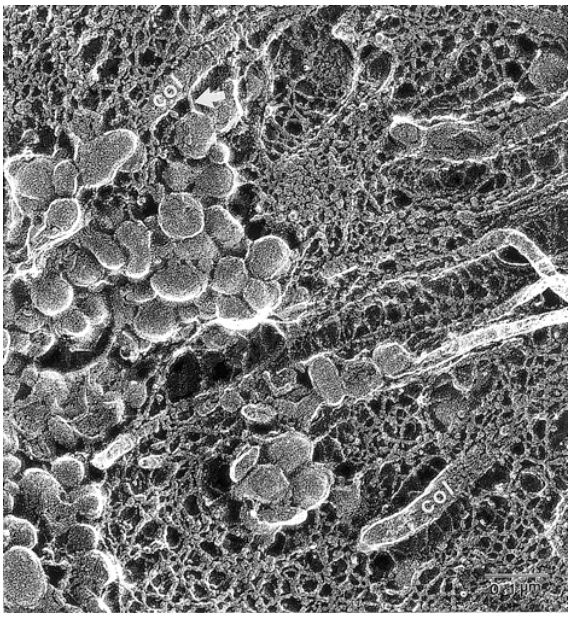
FATTY ACIDS INDUCE POTENTIALLY ATHEROGENIC CHANGES IN EXTRACELLULAR MATRIX PROTEOGLYCANS



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Cover image: Freeze-etch electron microscopy demonstrating LDL retention in the extracellular matrix of the intima of the apolipoprotein E–deficient mice (taken from [1] with permission of Prof. Joy Frank).

A mi querida familia,

ABSTRACT

Insulin resistance and type 2 diabetes (T2D) are associated with an increased prevalence of atherosclerotic cardiovascular disease (CVD). A key step in the initiation of atherogenesis is retention of low density lipoproteins (LDL) in the intima by chondroitin sulfate (CS)-rich proteoglycans (PG). In addition, heparan sulfate (HS)-containing PGs in liver have a major physiological function in the retention and internalization of chylomicron and very low density lipoprotein (VLDL) remnant particles. Elevated levels of circulating non-esterified fatty acids (NEFA) are part of the dyslipidemia of insulin resistance and T2D. The objective of this thesis was to investigate the impact of chronic NEFA elevation on extracellular matrix (ECM) PGs and its implications for diabetes-associated CVD.

Linoleate incubation decreased the PG secretion and gene expression of the core proteins of versican, and to a lesser extent, syndecan-1, in the human liver-derived cell line HepG2. The GAG chains of secreted PGs had a lower CS content. PGs extracted from livers of the insulin-resistant hyperlipidemic Zucker *fa/fa* rat carried GAG chains with increased CS content at the expense of HS compared with livers from their lean littermates. These changes resulted in a reduced capacity to bind β-VLDL remnant particles. Incubation of primary cultures of human arterial smooth muscle cells with linoleate or palmitate increased the gene expression of the core proteins of versican, biglycan, perlecan and decorin; and of CS synthase, CS-6 and CS-4 sulfotransferases, key enzymes for the polymerization and sulfation of chondroitin chains. These effects were accompanied by increased PG secretion. Secreted PGs were enriched in chondroitin/dermatan sulfate and with a higher extent of sulfation, resulting in a more efficient binding of LDL compared with PGs from control cells. Investigation of the signaling pathways involved in the linoleate-induced increase of versican expression showed that it was mediated by an extracellular signal-regulated kinase (ERK)-dependent activation of the transcription factor CCAAT-enhancer binding protein (C/EBP)β. These findings suggest a novel role for C/EBPβ in the fatty acid-induced changes of ECM.

Chronic NEFA elevation resulted in changes in the properties of matrix PGs that may contribute to generate a remnant-rich dyslipidemia and to pre-condition the arterial intima for lipoprotein deposition. Therefore, normalization of the fatty acid homeostasis should be considered a key target in the treatment of the atherogenic dyslipidemia of insulin resistance.

Keywords: proteoglycans, glycosaminoglycans, fatty acids, smooth muscle cells, hepatocytes, LDL binding, remnant clearance, insulin resistance, type 2 diabetes

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I Olsson U, Egnell A-C, Rodríguez-Lee M, Östergren-Lundén G, Lorentzon M, Salmivirta M, Bondjers G and Camejo G: Changes in matrix proteoglycans induced by insulin and fatty acids in hepatic cells may contribute to dyslipidemia of insulin resistance
Diabetes 50:2126-2132; 2001

II Rodríguez-Lee M, Östergren-Lundén G, Wallin B, Moses J, Bondjers G and Camejo G: Fatty acids cause alterations of human arterial smooth muscle cell proteoglycans that increase the affinity for LDL
Arterioscler Thromb Vasc Biol 26(1):130-135; 2006

III Rodríguez-Lee M, Wallin B, Ohlsson B, Bondjers G and Camejo G: Fatty acids induce potentially atherogenic versican expression in human arterial smooth muscle cells through ERK activation of CCAAT enhancer-binding protein beta
Submitted 2007

CONTENTS

ABBREVIATIONS

ADAMTS	a disintegrin and metalloproteinase with thrombospondin motif
C/EBP	CCAAT-enhancer binding protein
CS	chondroitin sulfate
CVD	cardiovascular disease
DAG	diacylglycerol
DS	dermatan sulfate
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
GAG	glycosaminoglycan
GalNAc	N-acetyl-galactosamine
GlcA	glucuronic acid
GlcNAc	N-acetyl-glucosamine
hASMC	human arterial smooth muscle cell
HS	heparan sulfate
IdoA	iduronic acid
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
Ndst	N-deacetylase/N-sulfotransferase
NEFA	non-esterified fatty acids
PG	proteoglycan
PI3K	phosphoinositol 3-kinase
PKC	protein kinase C
PKD	protein kinase D
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
sdLDL	small, dense LDL
SLRP	small leucine-rich proteoglycan
SMC	smooth muscle cell
T2D	type 2 diabetes
TRL	triacylglycerol-rich lipoprotein

INTRODUCTION

The Extracellular Matrix

Historical perspective

The extracellular matrix (ECM) is a complex structural entity surrounding and supporting the cells in tissues. Under a variety of other names, it preceded the discovery of cells by centuries, even thousands of years. Before the cell theory, the fiber theory (~1700) held that tissues and organs were composed of fibers that somehow were alive and that life arose by spontaneous generation. It was not until the middle of the 1800s that the observations of many histologists contributed to the cell theory. Rudolf Virchow, is perhaps best known for his theory *Omnis cellula ex cellula* ("every cell originates from another existing cell like it") which he published in 1858. He stated that cells arise only from cells*,* a revolutionary concept at the time, and suggested that intercellular substances were made by cells. At the end of the XIX century, the intercellular space was seen in the light microscope to contain fibers, mostly collagen but also elastin in some tissues, and a homogeneous background referred to as ground substance *(grundsubstanz).* It was only when various macromolecules began to be characterized in the 1930s by chemical and physicochemical methods that the term "extracellular matrix" came into use.[2]

Components and functions

The ECM determines the physical characteristics of tissues and many of the biological properties of cells embedded in it. The major components of the ECM are fibrous proteins that provide tensile strength (*e.g.*, various collagens and elastin), multi-adhesive matrix proteins (*e.g.*, fibronectin, laminin, and tenascin) that attach cells to diverse components of the ECM, and proteoglycans (PGs) that are largely responsible for the volume of the ECM. PGs constitute a hydrated gel which resists compressive forces and permit diffusion of small molecules and macromolecules between cells and tissues. Another important component of the matrix is hyaluronan, a large polysaccharide that forms a highly hydrated gel, making the matrix resilient to compression. Different combinations of these components tailor the strength of the ECM for different purposes. The ECM should not be viewed as merely providing strength and physical support for tissues and organisms, as this matrix exerts profound influences on

the embedded cells. The ECM is a reservoir for a wide range of biomolecules such as growth factors, enzymes, cytokines and hormones. In addition, it communicates directly or indirectly with the intracellular signaling pathways that direct a cell to carry out specific functions. The matrix also provides a mesh through which cells can move.[3, 4]

The cells in the matrix are immersed in the interstitial fluid. The interstitial fluid is that part of the extracellular fluid that is outside the vascular system, bathing the cells, and contains components filtered from plasma, lymph, and other components locally secreted by cells.[5, 6] The extracellular fluid is not considered to be part of the ECM, since the word "matrix" refers mainly to the macromolecular network.

