DIPLOMARBEIT

The role of low-density-lipoprotein receptor (LDLR) family members in human coronary artery endothelial cells in response to modified LDL

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Abstract

First mark of atherosclerosis is linked to endothelial dysfunction, with a massive injury of endothelium of the vascular intima of blood vessels.

Mechanical forces, as blood associated shear stress, play an important role in cardiovascular physiology. Atherosclerotic lesions can be detected in regions of low shear stress (LSS) and disturbed blood flow at curvatures and narrows of vessels and capillaries.

Various diseases such as diabetes, chronic renal insufficiency, and obesity come along with elevated levels of blood cholesterol and different modified low-density-lipoproteins (LDL). The modification of lipoproteins is a key event in early atherosclerosis, which leads to endothelial dysfunction and the formation of foam cells.

Human coronary artery endothelial cells (HCAEC) are used for studies of atherosclerosis, to observe the effect of modified LDL, such as oxidized, carbamylated, and glycated LDL. High-density-lipoprotein (HDL) and also the female hormone estrogen are defined to be vasoprotective and antagonize the formation of atherosclerotic lesions.

Lipotoxicity on endothelial cells could be demonstrated with increasing concentration of different modified LDL. We could determine that estrogen and HDL lower the cytotoxic effect of modified LDL on endothelial cells in cell culture experiments.

Previous data focused on the regulation of inflammatory sensors and upregulation of scavenger receptors. In this study the question was addressed to determine the role of LDL receptor gene family members, such as LDL receptor, LRP1 and LRP2 in HCAEC and the impact of modified LDL. More interest was also raised in sex specific differences and the impact of estrogen 17-beta estradiol (E2) in vasculature. The investigation was based on incubation of HCAEC with different modified LDL under static cell culture condition and also under subjection to shear stress. The Fiber Cell™ module is designed for cell culture experiments to study endothelial cells under shear stress.

However, LDL receptor expression seemed to be downregulated on the transcript level after exposure of endothelial cells to modified LDL under static conditions and under LSS. HSS affects the activation of LDLR in vasculature. Hence, LRP2 could be detected at low-level expression on the mRNA level in both conditions. LRP1 could not be detected under static conditions, but a slight upregulation under shear stress could be detected.

Moreover, estrogen induced a slight upregulation of the mRNA level of LRP1 and LRP2 under static conditions. The LDL receptor seemed to be significantly upregulated by estrogen administration, also published by Smith et al, 2004.

Concluding, the vasoprotective effect of estrogen and HDL and the lower incidence of toxic effects of modified LDL on HCAEC could be shown. The expression of LDL receptor gene family members, including LDLR, LRP1, and LRP2 seemed to be downregulated in atherosclerotic events.

Zusammenfassung

Erste Anzeichen der Atherosklerose kennzeichnen sich durch die endotheliale Dysfunktion in Folge von massiver Schädigung des Endothels in der Intima der Blutgefäße.

Der Blutfluss und die dabei wirkenden mechanischen Scherkräfte spielen eine entscheidende Rolle in der Gefäßhomeostase und der Aufrechterhaltung der Endothelfunktion. An Abgängen, Verzweigungen und Verengungen von Blutgefäßen wirken geringere Scherkräfte und der Blutfluss verlangsamt sich, infolgedessen treten an diesen Stellen vermehrt atherosklerotische Plauqes auf.

Verschiedene Krankheiten wie Diabetes, chronische Niereninsuffizienz und Übergewicht gehen mit erhöhtem Blutcholesterolspiegel einher. Patienten haben durch die Krankheit vermehrt modifizierte Lipoproteine im Blut. Modifikationsprozesse von LDL stehen mit ersten Anzeichen der Atherosklerose in Verbindung.

Humane koronare arterielle Endothelzellen (HCAEC) werden häufig im Forschungsbereich der Atherosklerose, insbesondere der endothelialen Dysfunktion eingesetzt, um die Wirkung von modifizierten LDLs und die dabei resultierende Schädigung der Endothelzellen herauszufinden.

HDL und das weibliche Hormon Östrogen wirken Schädigungen entgegen und werden als vasoprotektiv beschrieben.

Anhand von Toxizitätstests konnte die Lipotoxizität mit steigender Konzentration an modifizierten LDL auf Endothelzellen gezeigt werden. Die Vermutung konnte bekräftigt werden, dass HDL und 17-beta Estradiol (E2) den toxischen Effekt in Zellkulturexperimenten vermindern konnten.

Frühere Studien befassten sich mit der Regulation von inflammatorischen Markern und der Regulierung von Scavenger Rezeptoren, den sogenannten Fressrezeptoren. Bisher ist wenig über die Regulation von LDL Rezeptor Familienmitgliedern im vaskulären Endothelium bekannt.

Das Ziel meiner Diplomarbeit war, die Rolle von LDL Rezeptor Familienmitgliedern, wie LDL Rezeptor, LRP1 und LRP2, in diesem Zusammenhang zu definieren. Hierbei sollten HCAEC mit verschiedenen modifizierten LDLs (oxidiertem, glykiertem und carbamyliertem LDL) behandelt werden. Weiters sollten geschlechtsspezifische Unterschiede erfasst werden, indem Zellen zusätzlich zu modifizierten LDL mit E2 behandelt wurden.

Die Expressionsanalysen wurden unter statischen Bedingungen und unter Einwirkung von Scherkräften in Zellkartuschen (Fiber Cell™ Model) durchgeführt.

Zusammenfassend konnte gezeigt werden, dass der Transkriptionslevel von LDL Rezeptoren unter statischen Bedingungen und unter geringen Scherkräften verringert wird. Erhöhter Blutfluss und Scherkräfte wirken aktiv auf Rezeptoren der Endothelzellen. LRP2 mRNA konnte nur mit geringem Expressionstatus nachgewiesen werden. LRP1 konnte auf Transkriptionsebene in Zellkulturexperimenten nicht nachgewiesen werden, aber eine basale Aktivierung unter Einfluss von Scherkräften.

In Zellkulturexperimenten zeigte die Inkubation von Endothelzellen mit E2 und modifizierten LDL, einen geringen Antsieg von LRP1 und LRP2 auf mRNA Ebene. Eine signifikante Steigerung der LDL Rezeptor mRNA-Expression konnte bei E2 Zugabe erkannt werden.

Generell scheint die mRNA-Expression von LDL Rezeptor Familienmitgliedern unter Einwirkung von modifizierten LDLs in frühen atherosklerotischen Stadien verringert zu werden.

Chapter 1

Introduction

1.1 Cardiovascular disease and atherosclerosis

1.1.1 Cardiovascular disease (CVD)

The atherosclerotic cardiovascular disease accounts 2 million deaths per year in the European Union (EU) [Ref 1].

The traditional cardiovascular risk factors, defined by epidemiological studies such as the Framingham study, are high blood pressure, diabetes mellitus, dyslipidemia, male sex, smoking, and obesity. Patients with kidney disease as chronic renal insufficiency have an increased risk to develop atherosclerotic plaques [Longenecker, 2002].

The secondary effect of disease state is the disturbed lipoprotein metabolism. The initiating factor is the elevated level of blood cholesterol with high amounts of modified low-density-lipoproteins (LDL), which are associated with atherosclerotic plaque formation.

Atherosclerosis, and at least CVD syndromes, can also be caused by inherited genetic variances in family history with premature CVD.

Overall, several diseases and genetic variabilities are the main contributors for the development of cardiovascular pathologies. However, lifestyle, aerobic exercise, and body weight play an important role for health. Sports and a normal body mass index (BMI) can lower the incidence of CVD.

1.1.2 Atherosclerosis

Atherosclerosis is a severe disease substituted into different stages from early lesions to advanced plaque progression.

End stage atherosclerosis in coronary arteries contributes to development of CVD, including myocardial infarction and stroke. Considering this, atherosclerosis was also defined as an inflammatory disease [Ross, 1999].

As mentioned before, people who suffer from diabetes or patients with kidney disease have a higher incidence of developing atherosclerosis. Also obesity, smoking, and hypertension are associated with occurence of atherosclerotic lesions. Atherosclerosis is a disease initiated by insults to the endothelium in the vasculature. Endothelial dysfunction is described as an early marker and precedes the progression of atherosclerotic plaques [Hadi, 2007].

The main risk factor is the elevated cholesterol level and the accumulation of lipids in the subendothelial region of the arterial wall. Native and modified LDLs contribute mainly to atherothrombosis [Llorente-Cortes, 2005; Llorente-Cortes and Estruch, 2010; Badimon, 2009]. These LDL particles are retained in the subendothelial wall because of reduced turn-over and prolonged residence time, leading to modification of LDL. In further states of atherosclerosis, native LDL and also modified LDL can migrate through endothelial junctions into the vascular intima and further into the vascular media. The transcytosis of LDL leads to interactions with extracellular matrix (ECM) components such as proteoglycans, collagen, as well as elastin [Schwandt & Parhofer]. There, LDL can undergo modification processes through ECM residing proteolytic enzymes and oxidants (lipoxygenase, myeloperoxidase, free radicals) [Llorente-Cortes, 2005; Badimon, 2009]. LDL can be retained there for hours or days. LDL not only influences endothelial properties, but also modifies antithrombotic activities in the endothelium.

The first step starts with impaired function of the endothelium and comes along with inflammation and production of free radicals leading to massive endothelial cell damage by oxidative stress. The injury and dysfunction of the endothelium turns on remodelling processes for proliferation and migration of smooth muscle cells, which thicken the arterial walls. Inflammation causes harmful oxidative stress in vessel wall through defense mechanisms, such as producing hydrogenperoxide (H_2O_2) and

reactive oxygen radicals [Ross, 1999; Badimon, 2009]. Inflammatory response is potentiated by disturbed gene expression in endothelial cells and the mobilization of more inflammatory cells to the lesion site. Modified LDLs are chemotactic and can up-regulate the expression of genes for the macrophage colony stimulating factor and for the monocyte chemotactic protein derived from endothelial cells [Ross, 1999]. Macrophages and lymphocytes emigrate and their activation leads to the release of more hydrolytic enzymes, cytokines, chemokines, and growth factors [Ross, 1999]. Monocytes derived macrophages bind modified lipoproteins to their scavenger receptor and accumulate lipids. Accumulation of cholesterol esters results in the formation of foam cells (Fig.1.1).
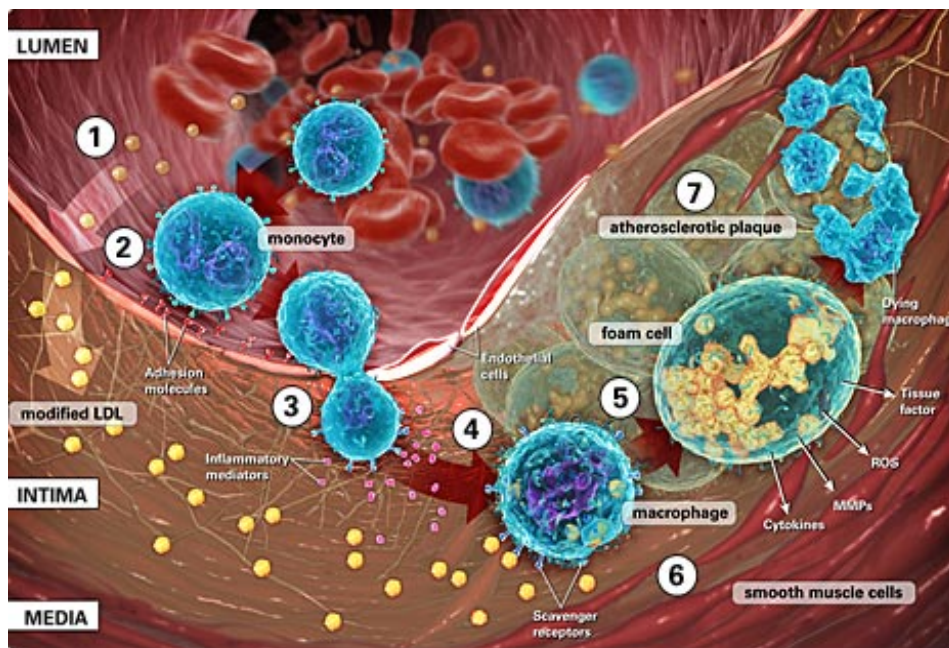
Fig.1.1 Several stages of atherosclerosis from transcytosis of LDL and the modification, to emigration of monocytes and the formation of foam cells in the endothelium of the vasculature [Ref 2].

Foam cells increase the vulnerability of the atherosclerotic plaque [Llorente-Cortes, 2005; Badimon, 2009]. Overload of foam cells can lead to necrotic processes. Building of foam cells is restricted to macrophages and also smooth muscle cells. Endothelial cells do not undergo foam cell formation and do not accumulate

cholesterol in plaques as macrophages and smooth muscle cells [Hassan, 2006]. They avoid the uptake and the accumulation of cholesterol intracellularly and are able to shut down the cholesterol synthesis and the further uptake of endogenous cholesterol.

An advanced complicated lesion is build by enlargement of fibrous tissue and formation of a fibrous cap that overlies the core of lipids [Ross, 1999; Badimon, 2009]. Arteries can no longer compensate by dilation, lesions intrude into lumen and alter the blood flow [Ross, 1999].

Fig.1.2 Atherosclerotic pathologies on aorta [Ref 3]

At end stage of atherosclerosis the blood vessel diameter decreases dramatically. Plaque rupture and thrombosis lead to complications, such as myocardial infarction and acute coronary syndromes (Fig.1.2) [Ross, 1999].
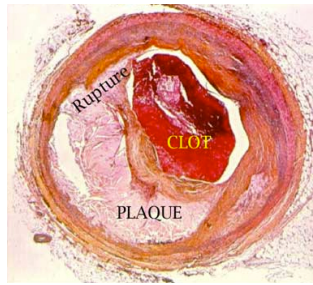
Fig.1.3 Plaque rupture [Ref 4]

1.2   Endothelium and endothelial dysfunction

1.2.1 Structure of endothelium

The vascular bed in coronary arteries is structured in three different layers, including the Tunica interna (Intima), Tunica media (Media), and Tunica externa (Adventitia) [4].

The endothelium is constituted of a monolayer of endothelial cells in tight connection to a large layer of smooth muscle cells (SMC) and an extracellular matrix (ECM) seen in Fig.1.3.
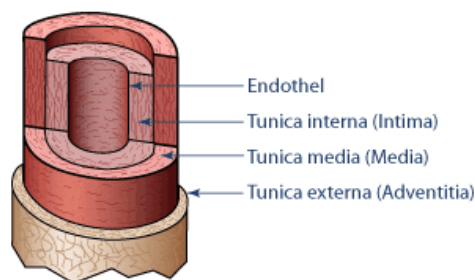
Fig.1.4 Structure of vasculature [Ref 5]

Permeability of endothelial cells is regulated by gap junctions and tight junctions, the so called functional connexins.

The tunica media, build up of smooth muscle cells, is responsible for the production of extracellular matrix components and diverse regulatory processes.

Endothelial cells act mainly as a barrier between blood and vessel wall. These cells react towards stimulation by blood flow, by activation of signalling pathways, and by the regulation of the tonus, coagulation, and proliferation of smooth muscle cells. Endothelial cells control the vascular homeostasis and the balance between vasodilation and vasoconstriction. When the balance is upset, endothelial dysfunction occurs causing damage of arterial walls and activation of pathological processes, which lead to plaque formation [Davignon, 2004].

1.2.2 Endothelial function

The activation of endothelial cells induces the production of endothelial derived regulating factors for vasodilation and vasoconstriction.

A major vasodilative substance released by the endothelium is nitric oxide (NO), originally identified as the endothelium derived relaxing factor (EDRF) [Schwandt & Parhofer; Vance & Vance]. Other vasodilators including prostacyclin and brandykinin stimulate the release of NO and inhibit platelet aggregation [Davignon, 2004].

NO is synthesized from L-arginin by endothelial nitric oxide synthase (NOS) by the formation of L-citrulline and NO in vasculature.

Three isoforms of NOS are defined, such as the two constitutive forms NOS-I or neuronal (nNOS), NOS-III or endothelial (eNOS), and the inducible form NOS-II (iNOS) [Kublickiene, 2008]. The constitutively expressed NOS requires calcium/calmodulin for activation, whereas iNOS gets activated by cytokines and inflammatory markers. Co-activators like tetrahydrobiopterin, nicotinamide-adenin-dinucleotidephosphate (NADPH), and coenzyme as calmodulin are involved in the production of NO (Fig.1.4) [Davignon, 2004; Hirata, 1995].

