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LRP1 AS A SOURCE OF THERAPEUTIC, DIAGNOSTIC AND PROGNOSTIC TOOLS IN CARDIOVASCULAR DISEASE

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1. Project Summary

Lipid accumulation in arterial and myocardial tissues causes cardiovascular alterations but is an unmet clinical need. Lipid accumulation is mainly caused by the uptake of atherogenic lipoproteins through a receptor named LRP1. In binding regions of LRP1 we identified a peptide sequence, P3, located in the CR9 domain, that interacts with atherogenic lipoproteins but not with other LRP1 ligands. We hypothesize that blocking CR9 by anti-P3 antibodies (Anti-P3 Abs) or sequestrating atherogenic lipoproteins by P3-based peptidomimetics (mP3) will specifically inhibit lipid accumulation in arterial and myocardial tissues. Intracellular lipids promote the release of a soluble form of LRP1, sLRP1. Circulating sLRP1 levels could thus reflect arterial and myocardial lipid accumulation. Our objectives are to determine the effects of Anti-P3 Abs and mP3 on cardiovascular alterations in a translational animal model, and to evaluate the diagnostic and prognostic value of circulating sLRP1 levels in patients with well characterized arterial and myocardial lipids. P3-immunized rabbits, mP3-treated rabbits, and control rabbits will be fed a high-fat diet. Blood samples will be regularly taken to determine lipidic and metabolic parameters. Aorta, heart, liver and skeletal muscle samples will undergo molecular and immunohistochemical analysis. We will evaluate atherosclerosis by imaging analysis and assess ventricular function by echocardiography. We will use molecular techniques to characterize lipid content, vascular procoagulant activity, and cardiac calcium-handling. Plasma sLRP1 concentrations will be determined using ELISA. The diagnostic value of sLRP1 as a biomarker of vascular and cardiac lipid deposition will be assessed in patients with atherosclerotic plaques characterized by computed tomography and in patients with myocardial lipids characterized by nuclear magnetic resonance. sLRP1 will be evaluated as a prognostic biomarker in a three-year follow-up study. We expect to develop P3-based molecules with in vivo validation of therapeutic efficacy and to validate sLRP1 as a diagnostic and prognostic biomarker in cardiovascular disease.

2. Results

INNOVATIVE LRP1-BASED POTENTIAL THERAPEUTIC TOOLS

Antibodies against the peptide P3 (LRP1 CR9 domain) show high efficacy to inhibit high-fat diet (HFD)-induced atherosclerosis in rabbits

Summary

In the rabbit model, we revealed a significant reduction of two surrogate markers of atherosclerosis, aortic [^{18}F]-FDG cellular metabolism and carotid resistance index, upon the P3 immunization of rabbits. Immunohistochemical, confocal, and molecular studies showed that P3 immunization efficiently counteracted the formation of fatty streaks due to the high efficacy of Anti-P3 Abs in preventing foam cell formation and their coupled pro-inflammatory signaling involved in monocyte recruitment and smooth muscle cell (SMC) migration.

Specific Results

Immunization with the P3 peptide efficiently induces the production of anti-P3 antibodies (Abs) in rabbits**:** ELISA analyses showed that P3 Abs were absent in the serum of controls along the immunization period while they strongly raised in the serum of P3-immunized rabbits.

Anti-P3 Abs counteract high fat diet (HFD)-induced atherosclerosis in rabbits: HFD induced a high percentage of fatty streak lesions in the aorta of the control animals but not in those with Anti-P3 Abs in their plasma. The reduction of atherosclerosis in P3 immunized rabbits occurred concomitantly with a reduced content of lipids, and reduced number of macrophages/SMCs in the arterial intima of aorta.