Proteoglycans

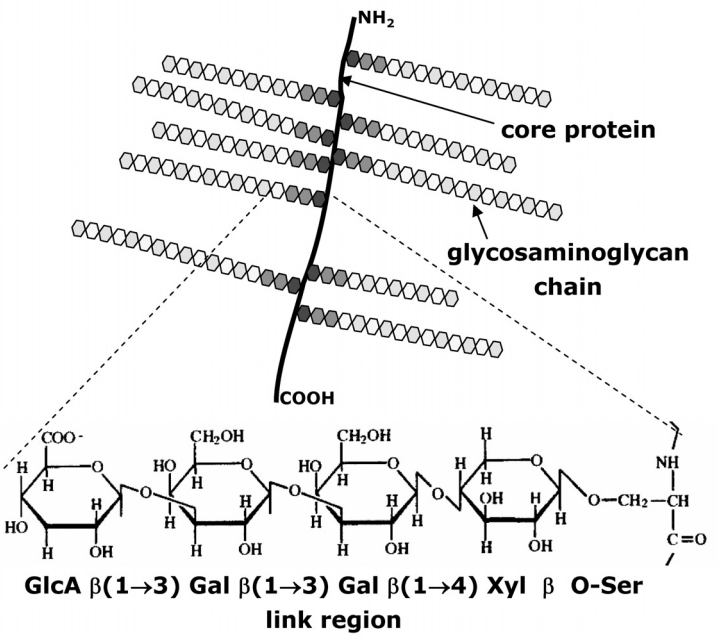
Historical perspective

The study of PGs dates back to 1861 when chondroitin sulfate (CS) was isolated from cartilage tissues. From 1930 to 1950, great progress was made in analyzing the chemistry of the polysaccharides of these preparations (also known as "mucopolysaccharides"), notably by Karl Meyer and colleagues, who described the structure of hyaluronan, dermatan sulfate (DS), keratan sulfate (KS), and different isomeric forms of CS. In 1948, Jorpes and Gardell described the chemical structure of heparin and heparan sulfate (HS). These polysaccharides eventually came to be known as glycosaminoglycans (GAGs) to indicate the presence of amino sugars and other sugars in a polymeric form. In 1958, Helen Muir provided the first evidence of a covalent association between GAGs and proteins. In 1966, Rodén and Lindahl established the structure of the GAG-core protein link region. These structural studies together paved the way for the biosynthetic studies that followed.[7-9]

Structure

PGs are macromolecules characterized by the presence of one, or more, long unbranched and highly polyanionic sugar side-chains called GAGs that are covalently attached to a core protein through a link region (Fig. 1). The repeating unit of the GAG consists of an amino sugar, either N-acetyl-glucosamine (GlcNAc) or N-acetyl-galactosamine (GalNAc); and a hexuronic acid, either glucuronic acid (GlcA) or iduronic acid (IdoA)[10]. One or both of the sugars in the repeating unit contain one or more sulfate groups. This basic structure of the PG has been found widely among vertebrates and invertebrates, suggesting essential functions in animal physiology.[11, 12]

Figure 1. Model of the basic structure of a proteoglycan.



In addition to the GAG chains, most core proteins carry N- and/or O-linked oligosaccharides, similar to those present in other glycoproteins.[13, 14] These oligosaccharides are much shorter than GAGs (*e.g.*, a 20-kD GAG chain contains ~80 sugar residues, whereas a typical biantennary N-oligosaccharide may contain 10–12 residues).

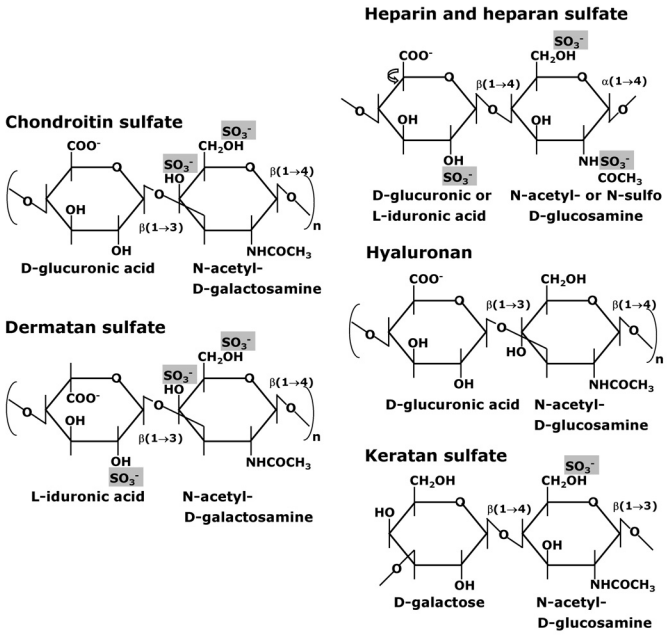
Classification and nomenclature

PGs are a very heterogeneous group of macromolecules. Even a single type of core protein can vary greatly in the number and type of attached GAG chains. The length of the chains also varies and the arrangement of sulfated residues along the chains differs.

Four main classes of GAGs are distinguished according to the structure of the repeating disaccharide unit: (1) CS and DS, (2) HS and heparin, (3) hyaluronan, and (4) KS[10] (Fig. 2).

Hyaluronan (also called hyaluronic acid or hyaluronate) is the simplest of GAGs. In contrast to all of the others, it contains no sulfated sugars, all its disaccharide units are identical, its chain length is enormous (thousands of sugar monomers), and it is not linked covalently to any core protein.[15]

Figure 2. Structure of the glycosaminoglycans.



KS is a sulfated polylactosamine chain identical to the type found in conventional glycoproteins and mucins. Their linkage to protein distinguishes two types of KS. KS I, originally described in cornea, is N-linked to asparagine residues in the core protein, whereas KS II or cartilage KS, is O-linked to serine or threonine residues.[16]

PGs can be classified according to several parameters: (1) attached GAG chain (CS/DS- or HS-containing PGs), (2) topographical distribution in relation to the cell (extracellular and basement membrane PGs, cell-associated PGs or intracellular PGs), and (3) core protein homology (hyalectans, small leucine-rich PGs (SLRPs), syndecans, glypicans among others).[10]

Chondroitin/dermatan sulfate proteoglycans

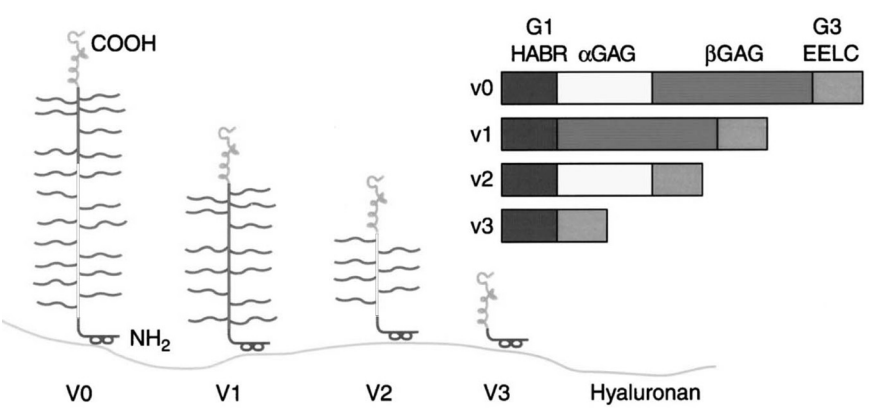
Versican belongs to the family of hyaluronan-binding proteoglycans that include aggrecan, neurocan and brevican, constituting a gene family collectively termed hyalectans.[10, 17, 18] Each family member has a characteristic distribution, with aggrecan prominent in cartilage, neurocan and brevican prominent in the central nervous system, and versican present in a variety of soft tissues. The gene and protein structure of versican follows a domain template. The amino-terminal globular end (G1) binds to the GAG hyaluronan, and the carboxy-terminal globular domain (G3) resembles the selectin family of proteins, consisting of a C-type lectin adjacent to two epidermal

growth factor (EGF) domains and a complement regulatory region. The middle region of the versican core protein is encoded by two large exons that specify the CS attachment regions of versican. The region encoded by exon 7 is called αGAG, the region encoded by exon 8, βGAG.[19] Four mRNA transcripts arise from alternative splicing, giving rise to V0, V1, V2 and V3 which differ in the length of the core protein and the number of attached GAGs[18] (Fig. 3). The consensus sequence for CS attachment sites reveals the number of potential GAG attachment sites in human versican to be 17–23 for V0, 12–15 for V1, 5–8 for V2, and 0 for V3.[20]

The amino- and carboxy-terminal features of versican together with the GAGs attached to the middle portion of the core protein make versican a highly interactive and versatile molecule, and that is the basis for its given name.[21]

Decorin and biglycan are members of a separate gene family, the SLRPs. The family comprises at least nine members grouped into three classes (I, II and III) and different subfamilies, and are all characterized by the presence of a central domain containing leucine-rich repeats to achieve strong protein-protein interactions. Decorin and biglycan, in class I, show the highest amino acid homology of the family (~57%) and are the only SLRPs with a propeptide. The propeptide is highly conserved across species and may function as a recognition sequence for xylosyltransferase, the first enzyme involved in the synthesis of the GAG chain. These two SLRPs contain CS side-chains attached to the amino-terminal domain of the core protein (one in decorin and two in biglycan) that are characterized by epimerization of some of the glucuronyl residues to iduronyl groups.[22]

Figure 3. Representation of the different isoforms generated by alternative splicing of the mRNA transcript for versican (taken from [20]).



Versican, decorin and biglycan are the major CS/DS PGs in the matrix of the mammalian arterial wall.[23] Versican V0 core protein (370 kDa) is roughly 10 times the size of the decorin (36 kDa) or biglycan (38 kDa) core proteins. Side-chains show a wide range of sizes, but generally average around 40-90 kDa each.