NO acts on guanylyl cyclase of SMC, to release cGMP following the activation of protein kinase G, which leads to a reduced calcium concentration. Reduction of the calcium release causes relaxation of SMC [Schwandt & Parhofer].
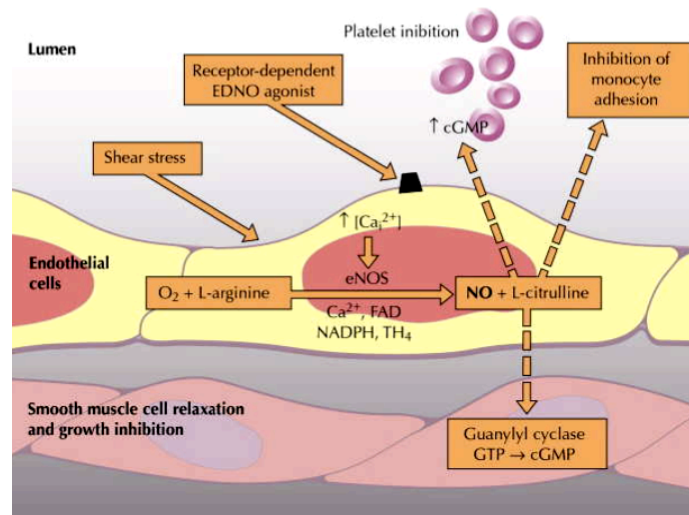
Fig.1.5 Endothelial function and the production of nitric oxide (NO) [Ref 6]

NO is not only anti-inflammatory and inhibits the adhesion of monocytes, it also controls the production of reactive oxygen species (ROS), platelet aggregation, and sustains the endothelium against lipoprotein permeability.

Vasoconstrictors in vascular system are endothelin, described as the most potent constrictor, and angiotensin II. Angiotensin II mainly activates the growth of SMC [Sendra, 2008].

1.2.3 Endothelial dysfunction

Imbalance of vasodilation and vasoconstriction to impaired vascular relaxing are early signs of endothelial dysfunction. The first step of development of atherosclerosis is the injury of the endothelium in coronary arteries.

As mentioned before, the progression of plaque formation comes from subendothelial retention of modified LDL particles and oxidative stress. Oxidative stress results in inflammatory response and migration of macrophages to the subendothelial region. Retaining native and modified LDL reduces the activation of NO synthase. Furthermore, NO can be faster degraded because of redox imbalance [Llorente-Cortes, 2005; Llorente-Cortes and Estruch, 2010]. Cell damage results in a reduced NO bioavailability, and comes along with the release of vasoconstrictive endothelin,

the aggregation of platelets and the expression of adhesive molecules. Meanwhile monocytes adhere to endothelial cells [Hirata, 1995].

During normal aging processes, the vasculature is also subjected to remodelling processes and a change in vessel diameter can be detected, correlating with higher blood pressure [Hirata, 1995].

Vascular aging shows an impaired endothelial mediated vasodilation and an overgrowth of smooth muscle cells causing atherosclerotic lesions. These factors increase the risk of cardiovascular events.

1.3 Lipoproteins

1.3.1 Low-density-lipoprotein (LDL)
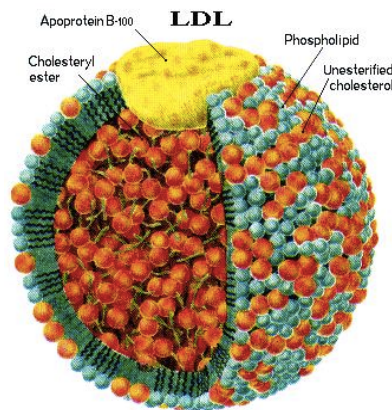
Fig.1.6 Basic structure and composition of LDL [Ref 7]

Lipids are water insoluble molecules with a highly hydrophobic nature and can therefore only be transported in circulation via lipoproteins. Plasma lipoproteins can vary in size, composition, and their different functions in lipid metabolism. Lipoproteins are spherical molecules with approximately 10-1200 nm diameter and show a density between 1.019 and 1.063 g/ml [Willnow, 2007; Llorente-Cortes, 2005]. Lipoprotein particles are build up by an amphiphatic shell of polar phospholipids and cholesterol, the inner core is filled with neutral lipids like cholesterolesters, triglycerides, and fat soluble vitamins. Apolipoproteins reside in the

shell of lipoproteins, which are important for assembly and the recognition by LDL receptors. The exclusive apolipoprotein of LDL is apoB 100, which is able to bind to LDL receptor binding domains. ApoB 100 facilitates the uptake and catabolism of LDL.

The lipid cargo is delivered by receptor-mediated endocytosis of LDL receptor gene family members. The apolipoprotein moiety is degraded into small peptides and the lipids are released intracellularly [Goldstein et al, 2001].
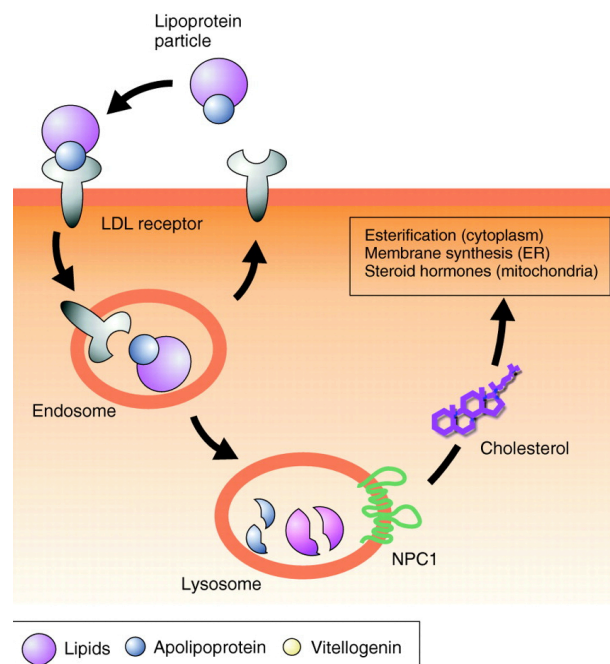
Fig.1.7 Concept of the LDL receptor mediated endocytosis [Willnow, 2007]

1.3.2 Modification of LDL

LDL does not only occur in its native form, but also in different modifications, such as lipid oxidized LDL, hypochlorite (HOCl) modified LDL from the myeloperoxidase-H_2O_2 halide system, glycated LDL and, carbamylated LDL. These modifications are thought to be even more proatherogenic.

Lipoproteins, which are retained in the subendothelial layer for a prolonged time, are susceptible for modification. Several types of modified LDLs can circulate in blood, also in a healthy person. Modifications occur spontaneously by nonenzymatic

reactions. Normally, they are recognized and eliminated without any atherogenic effect.

In patients the situation of modification is more problematic, because modified LDL cannot be cleared properly.

1.3.2.1 Oxidation of LDL

Oxidation occurs as a normal event in humans, through oxidants in blood circulation. LDL and also HDL are subjected to oxidation, which can cause pathologies.

Studies revealed that in minimally oxidative LDL, the apoB 100 has oxidized amino groups [Himmelfarb, 2002].

Oxidants are highly reactive compounds with a half life of seconds, but nevertheless lipids, which are oxidized by oxy-radicals have lifetimes ranging from hours to weeks [Locatelli, 2003]. Both H_2O_2 and oxygen (O_2) are potential precursors for the production of powerful oxidants [Locatelli, 2003]. O_2 is able to interact with NO in endothelial cells to form high reactive nitrogen species, while H_2O_2 produces hydroxyl radicals (OH^-) with intracellular iron, leading to lipid degradation in cell membranes [Locatelli, 2003].

Reactive aldehyds can be formed by oxidation of alcohol groups and via the addition of oxygen to unsaturated carbon double bonds in carbohydrates, lipids or amino acids [Himmelfarb, 2002]. Oxidized LDL (oxLDL) raises the production of reactive oxygen species (ROS), which can lead to endothelial dysfunction [Thum, 2007].

Oxidation is a potential source of atherosclerosis, because individuals are subjected to enhanced oxidative stress, but reduced anti-oxidant systems such as vitamin C, reduced vitamin E content, and decreased activity of the glutathione system [Locatelli, 2003]. OxLDL cholesterol inhibits the first step of production of NO by eNOS. Circulating oxLDL gets caught by scavenger receptors of macrophages and thereby modulates the remodelling of the vessel wall. Endothelial cell injury is accelerated and proliferation of smooth muscle cells is induced.

Even so, oxidative stress occurs in healthy individuals, it plays an important role in host defense mechanisms, but is excessively activated in pathological conditions.

1.3.2.2 Kidney disease and modification of LDL

Different diseases contribute to the genesis of atherosclerotic lesions in coronary arteries.

Chronic kidney disease in humans is associated with an increased risk factor for development of CVD. Dialysis patients represent the most relevant risk group regarding carbamylated LDL, since either the reduced renal clearance or the critical production of modified LDL. Thus, patients have a high concentration of LDL and moreover excessive urea remaining in the blood circulation with a long residence time. Carbamylation of LDL is generated by a nonenzymatic reaction of cyanate derived from urea. The active form, isocyanic acid, reacts with free nonprotonated amino groups of proteins [Ok, 2005]. Carbamylation results in neutralization of positive charges of modified lysine residues [Ok, 2005]. Carbamylated LDL induces dose dependent vascular cell injuries relevant to atherosclerosis and is the most abundant modified protein in human blood. The mortality in patients with advanced kidney disease is 10 to 20 times higher than in the general population [Ok, 2005].

1.3.2.3 Diabetes and modification of LDL

Diabetes is denoted by a high concentration of glucose in blood. Insulin resistance is associated to blocked uptake of glucose in muscle and therefore remaining in blood circulation.

High glucose concentration is assumed to be the key factor for autooxidation. Glucose is able to bind non-enzymatically to free amino groups on proteins or to lipids [DeVriese et al, 2000]. Hyperglycemia activates the modification of plasma proteins like LDL, but also other tissue proteins can be modified by glycation. Small dense subfraction LDL_3 is more susceptible to glycation in the apoB molecule, even in non-diabetic individuals [Younis, 2009]. Glycation products are called advanced glycation end products (AGE). AGE are irreversibly formed and accumulate in the region of atherosclerotic plaques [Toma, 2009]. AGE are known to quench the NO activity and impair endothelial function. Besides, also in diabetic patients an elevated level of oxidized LDL can be detected, the mixture of glycated and oxidized LDL amplifies the formation of lesions in vasculature. Glyoxidized LDL is known as

advanced lipid oxidation end product (ALE) [Younis, 2009; Locatelli, 2003]. Thus modified LDL has a slower catabolic rate than native LDL [Younis, 2009].

AGE and ALE can directly affect vasculature by oxidant and inflammatory response in endothelial cells [Toma, 2009]. AGE bind to specific cell receptors (RAGE), which in turn trigger oxidative stress and inflammatory responses.

Nevertheless, glycated LDL also accumulate in normal human body with age, but individuals with diabetes have high amounts of AGE molecules in their circulation.

1.3.3 High-density-lipoprotein (HDL)
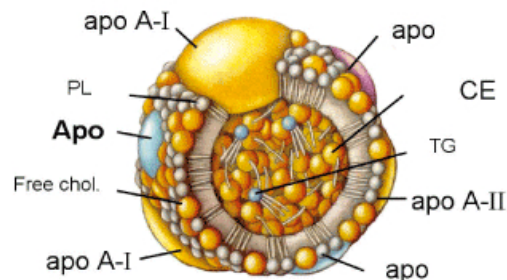
Fig.1.8 Basic structure and composition of HDL [Ref 8]

High-density-lipoprotein mediates the cholesterol efflux from peripheral cells via apoA-I and the reverse cholesterol transport to the liver [Schwandt & Parhofer]. The reverse cholesterol transport acts by clearing lipids from vessels and impairs the formation of atherosclerotic plaque [Willnow, 2007].

HDL is known as an anti-inflammatory and antioxidant particle, which can influence the development of cardiovascular disease and atherosclerosis.

1.3.3.1 Protective function of HDL in cardiovasculature

Many studies discovered the positive impact of HDL on atherosclerosis. HDL is considered as an antiatherogenic molecule because its protection of endothelial

cells. HDL protects against oxidative imbalance including modification of LDL particles in subendothelial regions.

Overall the major components of HDL molecules, which are apoplipoproteins, enzymes and sphingosine-1-phosphate (S1P) can influence the pathologies in the cardiovascular system. HDL associated S1P mediates the function of endothelial cells such as migration, proliferation, and endothelial integrity [Podrez, 2010]. Furthermore, the apoA-I part on HDL plays an antiatherogenic and antioxidant role, because it can remove oxidized phospholipids from oxidized LDL [Podrez, 2010; Ren, 2000]. HDL has the property to inhibit oxidative processes; HDL_3 has the most potent antioxidant activity [Podrez, 2010].

But overall, a reduced level of plasma HDL can be detected in disease state and correlates with increased catabolism of HDL.

1.4 LDL receptor gene family members

1.4.1 Overview

The low-density-lipoprotein receptor gene family constitutes of several structurally closely related cell surface receptors including LDLR, VLDLR, LRP1, LRP1b, LRP2, MEGF7/LRP4, and LRP8/apolipoprotein E receptor 2 (APOER2). They fullfil distinct biological functions in different organs, tissues, and cell types [Goldstein et al, 2001; Fisher, 2006].

The main function of these family members is the regulation of lipid metabolism. They are also involved in transport of nutrients, especially vitamins, and in neuronal development. Additional functions are described, for example in signal transduction during embryogenesis by transmitting signal molecules to target tissue [Schneider, 2003].

All family members share sequence homologies and are build of similar motifs for various functions. They consist of a large extracellular domain with cystein rich motifs for ligand binding and epidermal growth factor like repeats (EGF). An O- linked sugar domain is connected to the transmembrane domain. The intracellular domain has the

important YWTD (Tyr-Trp-Thr-Asp) motiv for pH-dependent release of ligands in endosomes. LDL receptor family members are clustered at sides of clathrin-coated pits on the cellular membrane by their NPXY (Asn-Pro-Xxx-Tyr) motifs [Willnow, 2007; Fisher, 2006; Schneider 1998].

Dysfunction of LDL receptor gene family members can be connected to diverse developmental defects.
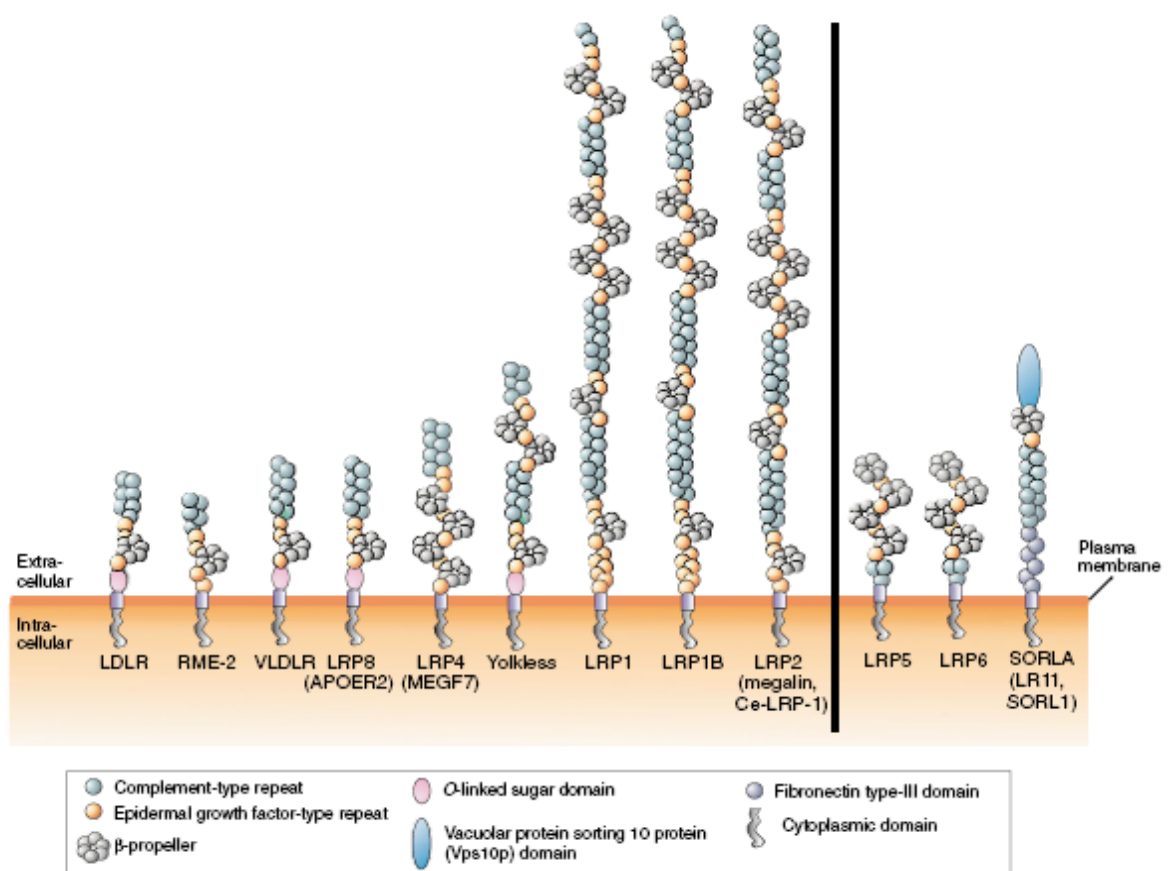

Fig.1.9 Overview of the LDL receptor gene family members. More distantly related members include LRP-5, LRP-6, and SorLA, which are characterized by unique combinations of domains [Willnow, 2007].

