LRP5 deficiency down-regulates Wnt signalling and promotes aortic lipid infiltration in hypercholesterolaemic mice

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Abstract

Low-density lipoprotein receptor-related protein 5 (LRP5) is a member of the LDLR family that orchestrates cholesterol homeostasis. The role of LRP5 and the canonical Wnt pathway in the vascular wall of dyslipidaemic animals remains unknown. In this study, we analysed the role of LRP5 and the Wnt signalling pathway in mice fed a hypercholesterolaemic diet (HC) to trigger dyslipidaemia. We show that Lrp5−/− mice had larger aortic lipid infiltrations than wild-type mice, indicating a protective role for LRP5 in the vascular wall. Three members of the LDLR family, Lrp1, Vldlr and Lrp6, showed up-regulated gene expression levels in aortas of Lrp5−/− mice fed a hypercholesterolaemic diet. HC feeding in Lrp5−/− mice induced higher macrophage infiltration in the aortas and accumulation of inflammatory cytokines in blood. Wnt/β-CATENIN signalling proteins were down-regulated in HC Lrp5−/− mice indicating that LRP5 regulates the activation of Wnt signalling in the vascular wall. In conclusion, our findings show that LRP5 and the canonical Wnt pathway down-regulation regulate the dyslipidaemic profile by promoting lipid and macrophage retention in the vessel wall and increasing leucocyte-driven systemic inflammation.

Keywords: LRP5 • atherosclerosis • plasma cholesterol • canonical Wnt signalling • macrophages

Introduction

Hypercholesterolaemia is a causal factor for atherosclerosis, which predisposes individuals to the development of clinical cardiovascular diseases [1]. High levels of low-density lipoprotein (LDL) in plasma rapidly infiltrate the vessel wall triggering an inflammatory-immuno-modulatory chain reaction [2, 3]. Members of the LDL receptor family are involved in lipoprotein transport and plasma LDL cholesterol clearance, modulating critical stages of atherosclerosis progression including inflammation, foam cell formation and endothelial activation [4, 5]. There is ongoing controversy on the role of LRP5 and the canonical Wnt/β-CATENIN pathway in lipid induced vascular damage and atherosclerosis. Indeed, activation of the β-CATENIN/lymphoid enhancer-binding factor 1 (LEF1) signalling in the endothelium occurs in response to atheroprone haemodynamic stimulations and precedes lesion development in ApoE−/− mice [6] and higher levels of active β-CATENIN are observed in disrupted atherosclerotic plaques compared to stable plaques from human carotid artery, suggesting a potential role for Wnt signalling in the evolution of atherosclerotic plaque [7]. However, ApoE−/−Lrp5−/− mice fed a high-fat diet developed bigger atherosclerotic lesions that their ApoE−/− littermates [8], although the exceedingly high levels of cholesterol in these animals (almost 750 mg/dl) could have shadowed any effect of the canonical Wnt receptor, LRP5.

The canonical Wnt/β-CATENIN pathway has also been described to regulate inflammatory reactions although its role remains controversial. Indeed, Wnt/β-CATENIN pathway seems to inhibit inflammation as β-CATENIN inhibitors increase the expression of inflammatory genes in human aortic endothelial cells [9] and administration of GSK3β, an inhibitor of Wnt/β-CATENIN pathway in human monocytes triggers Toll-like receptor-mediated pro-inflammatory cytokine production [10]. However, IL-1β and LPS induced nuclear β-CATENIN accumulation in human vascular endothelial cells [6] and activation of canonical Wnt genes have been found in endothelial cells of a rejected kidney model [11] suggesting that activation of the pathway triggers the inflammatory response.

We have recently reported that LRP5 is involved in monocyte to macrophage differentiation [12], it regulates macrophage motility and LRP5-expressing mononuclear cells are a fraction of the macrophages...
found in human advanced coronary atherosclerotic plaques [13]. Still, the presence of LRPS-positive cells in these coronary plaques does not imply causality. Thus, to better understand the role of LRPS and Wnt signalling in the early stages of lipid infiltration in the vessel wall, we studied the effects of a hyperlipidaemic diet inducing a mild increase in cholesterol serum levels in Lrp5\(^{-/-}\) mice and in wild-type (WT) controls. We hypothesized that LRPS and the Wnt signalling pathway have a role in the inflammatory process associated to atherosclerosis progression. Absence of LRPS, induced higher lipid infiltration in mouse thoracic aortas, increased the transcription of the LDLR family member Lrp6, induced higher macrophage infiltration and increased inflammatory cytokines secretion, supporting an anti-inflammatory role for LRPS and the target genes of the Wnt/beta-Catenin pathway.