Heparan sulfate proteoglycans

Five distinct classes of cell-associated and pericellular HSPGs account for at least 95% of the HS of mammalian cell surfaces, basement membranes and ECMs. The cell-associated HSPGs include four integral membrane syndecans and six glycosyl-phosphatidylinositol (GPI)-anchored glypicans. The three pericellular HSPGs include perlecan, agrin and the hybrid HSPG/collagen type XVIII. Although not directly linked with the plasma membrane, these PGs are sometimes termed as pericellular PGs rather than extracellular PGs because of their close association with the plasma membrane via integrins.[24]

Syndecans are a family of PGs consisting of a type I transmembrane core protein that bears HS chains distal from the plasma membrane, and sometimes CS chains near the membrane. All nucleated cells express one or more family members. Syndecan-1 is the major syndecan of epithelial cells including vascular endothelium, syndecan-2 is present mostly in mesenchymal, neuronal and smooth muscle cells (SMCs), syndecan-3 is the major syndecan of the nervous system, while syndecan-4 is ubiquitously expressed but at lower levels than the other syndecans. Each syndecan has a short cytoplasmic domain, a transmembrane domain (TM), and an extracellular domain with attachment sites for three to five GAG chains. The core proteins range in size from 20 to 45 kDa. Although their extracellular domains are highly divergent apart from regions bearing the GAG chains, the TM is highly conserved and plays a crucial role in syndecan oligomerization. All syndecans probably exist as homodimers and higher-order oligomers.[25, 26]

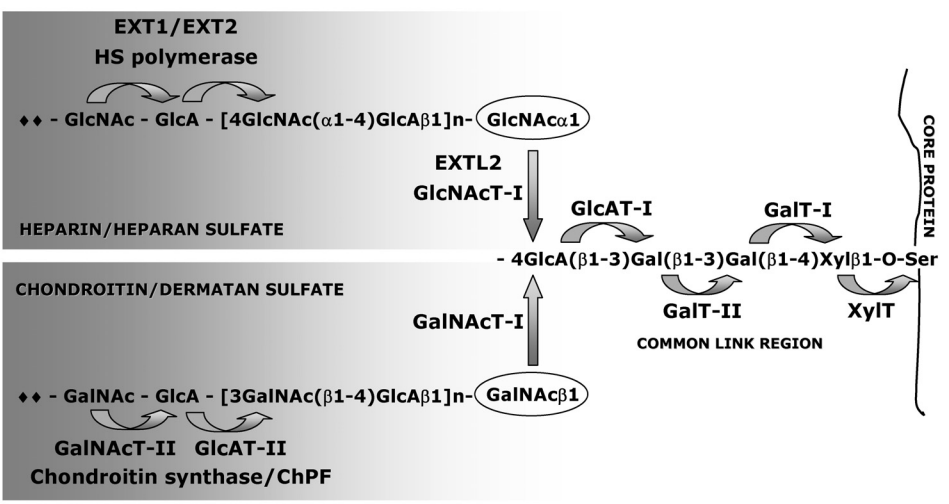
Perlecan is a modular HSPG that is expressed in nearly all basement membranes as well as mesenchymal organs and connective tissues, and is one of the largest single-chain polypeptides found in vertebrate and invertebrate animals. The five modules of perlecan and its HS chains can take part in a large number of molecular interactions such as with fibroblast growth factor-2, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and other matrix proteins. The core protein of human perlecan is ~470 kDa and, together with numerous O-linked oligosaccharides and as many as four HS chains (three in domain I and one potential chain in domain V), it can reach a molecular weight of over 800 kDa.[27, 28]

Biosynthesis and turnover

The cell takes up the building blocks for GAG synthesis, monosaccharides and sulfate, through specialized transporters in the plasma membrane. Sugars and sulfate are then activated by nucleotide consumption in the cytosol to form uridine diphosphate (UDP)-sugars and 3'-phosphoadenosine 5'-phosphosulfate (PAPS), respectively, that are then translocated into the endoplasmatic reticulum (ER) and Golgi lumens by specific transporters.[29, 30]

GAGs are built up on the so-called linkage region tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-, attached to specific serine residues of core proteins while they are adherent to the inner surface of ER/Golgi vesicles. The synthesis of this region is initiated by the addition of xylose (Xyl) to serine in the ER, followed by the addition of two galactose (Gal) residues and the first GlcA residue by specific glycosyl transferases in the early cis/medial regions of the Golgi. The first hexosamine transfer (*i.e.* the fifth residue) is critical in determining whether CS/DS or heparin/HS chains are selectively assembled. Chondroitin/dermatan sulfate is synthesized when GalNAc is transferred to the common link region, whereas heparin/HS is formed if GlcNAc is added (Fig. 4).[29, 30]

Figure 4. Biosynthesis of GAG chains (adapted from [30]).



Distinct sets of enzymes in different subcompartments of the Golgi complex are involved in HSPG and CSPG synthesis. The glycosyltransferases responsible for heparin/HS biosynthesis are encoded by five members of the *EXT* gene family, the hereditary multiple exostoses gene family of tumor suppressors. A family of four glycosyltransferases are responsible for initiation and elongation of chondroitin/dermatan sulfate chains (Table 1).

Table 1. Summary of the human glycosyltransferases involved in CS and HS polymerization.

Name	Specificity	Function
chondroitin / dermatan sulfate		
Chondroitin synthase [31]	GalNAcT-II GlcAT-II	chain elongation
Chondroitin GalNAc transferase-1 [32, 33]	GalNAcT-I GalNAcT-II	initiation chain elongation
Chondroitin GlcA transferase [34]	GlcAT-II	chain elongation
Chondroitin GalNAc transferase-2 [35]	GalNAcT-I GalNAcT-II	initiation chain elongation
Chondroitin polymerizing factor (ChPF) [36]	-	specific activating factor for chondroitin synthase
heparin / heparan sulfate		
EXT1/EXT2 [37-39]	GlcNAcT-II GlcAT-II	chain elongation
EXTL1[40]	GlcNAcT-II	chain elongation
EXTL2 [41]	GlcNAcT-I	initiation
EXTL3 [40]	GlcNAcT-I GlcNAcT-II	initiation chain elongation

GalNAcT: GalNAc transferase; GlcAT: GlcA transferase; ChPF: chondroitin polymerizing factor; GlcNAcT: GlcNAc transferase; EXT: exostosin; EXTL: EXT-like

Sulfation of the chondroitin polymer by specific sulfotransferases occurs as the polymer is being formed. Chondroitin 6-sulfotransferase (C6ST)[42, 43] and chondroitin 4-sulfotransferase (C4ST)[44] catalyze the sulfation of position 6 and 4, respectively, of the GalNAc residues of chondroitin. All the enzymes in the biosynthetic pathway have been cloned, with the exception of the glucuronyl to iduronyl epimerase involved in the formation of dermatan chains.

During HS polymerization, a series of modification reactions occurs by processing enzymes located in the Golgi apparatus. A family of four GlcNAc N-deacetylase/N-sulfotransferases (Ndsts) removes acetyl groups from subsets of GlcNAc residues, and adds sulfate to the free amino groups. An epimerase converts adjacent GlcA units to IdoA, and then a group of O-sulfotransferases adds sulfate to C6 and C3 of glucosamine units and C2 of uronic acids. These modifications occur in contiguous blocks of sugars along the chain in an incomplete manner, resulting in domains of variable size and sulfation.[24]

Extracellular proteinases of diverse gene families, principally metallo-, serine-, and cysteine- proteinases can degrade the core protein of PGs.

The matrix metalloproteinases (MMPs) are active at neutral pH and can therefore catalyze the normal turnover of ECM macromolecules such as the interstitial and basement membrane collagens, accessory ECM proteins such as fibronectin, and proteoglycans.[45] MMP-1, -2, -3, -7 and -9 are involved in the breakdown of versican.[46-48] MMP-2, -3 and -7 cleave decorin and MMP-3 and -13 perlecan.[49] The proteolytic activities of MMPs are precisely controlled during activation from their precursors and inhibited by endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).[50]

Members of the MMP family include the extracellular ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif).[45] An important difference between ADAMTSs and MMPs is the ability of many ADAMTSs, *e.g.* ADAMTS-1, -4 and -9, to bind to the ECM.[51] ADAMTS-1, -4, -5 and -9 have been reported to cleave either native versican or versican peptide substrates.[52-55] When processed at the C-terminus, ADAMTS-4 is also active against decorin core protein.[56] Like the MMPs, the ADAMTS members can be inhibited by TIMPs.

Plasmin has been shown *in vitro* to degrade native versican,[57] and endothelial cell-derived perlecan.[58] Cathepsins are also involved in the degradation of PGs.[59-62]

In addition to proteolytic cleavage of the core protein, the GAG chains are also enzymatically digested. Endoglycosidases generally initiate the GAG degradation pathway. Released oligosaccharides, 10–20 sugar residues long, are then substrate for sulfatases that remove sulfate groups, which is essential for the subsequent action of exoglycosidases.[63] The GAG-core protein link region can also be degraded by endo-β-xylosidase and endo-β-galactosidase.