Anti-P3 Abs reduce cholesterol accumulation and pro-inflammatory signals induced by high-fat diet (HFD) in the vasculature of rabbits without altering serum lipid levels or the lipoprotein profile: The biochemical analysis of serum evidenced that P3 immunization did not alter the plasma lipid levels and that cholesteryl esters (CE) in rabbits were mainly carried by LDL and VLDL lipoproteins in all rabbit groups. Consistently with the high impact of HFD on the VLDL and LDL cholesteryl ester content, HFD strongly increased the cholesteryl ester content of the aorta. Interestingly, HFD was unable to increase the aortic cholesterol content in P3 immunized rabbits. Molecular studies combined with confocal studies showed that HFD raised pro-inflammatory biomarkers such as tumor necrosis factor receptor 1 (TNFR1) and nuclear factor kappa beta (NF-kB) (p65) in the aorta of control group but not in the P3-immunized group. Together, our results indicate that anti-P3 Abs are highly efficient to inhibit HFD-induced cholesteryl ester accumulation and pro-inflammatory signaling in the vasculature of rabbits.

HFD serum from the P3-immunized rabbits, different from that of the control rabbits, failed to induce intracellular cholesteryl ester accumulation and pro-inflammatory signaling in human macrophages and human coronary vascular smooth muscle cells: Exposure of vascular cells to HFD serum (1%, 2 hours) from control rabbits but not from the P3-immunized rabbits dramatically raised the intracellular CE content in human macrophages (hMΦ) and human coronary vascular smooth muscle cells (hcVSMC). In addition, HFD serum from control rabbits promote a much higher induction of crucial inflammatory mediators, such as TNFR1 and pNF-kB (p65), than a chow serum in hMΦ and hcVSMC. Remarkably, P3 immunization efficiently restricted the pro-inflammatory effects of HFD serum. These results highlight the high efficiency of Anti-P3 Abs to inhibit HFD-induced pro-inflammatory signaling coupled with the intracellular cholesterol loading of hMΦ and hcVSMC.

PET/CT imaging studies show that Anti-P3 Abs reduce the cellular [^{18}F]-FDG uptake in the aorta of rabbits: PET-CT metabolic imaging performed in the Preclinical Platform of Vall d'Hebron Hospital showed that HFD significantly increased the mean of the standardized uptake value (SUVmean) in the upper and middle regions of the aorta of control rabbits. In contrast, in the P3-immunized rabbits, HFD only slightly induced the SUVmean in these aortic regions. The high impact of Anti-P3 Abs on the cellular [^{18}F]-FDG uptake confirmed molecular, confocal and immunohistochemical results showing an elevated anti-inflammatory potential of anti-P3 Abs in macrophages and smooth muscle cells in the vasculature.

Doppler ultrasonography imaging reveals that anti-P3 Abs prevent the HFD induced arterial resistance index in the carotids of rabbits: Doppler measurements were used to obtain the arterial resistance index (ARI), an indirect hemodynamic parameter, that reflects the resistance to blood flow in a vascular bed distal to the points of measurement. HFD increased ARI in both the external and internal carotid arteries of control rabbits. However, in the P3-immunized rabbits, HFD only slightly induced ARI in the internal carotid artery. As in the aorta, HFD dramatically induced cholesterol accumulation in the carotids of control rabbits and anti-P3 Abs significantly reduced cholesterol accumulation in the external but not in the internal carotid artery.

Stabilized P3 peptides (DP3) efficiently inhibit the process of LDL aggregation

Summary

Using a range of biochemical, biophysical and molecular experiments, here we demonstrate that the original version of P3 (LP3) and its retroenantio version (DP3) peptides, but not an irrelevant peptide (IP321) efficiently counteract LDL aggregation induced by the lipolytic enzymes sphingomyelinase (SMase) and phospholipase A2 (PLA_2). Although LP3 and DP3 efficiently counteracted alterations in LDL particle size, charge and sphingomyelin (SM) loss induced by SMase, they were unable to prevent the alterations induced in the same parameters by PLA_2. However, both peptides protected ApoB-100 from conformational alterations induced by SMase and PLA_2. Proteomics, *ab initio* modeling and molecular dynamics showed that these peptides establish stable electrostatic interactions with basic sequences of ApoB-100 keeping the conformation of ApoB-100 even under conditions of extreme lipolysis.