Materials and methods

Animals and experimental design

Lrp5\(^{-/-}\) mice, a kind gift from Dr. Bart Williams [14–16] were maintained in a C57BL/6 background. Mice were housed in cages under controlled temperature (21 ± 2°C) on a 12 hrs light/dark cycle with food and water ad libitum. Homozygous WT C57BL/6 mice (n = 22) and Lrp5\(^{-/-}\) C57BL/6 mice (Lrp5\(^{-/-}\); n = 22) were used for the protocols. The presence of Lrp5 alleles was assessed by PCR amplification from DNA extracted from tail biopsies in WT, heterozygous and homozygous littermates. Primers used were S17 (GGC TCG GAG GAC AGA CCT GAG), S23 (CTG TCA GTG C) and IRES31 (AGG GGC GGA ATT CGA TAG CT). Aortas were frozen in liquid nitrogen and aortic RNA was isolated with TRIzol\textsuperscript{TM} Reagent (Invitrogen; Carlsbad, CA, USA 7900HT) and the following specific probes from SABiosciences, Qiagen. Real time RT-PCR array was performed with RT2 Profiler PCR array PAMM-021 (SABiosciences, Qiagen).

Biochemical analysis and blood-derived mRNA

Blood samples were collected in serum separator gel tubes and PAX-tubes. Serum was obtained by centrifugation 1200g 20 min. at 4°C. Cholesterol, triglycerides and HDL levels were measured enzymatically by using commercially available kits (GERNON reagents) and read in a spectrophotometer (MC-15 SOFT; RAL). PAX-tubes were processed for preparation of blood-derived mRNA using PAXgene Blood RNA Kit (Qiagen Inc, Valencia, CA, USA). Real Time RT-PCR array was performed with RT2 Profiler PCR array PAMM-021 (SA Biosciences, Qiagen).

Quantification of atherosclerotic lesions

Mice were anaesthetized and aortas were removed, carefully cleaned of adventitial fat under a stereoscopic microscope, and longitudinally cut with the luminal surface facing up (n = 6–8 mice/group). Aortas were fixed overnight in 4% paraformaldehyde, washed with ddH\(_2\)O 1 hr in gentle shaking and stained with Oil-red-O (ORO) for 30 min. Aortas were rinsed with 70% ethanol and ddH\(_2\)O; images were captured by Nikon Instruments, Melville, NY, USA Eclipse 80i microscope and digitized by Retiga 1300i Qimaging, Surrey, BC, Canada Fast camera. ORO-stained area was quantified with Image J software and results are expressed as percentage of lipid area/total aortic area.

Thin layer chromatography

Aorta tissue (5 mg) was homogenized in NaOH 0.1 M. The organic solvent was removed under a N\(_2\) stream, the lipid extract was suspended in dichloromethane and separated by thin layer chromatography (TLC). TLC was performed on silica G-24 plates. Concentrations of standards (a mix of cholesterol, cholesteryl palmitate, triglycerides, diglycerides and monoglycerides) were applied to each plate. The chromatographic developing solution was heptane/diethylether/acidic acid (74:21:4, vol/vol/vol). The spots corresponding to cholesteryl esters (CE) were quantified by densitometry.