Only one mammalian heparanase appears to be responsible for digesting the HS chains.[64-67] The enzyme is an endo-β-D-glucuronidase (producing a GlcA at the newly formed reducing terminus), but the heparin/HS-sequence recognized and cleaved has not been established. Heparanase is mostly an intracellular lysosomal enzyme responsible for degrading internalized cell-associated HSPGs. However, it can also occur at the cell surface where it might digest adjacent HSPGs.[68]

Mammalian hyaluronidases are endo-β-acetyl-hexosaminidases that lack substrate specificity and can therefore act at a slow rate on CS chains.[69] The exoglycosidases in the mammalian degradation pathway of CS chains are β-glucuronidase, β-N-acetyl-galactosaminidase and α-L-iduronidase.[63]

GAG-lipoprotein interactions

Even though the PG core protein itself may display binding sites for specific ligands, the refined chemical structure of GAGs defines most of the PG binding properties, which are very diverse, leading to specific ligand recognition. Furthermore, multiple GAGs within a single PG molecule determine a large number of low-affinity high-capacity binding sites, turning PGs into extracellular reservoirs of their ligands.

Sulfate and carboxyl groups confer GAG chains with a highly negative charge. GAGs are the most anionic molecules produced by the animal cell. This property allows them to interact with various positively charged regions in many protein molecules, such as components of the coagulation cascade, growth factors, enzymes, cytokines and apolipoproteins exposing positive sequences in their surface.[70-74]

Role of liver heparan sulfate-containing proteoglycans on the clearance of triacylglycerol-rich lipoproteins

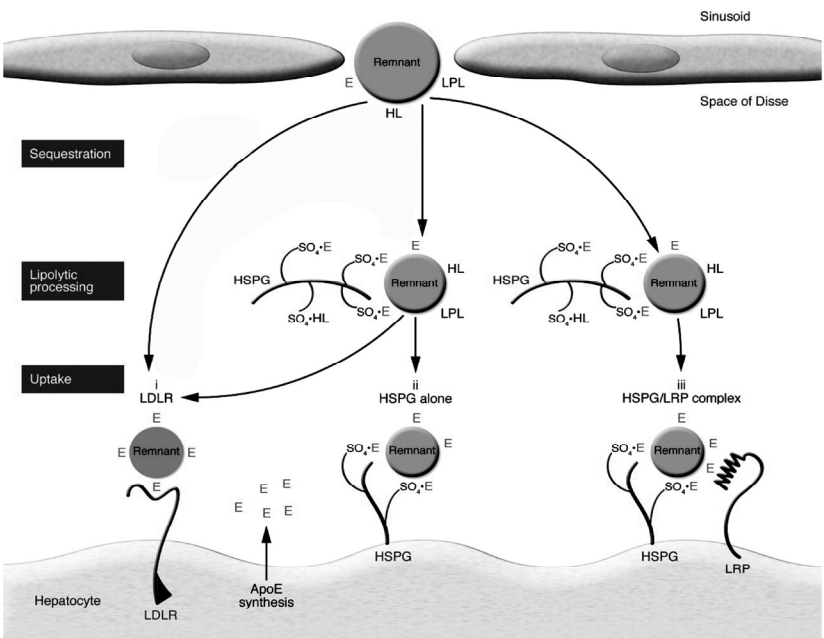
Exogenous lipids enter the circulation as large, buoyant chylomicrons, which arise in intestinal enterocytes and contain apolipoprotein (apo)B48. Endogenous lipids made in the liver appear in the circulation as very low density lipoproteins (VLDLs) that in humans contain apoB100 synthesized by hepatocytes. As these triacylglycerol-rich lipoproteins (TRLs) circulate, lipoprotein lipase (LPL) hydrolyzes the triacylglycerols in the core of the particles, resulting in the formation of chylomicron remnants (CRs) and intermediate-density lipoproteins (IDLs) from VLDLs. LPL also can remain associated with lipoprotein particles,[75] although most of the enzyme associates with HSPGs on the surface of endothelial cells.[76] TRL remnants also obtain apoE by transfer reactions from other lipoproteins in the circulation or by enrichment in the liver. ApoB48, apoB100, apoE, and LPL, have in common the capacity to bind HS.

Remnants are the largest of the lipoprotein particles, and they must enter the space of Disse. The space of Disse (or perisinusoidal space) is a location in the liver between a hepatocyte and a sinusoid. It is filled with membrane-bound HSPGs and secreted HSPGs from hepatocytes and endothelial cells and the microvillus projections from hepatocytes extending into this matrix.

HSPGs in the space of Disse bring all the participants in remnant clearance together (Fig. 5).[77-79] The first step is *sequestration*. The remnants enter the space of Disse through fenestrations in the endothelium and are captured there by interaction with HSPGs, the binding being mediated primarily through apoE. LPL and hepatic lipase (HL) may continue their *lipolytic processing*, preparing the particles for uptake by hepatocytes and assisting in binding the particles to HSPGs. ApoE synthesized and secreted by the liver serves as a reservoir for mediating binding and uptake. ApoE generated locally in the liver

functions better than peripherally produced apoE in mediating remnant clearance.[80] *Internalization* of the remnants involves several players: low density lipoprotein receptor (LDLR), HSPGs, and the HSPG/LDLR-related protein (LRP) complex that serve as receptors or co-receptors mediating remnant lipoprotein uptake. In the presence of LDLR, LPR has little or no physiological function in remnant clearance. It may function as a back-up system for LDLR.

Figure 5. Remnant lipoprotein clearance pathways (adapted from [79]).



Role of arterial wall chondroitin sulfate-containing proteoglycans on lipoprotein retention and atherogenesis

Several hypotheses have been proposed for the initiation of atherosclerosis in the last 150 years. The response-to-injury hypothesis,[81] stated that endothelial injury leads to an inflammatory response as part of a healing process in the arterial wall. Earlier experimental data linking arterial repair and atherosclerosis after mechanical injury were reported by Bondjers and Bjorkerud.[82-89] The response-to-oxidation hypothesis[90] proposed that lipoprotein oxidation is the important link in atherosclerosis.

Pioneering work by Mogen Faber[91] in 1949 showed that the interaction between cholesterol-containing lipoproteins and CS mucopolysaccharides accompanied lesion development in human arteries. Gero *et al.*[92] and Hollander *et al.*[93] provided additional experimental support to the importance of intima mucopolysaccharides

as agents that contribute to low density lipoprotein (LDL) retention and lesion development. In 1980, Camejo and Lalaguna[94] identified the main CS-containing PG contributing to complex formation in human arteries. This early work was extensively reviewed in 1982.[95] In 1995, Williams and Tabas[96] suggested that the subendothelial retention of cholesterol-rich lipoproteins within the arterial wall is the initiating step of atherogenesis and leads to oxidation, inflammation, and endothelial dysfunction, consistent with previous hypotheses.[81, 90] These concepts are known as the response-to-retention hypothesis. The hypothesis addresses the mechanism for the link between blood lipids and disease found in 1913 by Anitschkow and Chalatov in their cholesterol-fed rabbit model of atherosclerosis.[97]

Lipoproteins normally flux in and out the arterial wall. The retention of lipoproteins by CSPGs in the intima space determines the concentration of lipoproteins in the arterial wall. The interaction between lipoproteins and PGs involves an ionic interaction between basic amino acids in apoB100 and negatively charged sulfate groups on the GAG chains.[70, 98] Before lesions have appeared, lesion-prone arterial sites show enhanced lipoprotein retention,[99, 100] and purified CSPGs from these sites show enhanced binding affinities for atherogenic lipoproteins.[101] Several characteristics of CSPGs have been associated with higher *in vitro* LDL binding affinity such as longer side-chains,[102] and greater overall extent of sulfation.[103-105] Increased ratios of dermatan to chondroitin[104, 106] and of chondroitin 6-sulfate (C6S) to chondroitin 4-sulfate (C4S)[101, 103, 105-107] have also been associated with enhanced LDL affinity *in vitro*.

