaluating the clearance of apoE in the LDLR−/− background and the clearance of α2-M in MEFs on a wild-type background, where we found a significant reduction in the endocytosis capacity of LRPI by inactivation of the NPxYxxL motif. Nevertheless, next to the reduced endocytosis rate of LRPI, the decreased lipase activity in adipose tissue observed in the knock-in mice likely also contributes to the delayed postprandial lipid clearance. The decreased lipase activity is most likely attributable to a decreased expression of lipases instead of cell surface retention of the enzymes, because no differences in activity were observed between the total and heparin extract in white adipose tissue. These findings are in contrast to the observation in adipocyte specific LRPI knock-out mice.¹⁶ They are, however, in agreement with a recent study where was shown that LRPI-deficiency results in reduced LpL and hormonedependent lipase (HSL) expression and activity in adipocytes differentiated from MEFs.¹⁷ These results, together with our findings, could imply that the NPxYxxL motif is involved in the regulation of LpL and HSL expression in adipocytes.

The more atherogenic lipoprotein profile seen for the LDLR−/−LRP1n2/n2 mice on chow diet renders these mice more prone to develop atherosclerosis, and as such this correlated with significant higher atherosclerosis develop-

Discussion
In the present study previously described NPxYxxL knock-in mice, showing no distinct phenotype, were crossed into the LDLR−/− background to elucidate the role of the modified LRPI-ICD motif in the pathogenesis of atherosclerosis. On the LDLR−/− background, the NPxYxxL knock-in mutation resulted in a significant increase in total serum cholesterol and triglyceride levels compared to the background control. It has been described that LRPI in the liver is involved in clearance of postprandial lipoproteins from the circulation,² mediated via insulin induced LRPI translocation¹⁴ and subsequent binding of apoE and lipoprotein lipase to LRPI.¹⁵ In vitro studies using LRPI minireceptors with mutated LRPI-ICDs clearly determined that the YxxL motif, comprising the NPxYxxL motif, serves as the dominant endocytosis signal in the LRPI-ICD.⁷ Our in vivo observations support these in vitro results as shown by increased apoE, apoB48, CR, VLDL, and LDL concentration in the circulation of LDLR−/−LRP1n2/n2 mice, which are not linked to increased hepatic triglyceride

Figure 3. A, Effect of apoE inhibition on PDGF-directed SMC migration (LRP1+/+ [] and LRPI1232/G [■]; n=12 per condition and genotype). B, Determination of MMP2 and MMP9 activities via densitometry in aortas and a representative zymogram (LDLR−/− [] and LDLR−/−LRP11232/G [■]; n=5 per genotype). Data are mean±SEM. *P<0.05, **P<0.01.

Figure 4. TUNEL staining of atherosclerotic lesions of LDLR−/− (A) and LDLR−/−LRP1n2/n2 (B) mice and quantification (C; n=14 to 18, bars are 100 µm). D, TNF−α ELISA analysis of media from LPS-stimulated (200 ng/mL) macrophages after 4 and 20 hours (twice in duplicate; LDLR−/−[●] and LDLR−/−LRP11232/G [■]). Data are mean±SEM. *P<0.05, **P<0.005.
ment in the aortas on a high-cholesterol diet. The difference in atherosclerosis on the high-cholesterol diet between knock-in mice and control mice can in part be attributed to the altered lipoprotein profile even if only triglyceride levels were significantly higher and cholesterol levels were not different. Accumulation of lipoprotein remnants and, therefore, higher levels in the vessel wall could explain the differences in atherogenesis as seen for patients having type III hyperlipidemia. Hence, these results corroborate the atherogenic potential of triglyceride-rich lipoproteins.