Real time RT-PCR

Aortas were frozen in liquid nitrogen and aortic RNA was isolated with Trizol\textsuperscript{TM} Reagent (invitrogen; Carlsbad, CA, USA n = 5–7 mice/group). Concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and purity was checked by the A260/A280 ratio (ratios between 1.8 and 2.1 were considered acceptable). cDNA was synthesized from 0.5 μg RNA with cDNA Reverse transcription kit (Qiagen). The resulting cDNA samples were amplified with a RT-PCR thermal cycler (Applied Biosystems Carlsbad, CA, USA 7900HT) and the following specific probes from CONDA: Lrp5 (Mm.PT.49a.8045420), Lrp1 (Mm.PT.49a.7750137), Lrp2 (Mm.PT.49a.11916154), Lrp6 (Mm.PT.56a.6383636), Lrp8 (Mm.PT.49a.6553055), Ldr (Mm.PT.49a.9930556) and Cd36 (Mm.PT.49a.12111555). Viadr (Mm00443298_m1) was purchased from Applied Biosystems. Results were normalized with 18S probe from Applied Biosystems.

Immunohistochemistry

Immediately after surgical excision, aortas were immersed in fixative solution (4% paraformaldehyde) and embedded in paraffin, cut into 5 μm thick serial sections and placed on poly-L-lysine coated slides. Primary antibodies were used: Matrix Metalloproteinase-7, MMP-7 (Rabbit polyclonal; Abcam), Cambridge, UK (cATENIN (Rabbit polyclonal; Millipore) Bedford, MA, USA and HAMS6 (Mouse monoclonal; Dako Glostrup, Denmark). Before incubation with primary antibodies, sections were washed and endogenous peroxidase activity was impressed with H\(_2\)O\(_2\) and goat or horse serum block. Primary antibodies were detected using the avidin–biotin immunoperoxidase technique. Sections were incubated with an appropriate biotinylated secondary antibody (1:200; Vector Laboratories), Burlingame, CA, USA The chromogen...
used was 3,3'-diaminobenzidine. Haematoxylin was used for nuclear stain. Images were captured by Nikon Eclipse 80i microscope and digitized by Retiga 1300i Fast camera, magnification (×400).

Statistical analysis

Results are expressed as mean ± SEM. A Stat View statistical package was used for all the analysis. Comparisons among groups were performed by two-way ANOVA analysis. Regression analyses were performed by applying $Y = a + bX$ linear pattern selecting just highly adjusted equations. Slopes were compared by t-test. Statistical significance was considered when $P < 0.05$.

Results

Serum cholesterol profile

We analysed the differences in serum cholesterol profiles of WT and Lrp5/−/− mice fed a normocholesterolaemic (NC) or hypercholesterolaemic (HC) diet. Figure 1A shows an agarose gel with WT, Lrp5/−/− and Lrp5+/− alleles. Cholesterol serum levels of Lrp5/−/− mice fed a NC diet containing 3.5% (w/w) fat and 0% cholesterol were lower than those of their WT littermates (Fig. 1B). When mice were fed a HC diet containing 21% (w/w) fat and 0.25% cholesterol, total cholesterol levels were significantly increased in both Lrp5/−/− and WT mice (Fig. 1B). Interestingly, the overall increase in serum cholesterol levels in high-fat diet with respect to chow diet was double in Lrp5/−/− (125.69 mg/dl) than in WT (62.25 mg/dl, Fig. 1B).

Non-HDL cholesterol increased by 42.8% and 300.23% in HC WT and HC Lrp5/−/− mice with respect to their NC littermates (Fig. 1C).

Lipid deposition in aortas

The administration of a HC diet in WT and Lrp5/−/− mice led to a significant increase in early lesions in the aortic area with lipid infiltration in both groups. Indeed, the aortic lipid rich coverage increased by 7 ± 0.5% and by 17 ± 1% in WT and Lrp5/−/− mice respectively (Fig. 1D and E). The increased lipid deposition in HC Lrp5/−/− mice was over 70% higher than in HC WT mice indicating a protective role for LRP5 in mice aortas. Furthermore, HC feeding induced higher levels of CE accumulation in Lrp5/−/− (87 ± 8%) than in WT (51 ± 6%) aortas with respect to NC mice (Fig. 1F).