1.4.2 Low-density-lipoprotein receptor (LDLR)

Low-density-lipoprotein receptor was the first discovered receptor of the LDL receptor gene family [Schneider, 1989].

Familial hypercholesterolemia (FH) is caused by LDLR gene defects. People with FH have problems with the clearance of cholesterol rich LDL, leading to excessive levels of LDL in the blood [Goldstein, 2001].

The LDLR is involved in endocytosis of cholesterol-laden lipoproteins at clathrin-coated pits and binds the apolipoproteins apoE and apoB 100 on the cell surface of the lipoprotein shell. The human LDLR is approximately 160 kDa in size, but size depends on glycosylation status. The precursor protein is synthesized with a molecular weight of 120 kDa that is subsequently glycosylated to the mature form by N- and O-linked glycosylation.

Approximately 70% of LDL receptors in humans are expressed on liver cells [Mehta, 1999]. The transcription of LDL receptor is under tight control by ER associated sterol-responsive-element-binding-protein (SREBP). When sterols accumulate intracellularly, the synthesis of the receptor is repressed. In contrast, transcription of LDL receptor is induced when cells need increased demands of cholesterol [Mehta, 1999]. A decrease in LDL receptor on plasma membrane leads to accumulation of extracellular LDL in circulation.

1.4.3 Low-density-lipoprotein receptor related protein 1 (LRP1)

LRP1 is a large molecule composed of two subunits, a 515 kDa α-chain at the N-terminus and a smaller 85 kDa β-chain at the C-terminus of the protein.

The receptor plays a role in a variety of biological processes, including lipoprotein metabolism, protease degradation, activation of lysosomal enzymes, endocytosis of bacterial toxins, and viruses [Zhou, 2009].

LRP1 is widely expressed in distinct tissues and different cell types. It controls the clearance of dietary lipids in the liver [Willnow, 2007], but is not regulated by intracellular cholesterol. Lipoprotein remnants containing apoE are recognized and bound by LRP1 on the cell surface [Llorente-Cortés, 2004; Lilis, 2008]. LRP1 is

abundantly expressed in the vascular wall, in vascular smooth muscle cells (VSMCs), and macrophages. Patients suffering from coronary heart disease show an alteration in LRP1 gene expression.

Deficiency in LRP1 gene expression causes lethality in embryogenesis around mid-gestation and impaired function of the liver [Willnow, 2007; Hammes, 2006].

1.4.4　Low-density-lipoprotein receptor related protein 2 (LRP2)

LRP2 (Megalin/gp 330) is a transmembrane receptor, a type I cell surface receptor. This receptor is one of the largest LDLR family members with a molecular weight of 517 kDa in the non-glycosylated form.

The expression of LRP2 is distributed to distinct tissues, forming the apical epithelial cell borders. Main organs of expression include the yolk sac and the neuroepithelium of embryos, the proximal renal tubules, and the small intestine in adult organisms [Christensen & Birn, 2001].
LRP2, a multiligand binding receptor with scavenger function, regulates lipoprotein metabolism by binding LDL, filtrates vital substances from urine in the renal proximal tubules, is regulating the vitamin D homeostasis, and is essential as a messenger in certain organ systems during embryogenesis and brain development.
Another function is the response and binding of sex steroids in reproductive organs. Androgens and estrogens are bound and regulated by sex hormone binding globulin (SHGB). LRP2 mediates the endocytosis of SHGB into reproductive tissue, which has already been described in a mouse model [Hammes, 2005].

LRP2-deficient mouse embryos show to developmental defects, including malformation of reproductive organs and forebrain development [Willnow, 2007; Hammes, 2005].

1.4.5   Receptor associated protein (RAP)

Receptor associated protein acts as chaperon for LDLR family members, with 39 kDa in size. It binds LDLR- family members in the endoplasmatic reticulum (ER) for folding processes of these receptors and proper assessement to secretory pathways [Christensen & Birn, 2001]. RAP is associated to the ER by a carboxy-terminal tetrapeptide (HNEL), which is similar to ER-retention consensus sequence (KDEL) [Bu and Marzolo, 2000]. The expression is distributed to different tissues with the highest expression in kidney and brain. Bu and Marzolo described a model of how RAP functions as chaperon for LDL receptor family members. RAP associates with newly synthesized receptors and prevents the premature interaction of these receptors with ligands. During the secretory pathway and the maturing process, RAP dissociates in medial-Golgi from receptors because of low pH. A retrieval pathway transports RAP back to the ER compartment.

1.4.6   Scavenger receptors

Native LDL particles are normally internalized by binding to LDL receptors on cell membrane in clathrin-coated pits. In contrast, special scavenger receptors recognize aggregated modified LDLs and clear them from circulation. Such scavenger receptors are mainly expressed on macrophages, but could also be detected in SMCs and on platelets [Badimon, 2009]. The internalization of modified LDL is controlled by these scavenger receptors: SR-AI and SR-AII, CD36, LOX-1 or CXCL16 [Moore, 2006]. Endothelial cells express receptors specialized for uptake of oxidized LDL, such as CD36 transmembrane receptor [Carvalho, 2010].

In vasculature, modified LDL are also recognized and internalized by LRP1, especially in SMCs. LRP1 expression increases with higher accumulation of cholesterol-LDL in the intima of vasculature.

1.5   Blood flow associated shear stress

Endothelium in the cardiovasculature is subjected to chemical and mechanical stimulation; endothelial cells are continuously exposed to blood flow associated shear stress. The mechano-transduction working on endothelium, through mechanical force as shear stress, activates biochemical processes. The shear stress provides endothelial cell integrity and vascular function. Mechanical forces are vasoprotective and inhibit the apoptosis of endothelial cells.

Blood shear stress has an important impact on endothelial structure and regulation.

The hemodynamic shear stress conditions play a critical role in early atherosclerosis.

Mainly two types of shear stress conditions exist in vascular wall, the protective laminar high shear stress and the atherogenic low, oscillatory shear stress (LSS). Regions of arterial narrowing, curvatures, and bifurcations are especially susceptible to atherosclerotic lesion formation, because these regions are subjected to low and oscillatory shear stress [Pan, 2009]. LDL preferentially accumulate in regions of oscillatory shear stress, at bifurcations, and curvatures of arteries.

However, LSS tends to induce larger lesions with vulnerable plaque formation, whereas oscillatory shear stress tends to induce stable lesions [Pan, 2009]. At sides in vasculature were blood flows with high velocity meaning high shear stress (HSS), less formation of lesions is described by Pan, 2009. In contrast HSS or laminar blood flow preserves vascular homeostasis and the balance between vasodilation and vasoconstriction. HSS plays a key role in prevention of atherosclerosis.

Endothelial cells are continuous subjected to shear stress in vivo. Upon exposure to laminar shear stress, endothelial cells are deformed by mechanical forces [Pan, 2009].

The remodelling is important for transduction of signals. Changes in flow alter the expression of genes that have elements in their promoter regions that respond to shear stress. Some of these shear stress responding genes are: intercellular adhesion molecule 1, platelet derived growth factor B chain, and tissue factor in endothelial cells [Ross, 1999]. Thus, the expression is upregulated by reduced shear stress contributing to inflammation. Elevated levels of antiatherogenic eNOS are seen when blood flow is high. In addition increased NO release minimizes the permeability through endothelial tight junctions. Antiatherogenic molecule NO tightens the endothelial cell layer by sheer stress against the emigration of LDL and

macrophages. Depending on the kind of shear stress, different signalling pathways become activated and a various pattern in gene expression is induced. LSS promotes proatherosclerotic genes and HSS activates protective genes against atherosclerosis.

Laminar shear stress, previously mentioned, upregulates the expression of vasoactive substances such as the endothelial NO synthase, prostacyclin and antioxidants.

In contrast, LSS impairs the NO bioavailability, as well as increases the production of ROS and finally leads to endothelial dysfunction through apoptosis of endothelial cells [Pan, 2009].

Shear stress can be simulated and studied by the use of a chronic shear stress module. The Fiber Cell™ module is designed for cell culture experiments to study endothelial cells under subjection to shear stress conditions.

This allows the study of endothelial cells in a more physiological environment, compared to cell culture under static conditions.
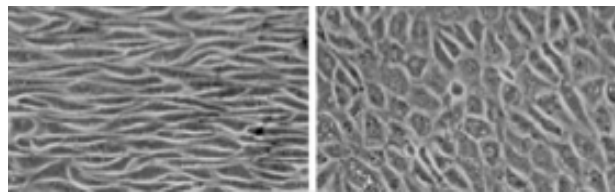
Fig.1.10 Endothelial cell morphology under different hemodynamic shear stress conditions of 0.5 dyne/cm^2 and high shear stress of 15 dyne/ cm^2 [Pan, 2009].

The cell morphology of a vasculature is imitated in a chronic shear module, as endothelium build up a monolayer and tight junctions are created. The cells can mimic their in vivo situation far better. It is known that gene expression pattern and protein expression profiles alter with various types of shear stress and can be different compared to static conditions.

1.6 Role of estrogen

1.6.1 Overview of estrogen

In recent years several studies demonstrated the effect of sex difference in diverse human physiological functions. Endothelium emphasizes the difference in gender in the cardiovascular physiology. Female sex is recognized as protective factor in cardiovascular disease and development of atherosclerosis. In younger age groups (25 to 49 years) coronary heart disease is two to five times more common in men than in women [Kublickiene, 2008]. Young men with a deficiency in the estrogen-receptor α show a higher incidence of atherogenic events at the age of 31 years [Kublickiene, 2008].

The first ovarian hormone estrone (E1) was isolated and its activity in biological samples was described in the early 1920 [Lieberman S, 2002].
Endogenous natural recycling estrogens are estrone (E1), estradiol (E2) and estriol (E3) [Smiley, 2009]. All steroid hormones are synthesized from cholesterol by steroidogenic enzymes residing in ER. E1 and E3 are formed in liver from E2. Half life of estrogen is about 3 hours, and it can be converted to the other forms E1 and E3 [Smiley, 2009].
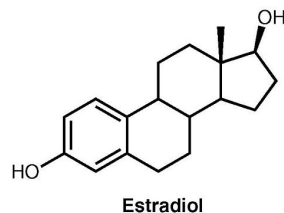
Estradiol

Fig.1.11 Chemical structure of estradiol [Ref 9]

Most steroids, like estrogen, are found in plasma bound to SHBG in a non-stoichiometric manner, only 2-3% exist as unbound free physiological active molecules [Smiley, 2009]. Free estrogen can diffuse rapidly through free diffusion into cells, but SHBG bound estrogen binds to specific receptors on the cell surface. Steroids bound to SHBG are internalized by LRP2 embedded in clathrin-coated pits or other appropriate hormone receptors on the cell membrane. The "free hormone hypothesis" states the free entry of hormones by unspecific diffusion through the

plasma membrane. Steroids can traverse the plasma membrane by diffusion because of their small size and lipid solubility [Willnow, 2009].

Specific estrogen receptors (ER) are found intracellularly as nuclear receptors namely, ERα and ERβ, which can form homodimers or heterodimers [Smiley, 2009]. In endothelium, both receptors ERα and ERβ are found. Endothelial injury results in a threefold upregulation of ERβ and stable low–level ERα expression in vascular tunica media [Watanabe, 2001]. Coronary arteries have an abundant amount of ERβ. In vasculature a specific G-protein coupled receptor has been identified to respond rapidly to free estrogen. GPR39 is a structurally unrelated protein, which binds E2 with high affinity and mediates G-protein signalling after estrogen binding [Smiley, 2009]. It is not yet clear, if this GRP39 is an endoplasmatic associated receptor or expressed in the plasma membrane [Smiley, 2009].

Estrogen is the predominant sex hormone in women, affecting the development. It promotes the growth and differentiation of tissues, including adipose, nerve, and musculoskeletal tissue and the cardiovascular system [Smiley, 2009].

The estrogen amount is depending on the menstrual cycle and is also influenced by life style. In menopausal women, the estrogen level declines and as a result they are more susceptible to CVD. Life style, as cigarette smoke and, free radicals impair the positive effect of estrogen in women. Estrogen and estrogen receptor expression vary from person to person and a significant change arises through aging.

Estrogen acts on vasculature, including endothelial cells, smooth muscle cells and also on extracellular matrix. Nevertheless, hormone replacement therapy is something controversial, because it seems to induce a progression of CVD and ischemic complications.

1.6.2   Role of estrogen in endothelium

It is known that the sex hormone estrogen has positive effects, for example in homeostasis of the endothelium in the cardiovascular region of the heart. Studies in animals and humans revealed the benefits of estrogen in cardiovasculature. In vivo studies on dogs and on isolated rat and rabbit hearts have shown, that estrogen treatment lowers the incidence of cardiovascular damage and enhances coronary blood flow [Smiley, 2009; Kublickiene, 2008].

Estrogen is able to activate the expression of protective molecules in vasculature as vasodilatory nitric oxide and prostacyclin and reduces the effect of vasoconstrictors as angiotensin and endothelin. Estrogen provides the relaxation of smooth muscle cells by attenuation of endothelin release. Bioavailability of endothelial NO is greater in arteries of female compared to male and estrogen may mediate the gender difference in NO production [Smiley, 2009]. Estrogen interacts also with mitochondria and decreases the production of reactive molecules, such as superoxide [Kublickiene, 2008].

The aromatic ring of estrogen molecules, which is absent in progestines and androgens, may play a key role as antioxidant moiety [Kublickiene, 2008].

Estrogen has multiple vascular effects including alteration of lipid serum concentrations, coagulation, fibrinolysis, and the antioxidant properties [Smiley, 2009]. The female hormone acts directly on endothelial cells. It activates angiogenesis and inhibits cell death. Furthermore, E2 stimulates the reendothelization in vascular healing [Billon, Lehoux, 2008]. Estrogen counteracts the negative correlations of atherosclerosis, such as vasoconstriction and oxidative stress.

Effects of Estrogen

Brain
Estrogen helps to maintain body temperature.

Estrogen may delay memory loss.

Estrogen helps to regulate parts of the brain that prepare the body for sexual and reproductive development.

Heart & Liver
Estrogen helps to regulate the liver's production of cholesterol, thus decreasing the build-up of plaque in the coronary arteries.

Ovary
Estrogen stimulates the maturation of the ovaries.

Estrogen stimulates the start of a woman's menstrual cycle – an indication that a girl's reproductive system has matured.

Vagina
Estrogen stimulates the maturation of the vagina.

Estrogen helps maintain a lubricated and thick vaginal lining.

Breast
Estrogen stimulates the development of the breasts at puberty and prepares the glands for future milk production.

Uterus
Estrogen stimulates the maturation of the uterus.

Estrogen helps to prepare the uterus to nourish a developing fetus.