Binding studies *in vitro* indicate that LDL has a higher affinity to versican than to decorin or biglycan,[108] in part due to the large number of side-chains on each molecule of versican. This effect could be magnified *in vivo* by the macromolecular organization of versican with hyaluronan that creates complexes that contain hundreds of CS side-chains. In addition, because of its high content within the prelesional and lesional arterial wall,[109] versican appears to have the largest number of LDL binding sites. Biglycan, with two side-chains as compared with decorin, has also been associated to retention of lipoproteins in the arterial wall.[110] Decorin, although implicated as a participant in lipoprotein binding *in vitro*,[111] appears to have the least direct participation in lipoprotein retention, with a low affinity for lipoproteins in the absence of LPL *in vitro* and a less consistent colocalization with lipid accumulation *in vivo*.[110]

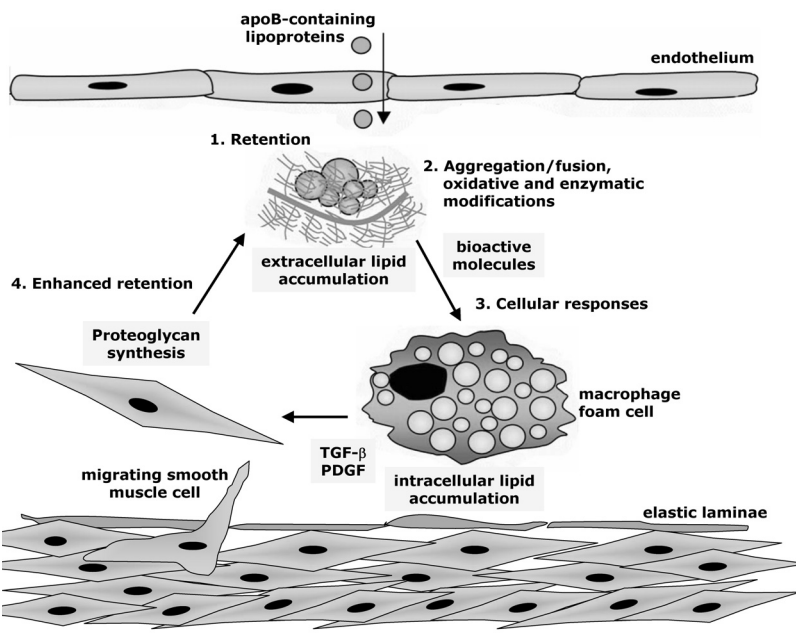
Once retained, atherogenic lipoproteins undergo several modifications with important pathophysiological consequences. The earliest modification is lipoprotein aggregation.[112, 113] Aggregation may occur when retained LDL is oxidized extensively[114, 115] or digested by several enzymes that are known to be present within the arterial wall, including secretory phospholipase A_2,[116-118] sphingomyelinase,[119, 120] cathepsins,[121] and mast cell-derived chymase.[122]

Whereas oxidation of PG-bound apoB100-containing particles triggers their release and subsequent uptake by macrophages, the non-oxidative modifications enhance the strength of particle binding to PGs.[123] In addition, such non-oxidatively modified particles tend to aggregate and fuse, which allows progressive extracellular accumulation of lipoprotein lipids. Both aggregated and other types of modified lipoproteins are avidly taken up by macrophages, leading to foam cell formation and intracellular lipid accumulation. Thus, local modification of apoB100-containing lipoproteins leads to both extra- and intracellular accumulation of lipids in the arterial intima.

The process may be self-augmenting as LDL aggregation substantially enhances its binding to arterial PGs, because of the creation of a multimeric ligand with better exposure of key positive residues on apoB.[124]

In addition, oxidative and enzymatic modifications of retained lipoproteins lead to the generation of biologically active molecules, such as lysophosphatidyl choline and ceramide that have been shown *in vitro* to provoke endothelial expression of cell-adhesion molecules, monocyte chemotaxis, and proliferation of SMCs that, in turn, increase their PG synthesis. Other lipoprotein-derived lipids such as lysophosphatidic acid and platelet-activating factor, block the conversion of monocytes into migratory cells and favor their retention in the subendothelium.[125] Figure 6 summarizes the events taking place in the arterial intima after retention of apoB-containing lipoproteins.

Figure 6. Subendothelial retention of atherogenic lipoproteins within the arterial wall.



Altogether, these processes are likely to promote local inflammation, enhanced lipoprotein retention, lesion progression, and ultimately, clinical events.

Insulin Resistance and Type 2 Diabetes

The world faces a pandemic of type 2 diabetes mellitus. The International Diabetes Federation anticipates that the worldwide incidence of diabetes among those aged 20 to 79 years will increase by around 70% in the next 20 years, from 194 million in 2003 to 333 million in 2025. The increase will affect all global regions, with projected increases ranging from 21% in Europe to 111% in Africa. Of particular concern is South East Asia, which will see an additional 40 million cases of diabetes by 2025.[126]

Although type 2 diabetes (T2D) impairs the quality of life and can lead to renal, ocular and neurological damage, the main complication is a 2-4 times increase in the risk of atherosclerotic cardiovascular disease (CVD). Even before diabetes becomes established, there is an increase in CVD risk in subjects with insulin resistance.[127, 128]

Pathophysiology

The evolution of T2D requires the presence of defects in both insulin secretion (β-cell dysfunction) and insulin action (insulin resistance). Both defects have a recognized genetic component as well as an acquired component that includes age, obesity, glucotoxicity and lipotoxicity.[129] The earliest detectable abnormality in T2D is impairment in the body's ability to respond to insulin. This is counterbalanced by a compensatory increase in insulin secretion by pancreatic β-cells (hyperinsulinemia) to maintain normal glucose tolerance. When the fasting plasma glucose concentration exceeds about 140 mg/dL (7.7 mmol/L), the β-cell can no longer maintain its elevated rate of insulin secretion, and the fasting insulin concentration declines progressively, resulting in impaired glucose tolerance (IGT) and, eventually, overt T2D.

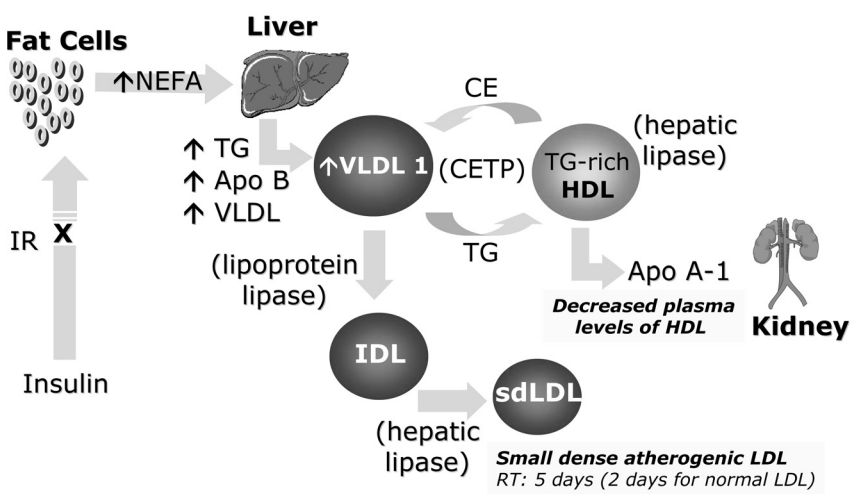
The current rise in prevalence of T2D and the metabolic syndrome is believed to be a result of increasingly sedentary life-styles combined with access to energy-rich food sources in genetically susceptible individuals. Healthy humans respond to positive energy balance primarily by storing excess energy as triacylglycerols in adipose tissue. While this response enables humans to cope efficiently with fluctuating energy availability, it predisposes persistently over-nourished individuals to weight gain, and ultimately, obesity. This condition, where energy intake exceeds the storage capacity of adipose tissue, leads to energy overflow inducing lipid accumulation in ectopic sites,

such as the liver and skeletal muscle, and possibly in pancreatic β-cells and kidney.[130, 131] Accumulation of fat within the liver and muscle leads to insulin resistance in these tissues, resulting in elevated fasting plasma glucose levels (due to accelerated hepatic glucose production) and postprandial hyperglycemia (due to decreased insulin-mediated glucose uptake by muscle).[132, 133]

Dyslipidemia of insulin resistance and type 2 diabetes

As mentioned, atherosclerotic vascular disease is the main cause of T2D mortality, these patients being exposed to a 2-4 higher risk than non-diabetic subjects with a comparable profile of other risk factors. The dyslipidemia of insulin resistance appears to contribute directly to arterial disease as it is a strong predictor for the elevated risk for CVD, even above that attributable to hyperglycemia and diabetes duration.[134-136] The lipid abnormalities of insulin resistance are characterized by elevated levels of triacylglycerols and cholesterol of VLDL, modest elevations of LDL cholesterol with increased fractions of small, dense LDL (sdLDL) particles, and decreased levels of high density lipoproteins (HDL) (Fig. 7). Increased levels of non-esterified fatty acids (NEFA) in plasma are an additional and frequently overlooked change in the circulating lipids in insulin resistance and T2D.[128, 137]

Figure 7. Dyslipidemia of insulin resistance and type 2 diabetes.



IR: insulin resistance; TG: triacylglycerol; CE: cholesteryl ester; CETP: cholesteryl ester transfer protein; RT: residence time of the particle in circulation

Free fatty acids and insulin resistance

Fatty acids are transported in the blood bound to albumin as NEFA or as part of triacylglycerols in lipoprotein complexes, which typically require triacylglycerol hydrolysis via LPL to deliver the fatty acids to the tissues. Plasma lipid concentrations play a role in determining the rate of uptake of NEFA into the muscle, particularly during conditions of hyperinsulinemia,[138] which is often present with insulin resistance. Fasting plasma NEFA concentrations in obese and T2D patients typically range from 600 to 800 μmol/L compared with 300–400 μmol/L in lean healthy persons.[139] This, coupled with reduced lipid oxidation due to loss of mitochondrial function often exhibited in skeletal muscle,[140] results in excessive intramyocellular lipid deposition. Consequently, the excess muscle NEFA are either stored in lipid droplets or converted to various signaling molecules. This NEFA conversion "spill over" is predominately due to increased availability of fatty acyl-CoA substrates.