Specific Results

DP3, the retroenantio version of P3, shows higher stability than the parent peptide P3 (LP3) in human serum**:** The retroenantio version of the peptide was synthesized using D-aminoacids, the sequence is thus not recognized by serum proteases and its half-life considerably extends.

LP3 and DP3 block SMase and PLA_2- induced LDL turbidity in a time- and dose-response manner: LDL turbidimetry reflects LDL aggregation. LDL turbidimetry induced by SMase and PLA_2 was almost completely counteracted in the presence of peptides.

LP3 and DP3 decrease the percentage of aggregated LDL particles in SMase-LDL and PLA_2-LDL: Electron microscopy experiments revealed a reduction in the percentage of aggregated LDL particles when LDL was modified in the presence of peptides.

LP3 and DP3 block the alterations in LDL phospholipid composition induced by SMase but not by PLA_2 treatment of LDL**:** HPTLC was used to separate LDL phospholipids (PLs). This procedure revealed that the main PLs in the LDLs were L-α-phosphatidylcholine (L-α-PC) and sphingomyelin (SM). SM was completely removed from LDL by its treatment with SMase. In contrast, in the presence of LP3 and DP3, SM was protected against SMase. In contrast, PLA_2 completely removed L-α-PC from LDL, leading to a sharp increase in the L-α-LysoPC content even in the presence of the peptides.

LP3 and DP3 block the alterations in the LDL electrophoretic profile of SMase-LDL but not those of PLA_2-LDL**:** Gradient gel electrophoresis (GGE) analysis showed that SMase caused a strong loss of the band corresponding to LDL. This is because the pore size of GGE gels precludes the entrance of the largest LDL aggregates. The band loss induced by SMase was efficiently counteracted by LP3 and DP3 but not by IP321. In contrast, LP3 and DP3 did not have any effect on the GGE pattern induced by the treatment of LDL with PLA_2.

DP3 blocks the conformational changes of ApoB-100 in SMase-LDL and PLA_2-LDL**:** Western blot analysis allowed the determination of conformational modifications of ApoB-100 due to phospholipolytic treatment. Both SMase and PLA_2 increased the exposure of reactive ApoB-100 epitopes to anti-ApoB-100 antibodies. LP3 and DP3 protected ApoB-100 against the higher reactivity to anti-ApoB-100 antibodies induced by these enzymes. IP321 had no effect on the increased ApoB-100 reactivity induced by the two enzymatic treatments.

LP3 and DP3 electrostatically interact with specific lysine enriched sequences in ApoB-100 protein**:** Limited proteolysis combined with mass spectrometry-based proteomics (LC-MS/MS) evidenced that DP3 protects specific sequences of ApoB-100 in LDL either by direct interaction or by remodeling the protein structure. The high coincidence between sequences protected by DP3 and those protected by aggregation indicates that most DP3-protected sequences participate in LDL aggregation, thereby explaining the high efficiency of LRP1-derived peptides to prevent LDL aggregation. An energy-based rigid body docking experiment was performed between ApoB-100 protein and DP3 peptide structures previously modeled alone by *ab initio* methods. Interestingly, we found that one of the lowest-energy poses of the docking simulations accommodated DP3 in such an orientation that ApoB-100 Lys3228, Lys3231 and Lys3233 (but not Lys3226 nor Lys3236) were deeply buried upon peptide binding. This finding is in strong agreement with our trypsin digestion data results.