Bone
Estrogen helps to preserve bone density.
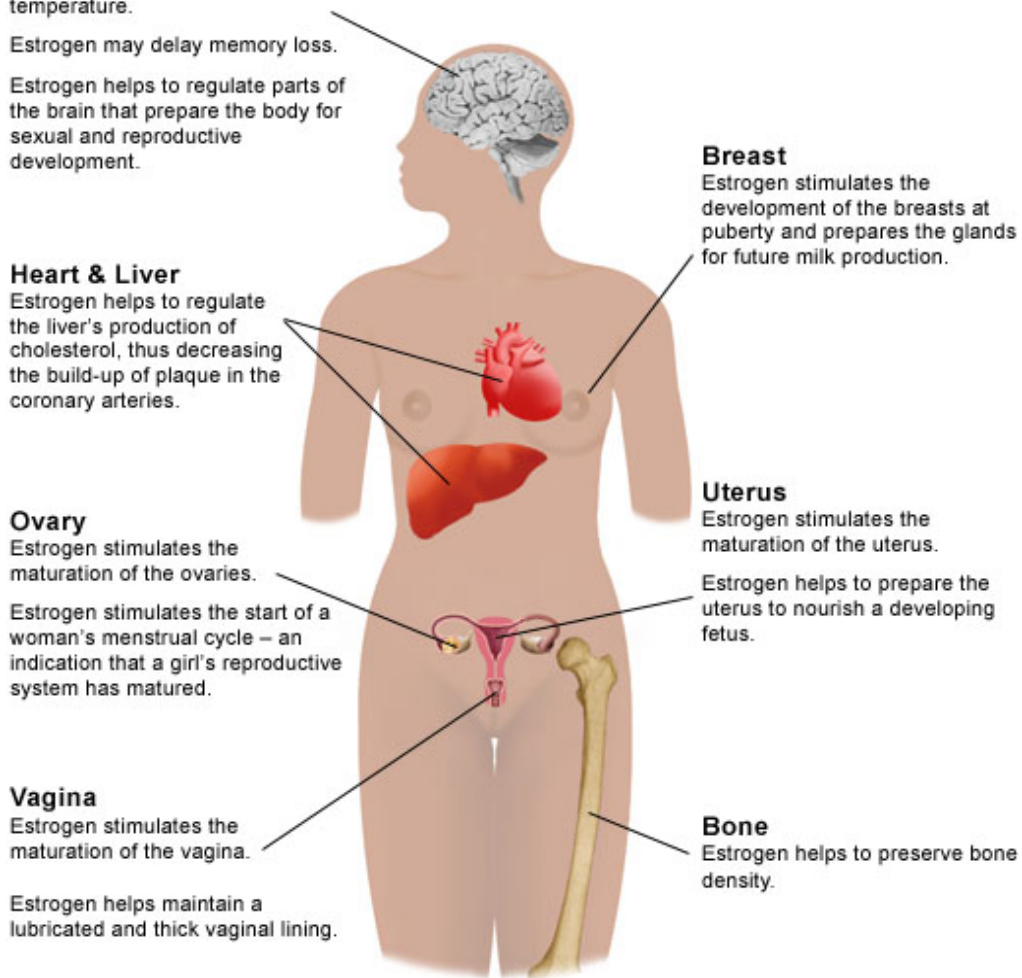
Fig.1.12 Positive effects of estrogen the in female body [Ref 10]

Chapter 2

Materials and Methods

2.1 Materials

Chemicals used for buffers and solutions were purchased from: AppliChem, BioRad, Fluka, GIBCO, Merck, Peqlab, Pierce, Qiagen, Roche, and Sigma-Aldrich.

2.1.1 Cell culture

Human coronary artery endothelial cells (HCAEC) were purchased from PromoCell and cultured as suggested by the supplier. Endothelial cell medium MV with growth supplement mix was also supplied by PromoCell. Cells were cultured in tissue culture flasks (25 cm^2 and 75cm^2) purchased from TPP and Nunc. For experiments cells were seeded in culture dishes (60.1 cm^2 and 22.1 cm^2) and on 96well cell-culture plates purchased from TPP and Iwaki. Cell culture cartridge was designed and delivered by FiberCell® Systems.

2.1.2 Oligonucleotide primers

The human oligonucleotides were synthesized by MWG-Eurofins.

Name of human primers	Sequence	Tm (°C)
Hu beta-actin_fw_16	5´ -GCGGGAAATCGTGCGTGACATT- 3´	62
Hu beta-actin_rev_17	5´ -GATGGAGTTGAAGGTAGTTTCGTG- 3´	61
LDLR_TMD1	5´ -GCTCACCACGGTGGAGATAGT- 3´	61.8
LDLR_TMD2	5´ -CARCGTGCTCCTCGTCTTCCTT- 3´	62.1
LRP1_fw_11	5´ -TGTCACCTCCAAGACAGTGCT- 3´	59.8
LRP1_rev_12	5´ -CGTTCCAAGCGGTAGACACTG- 3´	61.8
LRP2_fw_12	5´ -GCTGCAGAAAGTCTGGCTGTA- 3´	59.8
LRP2_rev_13	5´ -TACTCTCCCAATGTATGCGCGG- 3´	62.1

Table 2.1 Primers used for PCR

2.1.3 Antibodies

Human antibodies were purchased from Abcam, Calbiochem, and Sigma. A α-human LDLR polyclonal antibody was produced by our lab.

Name	Source	Reference
α-human LDLR	rabbit	Calbiochem
α-human LRP1	rabbit	Sigma
α-human LRP2	rabbit	Abcam
α-actin	rabbit	Abcam
α-rabbit- HRP conjungated	goat polyclonal	Sigma
α-rabbit- HRP conjungated	mouse polyclonal	Sigma

Table 2.2 Antibodies

2.2 Methods

2.2.1 Cell culture

Human coronary artery endothelial cells (HCAEC) were purchased from PromoCell and cultured as suggested by the supplier.

HCAEC were grown in Endothelial Medium MV (PromoCell) with special growth supplement mix (applied with the medium) and 100 units/ml penicillin, 100 µg/ml streptomycin in a humidified incubator at 37°C and 5% CO_2. Confluent cultures (70-80%) were passaged by aspirating the medium and cells were washed gently with 2ml PBS. Cells were detached with trypsin/EDTA. Detachment was controlled microscopically. To stop enzyme activity of trypsin solution, at least twice the volume of medium was added. Cells were centrifuged at 2 x g for 3 minutes (min) and the remaining pellet was resuspended in new medium. Cells were seeded on new culture flasks (T75) in an adequate splitting ratio of 1:3.

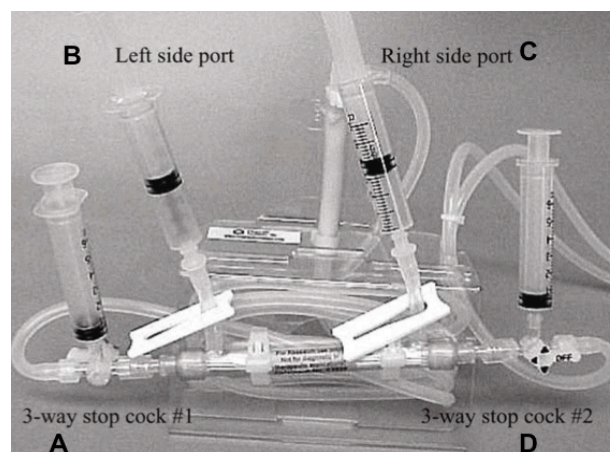### 2.2.2 Endothelial cell culture cartridges

Fig.2.1 Cell cartridge [Ref 11]

Endothelial cartridges were prepared in laminar flow hood by attaching 10 ml syringes to each of the ports A, B, C, D. The matrix of cartridge was activated with 20 ml of 70% ethanol in syringe on port A, 3 way stopcock at positions B and C were closed. The halfe volume of ethanol was rinsed to position D, waited for 60 seconds

(sec) and then the remaining 5 ml ethanol were flushed to syringe D. At position A, a new syringe with 10 ml H_2Odd was added and flushed to D with a new syringe and back to syringe A. This was repeated at least three times.

The matrix of the cartridge was coated with fibronectin (2.5 µg/ cm^2). The syringe with fibronectin solution was put on position A and a new, empty syringe was attached on D. Then the side port slide clamps C was opened, so that the "off" was facing to the cartridge. The stopcock on position D was turned to the "off " position facing to the cartridge. Fibronectin was flushed from A to syringe C through the fiber and flushed back to syringe A. The side port slide clamp on position C was closed and the D stopcock was opened facing away the cartridge. The fibronectin was then rinsed from syringe A to D. The side port slide clamp B was opened and stopcock on position A was closed to "off", facing away from cartridge. Fibronectin was then flushed from syringe D to B and back to syringe D. Side port slide clamp B was reclosed. The half fibronectin was rinsed from D to A and coating was performed for 1h at 37°C, 5% CO_2. All side port slide clamps were closed and the stopcocks were facing towards the cartridge.

A sterile flask (125 ml) was filled with Endothelial cell medium (PromoCell) with a sterile flask cap and cartridges were connected to the flask via plastic hoses for medium flow. The fiber matrix was filled with medium by flushing it from syringe A to D. All ports were closed with sterile luer caps. Medium was pumped to the system until the cartridge was completely filled, preculture was done with pump on low stroke 1 over night (oN).

The next day, the cartridge was put back to the laminar flow hood and the medium in the flasks was changed to serum free endothelial cell medium-SFM (PAA), and pumped manually to the system.

Cells from a 75 cm^2 flask were used for one cell culture cartridge. 10 ml cell suspension was filled in a new syringe on position A. New syringes were also attached to positions B, C, and D side port slide clamps on B and C were closed and stopcocks A and D facing away from cartridge. Cell suspension was gently flushed three times between position A and D, so that half of the volume was remaining in both syringes A and D. Stopcock on position A was closed, facing "off" towards the cartridge and slide clamp B was opened. Cells were rinsed from syringe D to syringe B. Side port slide clamp B was closed and slide clamp C was opened instead. Cells were flushed from syringe D to syringe C. Followed by closing position D and

opening of position A. Cell suspension was flushed from syringe A to syringe C. All ports were closed with sterile luer caps. Cell cartridges were incubated at 37°C in the incubator, and each 30 min the cartridge was turned 90 ° for cell attachment all over the fiber matrix.

The endothelial cartridge was connected to the perfusion equipment with a 50 ml perfusion syringe filled with SMF medium. The perfusion-syringe is attached via port A to the cell cartridge, with stopcock "off" in direction towards the tapered plastic hose. The perfusion velocity was 10 ml per hour (h).

The outflow plastic hose was connected to a small T25 flask for collection of perfusionsolution, to determine the amount of cells, which could not attach to the fiber matrix. All ports were closed with sterile luer caps. Endothelial cartridge was connected to the pump and low stroke 1 was simulated oN. Another cell cartridge was connected to pump and high stroke was simulated oN. HCAEC were subjected to high stroke-HSS (25 dyne/cm^2) and low stroke-LSS (2.5dyne/cm^2). After 24h the medium was changed. Chronic sheer stress was simulated for 9 days and then cells were harvested by trypsin/EDTA solution.

First, cells in cartridge were washed with PBS from port A to port C. Trypsin /EDTA solution in syringe on port A was flushed in cartridge and incubated for 4 min, then flushed between A and C for 1 min. The cells were collected in syringe C. Cell suspension was centrifuged at 2 x g, 5 min at room temperature (RT). Cell pellet was stored at -20°C.

2.3 Molecular Biological Methods: RNA/DNA

2.3.1 Isolation of total RNA with TRI Reagent

HCAEC cells were directly lysed in 1ml TRI Reagent (Sigma) per 3.5 cm diameter dish, by passing it several times through a pipette. Tissue samples were homogenized in 1 ml TRI Reagent per 50-100 mg of tissue with a polytron.

The samples were incubated for 5 min at RT for dissociation of nucleoprotein complexes. After incubation, per 1 ml of TRI Reagent, 0.2 ml of chloroform were added and mixed vigorously before an incunation at RT for 3 min. The samples were centrifuged at 12,000 x g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and mixed with 0.5 ml isopropyl alcohol per 1 ml TRI Reagent. Samples were incubated 10 min at RT and centrifuged at 12,000 x g for 10 min. The RNA precipitate was washed with 75% ethanol followed by centrifugation at 7,500 x g for 5 min at 4°C. The RNA pellet was air-dried and resuspended in ultra pure water (H_2O up). Finally, RNA was incubated at 55°C for 10 min and stored at -70 °C. RNA concentration was measured in ng/µl with Nanodrop 2000 based on the absorbance at 260 nm.

2.3.2 cDNA Synthesis

Synthesis of cDNA was performed by using the SUPERScript™ RNase H$^-$ Reverse Transcriptase from Invitrogen. Total RNA (1-5 µg) was used for reverse transcription and diluted to a total volume of 10 µl. Following components were added and mixed in a sterile eppendorf tube.

1-5 µg total RNA

1 µl   oligo (dT) primer (500 µg/ml)

1 µl   dNTPs (10nM)

x µl H_2Oultra pure (up) to a final volume of 10 µl

The mixture was heated to 65°C for 5 min and then chilled on ice for some sec.

The following components were added and gently mixed:

4 µl   5x First Strand Buffer
2 µl   DTT (0.1 M)

The content was incubated at 42°C for 2 min, and 1 µl of SUPERScript ™ II RT (reverse transcriptase (200 U/µl) was added and cDNA synthesis was performed at 42°C for 50 min. The reaction was stopped by heating to 70°C for 15 min.

2.3.3   Polymerase Chain Reaction (PCR)

A reaction volume of 50 µl was used for PCR. The following components were pipetted on ice in a sterile PCR-tube:

39 µl $H_2 0$up
5 µl   10 x High Fidelity PCR Buffer containing 15mM $MgCl_2$
2.5 µl   DMSO
1 µl   dNTP mix (10 mM)
1 µl   forward primer (20pM)
1 µl   reverse primer (20pM)
1 µl   cDNA template
0.5 µl   Expand High Fidelity enzyme mix (5U/µl)

The PCR reaction was performed in T3000 thermocycler (Biometra) and different PCR conditions were used, depending on the primer set. A touch-down thermocycling program was performed for optimization of PCR conditions. PCR products were separated by agarose gel electrophoresis.

Denaturation	94°C	30 sec
Annealing	65°C	30 sec
Extension	72°C	30 sec
Final extension	72°C	10 min
Pause	4°C	∞

Table 2.3 PCR conditions (40 cycles)

Lid temperature	94°C	∞
Denaturation	94°C	1 min
Annealing	65°C	1 min
Extension	72°C	30 sec
Denaturation	94°C	1 min
Annealing	59°C	1 min
Extension	72°C	30 sec
Final extension	72°C	6 min
Pause	4°C	∞

Table 2.4 Touchdown PCR conditions (40 cycles)

2.3.4 DNA Gel-Electrophoresis

Separation of DNA was obtained by agarose gel electrophoresis on a 1% agarose gel. DNA samples were mixed with 5x DNA loading buffer and for size determination a 1kB Plus DNA ladder (Invitrogen) was used. Gel electrophoresis was performed at 100 Volt (V) for 20 min. For visualization of DNA products under ultraviolet light (366 nm), the agarose gel contained the intercalating dye ethidium bromide (10 mg/ml).

50 x TAE, pH 8.0   5x DNA loading buffer (xylencyanol)

2 M Tris-HCl   50% glycerol

1 M acetic acid   0.1M EDTA

0.1 M EDTA   bromphenol blue

2.3.5   DNA Gel Extraction

The Xact DNA Gel Extraction Kit (Genexpress) was used for the elution of DNA fragments from agarose gel.

DNA bands were excised from the gel with a sterile scalpel and transferred into an eppendorf tube. The gel fragment was dissolved in 400 µl binding buffer II provided by the gel extraction kit and incubated at 55°C until the gel was dissolved.

The solution was transferred to a Xact column and incubated for 2 min and afterwards centrifuged at 10,000 rpm (table-top centrifuge-eppendorf) for 2 min. The flow through was discarded. The samples were washed with 300 µl wash buffer and centrifuged at 10,000 rpm for 2 min, another washing step with 200 µl was done and another centrifugation step for 2 min, 10,000 rpm was performed to remove all wash buffer.

The Xact column was transferred to a new eppendorf tube and 40 µl of elution buffer was pipetted on the center of the column and incubated for 2 min at RT. To elute the DNA, centrifugation at 10,000 rpm for 2 min was performed. The purified DNA was stored at -20°C.

2.3.6 Real Time PCR

Real time quantitative PCR (qPCR) was performed with LightCycler® 480 system (Roche Applied Science) in LightCycler® 480 Multi-well plates 96.

A 1:10 serial dilution was prepared with PCR gel elution of a housekeeping gene.

Primers were used in a concentration of 20 pmol.The template was not diluted and 1.5 µl were pipetted per well.

Mastermix components:

4.5 µl	H_2Oup
0.75 µl	forward primer
0.75 µl	reverse primer
7.5 µl	SYBR LC FAST (Peqlab)
13.5 µl per well	

After an initial step of denaturation at 95°C for 10 s, an annealing temperature (65-66°C) depending on primer pairs, was used for 15 sec, followed by extension at 74°C for 15 sec and at least a single elongation step at 79°C for 1 sec was performed. Fluorescence was measured and signals were quantified within the linear range of the standard curve with LightCycler® 480 Software.

2.4 Molecular Biological Methods: Proteins

2.4.1 Preparation of lipoproteins

Native LDL was isolated from human plasma by sequential density gradient ultracentrifugation with potassium bromide (density 1.019- 1.063).
Blood plasma (density=1.006) was adjusted to VLDL density of 1.019 with potassium bromide (KBr) using the formula and KBr/EDTA solution.