LRP1, however, is not only expressed in liver and adipocytes, therefore it is conceivable that loss of a functional NpxYxxL motif could have an effect on signaling functions of LRP1 in other tissues which can contribute to the atherogenic potential. As reported, mice lacking LRP1 in vascular SMCs have higher PDGFR-β expression and signaling activity. Additionally it has been shown that incubation of vascular SMCs in vitro with apoE inhibits the stimulatory effect of PDGF-BB on cell migration, and loss of LRP1 expression is preventing this inhibitory activity of apoE. Evaluation of the plaque phenotype showed for the LDLR<sup>−/−</sup>/LRP1<sup>n2/n2</sup> mice a 2-fold increase in relative SMC content associated with increased collagen content in the lesions. It is known that SMCs in the atherosclerotic lesion are involved in matrix deposition by the production of molecules like collagen, hence explaining the concomitant increase. The relative increased SMC content in the lesions of the LDLR<sup>−/−</sup>/LRP1<sup>n2/n2</sup> mice could have been caused by a stimulatory effect of the mutations on the migratory properties of SMCs out of the tunica media. Nevertheless, we observed in vitro only a limited decrease in sensitivity for the apoE-mediated inhibition of PDGF-stimulated cell migration in SMCs. So, based on these analyses, only subtle impairment of regulation of PDGFR-β signaling by LRP1 could possibly contribute to the change of plaque phenotype. Unfortunately, a possible impact of the NpxYxxL inactivation on PDGFR-β expression and signaling in the aorta seems to be too subtle to measure in our analyses. Alternatively, maybe other LRP1 intracellular motifs are involved in this intracellular crosstalk between LRP1 and PDGFR-β and can compensate for the loss of the functional NpxYxxL motif.

The results impelled us to assess the levels of other LRP1 ligands which are shown to be involved in migration of SMCs. Therefore, the activity of MMPs are good candidates for the change of plaque phenotype. Unfortunately, a possible impact of the NpxYxxL inactivation on PDGFR-β expression and signaling in the aorta seems to be too subtle to measure in our analyses. Alternatively, maybe other LRP1 intracellular motifs are involved in this intracellular crosstalk between LRP1 and PDGFR-β and can compensate for the loss of the functional NpxYxxL motif.

phorylation, reducing both MMP9 clearance and production resulting in unaltered levels in the aorta.

It has been suggested in recent publications that LRP1 is involved in the regulation of inflammatory responses and apoptotic pathways. Because atherosclerosis is an inflammatory disease and apoptosis is attributed to lesion vulnerability, the amount of apoptosis in atherosclerotic lesions was quantified and showed a significant 2-fold increase in apoptotic cells for the lesions of LDLR<sup>−/−</sup>/LRP1<sup>n2/n2</sup> mice. Because sensitivity of peritoneal macrophages for induction of apoptosis by oxysterols was unchanged, the LRP1-mediated secretion of the proapoptotic cytokine, TNF-α, was evaluated in these macrophages in search of an alternative explanation. Our results demonstrated that disruption of the NpxYxxL motif significantly increases the LPS-induced secretion of TNF-α, and this might indeed explain the increase in apoptotic cells in lesions of the LDLR<sup>−/−</sup>/LRP1<sup>n2/n2</sup> mice. These data are corresponding to the results obtained in the LRP1-deficient macrophages, where a similar increased TNF-α secretion was speculated to be due to reduced clearance of TNF-α bound to α<sub>M</sub>. However no effect was seen on the secretion of the monocyte recruitment factor MCP-1. This observation is consistent with the unaltered macrophage content observed in lesions of LDLR<sup>−/−</sup>/LRP1<sup>n2/n2</sup> mice.