Low-density lipoprotein receptor-related protein 1 deficiency in cardiomyocytes reduces susceptibility to insulin resistance and obesity in a murine model

Summary

A murine model developed by our group with conditional and specific induction of *Lrp1* deficiency in cardiomyocytes was essential to demonstrate that *Lrp1* expression in cardiomyocytes regulates the circulating levels of atrial natriuretic peptides and that, through this mechanism, controls NPRA-dependent fatty acid metabolism in the liver and whole-body metabolism.

Specific Results

Cardiomyocyte *Lrp1* deficiency prevents diet-induced overweight and glucose intolerance by facilitating increased energy expenditure: The body weight of *cm-Lrp1*−/− mice was lower than that of the controls at all tested times. The weight-reducing effect of *Lrp1* deficiency was associated to decreased white adipose tissue and white/brown adipocyte size. While the triglyceride (TG) and the fatty acid (FA) content was reduced in the liver of *cm-Lrp1*−/− mice, no differences were found in the heart and skeletal muscle between groups. To know whether these phenotypic changes elicit a favorable metabolic profile, we conducted a tolerance test for glucose (TTG) test. Compared with controls, *cm-Lrp1*−/− mice had lower glucose intolerance and lower area under the curve (AUC) values. Glucose, insulin and insulin resistance (IR) index (HOMA-IR) were also reduced in *cm-Lrp1*−/− compared to control mice. Comprehensive Lab Animal Monitoring System (CLAMS) experiments evidenced that *cm-Lrp1*−/− mice showed higher VO_2 and energy expenditure than controls during both light and dark phases.

Lrp1 deficiency cardiomyocytes show enhanced corin activity that favors higher ANP release to the plasma: To identify cardiac proteins potentially involved in the favorable metabolic phenotype of *cm-Lrp1*−/− mice, we performed proteomic analysis of hearts from both *cm-Lrp1*−/− and *cm-Lrp1*+/+ mice groups. Western blot analysis confirmed the differences obtained by proteomics (decreased levels of proANP in hearts from *cm-Lrp1*−/− mice) and suggest that there is higher release of ANP from LRP1- hearts. Immunoprecipitation studies evidenced that serpins form complexes with corin in the murine heart and that the number of serpinA1/corin complexes were higher in the heart of *cm-Lrp1*−/− mice compared to that of controls. Serpin1 binding to corin creates steric impediments to the union of effective inhibitors and allows prolonged corin activation. Consequently, we observed higher ANP levels in the plasma of *cm-*

Lrp1−/− than in that of *cm-lrp1*+/+ mice. Our results suggest that cardiac Lrp1 deficiency facilitates corin activation and ANP release.

The ANP-NPR-A signaling activation is linked to AMPK activation and increased FA oxidation in the liver of *cm-Lrp1*−/− mice: the levels of crucial mediators of the ANP receptor, natriuretic peptide receptor A (NPRA), such as cyclic guanosine monophosphate (cGMP) and vasodilator-stimulated phosphoprotein (pVASP) were analyzed in peripheral tissues including the liver, the skeletal muscle and the heart of *cm-Lrp1*+/+ and *cm-Lrp1*−/− mice. We found increased levels of cGMP and pVASP in the liver and skeletal muscle of *cm-Lrp1*−/− mice that were abolished by A71915, an antagonist of NPRA, totally in the liver and partially in the skeletal muscle. In addition, cGMP/ pVASP signaling was linked to increased phosphorylation of AMP-activated protein kinase (AMPK) in the liver. The activation of AMPK was confirmed by the increased phosphorylation of the acetyl-CoA carboxylase (ACC), the downstream target of pAMPK, in the liver of untreated *cm-Lrp1*−/− compared to the control mice. To explore the potential impact of pAMPK activation on hepatic FA oxidation, the levels of carnitine palmitoyltransferase I (CPT1), a key mitochondrial enzyme responsible for FAO and those of the main components of the oxidative phosphorylation system (OXPHOS), responsible for mitochondrial respiration, besides those of uncoupling protein (UCP3), an indicator of mitochondrial response to enhanced intracellular FA, were analyzed by Western blot. Increased levels of these mediators were found in the liver of *cm-Lrp1*−/− and abolished by treatment of the animals with the NPRA antagonist. These results demonstrate increased ANP signaling underlies the exacerbated fatty acid oxidation in the liver of *cm-Lrp1*−/− mice.