Formula: g (KBr) = Vol (plasma) x (D2-D1) / 1 – (0.321 x D2)

Vol = volume in ml   D = density

Plasma with density 1.019 was transferred to centrifugal tubes (Beckman 2 mm x 89 mm) with flow through a syringe needle (18 gg). The tubes were heat sealed and centrifuged for 20h at 55 K at 14°C with 50,2 Ti rotor in an ultracentrifuge (Beckman). The top VLDL fraction was removed and bottom LDL and HDL section was adjusted to a density of 1.063 with potassium bromide and KBr/EDTA solution. After ultracentrifugation at 55 K for 24h the top LDL fraction was removed and desalted using a PD-10 desalting column (GE healthcare) to manufacturer's recommendation. The equilibration of the column was done with LDL buffer (density 1.006). The HDL fraction was obtained from the bottom by adjusting the density to 1.21 with potassium bromide and KBr/EDTA solution and ultracentrifugation at 55 K for 36h [Goldstein et al, 1998].

LDL buffer (1x) pH 7.4

150 mM NaCl

0.24 mM EDTA

2.4.2 Modification of lipoproteins

Modification of lipoproteins was done in collaboration with the department of medical chemistry Vienna.

LDL (200 to 500 µg/ml) was oxidized by incubation with copper ions (60 µM/mg protein) at 37°C in PBS pH 7.4 for 4h and 24h. The oxidation reaction was stopped by addition of EDTA (120 µM/mg protein) and the preparation was subjected to gel chromatography to eliminate copper ions.

Carbamylated LDL (up to 5mg/ml) was prepared using potassium cyanate (20 mg/mg protein) in PBS pH 7.4 containing 100 µM EDTA at 37°C. Subsequently, the preparation was subjected to gel chromatography to eliminate unreacted cyanate.

Glycation of LDL (up to 5mg/ml) was performed with 100 mM glycolaldehyde in PBS pH 7.4 containing 100 µM ETDA for 24 h at 37°C. At the end of incubation, the preparation was subjected to gel chromatography to eliminate free aldehyde.

The final modification preparations were stored at 4°C in presence of 50 µM EDTA.

The modification process of LDL was estimated by relative electrophoretic mobility (REM) on a 1.2% agarose gel.

The extent of LDL oxidation was determined by quantification of thiobarbituric acid-reactive substances (TBARS). Malondialdehyde (E. Merck, Darmstadt, FRG) was used as a standard.

Electrophoresis was run at 90V for 60 min. The gel was fixed in ethanol-acetic acid for 10 min and afterwards shortly rinsed in destaining solution, and stained in fixing solution with 0.1% coomassie for 2h. Then the gel was destained with destaining solution.

Fixing solution

ethanol-acetic acid (50%:10%; v:v)

ethanol-acetic acid (5%:7%; v:v)

2.4.3 Cytotoxicity assay

Human coronary artery endothelial cells (HCAEC) were cultured in 96 well plates with a density of 2,000-5,000 cells per well. Cells were treated with different modified LDL (25 µg or 100 µg) or with native LDL (25 µg or 100 µg) for 19h in serumfree endothelial cell medium (SFM), containing 2mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Another set of cells was incubated with 17-beta estradiol (50 nM) to estimate the vasoprotective effect and treated with different modified LDL (100 µg) for 19h in SFM. To verify protective effect of HDL, another set of cells were pretreated with both HDL (30 µg or 100 µg) and different modified LDL (100 µg) for 19h in SFM.

After incubation, cell cytotoxicity was estimated by measuring lactat dehydrogenase (LDH) release in medium and total lysed cells using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit purchased from Promega, according to the manufacturer`s protocol. 2 µl of LDH positive control, delivered with the kit, was diluted in 10 ml PBS with 1% BSA (1:50 000 dilution). Substrate mix was reconstituted, by mixing 12 ml of assay buffer with a bottle of substrate mix.

After incubation time, 50 µl of medium were transferred to an enzymatic assay plate (Greiner) and 50 µl of reconstituted substrate mix were added per well and incubated for 30 min at RT, protected from light. The reaction was stopped by addition of 50 µl stop solution to each well. Meanwhile, total LDH release was measured by total lysis of cells with 10 µl of lysis solution per 100 µl remaining medium for 45 min at 37°C.

The total LDH release was measured by the same procedure as the spontanous LDH release. The absorbance of the LDH mediated conversion was measured at 490nm using the Viktor[3] V microplate reader (Perkin Elmer).

Percent cytotoxicity was calculated as ratio of experimental LDH release in medium to the total LDH release of lysed cells.

All experiments were performed in triplicates (n=3).

2.4.4 Protein measurement

Protein was measured with Quick Start Bradford 1x Dye Reagent (BIORAD) and bovine serum albumin (BSA, 2 mg/ml) as standard as followed with the Bradford method. 10 mg of BSA were used as standard in 1 ml of Bradford reagent. 1 µl of protein-extract was diluted in 1 ml of Bradford reagent and then analysed with UV spectrometer at 595 nm. Protein concentration was calculated as followed:

$$\frac{\text{µg standard}}{\text{OD standard}} \times \frac{\text{OD sample}}{\text{µg sample}} = \text{µg/µl protein}$$

LDL concentration was measured by Modified Lowry Protein Assay Kit (Thermo scientific, Pierce Protein Research Products).

Protein concentration of native LDL was determined by using a Modified Lowry Protein Assay Kit (Pierce), performed as manufacturer's recommendations.

A set of protein standard with concentrations of 10, 20, 40, 80 mg/ml were prepared with BSA (1 mg/ml). Lipoprotein-sample was prediluted 1:10 in H_2Odd, and 20 µl and 40 µl of LDL dilutions were used for measurement.

To total volume of 200 µl, 1 ml of Modified Lowry Reagent was added, mixed and incubated at RT for 10 min. At the end of incubation, 100 µl of prepared 1X Folin-Ciocalteu Reagent were added, mixed and incubated for 30 min at RT. Absorbance was measured with spectrophotometer at 750 nm, after blank correction. For protein determination blank corrected value of lipoprotein sample was plotted against BSA standard curve.

2.4.5 SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cell extracts were separated by SDS-PAGE with a thickness of 1 mm using the BIORAD system. The components of the separating gel were mixed and poured into a cleaned gel unit and overlaid with isopropanol. Stacking gel was poured onto polymerized separating gel after removal of isopropanol.

The combs were inserted and the stacking gel was allowed to polymerize. After removal of the comb, the gel was put into the buffer chamber and filled with electrophoresis buffer.

Components for 2 gels	Separation gel 6%	Stacking gel 4%
H_2O dd in ml	5.35	3.05
0.5 M Tris (pH 6.8) in ml	-	1.25
1.5 M Tris (pH 8.8) in ml	2.50	-
Polyacrylamide (30% PAA) in ml	2	0.65
SDS (10%) in µl	100	50
APS (10%) in µl	50	25
TEMED in µl	10	10

Table 2.5 Composition of gels used for SDS-PAGE

0.5 M Tris-HCl Buffer
0.5 M Tris-HCl pH 6.8

1.5 M Tris-HCl Buffer
 1.5 M Tris-HCl pH 8.8

30% Polyacrylamide
29.2% Acrylamide
0.8% N,N`-Methylenbisacrylamide

1x Electrophoresis Buffer
250 mM Tris-HCl
192 mM Glycine
0.1% Glycine

2.4.6 Westernblotting

Cell lysates were loaded on the SDS-gel under reducing (β-mercaptoethanol) or non-reducing conditions with 4x Laemmeli loading buffer. In the case of reducing conditions, the samples were heated to 95°C for 5 min before loading. One slot was loaded with 10 µl of Precision Plus Protein™ Unstained Standard (BIORAD), which served as size marker.

The gel was run at 180V for ~1h, until the bromphenolblue front reached the bottom of the gel. The gels were used for western blotting or direct staining with Coomassie blue or Silver Stain.

Proteins were transferred to nitrocellulose membrane (Hybond™-C Extra, Amersham Bioscience). The blot was prepared as followed: 1 fibre pad was applied to the blotting unit with 3 Whatman papers soaked with transfer buffer and followed by nitrocellulose membrane and the gel. Finally three whatman paper and 1 fibre pad were added on the other side of the blotting unit. Air bubbles were removed by rolling a tube over the blot. The blotting unit was put into the blotting chamber filled with transfer buffer in an ice box. Wet tank blotting was done at 200 mA per gel for 1-1.30 min.

Transfer efficiency was checked by Ponceau S staining of the membrane, blotted proteins and the standard were visualized by destaining with H_2Odd. The protein standard and the lanes were marked with a membrane pencil and excess parts of the membrane were cut off.

The membrane was blocked in blocking solution for 1h at RT. After blocking, incubation with primary antibody was done oN at 4°C on a shaker. Dilutions of the primary antibody were dependent on the primary antibody affinity. The membrane was washed three times with TBS-T each 10 min, followed by incubation with the secondary antibody-HRP conjungated for 1h at RT by gentle agitation. The membrane was washed three times with TBS-T, each 10 min. Finally, the immunoblot was incubated with ECL solution I and II (mixture 1:1) for 5 min at RT and put into the exposure cassette. Films were exposed in dark room for a few seconds up to 30 minutes, developed by AGFA developing maschine.

4x Laemmli buffer (reducing)	4x Laemmli buffer (non reducing)
31.2 % Glycerine	31.2% Glycerine
6 % SDS	6% SDS
20 mM Tris-HCl, pH 7.4	20 mM Tris-HCl, pH 7.5
Bromophenol-blue	Bromophenol-blue
25 mM Dithiothreitol (DTT)	Add H_2Odd to 20 ml
Add H_2Odd to 20 ml	

1x Electorphoresis buffer

25 mM Tris-HCl

192 mM Glycine

1% SDS

1x Transfer buffer

25 mM Tris-HCl

192 mM Glycine

Ponceau S

0.2 % Ponceau S

3% trichloracetic acid

Dilute with 1x TBS

Bloking solution

5% non fat dry milk

1x TBS-T

TBS-T

1x TBS

0.1% Tween 100

10x TBS

1.37 M NaCl

0.027 KCl

0.25 M Tris-HCl

With HCl to pH 7.4

2.4.7 Co-Immunoprecipitation

Co-Immunoprecipitaion is based on the principle of the interaction of proteins.

Ni-NTA agarose (QIAGEN) with a binding capacity of 50 mg His-tagged protein/µl bead suspension was used for binding of recombinant His-tagged RAP protein. 500 µl of RAP protein were added to 100 µl of Ni-NTA beads in TBS-T with 2 mM CaCl$_2$ and protease inhibitor and incubated oN at 4°C on a rotator. The next day, the samples were centrifuged at 3,500 rpm (table-top centrifuge-eppendorf) for 5 min at 4°C and the supernatant was discarded. The beads were washed three times with TBS-T with 2 mM CaCl$_2$. Then appropriate amount of cell lysate was added to the RAP-linked Ni-NTA beads in TBS-T with 2 mM CaCl$_2$ and incubated oN at 4°C on a rotator. The next day, the samples were centrifuged at 3,500 rpm for 5 min at 4°C and the supernatant was discarded. The beads were washed three times with TBS-T with 2 mM CaCl$_2$. . The beads were mixed with 4x Laemmli buffer (reducing) and heated to 95°C for 5 min to separate the proteins from the beads. Samples were applied to SDS-PAGE and analysed by silver staining.

2.4.8 Coomassie–blue staining

After SDS-PAGE the gel was removed from the glass plates and covered with Coomassie staining solution for 1h at RT with gentle agitation. The gel was destained with destaining solution with gentle agitation, changing the destaining solution at least three times. To preserve the gel, it was dried by vacuum (BioRad) at 60°C for 1h.

Coomassie Solution

10% acetic acid

25% isopropanol

0.862 g Coomassie Blue R250

up to 2L with H_2Odd

Destaining solution

10% acetic acid (v:v)

30% methanol (v:v)

2.4.9 Silver Stain

Silver stain of proteins after SDS-PAGE was performed by SilverSNAP Stain Kit II from Pierce. All steps were performed in a clean plastic tray with gentle agitation.

After electrophoresis, the gel was washed two times in sterile H_2Odd for 5 min. Fixation of proteins was done by incubation of the gel in fixing solution two times for 15 min. Afterwards, the gel was washed with two changes of 10% ethanol and two changes of sterile H_2Odd for 5 min. The gel was incubated in Sensitizer Working Solution for 1 min and then washed with two changes of sterile H_2Odd for 1 min. The Work Stain Solution was added to the gel and incubated for 30 min and subsequently washed two times with sterile H_2Odd for 20 sec. Developing was performed with Developer Working Solution, until protein bands appeared. Stop Solution, 5% acetic acid, was added to the gel tray to stop developing reaction. At least the gel was incubated in H_2Odd for 10 min. For storage, the gel was dried using the vacuum dryer at 70°C for 1h.

Fixing solution

30% ethanol

10% acetic acid

Sensitizer Working solution

50 µl of sensitizer

up to 25 ml H_2O steril

Work stain solution	Developer Working solution
0.5 ml Enhancer	0.5 ml Enhancer
25 ml Stain	25 ml Developer

2.5 Statistical Analysis

Data are expressed as +/- standard deviation. The Student's t-test was employed to explore whether differences in parameters measured in cytotoxicity assays were statistically significant. The significance value was set to $p < 0.05$ and calculated as triplicates n=3.

Chapter 3

Results

Endothelial dysfunction is an early marker of atherosclerosis. Several diseases are marked by atherosclerotic plaque progression in coronary arteries through modified lipoproteins.

Different modified lipoproteins were already observed as oxidized LDL, glycated LDL (diabetes), and carbamylated LDL (kidney disease), depending on disease state. Even so, during aging modified LDL exist also in healthy people, but the extent of different modified LDL increases with disease mentioned above.

The focus of this study was to investigate, how human coronary artery endothelial cells alter their gene expression after exposure to different modified LDL. Published data revealed the increase of inflammatory sensors as I-CAM and E-selectin in first atherosclerotic events. In addition, previous studies focused on the upregulation of scavenger receptors for clearance of modified LDL.

In this study the interest was focused on the role of LDL receptor gene family members including LDLR, LRP1, and LRP2 in HCAEC and further to investigate the effect of E2 and HDL on HCAEC.

3.1 Modification of lipoproteins in vitro

For in vitro modification of lipoproteins, native LDL from blood of healthy individuals was isolated by sequential gradient ultracentrifugation with potassium bromide. Blood was exclusively isolated from men.

Lipoproteins could be separated into different fractions by their density. After ultracentrifugation for 20h with a density of 1.019, the VLDL fraction could be isolated at the top of the centrifugal tube (Fig.3.1-A).

Native LDL was isolated after additional centrifugation for 24h with a density of 1.063 (Fig.3.1-B). At least HDL was isolated after centrifugation for 36h with a density of 1.21, seen as fraction also at the top of the centrifugal tube (Fig.3.1-C).
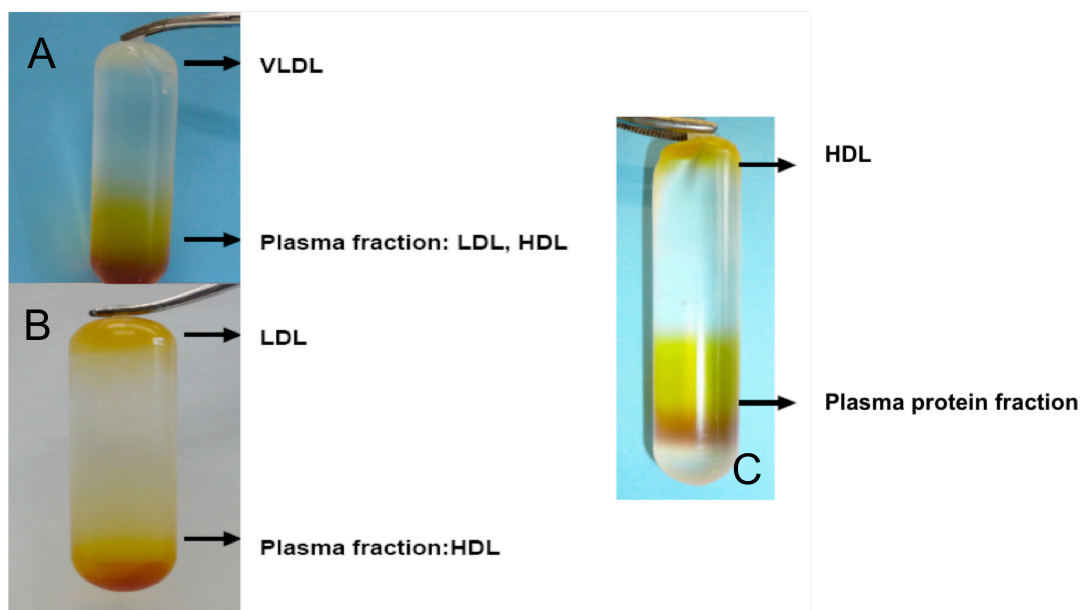
Fig.3.1 Isolation of lipoproteins from healthy individuals. All lipoproteins including VLDL, LDL, and HDL were isolated from the top of the tube after density gradient centrifugation. VLDL with density of 1.019 (A), LDL with density 1.063 (B), and at least HDL with density of 1.21 (C).