Positive correlation between plasma cholesterol levels and aortic lipid deposition in WT and Lrp5/−/− mice

To analyse the effect of total plasma cholesterol on aortic lipid coverage, regression analyses were performed for WT and Lrp5/−/− mice results. Significantly higher aortic lipid deposition was observed with higher plasma cholesterol levels (Fig. 2A and B). The slope for this correlation was steeper for Lrp5/−/− mice. Correlation analyses remained positive and significant between non-HDL cholesterol and aortic lipid coverage and slopes were significantly different with higher change in Lrp5/−/− mice (Fig. 2C and D).
Increased macrophage infiltration in Lrp5\(^{-/-}\) mice

Lrp5 mRNA expression in white blood cells showed a 77.5 ± 0.2% increase because of hypercholesterolaemia in Wt mice (Fig. 3A). Lrp5 mRNA expression was negligible in white cells of Lrp5\(^{-/-}\) mice. Immunostaining with HAM56 for macrophages (Fig. 3B) showed a 47.6 ± 2% increase in macrophage infiltration to the intima in HC Lrp5\(^{-/-}\) mice compared with HC Wt mice (Fig. 3C). Monocytes–macrophages in aortic tissue were more abundant in Lrp5\(^{-/-}\) mice.
Expression of pro-inflammatory cytokines in white blood cells

We then analysed gene expression levels of pro-inflammatory cytokines in white blood cells from HC Wt and HC Lrp5−/− mice. Results show an increase of Il1b, Il15 and Il1β mRNA expression by 62.5 ± 2%, 43 ± 1% and 91 ± 2% respectively in HC Lrp5−/− compared with HC Wt mice (Fig. 3D). These results show increased inflammation in HC Lrp5−/− mice respect to their Wt littermates.

Wnt pathway activation in mice aortas

Levels of two proteins of the canonical Wnt pathway, β-CATENIN and MMP-7 were analysed in macroscopically ORO-stained areas of the aortas of Wt and Lrp5−/− mice. β-CATENIN staining was increased in aortas of HC Wt mice compared to NC Wt mice indicating an activation of the Wnt/β-CATENIN pathway by hypercholesterolaemia. As expected, there was no increase in β-CATENIN staining in HC Lrp5−/− mice (Fig. 4A and B). Similarly MMP-7, another downstream protein of the pathway, was up-regulated in HC Wt compared with NC Wt mice, while in aortas from HC Lrp5−/− mice, the area covered by MMP-7 staining was smaller (Fig. 4C and D). These results suggest that the canonical Wnt/β-CATENIN pathway is triggered by mild dyslipidaemia as a protective response and that this effect cannot be produced when LRP5 is absent.

LDL receptors in WT and LRP5−/− mice aortas

LRP5 belongs to the LDL receptor superfamily of proteins involved in lipoprotein trafficking [5]. To determine if other receptors were causing the lipid infiltration observed in aortas of Lrp5−/− mice, we analysed gene expression levels of receptors described to be involved in the initial stages of atherosclerosis lesions. Results show that Lrp2 gene expression was strongly down-regulated in Lrp5−/− mice independently of diet (Fig. 5A), while Lrp8 gene expression was down-regulated in Wt and Lrp5−/− mice after HC diet (21.05 ± 2% and 57.14 ± 2% respectively, Fig. 5B). Similarly, the classical Ldlr mRNA expression levels were reduced with HC feeding in both genotypes (21.4 ± 0.5% in HC Wt respect to NC Wt and 23.6 ± 1% in Lrp5−/−, Fig. 5C). Cdo36 increased its mRNA expression by 48.2 ± 1% in HC Wt mice with respect to NC Wt animals, but showed a 41.6 ± 0.5% decrease in HC Lrp5−/− mice with respect to NC Lrp5−/− mice (Fig. 5D). On the contrary, Lrp1 significantly increased by 37 ± 2% in HC Wt mice and 21 ± 9% in HC Lrp5−/− mice aortas with respect to their NC littermates, but there was no significant effect because of HC in the Lrp5−/− mice (Fig. 6A). The Vldlr mRNA levels were up-regulated by the HC diet in Wt and Lrp5−/− mice, although in a non-significant manner (Fig. 6B). Finally, Lrp6 mRNA expression levels were increased by 31 ± 2% in HC Lrp5−/− mice with respect to NC Lrp5−/− mice (Fig. 6C). Regression analyses revealed a significant positive correlation between Lrp6 gene expression levels and lipid rich coverage in aortas of Lrp5−/− mice, but not for Lrp1 (Fig. 6D).
Over the last decades, LRP5 has been involved in several pathways, including bone development, impaired fat tolerance and glucose metabolism [8, 17–19]. Impaired plasma clearance of chylomicron remnants and reduced glucose tolerance was observed in LRP5-deficient mice fed a high-fat diet compared with their WT littermates [17]. Here, we have studied the role of LRP5 and the canonical Wnt pathway in mildly HC Lrp5/C0/C0 mice. Total cholesterol and non-HDL plasma levels increased after HC feeding in WT and Lrp5/C0/C0 mice. However, total cholesterol levels in HC Lrp5/C0/C0 mice that had a significantly higher aortic lipid infiltration were higher than those in HC WT mice, suggesting an involvement for LRP5 during dyslipidaemia, at the initial stages of atherosclerosis development. Similarly in humans, LPR5-rs3736228T alleles that cause loss-of-function in LRP5 protein show increased plasmatic cholesterol levels in Chinese Han population [20] and is considered an independent risk factor for hypercholesterolaemia in the male Japanese population [21].