Activated ANP-NPR-A signaling is linked to increased FA uptake by the liver: Oral fat gavage (OFG) experiments showed an increase in [^{3}H]-TG and [^{3}H]-FFA uptake by the liver of *cm-Lrp1*−/− mice that was blocked by the NPRA antagonist A71915. In contrast to liver, there was a reduced uptake of [^{3}H]-TG and [^{3}H]-FFA by white adipose tissue of *cm-Lrp1*−/− mice. A71915, an NPRA inhibitor, equaled weight and adipocyte size of white adipose tissue between *cm-Lrp1*−/− and *cm-Lrp1*+/+ mice. CLAMS experiments showed that treatment of *cm-Lrp1*−/− mice with the NPRA antagonist counteracted increased lipid oxidation during the light phase and increased glucose oxidation during the dark phase. There were no differences in oxygen consumption and energy expenditure between *cm-Lrp1*−/− and control mice treated with the NPR-

antagonists. These results clearly evidenced that ANP signaling underlies the increased capacity of the liver of *cm-Lrp1−/−* to take and oxidize fatty acids, and through this mechanism, control the whole-body metabolism.

INNOVATIVE OMIC DIAGNOSTIC TOOLS

sLRP1 is a biomarker of cardiovascular risk: In collaboration with the IMIM Group of Cardiovascular and Genetic Epidemiology (Dr. Marrugat and Dr. Elosua), our group demonstrated that the circulating levels of lrp1 (slrp1) predict future cardiac events in the REGICOR population (Registre Gironí del Cor). The increase of one unit in the circulating levels of sLRP1 receptor translates into a 40% increase in cardiovascular risk independently of other risk factors. This is because this biomarker reflects complementary, local mechanisms to which this receptor is connected.

sLRP1 is a biomarker of epicardial fat extension in general population and type 1 diabetics: Molecular studies in plasma combined with atherosclerotic plaque characterization by coronary tomography angiographic studies showed that sLRP1 reflects the extent of epicardial fat in different populations.

microRNAs as new biomarkers of subclinical atherosclerosis and epicardial fat extension: By complementary molecular studies performed by our group combined with multidetector computed tomography studies performed by the Cardiovascular Imaging Unit of Sant Pau hospital, we have identified innovative biomarkers in plasma reflecting subclinical atherosclerosis and epicardial fat extension.

Non-coding RNAs as biomarkers of intramyocardial lipid accumulation: Molecular studies carried out with plasma samples from patients undergoing determination of intramyocardial lipid determination and cardiac remodeling using magnetic resonance imaging in the context of the international study PIRAMID (Pioglitazone Influence on triglyceride Accumulation in the Myocardium In Diabetes) allowed us to identify new circulating biomarkers of intramyocardial lipid accumulation thanks to the collaboration with the group of Dr. Hildo Lamb (Leiden University Medical Center, Leiden, The Netherlands).

3. Relevance of possible future applications

Much of the results obtained throughout this project have been obtained by collaboration between our core group and clinical groups attracted by the potential clinical impact and translational nature of our research. To highlight our active collaboration with Dr. Bayes Genis, Director of the Institute of Cardiology and the Department of Cardiology at the Germans Trias i Pujol Hospital. Our close collaboration with the Cardiac Imaging Unit (Dr. Carreras; Dr. Leta and Dr. Vilades) and with the Endocrinology Unit (Dr. Pérez and Dr. Gil) of the Hospital de la Santa Creu i Sant Pau has also been highly productive. Lastly, it is worth highlighting the relevance of the results obtained by the collaboration with the IMIM Cardiovascular Epidemiology group (Dr. Elosua and Dr. Marrugat) and with clinical specialists in Nuclear Medicine (Dr. Castell) at the Hospital Universitari Vall D'Hebrón. The active participation of clinicians in this project has contributed to the high translational nature of the results obtained.