So, in conclusion, the obtained results support the hypothesis by which loss of the NpxYxxL motif is affecting LRP1 internalization properties negatively leading to a raise in apoE containing lipoprotein particles, MMP2, t-PA, FVIII, and TNF-α and consequently in a more atherogenic lipoprotein profile and increased atherosclerosis development in the LDLR<sup>−/−</sup>/LRP1<sup>n2/n2</sup> mice. These findings are relevant in relation to reports where polymorphisms in the LRP1 gene, LRP1 mRNA, and LRP1 protein expression levels are associated with an increased risk of coronary artery diseases (CAD). Furthermore, there is evidence that LRP1 polymorphisms might effect the plasma lipid concentrations in the postprandial period. Altogether, our study provides experimental evidence in an animal model that subtle partial loss of LRP1 functionality leads to the accumulation of small defects at different levels, all contributing to an increased susceptibility for the development of CAD. In the human population the underlying mechanism is more likely the direct impact of LRP1 allelic differences itself on the expression level of LRP1 or indirectly the impact of modifier genes on the expression level of LRP1, rather than mutation of the protein coding domains. In this view our results emphasize the importance for further investigations toward LRP1 function in relation to postprandial dyslipidemia and the other risk factors for CAD.

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Disclosures

None.

References

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Supplement Material

Supplement Figures

Figure I. Lipid levels and apolipoprotein distribution. A-B, Serum cholesterol (A) and triglyceride (B) levels in LDLR<sup>−/−</sup> (□) and LDLR<sup>−/−</sup>LRP1<sup>n2/n2</sup> (■) mice on chow diet. C-D, Immunoblot analysis of serum apolipoproteins (C) and the relative expression levels of apoB100, B48, E and AI as determined by densitometry (n=6 per genotype) (D). Data are mean±SEM. *<i>P</i>&lt;0.05 and **<i>P</i>&lt;0.005.
**Figure II.** Lipase activity levels in post-heparin plasma. Plasma was diluted 10-fold and 10 µl of the dilutions were used for determination of lipoprotein lipase activity. Activity was determined after incubation for 30 minutes at 37°C. Lipase activity levels in LDLR^{−/−} (□) and LDLR^{−/−}LRP1^{n2/n2} (■) mice did not show a significant difference. Data are mean±SEM.
Figure III. Endogenous mouse plasma t-PA (E) and FVIII (F) levels of 5h fasted LDLR^+/-(□) and LDLR^-/-LRP1^n2/n2 (■) mice (resp. 7.4±0.6 nM versus 10.7±0.9 nM t-PA and 1.2±0.1 U/ml versus 2.0±0.2 U/ml FVIII; n=18 per genotype; data are mean±SEM). *P<0.01.
Figure IV. Lipid levels and lipoprotein distribution. A-B, The serum lipoprotein distribution of cholesterol (A) and triglycerides (B) by HPLC in LDLR−/− (Δ) and LDLR−/−LRP1n2/n2 (■) mice put on the atherogenic diet. C-D, Immunoblot analysis of serum apolipoproteins (C) and the relative expression levels of ApoB100, B48, E and AI as determined by densitometry (n=6 per genotype) (D). Data are mean±SEM. *P<0.05 and **P<0.005.
Figure V. Qualitative assessment of atherosclerotic lesions. Lesions at the level of the aortic sinus were categorized according to the lesion severity (early, moderate and advanced) according to the literature\(^1\). Lesion classification of LDLR\(^{-/-}\) (□) and LDLR\(^{-/-}\)LRP1\(^{n2/n2}\) (■) mice are shown as percentage of the total number of lesions analyzed (n=24-27 per genotype). Data are mean±SEM. *\(P<0.05\) and **\(P<0.005\).
Figure VI. PDGF signaling. A-B, Aortas extracts were analyzed by immunoblotting for expression of PDGF-β, LRP1-α and ERK and for the phosphorylated status of PDGFR-β (p-PDGFR-β) and ERK1/2 (p-ERK) (A). Bar graphs showing the relative expression level as determined by densitometry (B) (LDLR−/− (□) and LDLR−/−LRP1−/− n2/n2 (■); n=6 per genotype). Data are mean±SEM.
Figure VII. Effect of apoE inhibition on PDGF-directed migration. Quiescent MEFs² were incubated with or without apoE for 30 min at 37°C prior to addition into the upper chamber of the Cell Culture Inserts. The lower chamber contained basal medium and 0 or 10 ng/ml human recombinant PDGF-BB. Migratory cells were quantified in duplicate after 6h (wild type (□), LRP1^a2/n2 (■) and LRP1^−/− (□); n=12 for each condition and genotype). The results in the derived MEFs² and the LRP1-deficient PEA-13 cells (LRP1^−/−)³, showed that the NPxYxxL mutation had no effect on the anti-migratory property of apoE while the LRP1-deficient PEA-13 cells revealed, as expected, a loss of apoE mediated anti-migratory effect. When these experiments were performed in SMCs a slightly different result was obtained. In the SMC’s, the NPxYxxL mutation revealed at low concentrations of apoE a loss of the anti-migratory effect, while higher concentrations of apoE were reducing the migratory property of PDGF-BB as comparable to the wild-type control cells. Data are mean±SEM. *P<0.05, **P<0.01.
Figure VIII. *In vitro* TUNEL-staining of peritoneal macrophages after a 20-hour treatment with Staurosporine (STS), 7-ketocholesterol (7-KC) and 25-hydroxycholesterol (25-OHC). (LDLR<sup>+/−</sup> (□) and LDLR<sup>+/−</sup>LRP<sub>1<sup>n2/n2</sup> (■)). Results represent the mean±SEM of two independent experiments performed in duplicate.
**Figure IX.** MCP-1 ELISA analysis of media from macrophages stimulated with LPS (200 ng/ml) after 4 and 20 hours (LDLR⁻/⁻ (Δ) and LDLR⁻/⁻LRP1²/² (■)); Results represent the mean±SEM of two independent experiments performed in duplicate.
Expanded Methods