Modifications of lipoproteins were performed in vitro by chemical reactions. Oxidized LDL was attained by chemical reaction with copper ions, carbamylated LDL was

obtained by incubation with KOCN, and glycated LDL by incubation with glycerolaldehyd.

To estimate the modification processes relative electrophoretic mobility (REM) and thiobarbituric acid-reactive substances (TBAR) assay were performed (data not shown). REM of lipoproteins was monitored on an agarose gel and subsequently by Coomassie staining. Different migration through the gel could be used to estimate modification status.

Native unmodified LDLs (Fig.3.2, lane 1-3) did not migrate as far as modifed LDL (Fig.3.2, lane 4-8), because its positive charge on the lipoprotein shell. The modification neutralizes modified LDL, therefore it can migrate further on the gel. Neutralization of positive charge allows the migration of oxidized, glycated, and carbamylated LDL.

Oxidation of LDL leads to reactive aldehydes via the addition of reactive oxygen to unsaturated carbon double bonds of lipids or amino acids and thereby obtaining a negative charge [Himmelfarb, 2002]. LDL oxidized for 24 h (Fig.3.2, lane 5) migrates further compared to LDL oxidized for 4h (Fig.3.2, lane 4), because of more oxidized apoB 100 after 24h. Carbamylation results in neutralization of positive charge of modified lysine [Ok, 2005] and glucose modification also neutralizes positive charge of lipids and amino acids.

Each modification experiment has its varitions, because of different blood donors and also chemical modifications can fluctuate. Modification reaction such as carbamylation and glycation resulted in no change in REM, compared to oxidized LDL.
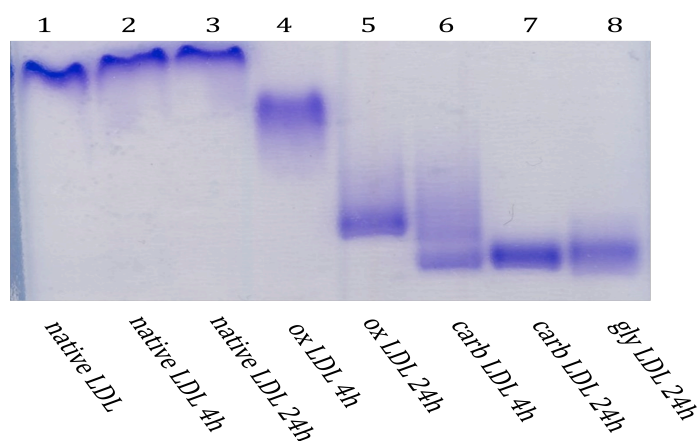
Fig.3.2 Analysis of modification processes by REM on an 1.2 % agarose gel with subsequent Coomassie staining (0.1%). Lane 1-3 native LDL, lane 4 and 5 oxidized LDL (ox LDL 4h and 24h), lane 6 and 7 carbamylated LDL (carb LDL 4h and 24h), and lane 8 glycated LDL (gly LDL 24h).

Further the formation of tiobarbituric acid reactive substances in the case of oxidized LDL was measured. Malondialdehyd was used as standard. Through oxidation reaction reactive radicals were built. Data of TBAR assay are not shown.

3.2   Human coronary artery endothelial cells (HCAEC)
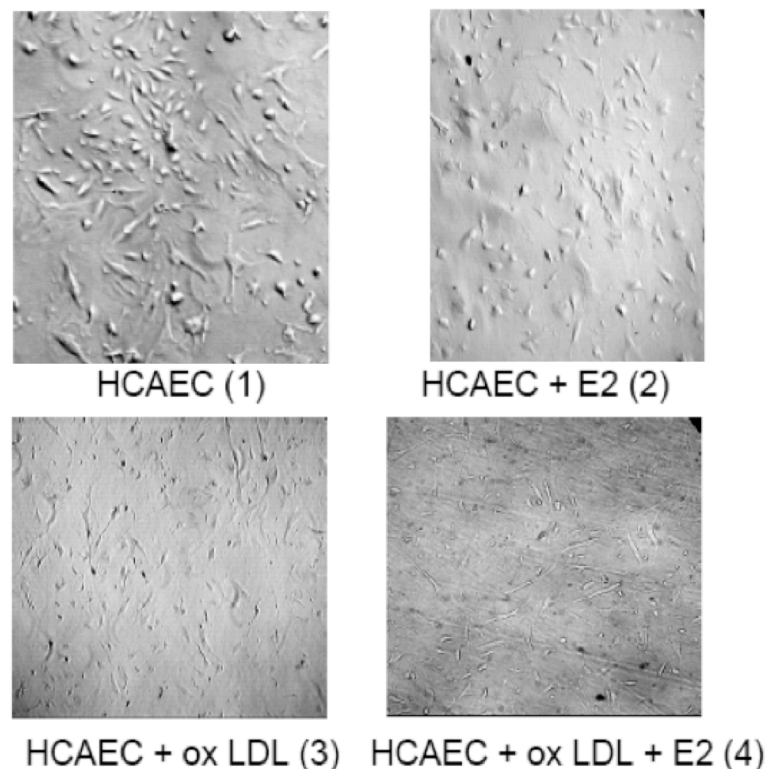
Fig.3.3 Morphology of HCAEC incubated with different modified LDL and E2 compared to control cells Cell morphology and growth to confluency in HCAEC control in SFM without incubation (1), incubated with E2 (2), with ox LDL (3), and with E2 and ox LDL (4) HCAEC were cultured in 60 mm dishes. 20 fold magnification.

The interest was, if human coronary endothelial cells (HCAEC) alter their morphology after exposure to different modified LDL for 19h. Studies showed the remodelling of endothelial cells in contact with modified LDL. Further, if incubation with E2 had a positive effect on cell behaviour, when incubated with modified LDL as well.

Normal untreated cells in SFM (Fig.3.3-1) and also cells with E2 (Fig.3.3-4) seemed to proliferate and grow to confluency, but cells incubated with modified LDL (Fig.3.3-3) seemed to change their cell morphology. As seen in Fig.3.3 (3 and 4) cells formed stretches and lost their round shape. Also cells tended to form cell islands.

3.3   Determination of cytotoxicity of modified LDL to HCAEC

Based on the change of cell growth behaviour and alteration in morphology after treatment with modified LDL, the cytotoxic effect of modified LDL was determined.

A cytotoxicity assay was performed to measure the release of lactat dehydrogenase from cells. Experimental LDH release could directly be associated with cell damage and apoptosis.

For incubation of HCAEC with different modified LDL, concentrations of 25 µg and 100 µg were used. Further investigation was done, to analyze if E2 and HDL can antagonize the cytotoxic effect of modified LDL.
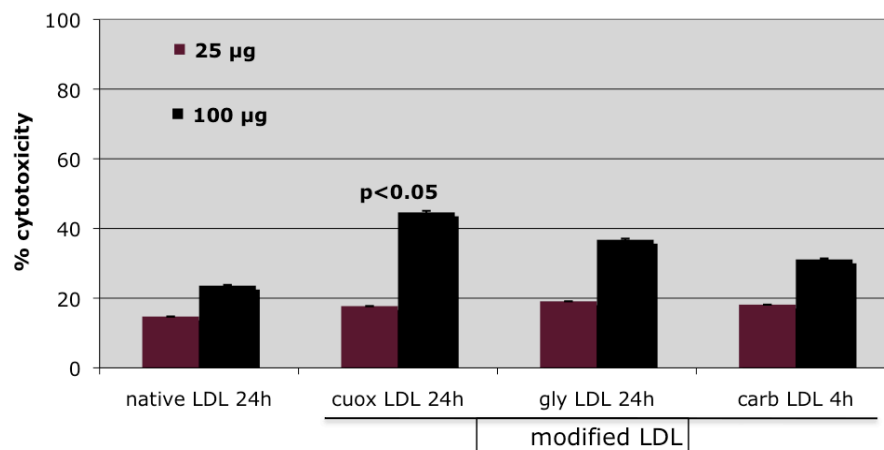
Fig.3.4 Cytotoxicity of 100 µg compared to 25 µg of modified LDL HCAEC were incubated with modified LDL (100, 25 µg) in SFM, average experimental LDH release in medium of cells was determined. Total LDH release could be measured by lysing the cells. Percent cytotoxicity was calculated as ratio of experimental LDH release to total LDH release. p values were calculated to native LDL as triplicates n=3.

An incubation of HCAEC with 25 µg compared to 100 µg was not highly toxic. However a baseline apoptosis of cells about ~15%-20% could be estimated (Fig.3.4). Copper oxidized (ox LDL 24h), carbamylated (carb LDL 4h), and glycated LDL (gly LDL 24h) showed a higher cytotoxic effect with a treatment concentration of 100 µg. Strongest cytotoxic effect was detected with 100 µg of oxidized LDL. For the following experiments a concentration of 25 µg of modified LDL was used.
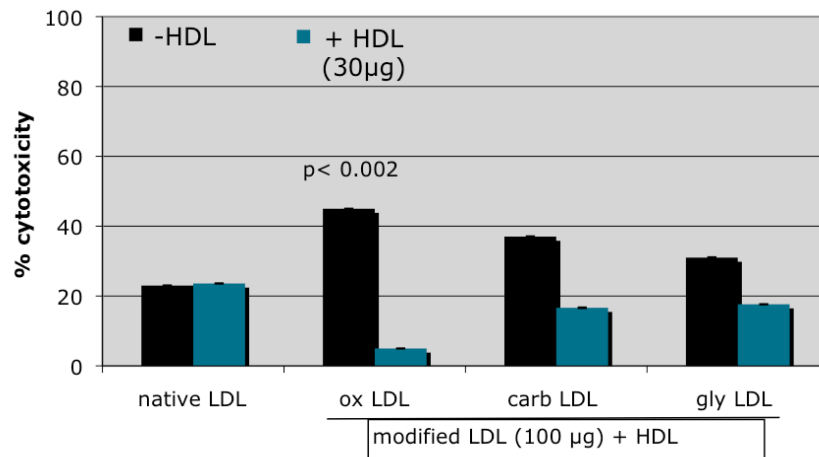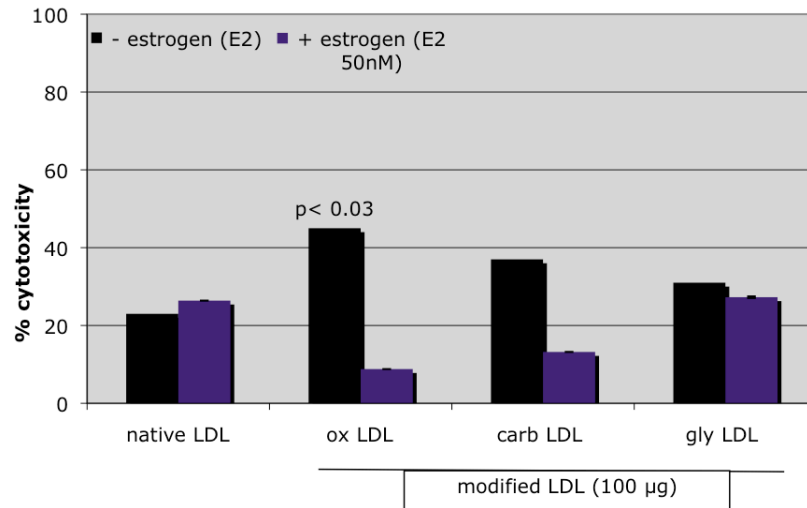
Fig.3.5 Cytotoxicity of modified LDL and protective effect of HDL Cells were incubated with 100 µg of different modified LDL with or without HDL (30 µg). The HDL/LDL concentration was used in a ration 1:3, according to human physiology. The incubation of HCAEC was done for 19h in SFM and LDH release was measured as described in Fig 3.4. p values were calculated to native LDL as triplicates n=3.

HDL was defined as protective molecule that antagonizes the oxidative stress in vasculature. Cytotoxic effect was lower after incubation of HCAEC with modified LDL (100 µg) in the presence of HDL (30 µg) seen in Fig.3.5. The concentration ratio of HDL (30 µg) to LDL (100 µg) reflects human blood circulation.

Fig.3.6 Cytotoxicity of modified LDL and protective effect of E2 HCAEC were incubated with 100 µg of different modified LDL and also with or without E2 (50 nM) for 19h in SFM. The LDH release was measured as described in Fig 3.4. p values were calculated to native LDL as triplicates n=3.

Moreover, incubation with E2 showed a potent decrease in cytotoxicity when incubated with oxidized and carbamylated LDL. Interestingly, no decrease in cytotoxicity could be determined in the case of glycated LDL with E2 (Fig.3.6). The explanation could be that diabetes comes along with elevated levels of glycated LDL and that the effect of estrogen is abolished in this disease.

Concluding the data, 25 µg of modified LDL showed no detrimental cytotoxic effect to HCAEC in cell culture compared to 100 µg modified LDL. A 1.5 to 2 fold increase in cytotoxicity could be detected with a concentration of 100 µg of different modified LDL. To confirm the positive effect of estrogen and HDL, cytotoxicity decreased after incubation with modified LDL (100 µg) seen in Fig.3.5 and Fig 3.6.

3.4 Expression analysis of LDL receptor gene family members in HCAEC

The aim of this study was to investigate the expression profile of LDL receptor gene family members including the LDL receptor, LRP1, and LRP2 in human coronary artery endothelial cells. Further, we wanted to estimate possible alteration in gene expression after exposure to different modified LDL, as in atheriosclerotic events. The question was if these receptors have a specific function in coronary arteries for regulation of lipid metabolism in early atherosclerosis.

First RT-PCR analysis was performed followed by determination of quantitative expression level (qPCR) of LDL receptor, LRP1, and LRP2 in HCAEC. The absolute quantification was used to compare mRNA expression of different receptors.

3.4.1 RT-PCR analysis

PCR analysis showed an overall detetction of LDL receptor, LRP1 and LRP2 DNA fragment in human coronary artery endothelial cells. Total RNA was isolated from HCAEC after incubation with modified LDL and also after doubletreatment with E2. RNA was reverse transcribed into cDNA, which was used as template for RT-PCR analysis and qPCR. The data obtained from RT-PCR corresponded only partially with the results obtained by quantitative PCR analysis.
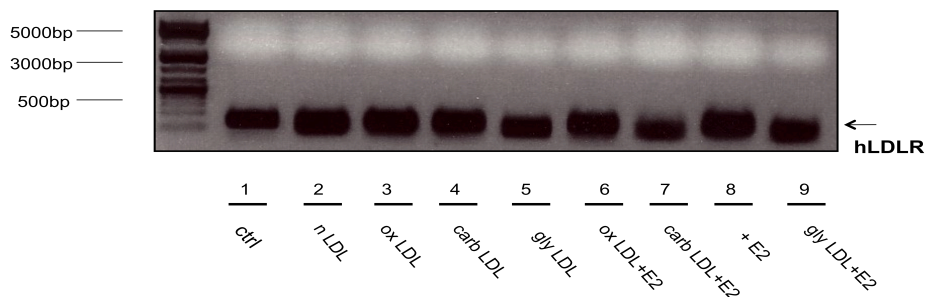
Fig.3.7 RT-PCR analysis of LDLR fragment in HCAEC using a touchdown PCR program. RNA was isolated and reverse transcribed into cDNA, which was used as template for RT-PCR. The forward primer LDL_TMD1 and reverse primer LDL_TMD2 were used. Primers were designed for transmembrane domain of LDLR and generated a fragment of 133bp. Lane 1 untreated cells (ctrl), lane 2 HCAEC incubated with native LDL (n LDL), lane 3-5 detected PCR products with incubation of HCAEC with modified LDL (25 µg) and lane 6-9 detected LDLR fragments in HCAEC treated with modified LDL and E2 (50nM).