The main advances resulting from this project are: 1) new therapeutic tools (antibodies and peptides) to specifically modulate the pathological function of LRP1 without altering its essential functions, useful in arteriosclerosis and probably in other diseases in which tissue accumulation of cholesterol is determinant in the progression of the disease; 2) new *in vivo* models such as a murine model of specific and conditional *Lrp1* deficiency in cardiomyocytes and a new rabbit model with protective immunization against arteriosclerosis; 3) *in vivo* evidence of a new heart-liver axis controlled by *Lrp1* expression levels in cardiomyocytes that determines ANP signaling, favoring greater uptake and oxidation of fatty acids by the liver; 4) new biomarkers of cardiac remodeling and 5) new omic and protein biomarkers with predictive value of coronary risk in the general population, closely associated with subclinical atherosclerosis and epicardial fat extension.

The high productivity, quality and translational nature of the publications obtained by the research carried out give this project a key role in the evolution of our lines of research towards the clinic, since they provide new tools to intervene in new crucial mechanisms underlying atherosclerosis, diabetic and dilated ischemic heart disease, pathologies of increasing prevalence in western societies. Also note that this project has placed our group in a favorable position to generate new economic resources from both public and private agencies.

To advance with the results of the 201521-10 project of the Fundació La Marató TV3, we intend to carry out the following experimental developments in the short-medium term: 1) purify anti-P3 antibodies and validate their anti-atherosclerotic efficacy in preclinical models (PCSK9 mini-pigs) of arteriosclerosis; 2) use the purified antibodies as a new tool for the detection of therapeutic peptides in new studies of peptide biodistribution and efficacy that we plan to carry out in preclinical models; 3) to compare the efficacy of the antibodies and peptides developed in the current project with the lipid-lowering drugs normally used in clinical practice, as well as to determine if the new therapeutic tools work in a complementary way to the usual ones. To progress in the study of extracellular matrix biomarkers carried out in collaboration with Dr. Samouillan's group (Toulouse University), we plan to validate the new biomarkers identified in a porcine model in human ventricles obtained from autopsies and explanted hearts. Our collaboration with the Pathological Anatomy and Cardiology Services of the Hospital de la Santa Creu i Sant Pau makes these samples already available to analyze the potential of these new biomarkers in human samples. In collaboration with Dr. Laurent Duca, we plan to explore the role of elastin degradation, and particularly elastin-derived peptides, in the generation of foam cells in *in vitro* and *in vivo* models. In order to validate the new omic and proteomic biomarkers identified, we have established new collaborations with clinicians who have available samples of patients characterized from the cardiovascular and metabolic point of view. We have already started collaborations with Dr. González Juanatey (Servei de Cardiologia del Complex Hospitalari Universitari de Santiago) with Dr. Masana (Facultat de Medicina i Ciències de la Salut de Reus) and Dr. Guerra (Departament de Cardiologia de l 'Hospital de la Santa Creu i Sant Pau).

4. Scientific Publications

Olga Bornachea, Aleyda Benitez-Amaro, Angela Vea, Laura Nasarre, David de Gonzalo-Calvo, Juan Carlos Escola-Gil, Lidia Cedo, Antoni Iborra, Laura Martínez-Martínez, Candido Juarez, Juan Antonio Camara, Carina Espinet, Maria Borrell-Pages, Lina Badimon, Joan Castell, Vicenta Llorente-Cortés. Immunization with the Gly^{1127}-Cys^{1140} amino acid sequence of the LRP1 receptor reduces atherosclerosis in rabbits. Molecular, immunohistochemical and nuclear imaging studies. *Theranostics* 2020; 10: 3263-3280. IF:8.712

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