In order for lipids to be used as fuel by skeletal muscle, NEFA must be taken up by fatty acid transport proteins (FATPs) and converted intracellularly to long-chain fatty acyl-CoAs (LCACoAs), imported into the mitochondria by carnitine acyltransferases, and subjected to β-oxidation. However, LCACoAs also serve as a source of second messengers, such as diacylglycerol (DAG) or ceramide, either by *de novo* synthesis or through phospholipase C activation or phospholipid hydrolysis.

Mechanism of fatty acid-induced insulin resistance

When insulin binds to the insulin receptor (IR) on the cell surface of insulin target tissues, second messengers are generated that initiate a series of events involving a cascade of phosphorylation-dephosphorylation reactions that result in the stimulation of intracellular glucose metabolism. The first step is IR phosphorylation of the β subunit, with subsequent activation of its intrinsic tyrosine kinase. Activated IR tyrosine kinase phosphorylates specific intracellular proteins, including insulin receptor substrate-1 (IRS-1) (pivotal for glycogen synthesis in muscle) and IRS-2 (which mediates the effect of insulin on hepatic gluconeogenesis and glycogen formation). When IRS proteins become tyrosine phosphorylated, they recruit a number of signal transducers such as phosphoinositol 3-kinase (PI3K) leading to translocation of specific glucose transporters.

Insulin resistance has been associated with reduced tyrosine phosphorylation of IRS-1, leading to diminished activity of PI3K.[141] Serine/threonine phosphorylation of IRS-1 has been implicated in diminished insulin action.[142] Furthermore, elevated NEFA concentrations within the muscle itself have been linked with increased serine phosphorylation of IRS-1,[143] indicating one specific mechanism of fatty acid-induced insulin resistance. The mechanism appears to

involve IRS protein dissociation from the IR by inducing conformational changes impeding access to tyrosine phosphorylation sites.[144] This may facilitate IRS release from the intracellular complexes that maintain the proteins in close proximity to the IR.[145] The result is reduced IRS-1 activation of PI3K and, consequently, diminished glucose transporter isoform-4 (GLUT4) translocation to the membrane surface.

Serine phosphorylation of IRS-1 occurs at specific serine residues[146] and seems to be the result of increased activation of protein kinase C (PKC) isoforms.[147] Because chronically elevated plasma NEFA concentrations correspond to increased lipid deposition within the muscle, the intracellular concentration of DAG increases and is associated with the blunting of insulin signaling.[148] The accumulation of intracellular lipid metabolites (*e.g.*, DAG) is the trigger of insulin resistance.

In contrast to the marked impairment of the PI3K signaling pathway, the ability of insulin to stimulate mitogen-activated protein kinase (MAPK) pathway activity in insulin-resistant T2D individuals remains intact. Hyperinsulinemia increases mitogen-activated protein kinase kinase (MEK) 1/2 activity and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation and activity to the same extent in lean healthy subjects as in T2D patients.[149]

Peroxisome proliferator-activated receptor agonists and insulin resistance

Since their discovery in the early 1990s, the members of the peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors have been recognized as key elements in glucose homeostasis. In addition, they are therapeutic targets against dyslipidemia and insulin resistance. Three distinct PPARs termed alpha, beta/delta and gamma, each encoded by a separate gene, have been described. Each isoform has its own tissue distribution pattern. Whereas PPARα is predominantly expressed in tissues with a high oxidative capacity such as the heart and liver, PPARγ is highest expressed in adipose tissue, and the expression of PPARβ/δ is more ubiquitous. All PPARs are activated by endogenous and natural ligands consisting of dietary lipids and their metabolites. In addition, there exist synthetic and specific ligands that are used as drugs in clinical applications. The PPARs regulate gene expression by binding as heterodimers with the 9-cis retinoic acid receptor (RXR), an obligate partner, to specific DNA sequences known as peroxisome proliferator response elements (PPREs) located in the promoters of target genes.[150]

Dyslipidemia of insulin resistance and vascular extracellular matrix

Most of the microangiophaties and macrovascular complications of T2D and insulin resistance are associated with profound changes of the ECM.[151-154] Despite the key participation of the metabolic abnormalities induced by fatty acids in the generation of insulin resistance and its association with CVD, it is still largely unknown if fatty acids are involved in the matrix alterations accompanying atherosclerosis. This lack of knowledge has been the main motivation of the experiments that constitute the main body of this thesis.

AIMS

The overall objective of this thesis was to investigate the impact of chronic elevation of non-esterified fatty acids, a component of the dyslipidemia of insulin resistance and type 2 diabetes, on extracellular matrix proteoglycans and its implications for diabetes-associated vascular disease.

More specifically, the major objectives were:

- To characterize the proteoglycan production of hepatic cells after exposure to non-esterified fatty acids and insulin, and to evaluate its implication for hepatic remnant clearance.
- To characterize the proteoglycan production of vascular smooth muscle cells after exposure to non-esterified fatty acids and insulin, and to evaluate its implication for intima LDL retention.
- To investigate the signaling pathways behind the fatty acid-induced upregulation of versican in vascular smooth muscle cells.

RESULTS AND DISCUSSION

PAPER I – Heparan sulfate proteoglycans and hepatic remnant clearance

The objective of this study was to evaluate the effects of a continuous influx of NEFA and insulin on the PG biosynthesis of hepatic cells.

For this purpose, we used as an *in vitro* model a human hepatoma cell line, HepG2 cells, exposed to linoleate, insulin or both. This cell line retains many of the differentiated properties seen in hepatocytes and has been used for many years as an alternative to primary hepatocytes.

We found that linoleate and insulin induced quantitative and qualitative changes on the PG production. HepG2 cells incubated with linoleate decreased their PG secretion by 10% whereas cells incubated with insulin secreted ~15% more compared with control cells. Analysis of the GAG composition of the secreted PGs revealed that linoleate markedly decreased CS secretion whereas insulin increased it. The PKC inhibitor, bisindolylmaleimide, partially interfered the linoleate-induced shift in GAG composition.

These opposing effects of linoleate and insulin on HepG2 cells' secretion of PGs and the relative content of CS, could reflect fatty acid-induced cellular insulin resistance. Although little is known about the mechanisms of insulin regulation of ECM components, it is possible that activation of PKC by fatty acids impairs the insulin signaling involved in PG and GAG biosynthesis.

We speculate that NEFA could activate PKC isoforms and thereby activate a set of downstream cell-type specific transcription factors regulating the expression of PG core proteins. More than 25 enzymes are responsible for HS biosynthesis. Changes in expression or activity in any of these enzymes could alter liver HS fine structure and affect lipoprotein catabolism.

The linoleate-induced changes in the CSDS:HS ratio of GAG chains of secreted PGs in HepG2 cells were accompanied by changes in the expression of the core protein of the CSDS-containing PG, versican. Linoleate decreased versican mRNA whereas insulin increased it. Similar changes were observed on the expression of syndecan-1, but to a lesser extent and were not reflected on changes in the CSDS:HS ratio of GAG chains of cell-associated PGs. The effects of linoleate on versican expression were mimicked by a PPARα agonist. Bezafibrate, as linoleate, decreased versican mRNA, whereas darglitazone had no effect. PPARα is the primary PPAR subtype expressed in the liver. Upon

activation, PPARα favors fatty acid uptake in hepatocytes and inhibits triacylglycerol synthesis and VLDL production. This suggests that decreasing the intracellular levels of fatty acids, and possibly those of fatty acyl-CoA derivatives, may restore basal PG expression.

As an *ex vivo* model we used livers from obese Zucker *fa/fa* rats and their lean *fa/+* littermates. The obese animals had insulin, NEFA and triacylglycerol levels roughly 10 times higher compared with their lean littermates. The metabolic syndrome in the Zucker fatty (*fa/fa*) rats is due to a chronic hyperphagia, and thus can be an excellent representation of the human condition. The *fa* mutation (Glu→Pro) in the extracellular domain of the leptin receptor leads to a reduced leptin-binding affinity and signal transduction.[155, 156]

PGs extracted from whole liver (secreted and cell-associated) of the obese rat had GAG chains with an increased CS content at the expense of HS, when compared with liver PGs from the lean rat. In addition, these animals had increased expression of versican and syndecan-1 core proteins. The complete versican core protein sequence contains 17–23 potential attachment sites for CSDS chains. Analysis of syndecan-1 core protein indicates it can carry 2-3 HS chains near the N-terminus and 1-2 CS chains close to the TM domain.[157] If these changes are also apparent at the protein level, and taking into account the stoichiometry GAG chain to core protein, it is possible that the increase in CSDS:HS ratio of PGs from obese rats was due to an increase in CS chains.

Using apoE-enriched rabbit β-VLDL as a model of remnant particle, we estimated lipoprotein-binding to PGs isolated from HepG2 and rat livers. The increase in the relative content of CS (hence, decrease of HS), both in insulin-treated HepG2 cells' secreted PGs and obese rat liver total PGs, resulted in a reduced capacity to bind β-VLDL remnant particles.

HSPGs are the predominant PG class found in the liver, accounting for more than two-thirds of total tissue GAG content, followed by CS/DS PGs; no hepatic KS PGs have been reported so far.

The HS content in the liver is not only important for lipoprotein catabolism. CS have been reported to be increased under other physiological and pathological conditions, such as hepatic regeneration,[158] cholestasis,[159] and liver cirrhosis.[160] These changes can affect the normal interaction between several growth factors with their respective liver cell surface receptors, thus modulating hepatocyte proliferation and fibrogenesis.