LDL receptor PCR products could be detected by RT-PCR seen in Fig.3.7, also LRP2 could be observed in HCAEC seen in Fig.3.8. PCR production of LRP1 (Fig.3.9) was not as strong compared to the two other receptors, only in the case of oxidized LDL treated HCAEC Fig.3.9 (lane 3). LRP1 seemed to be stronger with E2 as seen in Fig.3.9 (lane 6-11).
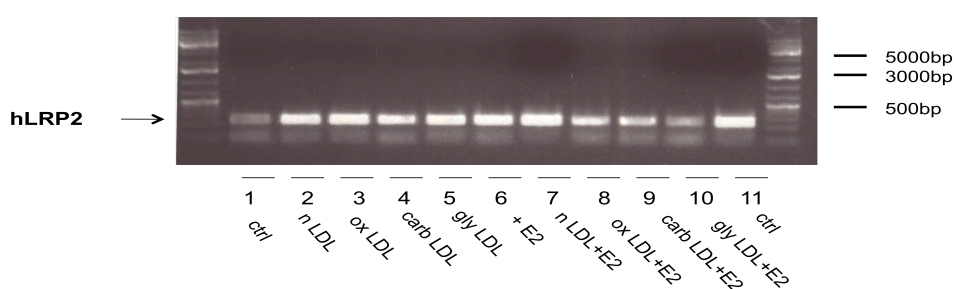

Fig.3.8 RT-PCR analysis of LRP2 fragment in HCAEC using a touchdown PCR program. RNA was isolated and reverse transcribed in cDNA used as template for RT-PCR. PCR product with fragment size of 237bp was generated with the primer hu_LRP2 (12fwd) and hu_LRP2 (13 rev). Lane 1 untreated HCAEC (ctrl), lane 2 HCAEC incubated with native LDL (n LDL). Lane 3-5 show that LRP2 PCR product in HCAEC with modified LDL (25 µg) and lane 6-10 showed LRP2 fragment in HCAEC with modified LDL (25µg) and E2 (50nM). Lane 11 untreated cells as control (crtl).
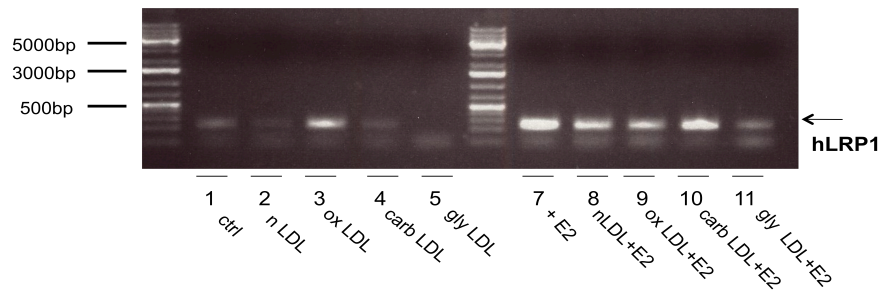
Fig.3.9 RT-PCR analysis of LRP1 fragment in HCAEC using a touchdown PCR program. RNA was isolated and reverse transcribed in cDNA, which was then used as template for RT-PCR. PCR product with fragment size of 234bp was generated with the primer hu_LRP1 (11fwd) and hu_LRP1 (12 rev). Lane 1 demonstrated LRP1 PCR product of untreated cells (crtl) Lane 2-5 showed HCAEC with modified LDL (25 µg), no band could be detected with glycated LDL lane 5. Lane 7-11 showed LRP1 PCR fragment of HCAEC with E2 (50nM) and incubation with modified LDL (25µg).

The mRNA expression level of LRP2 should be elucidated under static conditions in cell culture and further by using the chronic shear stress module (Fiber Cell™ module). HCAEC were grown in this cell cartridge and subjected to LSS and HSS. Cells were harvested and RNA was isolated followed by reverse transcription. Two conditions were compared as HSS and LSS, also when cells were additional incubated with carbamylated LDL. LRP2 is abundantly expressed in human epithelial kidney cells (HEK) (Fig.3.10, lane 1), which were used as positive control. Transcription level of LRP2 increased after treatment with E2 (Fig.3.10, lane 2), which was discovered in our lab. RT-PCR revealed a low signal of LRP2 under shear stress seen in Fig.3.10 in lane 4-7 compared to HEK positive control (Fig.3.10).
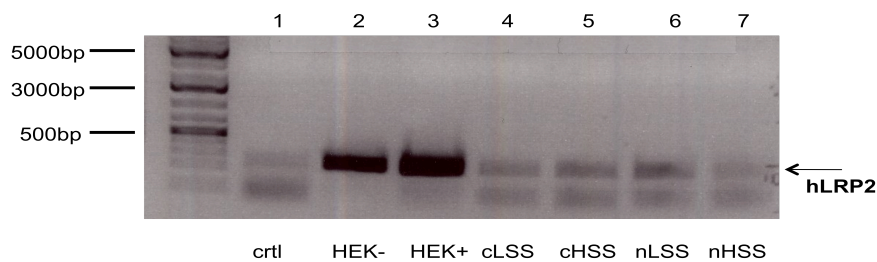
Fig.3.10 RT-PCR of HCAEC under shear stress RNA was isolated and reverse transcribed in cDNA, which was used as template for RT-PCR. PCR product with a fragment size of 237bp was generated with the primer hu_LRP2 (12fwd) and hu_LRP2 (13 rev). Lane 2 showed HEK (human epithelial kindey cells), which were used as positive control and in lane 3 were HEK + (human epithelial kindey cells after E2 treatment for 24h). Crtl (untreated HCAEC) could be seen in lane 1, carbamylated LDL under LSS and HSS (cLSS and cHSS) and native LDL under HSS and LSS (nLSS, nHSS)

3.4.2. Quantitative analysis of mRNA levels under static conditions

Quantitative real time PCR should reveal the differences in the mRNA level of LDL receptor family members in normal untreated HCAEC and in HCAEC incubated with oxidized, glycated and carbamylated LDL. Further, we wanted to know if the genexpression-level changes when cells were doubletreated with E2 and modified LDL.

Real time PCR was performed with LightCycler® 480 and using SYBR Green, 45 cycles were run. LightCycler® 480 software (version 1.2) was used for quantification. The absolute quantification was used to compare mRNA levels under various conditions.
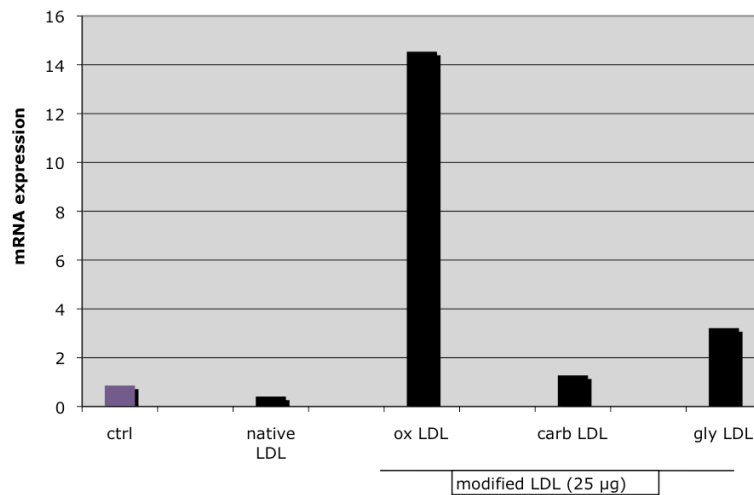
Fig.3.11 Quantitative PCR analysis of LDLR mRNA in HCAEC incubated with modified LDL Undiluted cDNA was used as template for qPCR with the forward primer LDL_TMD1 and the reverse primer LDL_TMD2. Gene expression was normalized to human-beta actin housekeeping gene.

As seen in Fig.3.11, the LDLR mRNA level increased after treatment with ox LDL, an approximately 9.3 fold increase compared to control was detectable. Transcription of LDLR in samples treated with carbamylated and glycated LDL was slightly higher compared to control cells.

Interestingly, after administration of E2, a significant increase of LDLR transcript in control group (Fig.3.12, left panel) was detected. Smith et al, 2004 published that the LDLR mRNA level is responding to estrogen treatment in HepG2 cells. Also HCAEC showed an effect after E2 treatment. LDLR transcription was arbitrarily set to 100%, the LDLR transcription in HCAEC incubated with native LDL decreased to 0.02%.
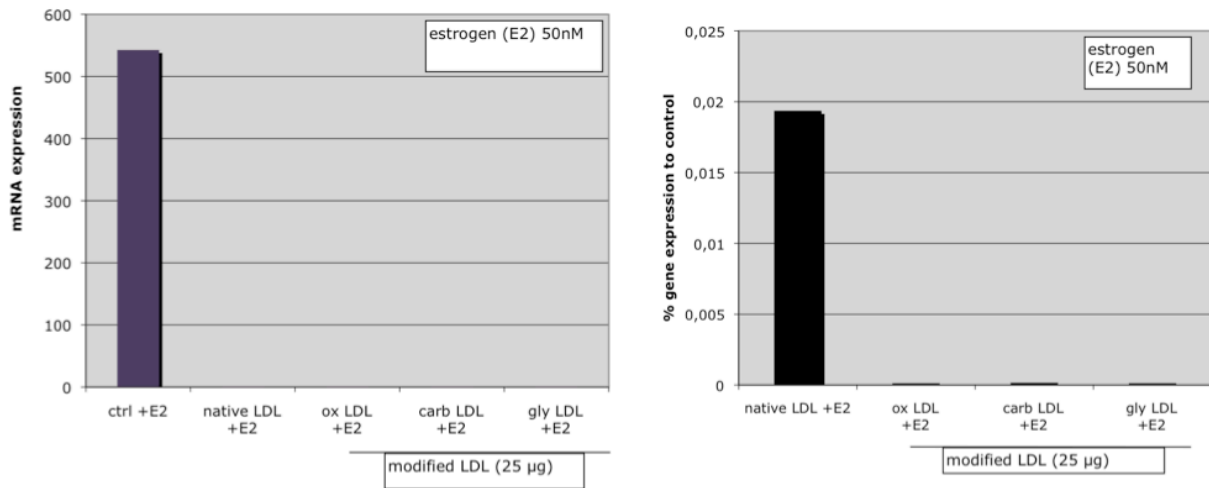
Fig.3.12 Quantitative PCR analysis of LDLR mRNA in HCAEC incubated with modified LDL and E2 Undiluted cDNA was used as template for qPCR with the forward primer LDL_TMD1 and the reverse primer LDL_TMD2. Gene expression was normalized to human-beta actin housekeeping gene. Percentage of gene expression was calculated in comparison to the control (ctrl + E2).
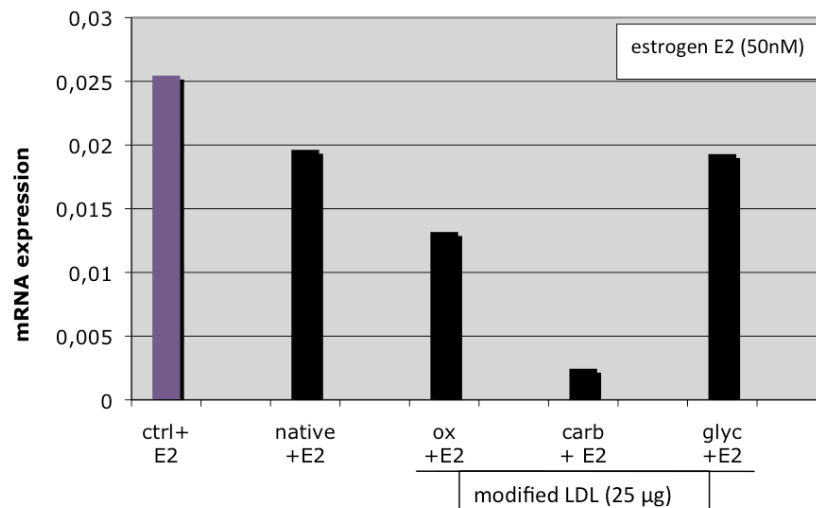

Fig.3.13 Quantitative PCR analysis of LRP1 mRNA in HCAEC incubated with modified LDL and E2 Undiluted cDNA was used as template for qPCR with the primers hu_LRP1 (11fwd) and hu_LRP1 (12rev). Gene expression was normalized to human-beta actin housekeeping gene.

LRP1 mRNA could not be detected with real time PCR in HCAEC incubated with modified LDL without E2 (data not shown). After E2 treatment and incubation with modified LDL, LRP1 mRNA level was slightly upregulated (Fig.3.13).

LRP1 was also detected with RT-PCR at low-level (Fig.3.10), which correlated with quantitative mRNA levels of LRP1. LRP1 transcription was higher in control cells treated with E2 compared to cells after exposure with ox LDL, carbamylated LDL, and glycated LDL also after E2 treatment. A stronger downregulation could be seen in samples with carbamylated LDL and E2.
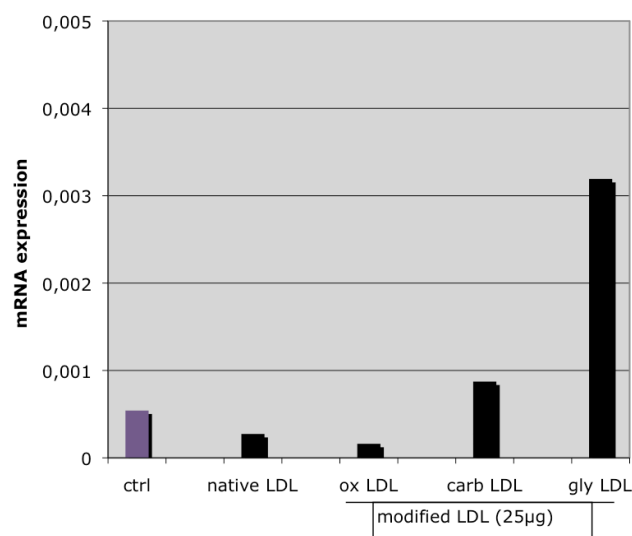

Fig.3.14 Quantitative PCR analysis of LRP2 mRNA in HCAEC incubated with modified LDL Undiluted cDNA was used as template for qPCR with the primers hu_LRP2 (12fwd) and hu_LRP2 (13rev) Gene expression was normalized to human-beta actin housekeeping gene.
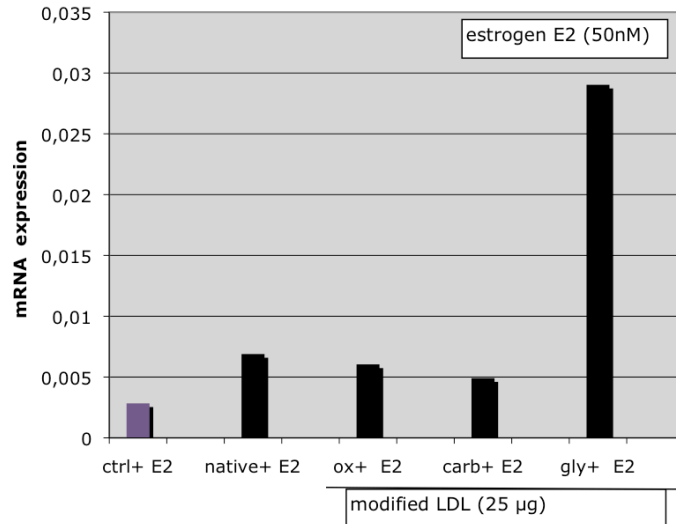
Fig.3.15 Quantitative PCR analysis of LRP2 mRNA in HCAEC incubated with modified LDL + E2 Undiluted cDNA was used as template for qPCR with the primers hu_LRP2 (12fwd) and hu_LRP2 (13rev) Gene expression was normalized to human-beta actin housekeeping gene.

LRP2 transcription in HCAEC was similarly correlating to the mRNA level of LRP1 in HCAEC after E2 treatement. In contrast, LRP2 transcription level was upregulated by glycated LDL. No response of LRP2 in HCAEC control cells treated with E2 could be detected. Estradiol seemed to upregulate LRP2 (5 to 10 fold increase) when cells were incubated with modified LDL (Fig.3.15)

In HCAEC a very low-level of mRNA expression of LRP1 and LRP2 could be detected. Hence, a change in the transcriptional level could be seen after doubletreatment with different modified LDL and E2. In the case of treatment with glycated LDL, an increase could be detected in both receptors, LRP1 and LRP2.

LDL receptor is normally expressed in HCEAC, but downregulated in cells treated with modified LDL. An abundant upregulation of LDL receptor transcription in HCAEC control could be observed when incubated with E2.

Consequently, modified lipoproteins could not be recognized by LDL receptors anymore. The uptake of chemically modified LDL is restricted to scavenger receptors on endothelial cells, macrophages and, smooth muscle cells.