Animals and Diets

Homozygous LRP1 knock-in mutant mice (mixed C57Bl/6J and 129 background) containing the previously described NPxYxxL knock-in mutation (PTNFTNPVYATL → PTNFTAATAATL, LRP1\(^{n2/n2}\))^2 were crossed with homozygous LDL receptor knock-out mice (LDLR\(^{-/-}\))^4 on a C57Bl/6J background, which were kindly provided by Dr. P. Holvoet. The F1 progeny of this mating (LDLR\(^{-/-}\)LRP1\(^{n2/+}\)) were again crossed with LDLR\(^{-/-}\) mice to obtain offspring, heterozygous for the LRP1 mutation and LDLR-deficient (LDLR\(^{-/-}\) LRP1\(^{n2/+}\)). Subsequent intercross of these mice resulted in offspring that had either zero, one or both LRP1 knock-in alleles in a LDLR-deficient background (LDLR\(^{-/-}\)LRP1\(^{n2/n2}\), LDLR\(^{-/-}\)LRP1\(^{n2/-}\) and LDLR\(^{-/-}\)LRP1\(^{+/+}\) respectively). The obtained LDLR\(^{-/-}\)LRP1\(^{n2/n2}\) and LDLR\(^{-/-}\)LRP1\(^{+/+}\) (= LDLR\(^{-/-}\)) mice (87.5 % C57Bl/6J) and their offspring were used for further experimental analysis. At 12 weeks of age mice were fed a high-fat/high-cholesterol diet (atherogenic diet) (Teklad, TD.04418, containing 21% (w/w) milk fat, 1.25% (w/w) cholesterol and 0.5% (w/w) cholic acid) for an additional 12 weeks. The research was approved by the Institutional Animal Care and Research Advisory Committee of the K.U. Leuven.