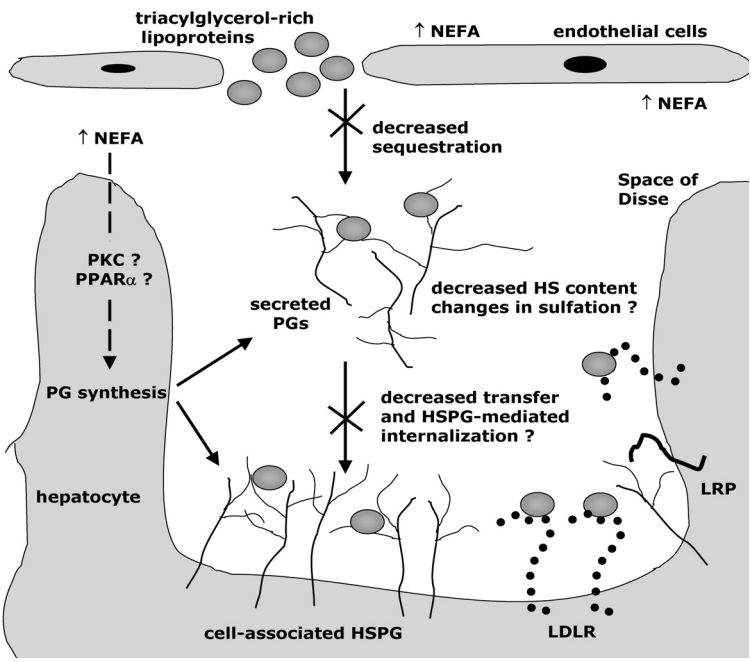
The structural features of GAGs are cell type-specific and tissue-specific. Liver HSPGs hold almost 2-fold more sulfate content than those derived from other tissues. The particular distribution of sulfate groups along the liver HSPGs confers increased affinity for apoE compared to HSPGs expressed in the vascular bed.

Mahley and colleagues[77] have suggested that HSPGs are key players in the hepatic remnant clearance. Remnant particles are sequestered

near the hepatocyte cell surface via apoE-HS or lipase-HS bridging on secreted PGs, and after lipolytic processing, the particles are transferred for internalization to the LDLR or LRP in association with or independently of cell-bound HSPGs. The observed decrease in the relative content of HS of liver PGs could therefore account for the observed reduced capacity to bind β-VLDL remnant particles. This could lead to reduced sequestration in the space of Disse. As LDLR-independent clearance accounts for 25-50% of the hepatic remnant uptake, these changes could probably reduce the internalization of the particles (Fig. 8).

In addition to GAG composition, sulfation determines the GAG binding capacity. We did not observe changes in the degree of 6-O (NAcGlc) and 2-O (GlcA/IdoA) sulfation of the secreted or cell-associated HS chains from HepG2 cells. However, GAGs from obese rat livers exhibited a lower anodic electrophoretic mobility in agarose gels, suggesting a lower negative charge density compared with lean rat extracts. This probably reflects a decreased relative content of HS.

Figure 8. Consequences of overexposure of hepatic cells to chronic high levels of circulating NEFA.



Interestingly, livers from diabetic rodents show reduced HS production,[161] reduced sulfation of the chains,[162] and decreased expression of *Ndst1*.[163, 164] The phenotype of Ndst1-deficient mice is similar to that of patients with hypertriglyceridemia associated with

elevated levels of VLDL particles, as present in insulin resistance and T2D.

If HSPGs are critical players in controlling lipoprotein metabolism, polymorphism analysis of genes encoding HSPG core proteins and/or GAG biosynthetic enzymes may define novel markers of predisposition to dyslipidemia or response to lipid-lowering therapy.

In summary, these results indicated that insulin and NEFA modulate the expression of PGs in hepatic cells. The exchange of CS for HS may reduce the clearance of remnant β-VLDLs and contribute to the dyslipidemia of insulin resistance. Whether these findings are also present and pathogenically relevant in patients with T2D and metabolic syndrome remains to be established.

PAPER II – Arterial smooth muscle cell chondroitin sulfate proteoglycans and LDL binding

The objective of this study was to investigate the effects of high NEFA levels on the PGs secreted by human arterial smooth muscle cells (hASMCs) and whether these effects could modify the PG affinity for LDL.

Primary cultures of hASMCs were exposed to either linoleate or palmitate, alone or in combination with insulin. We found that linoleate and palmitate increased the expression of genes for the core proteins of versican, the SLRPs with either 1 (decorin) or 2 (biglycan) CS/DS side chains, and perlecan, the main HS-containing PG of the basement membrane.

The effect of NEFA on core protein mRNAs was accompanied by increased PG secretion, as evaluated by ion exchange chromatography (Rodríguez-Lee *et al*. 2005, unpublished data). In addition, linoleate-treated cells secreted PGs carrying GAG chains with higher content of CS. The linoleate-induced increase in the CSDS:HS ratio of secreted PGs was blocked by PKC inhibition.

CS synthase and CS-6 and CS-4 sulfotransferases are key enzymes for the polymerization and sulfation of chondroitin chains. Therefore, we investigated whether the observed upregulation of core proteins for CS-containing PGs induced by fatty acids was accompanied by changes in the mRNA for these enzymes. We found that linoleate increased the expression of the 3 enzymes compared with control cells.

Negative charge density and chain length of the GAGs are the main properties controlling the binding of PGs to many proteins, including LDL. We found no differences in GAG chain length, as evaluated by PAGE. However, GAGs from linoleate-treated cells had higher anodic electrophoretic mobility and $[^{35}S]$:$[^{3}H]$ ratio, suggesting an increase in

negative charge. The upregulation of CS-4 and CS-6 sulfotransferases observed upon linoleate stimulation supports this conclusion.

The changes induced by linoleate in GAG composition and negative charge density led to a more efficient binding of LDL compared with GAGs from control cells. Insulin and rosiglitazone abrogated this fatty acid-dependent increase.

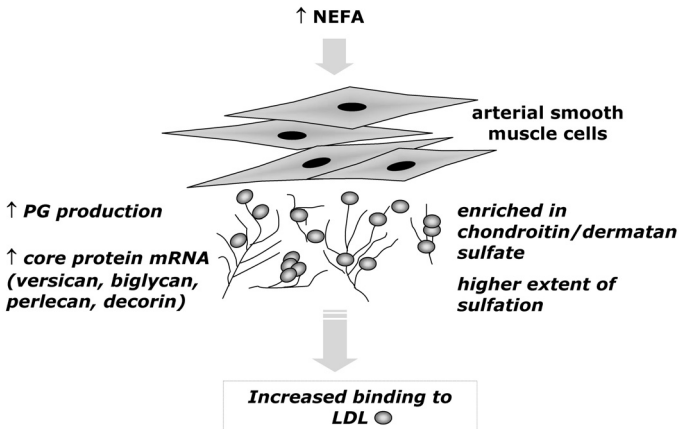
Under our culture conditions, insulin on its own added on top of the basal level of <10 pmol/L had no effect on any of the PG or GAG properties. On the other hand, the changes caused by the fatty acids on genes encoding the PG core proteins, the substantial changes in GAG structure, and increased affinity for LDL were blunted by co-incubation with insulin levels >1 nmol/L. These results suggest that levels of insulin in the picomole range were sufficient to maintain a basal rate of PG synthesis and composition. However, under overexposure to fatty acids, higher concentrations of insulin or the sensitizing action of rosiglitazone were required to restore the basal biosynthesis and structure of secreted PGs. Again, this could reflect fatty acid-induced cellular insulin resistance, possibly mediated by PKC activation.

After incubation with linoleate, the triacylglycerol and DAG content of the cells increased compared with control cells. Oil Red O staining revealed lipid droplets in the cytoplasm of fatty acid-treated cells.

The PPARγ agonist, rosiglitazone, blocked the effects of linoleate on PGs, GAGs, and LDL binding. We speculate that it may oppose the actions of NEFA by accelerating their conversion into stored triacylglycerols. This could limit the concentration of DAG or other bioactive lipids that could modulate the insulin-signaling cascade. It may also limit the actions of fatty acyl-CoA on glucose utilization and lower the contribution of the hexosamine pathway. The end products of this pathway, UDP-GlcNAc and UDP-GalNac, are the building blocks for the synthesis of the GAG chains of PGs.

In summary, this study indicated that exposure of hASMC to high levels of fatty acids caused structural changes in secreted PGs that explain the increased binding of LDL (Fig. 9). If similar effects occur in the human arterial intima in conditions of insulin resistance, overexposure of the arterial smooth muscle to fatty acids could induce ECM changes favoring apoB-lipoprotein deposition and atherogenesis.

Figure 9. Consequences of overexposure of arterial SMCs to chronic high levels of circulating NEFA.



PAPER III – Versican expression and ERK-dependent activation of CCAAT-enhancer binding protein beta in arterial smooth muscle cells.