Hypercholesterolaemic Lrp5/C0/C0 mice show larger lipid infiltration in the thoracic aorta compared with HC WT animals. Accordingly, Immunohistochemistry analyses revealed an increase in macrophage staining in the intima layer of HC Lrp5/C0/C0 mice confirming our previous in vitro results showing that monocytes that overexpress LRP5 show a down-regulation of the differentiation processes by the sequestration of β-CATENIN to the cell membrane [12]. Consequently, here we show that mice lacking LRP5 have more macrophag

**Discussion**

![Fig. 5](image_url)

Fig. 5 Receptors expression in mice aortas. mRNA expression levels of Lrp2 (A), Lrp8 (B), Ldlr (C) and Cd36 (D) in aortas from WT and Lrp5/C0/C0 mice fed a normocholesterolaemic (NC) or a hypercholesterolaemic (HC) diet. *P < 0.05; ***P < 0.005.

![Fig. 6](image_url)

Fig. 6 Receptors expression in mice aortas. mRNA expression levels of Lrp1 (A), Vldlr (B) and Lrp6 (C) in aortas from WT and Lrp5/C0/C0 mice fed a normocholesterolaemic (NC) or a hypercholesterolaemic (HC) diet. *P < 0.05; ***P < 0.005. (D) Regression analyses between aortic lipid coverage and Lrp6 or Lrp1 gene expression levels in aortas of Lrp5/C0/C0 mice with their statistical significances (p), correlation coefficients (r) and slopes (b).
es in the vascular wall. Activated monocytes/macrophages stimulate cytokine secretion promoting pro-inflammatory chronic stimulation [1, 22, 23]. Therefore, we analysed the expression of pro-inflammatory and pro-atherogenic cytokines (IL18, IL15 and IL10) in white blood cells from HC Wt and HC Lrp5−/− mice [24–27]. Wnt/β-CATENIN activation inhibits the inflammatory response in endothelial cells [9] and enhances injury repair and healing responses in other inflammatory diseases including rheumatoid arthritis and colitis [28]. We found increased inflammatory genes expression in white blood cells from HC Lrp5−/− mice indicating increased inflammation.

IHC analyses revealed a down-regulation of the Wnt canonical pathway members, β-CATENIN and MMP7, in the absence of LRP5 evidencing a down-regulation of the canonical Wnt signalling pathway in HC Lrp5−/− mice aortas. This supports our previous in vitro findings showing that LRP5 silencing in human macrophages abrogates Wnt/β-CATENIN pathway activation [13].

Aortic Lrp6 expression increased in Lrp5−/− animals fed the HC diet and directly correlated with aortic lesion area. LRP6 has been found overexpressed in human atherosclerotic lesions [29] and regulates LDLR-mediated LDL uptake as LDLR internalization is diet and directly correlated with aortic lesion area. LRP6 has been Lrp5

Cd36

reduced uptake of oxidized LDL [35]. Our results do not support [34], but cultured macrophages from these patients present a associated with enhanced atherosclerotic cardiovascular diseases

Wnt

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