Serum Lipids and Lipoprotein Distribution Analysis

Serum samples were obtained by cardiac puncture from mice fasted for 16 hours. Serum total cholesterol and triglyceride levels were measured by commercially available enzymatic kits (N.V. Invitrogen SA, Merelbeke, Belgium and Wako Chemicals GmbH, Neuss, Germany). In addition, 50 µl pooled serum was used for lipoprotein profiling by high-performance liquid chromatography using molecular sieve columns with the LipoSEARCH system (Skylight Biotech Inc, Tokyo, Japan)^5.
Triglyceride Secretion Rate and Postprandial Triglyceride Response

Six mice of each genotype were fasted for 5h prior to a tail vein injection of Tylooxapal (Triton WR-1339, Sigma Chemical, Co.) at a dose of 0.5 mg/g body weight. Plasma was collected by tail bleeding for triglyceride measurements at time points 1, 15, 30, 60 and 120 minutes after injection. Ten mice of each genotype were fasted for 5h prior to receiving an intragastric load of olive oil (10 µl/g body weight). Plasma was collected by tail bleeding for triglyceride measurements at time points 0, 60, 120, 180 and 240 minutes after injection.

LpL Activity Assay

For determination of LpL activity in plasma mice were injected IP with 300U heparin /kg body weight and plasma was collected by tail bleeding 10 minutes after heparin infusion. Plasma was diluted 10-fold and 10 µl of the dilutions were used for determination of lipoprotein lipase activity using the LpL activity assay (Roar biochemical Inc., NY, USA). Activity was determined after incubation for 30 minutes at 37°C according to the manufacturer’s instructions. For determination of LpL activity in total cell extracts, extracts were prepared by homogenizing 90 mg of tissues in buffer containing 150 mM NaCl, 10 mM Tris, 2 mM EDTA, and pH 7.4. Cell-surface LpL was isolated from the minced tissues by collecting supernatants from centrifugation of samples for 10 minutes at 20,000 g after incubation for 1 hour at 37°C in buffer 150 mM NaCl, 10 mM Tris, 2 mM EDTA, pH 7.4, 10 U/ml heparin and 0.5% BSA. LpL activities in undiluted tissue homogenates and in the heparin-released supernatants were determined as described above.
Isolation and Culture of Fibroblastic, Aortic Smooth Muscle Cells and Peritoneal Macrophages

The previously described Lrp1 wild-type MEFs, the NPxYxxL mutant MEFs and the homozygous LRP1-deficient PEA-13 fibroblasts were used for comparative analysis. Aortic SMC were isolated from Lrp1 wild-type and NPxYxxL mutant mice using a modification of Mimura’s procedure. Cells between passage 2 and 6 were used for experiments. Mouse peritoneal macrophages were isolated 3 days after IP injection of 1ml thioglycolate broth via a peritoneal lavage as described by Overton et al. All the cells were cultured in DMEM containing 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Prior to stimulation with PDGF-BB (BD Biosciences, NJ, USA), cells were cultured for 24 hours in DMEM containing 0.4% FBS.

Internalization Assay

The endocytosis assay was adapted from the procedure performed by Dedieu et al. Briefly, mouse peritoneal macrophages (seeded at 2x10^6 / well) or confluent MEFs were washed twice with PBS and incubated for 1 hour in fresh serum-free medium containing 50 µg/ml Cy3 labelled human recombinant apoE3 (PeproTech EC Ltd, London, UK) or 50 µg/ml FITC labelled human α2M (Biomac, Liepzig, Germany) in the presence of 100 µM chloroquine to inhibit lysosomal activity. Cells were next washed three times with ice-cold PBS and the remaining surface bound Cy3-apoE or FITC-α2M was removed by incubating the cells for 2 minutes with PBS containing 10U/ml heparin on ice. Finally the cells were solubilised in ice-cold lysis buffer (NaF 50mM, Na2-EDTA1mM, EGTA 1mM, 20µM phenylarsine oxide, 5 mM Na3VO4, 1% Triton X-100 and proteinase inhibitors [Complete™, Roche Applied Science GmbH, Mannheim, Germany]). Cell homogenates were centrifuged for 20 min at
15000 x g and the supernatant was collected to measure the intra-cellular fluorescence and the protein concentration.