Our earlier studies show increased versican expression induced by fatty acids accompanied by increased binding affinity for LDL (Paper II).[165] Versican is the most abundant PG of the human intima and, when isolated from human arteries or SMC cultures *in vitro*, shows a high affinity for LDL.[102, 166] Its unique structural features create a highly interactive molecule that binds growth factors, enzymes, lipoproteins, and a variety of other ECM components to influence fundamental events involved in vascular disease. Because of the critical importance of this PG in atherosclerosis, we decided to investigate in this study the signaling pathways involved in the fatty acid-induced upregulation of versican in hASMCs.

We previously reported that hASMCs exposed to fatty acids have a significantly higher intracellular content of DAG.[165] In this study, we observed that conventional ($\alpha\beta\gamma$), novel (ε) and atypical (ζ) PKC isoforms were all expressed in hASMC but only conventional and novel kinases with functional DAG binding sites were activated by linoleate. Moreover, inhibition of PKC blunted the linoleate-induced upregulation of versican expression. Protein kinase D (PKD1) was also activated by linoleate treatment. The detected activation of PKD1 could be a direct or indirect effect (via PKC activation) of the increased DAG content. In addition, linoleate-treated cells increased the relative content of phosphorylated MEK1/2, and of its downstream substrate, ERK1/2.

Our earlier studies showed that linoleate-induced increase in CSDS content was blocked by PKC inhibition. In this study, we demonstrated

that the linoleate-induced increase in versican expression could be also blunted by inhibition of ERK1/2 activation.

ERK1/2 activation modulates the transcription of several target genes by *i*) direct phosphorylation of transcription factors such as human CCAAT-enhancer binding protein (C/EBP)β at Thr235,[167, 168] or *ii*) phosphorylation of other downstream kinases such as the p90 ribosomal S kinase.[169] C/EBPs are a family of transcription factors that play important roles in energy metabolism.[170]

We identified a putative C/EBP binding site at position -471 (relative to the transcription start) in the 5'-flanking region of the human versican gene. Therefore, we conducted experiments with an inducible reporter plasmid containing the luciferase reporter gene driven by a basic promoter element (TATA box) plus 3 repeats of the C/EBP binding site (pC/EBP-Luc). Results showed that linoleate induced greater luciferase activity than transfected control cells.

We also evaluated binding to a biotin-labeled double-stranded DNA (dsDNA) containing the consensus binding site for C/EBPs in the nuclear extracts. We found increased C/EBP DNA-binding activity when hASMC were treated with linoleate.

Importantly, linoleate activation of ERK1/2 preceded the induction of both C/EBP-driven luciferase activity and C/EBP DNA-binding activity. Inhibition of ERK1/2 not only blunted the effect of linoleate on C/EBP-driven luciferase activity, but also on C/EBP DNA-binding activity to both C/EBP consensus dsDNA and versican dsDNA. These data indicate that C/EBP is a fatty acid-responsive factor and suggest that the linoleate-induced C/EBP activation is dependent on ERK1/2 activation.

The phosphorylation state of this transcription factor can modulate the transactivation capacity and/or DNA-binding activity of existing C/EBP, and in addition can control the translocation of the protein to the nuclear compartment.[170] We found that linoleate increased the cellular content of phospho-C/EBPβ (Thr235), and in addition decreased the cytosolic content and increased the nuclear content of C/EBPβ. Taken together, these data suggest that the increase in C/EBPβ nuclear content could be due to increased C/EBPβ phosphorylation and translocation to nucleus upon linoleate stimulation.

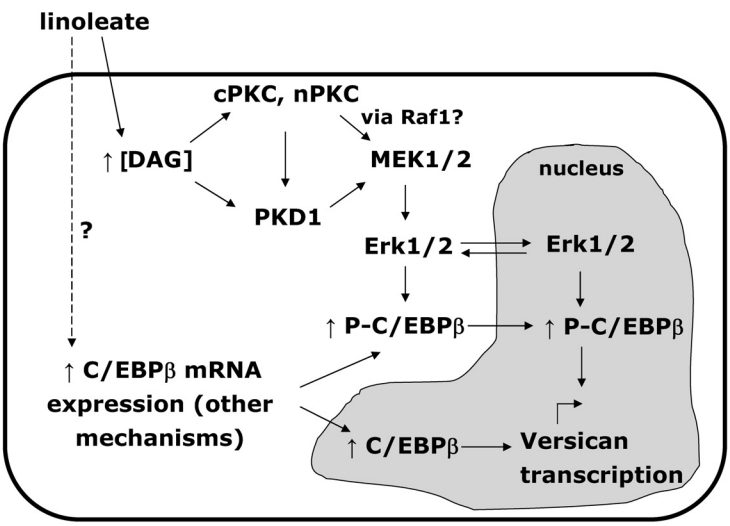
We also found that linoleate treatment upregulated C/EBP α and β, whereas the expression levels of C/EBP isoforms γ, δ, or ζ did not differ between control and linoleate-treated cells. If these changes are also apparent at the protein level, they could contribute to the linoleate effects on versican expression.

Most importantly, we demonstrated specific C/EBP binding to the putative binding site in the versican promoter. Super-shift assay confirmed the presence of C/EBPβ in the DNA-protein complexes induced by linoleate. To a lesser extent, anti-C/EBPα antibody caused the formation of super-shifted DNA-protein complexes, suggesting that

this isoform could also be recruited to this fatty acid-responsive C/EBP site in versican promoter.

In summary, this study defined an ERK-dependent pathway that mediates phosphorylation and translocation of C/EBPβ to the nucleus in response to fatty acids, and that results in increased expression of versican in hASMC (Fig. 10). These findings suggest a novel role for C/EBPβ in fatty acid-induced changes of ECM. If this mechanism occurs in the human arterial intima, it could contribute to LDL deposition in insulin resistant subjects.

Figure 10. Linoleate induces versican expression in arterial smooth muscle cells through ERK1/2 activation of C/EBPβ.



CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, the focus has been to investigate the impact of chronic NEFA elevation, a component of the dyslipidemia of insulin resistance and T2D, on ECM PGs and its implications for diabetes-associated CVD.

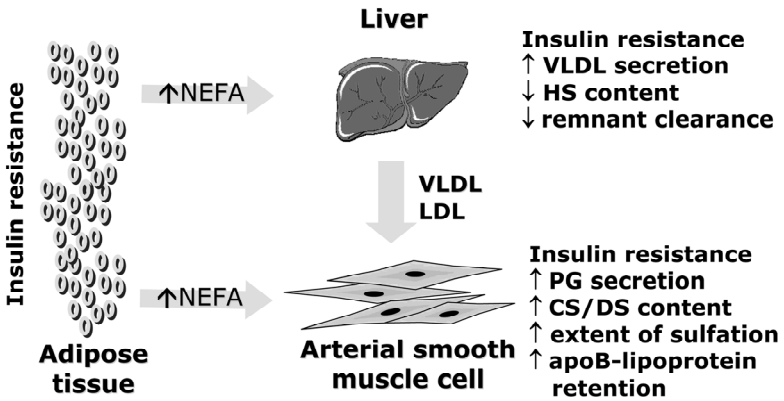
Overexposure of liver to NEFA released from insulin-resistant adipose tissue results in the development of atherogenic dyslipidemia, including overproduction of VLDL particles.[171] The central role for cholesterol-rich apoB100-lipoproteins in the development of atherosclerosis has been extensively documented. Postprandial TRLs also carry a substantial amount of cholesterol,[172] and despite their particle size, penetrate into the arterial wall where they become retained through interactions with arterial PGs.[173-175]

We found that the synthesis of hepatocytes and arterial SMCs was modified by exposure to increased levels of NEFA. Quantitative and qualitative changes in the structure of the GAG chains resulted in altered lipoprotein binding. GAGs from hepatic cells exchanged CS for HS, which may reduce the clearance of remnant β-VLDLs and contribute to the dyslipidemia of insulin resistance. Arterial SMCs secreted PGs enriched in chondroitin/dermatan sulfate and with a higher extent of sulfation, favoring apoB-lipoprotein deposition and atherogenesis.

In addition, NEFA appeared to compromise a basal control exerted by insulin in matrix PG production by hepatocytes and SMCs. Physiological insulin signaling contributes to the maintenance of the quiescent phenotype of SMCs in healthy arteries by opposing the action of growth factors that could promote de-differentiation and cell migration.[176] Increased biosynthesis of PGs, as induced by NEFA, is characteristic of the change from the quiescent to the synthetic phenotype. This may precede cell migration from media to intima and the increased matrix production reported in atherogenesis and restenosis.[166, 177]

We suggest that the observed NEFA effects on ECM PG could contribute to the increased risk of CVD in individuals with insulin resistance by worsening the dyslipidemia and by acting directly on arterial cells (Fig. 11).

Figure 11. Summary of the effects of NEFA on hepatocytes and SMCs that may contribute to generate the dyslipidemia of insulin resistance and increase its atherogenicity.



These observations indicate that reduction of circulating levels of NEFA should be considered an important target for any therapeutic intervention intended to decrease arterial disease accompanying insulin resistance. Hence, a combination of healthy eating habits, balanced physical activity and moderate pharmacological activation of the transcription factors PPARα and PPARγ may offer an approach to the treatment of patients with insulin resistance and T2D.

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