**Quantification of Mouse Plasma Factor VII and t-PA**

Blood was obtained by tail bleeding. Samples were collected in polypropylene eppendorf tubes containing 1/10 vol of 3.2% (wt/vol) trisodium citrate. Plasma was prepared by centrifugation of blood at 2000g for 10 minutes at RT, immediately snap-frozen in liquid nitrogen, and stored at −80°C prior to analysis. Mouse plasma FVIII activity was measured by means of the Coatest FVIII Chromogenic assay (Chromogenix, Milano, Italy) and t-PA levels by means of an ELISA (Innovative Research Inc, Michigan, USA) as described by the suppliers.

**En Face Quantification of Aortic Lesions**

Mice euthanized by CO₂ intoxication were perfused with 10 ml phosphate-buffered saline (PBS) followed by 20 ml freshly prepared paraformaldehyde (4% [wt/vol] in PBS). The heart and ascending aorta up to the iliac bifurcation were removed and incubated in paraformaldehyde. The heart and adventitial tissue were removed; the aortas were cut open and pinned flat. Next the aortas were incubated with Sudan IV staining solution (0.5% Sudan IV, 35 % ethanol, 50% acetone) for 15 minutes and de-stained with 80% ethanol. Images were acquired using a Leica IC A video camera attached to a Leica MZ FLIII stereomicroscope and lesions were measured using image analysis software Leica IM1000 (Leica Microsystems Ltd, Heerbrugg, Switzerland). The data are expressed as the percentage of the total Sudan IV positive lesion areas compared to the total aortic area.
**Immunohistochemistry**

For immunohistochemical analysis of atherosclerotic lesions, hearts together with the proximal aorta were isolated after a single perfusion with PBS. The upper part of the hearts were placed onto a tissue mold, covered in OCT (Tissue-Tek), and frozen. Serial 10-µm cryosections from similar parts of the aortic sinus were stained with a rabbit anti mouse macrophage antibody (AIA-31240, 1/200 dilution, Accurate Chemical & Scientific Corp., NY) and a monoclonal mouse anti-α-smooth muscle cell actin-monoclonal antibody (clone 1A4, 1/500 dilution, Sigma-Aldrich N.V./S.A., Bornem, Belgium). Sections were fixed using 4% paraformaldehyde in PBS for 30 min and blocked in 2% serum-2% bovine serum albumin in TNB buffer (0.1M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent) and reacted for 1 h at room temperature with the primary antibody in the same solution. Bound primary antibodies were detected with horseradish peroxidase-tagged secondary antibodies. Sections were treated with DAB and counterstained with haematoxylin. Quantification of SMC positive areas was done using Leica IM1000 software (Leica Microsystems Ltd, Heerbrugg, Switzerland) at the level of 14-18 individual lesions overlapping in size ranging from 50,000 to 500,000 µm² and expressed as a percentage of the size of the individual lesion. Collagen was stained for with Masson’s Trichrome (Merck KGaA, Darmstadt, Germany). All the lesions (n=24-27) were classified according to severity as described by Van Vlijmen et al.¹ The numbers in each category are expressed as the percentage of the total number of lesions analyzed within the corresponding group.

**Immunoblot Analysis**

Homogenates were made using ice-cold lysis buffer (NaF 50mM, Na₂.EDTA1mM, EGTA 1mM, 20µM phenylarsine oxide, 5 mM Na₃VO₄, 1% Triton X-100 and proteinase inhibitors [Complete™, Roche Applied Science GmbH, Mannheim, Germany]). For immunoblot
analysis in aortas, the aortas after removal of the adventitia and connective tissue were
ground to a fine powder under liquid nitrogen and incubated in ice-cold lysis buffer for 1
hour. Insoluble matter was removed by centrifugation for 10 min at 500 x g. For analysis of
cells, MEF’s and vascular smooth muscle cells were washed twice with chilled PBS and
lyzed in ice-cold lysis buffer. Cell homogenates were centrifuged for 20 min at 15000 x g and
the supernatant was collected. Equal amounts of homogenates or postnuclear supernatant
were run on SDS-PAGE 4-12 % Bis-Tris NuPage (N.V. Invitrogen SA, Merelbeke,
Belgium) gels as previously described2. Rabbit antibodies recognizing the LRP1 protein
were raised against a mixture of two LRP1 α-subunit-specific peptides
Antibodies against ERK1/2 and p-ERK1/2 are from Cell Signaling Technology Inc (Danvers,
US). Antibodies recognizing PDGFR-β and phospho-PDGFR-β were purchased from Upstate
and CHEMICON (Temecula, US). For quantification, images were analyzed with NIH image
software (Image J, 1.36b; NIH, Bethesda, Maryland).

Cell Migration Assay

Examination of migration by MEFs and mouse aortic smooth muscle cells towards a PDGF-
BB gradient was performed according to the procedure described by Law et al.9. Briefly, 5 x
10^5 cells were made quiescent by incubation with DMEM containing 0.4% FBS for 48 hours
and then incubated in a 1 ml solution with or without human recombinant apoE3 (PeproTech
EC Ltd, London, UK) for 30 min at 37°C. A 350 µl aliquot of the cell suspension was added
to the top chamber of tissue-treated Transwell polycarbonate membranes with 8 µm pores in
24-well plates (Innocyte™ Cell migration assay, Calbiochem, Germany). The lower
compartment contained 0.5 ml of DMEM containing 0.4% FBS, 0.2% bovine serum albumin
with or without 10 ng/ml PDGF-BB. After incubation for 6h at 37 °C, the number of cells
that migrated to the lower surface of the filter was determined as described by the manufacturer.

**Zymography**

MMP9 and MMP2 enzyme activity was assayed by gelatin zymography. Equal amounts of homogenates were separated by electrophoresis, under non-reducing condition, through a Novex® 10% Zymogram Gelatin Gel (N.V. Invitrogen SA, Merelbeke, Belgium). Gels were washed in Novex® Zymogram Renaturing Buffer three times, and then incubated overnight at 37°C in Novex® Zymogram developing buffer. Gels were subsequently stained with 0.25% Coomassie brilliant blue R250 and next destained with 15% acetic acid and 15% ethanol. Enzymatic activity attributed to MMP9 and MMP2 were visualized in the zymogram as clear bands against a dark blue background. A human MMP9 and MMP2 zymography standard (10 ng) (Calbiochem, Germany) was loaded as control. For quantification, images were analyzed with NIH image software (Image J, 1.36b; NIH, Bethesda, USA).

**TUNEL Studies**

Mouse peritoneal macrophages were cultured in Laboratory-Tek chamber slides (Nalge Nunc International). After overnight incubation in DMEM containing 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin, the cells were treated with 0.5 µg/ml staurosporine (STS), 10µg/ml 7-ketocholesterol or 20µg/ml 25-hydroxycholesterol for 20 hours. The cells were labeled with the In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics Belgium) according to the manufacturer’s instructions. TUNEL positive cells were counted in 10 fields (x400) using the CellR Fluorescence microscope (Olympus UK Ltd). The same kit was used for the 10-µm cryosections from the aortic sinus and three serial sections from each mouse were stained.
Cytokine Secretion

Mouse peritoneal macrophages were seeded at 2x10^6/well in DMEM containing 10% FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Two hours after seeding the cells were stimulated with 200ng/ml lipopolysaccharides (LPS) in DMEM containing 100 units/ml penicillin and 0.1 mg/ml streptomycin. Media samples were collected after 4 and 20 hours and the cytokines (TNF-α and MCP-1) were measured by specific ELISA kits according to the manufacturer’s instructions (BD Biosciences, NJ, USA). Media were normalized for cellular protein content.
References