3.4.2. Quantitative analysis of mRNA levels under shear stress

As mentioned before, mRNA levels of LDLR, LRP1, and LRP2 should be studied under static conditions in cell culture experiments. Secondarily, these receptors should be detected with cell culture cartridges, where cells can be subjected to shear stress. This method could simulate the in vivo situation of human physiology even better.
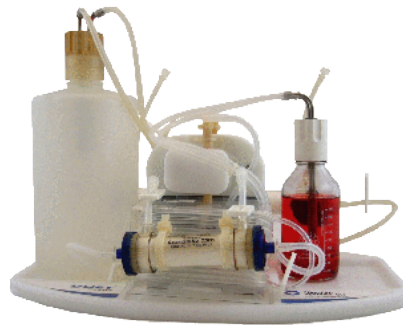

Fig.3.16 Fiber Cell™ cartridge [10]

Before use, the fibers of the cell cartridge had to be coated with extracellular matrix protein fibronectin, to permit the attachment of endothelial cells to the interior wall of the fibre. A special microprocessor controlled Fiber Cell™pump produced consistent shear stress by regulating the flow of SF-medium through the cartridges. The pump generated shear stress of 2.5 dyne/cm^2 (LSS) and 25 dyne/cm^2 (HSS). Chronic shear stress was simulated for 9 days; on day 8 the modified LDL were added to the fibre for 19h. HCAEC were harvested and RNA was used for reverse transcription to cDNA.

Real time PCR was performed with Applied Biosystem by using fluorescent primers designed and purchased from Applied Biosystem. Results were obtained by cooperation with Vienna General Hospital.
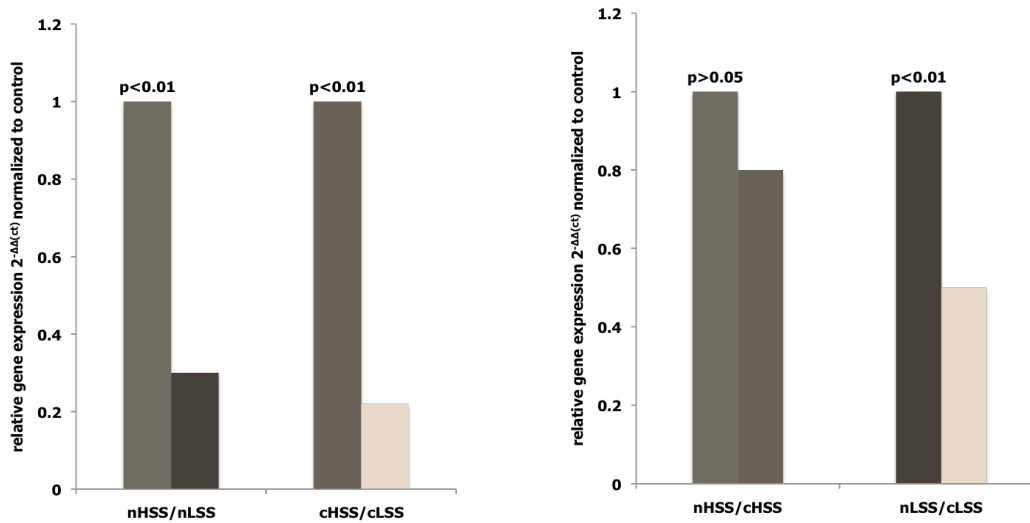
Fig.3.17 Quantitative PCR analysis of LDLR mRNA in HCAEC incubated with native and carbamylated LDL under shear stress Relative gene expression was normalized to control of human beta actin, native LDL under high shear stress (nHSS), native LDL under low shear stress (nLSS), carbamylatd LDL under high and low shear stress (cHSS and cLSS). P values were calculated as triplicates n=3; $p > 0.05$ and $p < 0.01$

Shear stress differentiated coronary artery cells seemed to downregulate the LDLR relative gene expression under LSS. Sites of LSS in cardiovascular regions of the heart are more susceptible to atherosclerotic plaque progression. HSS was protective and no change in LDLR mRNA with incubation of native or carbamylated LDL was seen (Fig.3.17, left panel). Chronic high shear stress could modulate the negative effect of modified LDL. Comparison of LSS either with native or carbamylated LDL elucidated, that LSS downregulted the LDLR transcription.
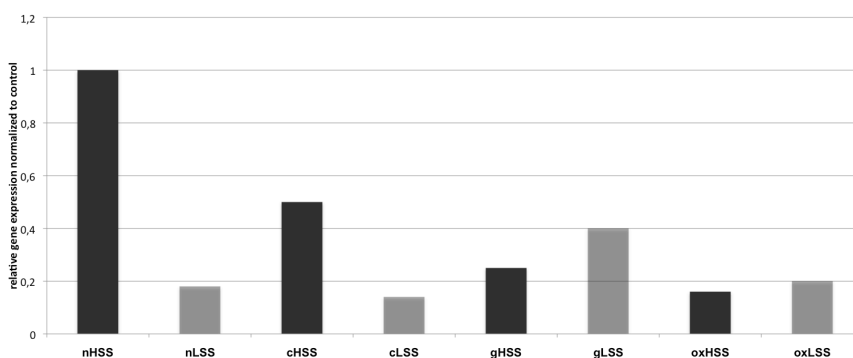
Fig.3.18 Quantitative PCR analysis of LRP1 mRNA in HCAEC incubated with different modified LDL under HSS and LSS Relative gene expression was normalized to control of human beta actin, native LDL high shear stress (nHSS), native LDL low shear stress (nLSS), carbamylatd LDL high and low shear stress (cHSS and cLSS), glycated LDL high and low shear stress (gHSS and gLSS), oxidized LDL high and low shear stress (oxHSS and oxLSS)

Quantification of relative LRP1 mRNA expression revealed a downregulation under low shear stress, as seen in LDLR gene expression (Fig.3.17). Interestingly, mRNA level of LRP1 seemed to be upregulated in cells under LSS treated with glycated LDL, also seen under static conditions. The relative LRP1 expression is higher in native compared to different modified LDL under HSS. LRP1 could not be detected under static condition in cell culture experiments, but after subjection to shear stress. This means that shear stress alters transcription and has a fundamental influence on mRNA pattern.

As shown in Fig.3.18, LRP2 mRNA expression did not change under static conditions and also a low-level expression could be estimated under LSS and HSS. No significant alteration of LRP2 expression could be detected under different conditions.
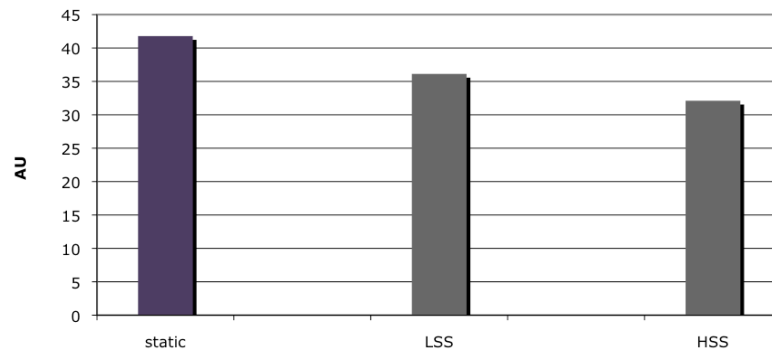
Fig.3.19 Quantitative PCR analysis of LRP2 mRNA in HCAEC under HSS and LSS HCAEC subjected to low shear stress (LSS) or high shear stress (HSS), compared to HCAEC under static (static) in vitro cell culture conditions. Gene expression was definded as arbitrary units (AU).

3.5 Determination of protein expression in HCAEC in response to modified LDL

After detection at the mRNA level, the protein level remained to be elucidated. HCAEC cell lysates were prepared under the same conditions and western blotting was performed by transfer of the proteins to a nitrocellulose membrane.
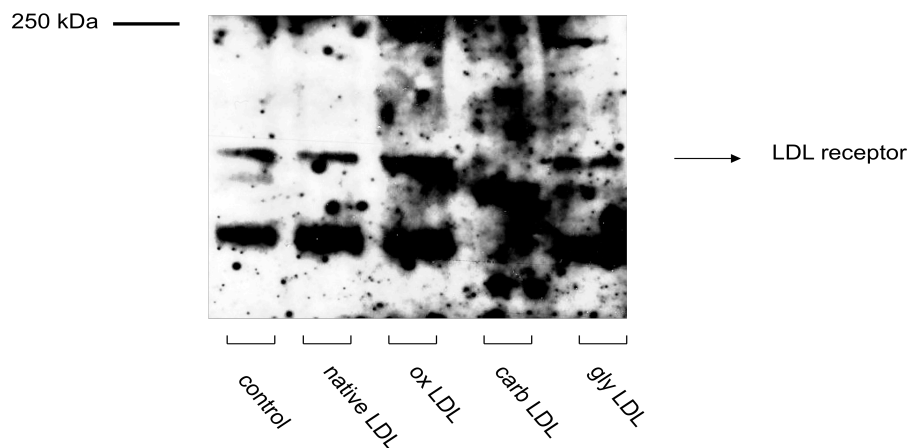

Fig.3.20 Western blot analysis of LDLR expression in HCAEC treated with different modified LDL HCAEC cell lysates (70µg) were subjected to a 8% SDS PAGE under non-reducing conditions and blotted to a nitrocellulose membrane. For detection, an α-LDLR antibody produced by our lab was used with a dilution of 1:700. HRP-conjugated goat α-rabbit antibody (1:50000) was used as secondary antibody and visualization was performed using ECL detection.

LDLR protein expression could be detected in control as well as after treatment with different modified LDL. A stronger signal could be detected with oxidized LDL, which correlates with results from quantitative PCR (Fig.3.11). Carbamylated LDL resulted in a fainter band of LDLR.

Unfortunetly, these antibodies had a massive background signal. Different LDLR antibodies were used as the polyclonal α-rabbit-LDLR produced by our lab and the monoclonal α-mouse-LDLR (Calbiochem). LDLR expression could not be detected in HCAEC with E2 treatment (data not shown), correlating with the results of downregulation of LDLR mRNA by qPCR.

Another possible explanation for the impossibility to detect the protein expression of LRP1 and LRP2 might be the low detection of these receptors on mRNA level. LRP1 only could be detected in positive control of mouse liver extract (data not shown).

To by-pass the problems with the detection of LRP1 and LRP2 westernblotting, LDL receptor gene family members were pooled by Co-Immunoprecipitation with RAP. With this method LRP1 and LRP2 could not be detected by western blotting after Co-Immunoprecipitation as well. A silver stain was performed, which is a sensitive method for showing proteins, to visualize LRP1 and LRP2 after Co-Immunoprecipitation with RAP (Fig.3.22).
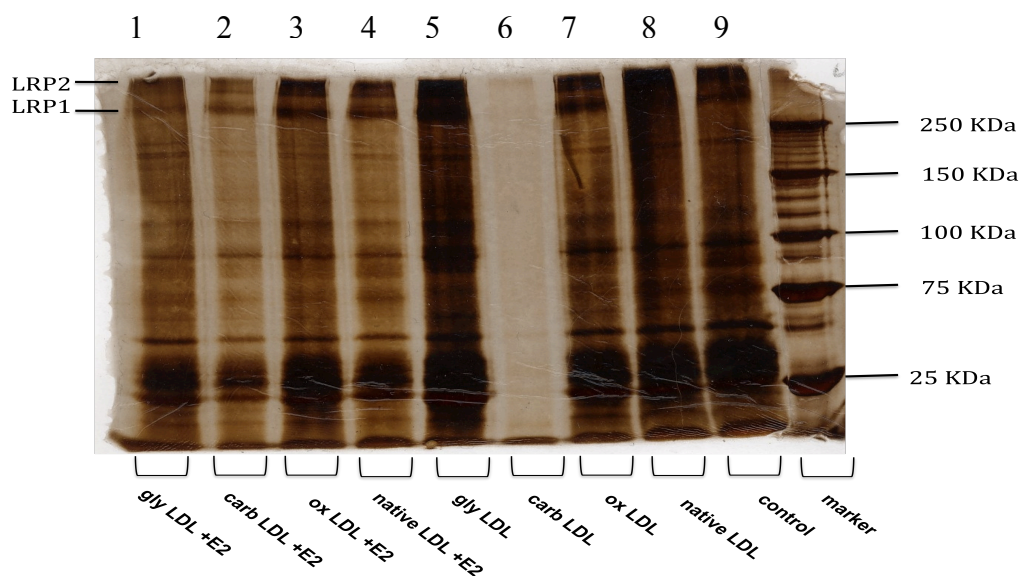

Fig.3.21 Co-Immunoprecipitation of LDL receptor family members using cell lysates and RAP chaperon After Co-Immunoprecipitation with RAP-His tag to agarose beads oN, cell lysates of HCAEC treated with modified LDL or with E2 were added to the beads to accumulate LDL receptor family members as LRP2, LRP1. After SDS-PAGE under non reduced conditions on a 8% SDS-gel, a silver stain was performed. Lane 1-4 show HCAEC treated with native and different modified LDL with E2, lane 5-8 show HCAEC tretaed with modified LDL and native LDL, lane 9 untreated cells (control).

The advantage of silver stain is that it detects small amounts of protein. Because of the relatively similar size of LRP1 (515 kDa) and LRP2 (600 kDa) in glycosylated status, it was difficult to distinguish these two receptors. Detection of LDLR and LRP2 in HCAEC remains to be elucidated because of the problems with antibodies. Our lab is now working to produce a human LRP2 antibody.

Chapter 4

Discussion

Atherosclerosis and early markers like endothelial dysfunction can be associated with several pathological conditions. The traditional cardiovascular risk factors are high blood pressure, dyslipidemia, smoking and, obesity. Patients suffering from diabetes mellitus or kidney disease come along with a higher risk of progression of atherosclerosis.

Disease patients have elevated cholesterol levels in their blood circulation. The accumulation and subendothelial retention of LDL particles in vasculature contribute to modification processes. Depending on the disease, different modified lipoproteins were already observed as lipid oxidized LDL, hypochlorite modified LDL, glycated LDL, or carbamylated LDL. Besides, modification processes take place during aging. General populations have oxidized LDL, normally through oxidants in blood circulation. Smoking increases the risk of oxidation of lipoproteins. Hypochlorite modified LDL is formed by the myeloperoxidase H_2O_2-halide system in oxidative stress situations. Glycation of LDL increases in patients with diabetes and carbamylated LDL devates in patients with chronic renal insufficiency. Modified lipoproteins are thought to be even more proatherogenic. Modification of lipoproteins, especially LDL, is a key event in atherosclerosis leading to endothelial dysfunction.

Vascular intima, a monolayer of endothelial cells in coronary arteries, regulates the tonus, coagulation, and the proliferation of smooth muscle cells. Endothelial imbalance contributes to oxidative stress and upregulation of vasoconstriction towards overgrowth of smooth muscle cells. Imbalance of endothelium is associated with endothelial dysfunction described as injury of endothelial cells. During this step, a change in gene regulation is triggered and remodelling processes get activated.

The aim of this project was to get some insights in lipotoxicity of different modified LDL on human coronary artery endothelial cells. To understand the situation of endothelial dysfunction and the influence of oxidized, glycated, and carbamylated LDL even better.

Further interest was to investigate possible changes in gene expression associated with the exposure of HCAEC to different modified LDL under static conditions and under shear stress.

Much data is known about the cytotoxic effect of modified lipoproteins in different concentrations on endothelial cells. Previous studies focused on inflammatory sensors such as ICAM-, monocyte chemotactic protein (MCP-1), NFκB and the upregulation of specific scavenger receptors such as LOX-1 and CD36 [Moore,2006; Zahler, 2000; Zu, 2010]. Scavenger receptors regulate the uptake of modified aggregated LDL and clear them from circulation. Such scavenger receptors are mainly expressed on macrophages and SMC in advanced atherosclerotic progression.

In this study, the question was addressed about early steps of endothelial cell damage. Few is known about the regulation and role of LDL receptor family members in endothelial dysfunction. The interest was, how endothelial cells alter their gene expression pattern in concern to the focus on LDL receptor family members such as LDLR, LRP1, and LRP2 after exposure to different modified LDL.

First, I wanted to determine the cytotoxic effect of different modified LDL as oxidized, glycated and carbamylated LDL on HCAEC, to define a non-toxic concentration for further experiments. We wanted to study the influence of vasoprotective molecules as HDL and estrogen. Many studies revealed the positive effect of estrogen in vasculature and the later incidence of CVD in women. Estrogen has multiple vascular effects including the alteration in lipid serum levels, fibrinolysis, and antioxidant properties [Kublickiene, 2008]. HDL particle and components were characterized to have antioxidant properties and counteract processes of modified LDL.

Incubation of HCAEC with 100 μg of different modified LDL resulted in a higher cytotoxic effect than with 25 μg of modified LDL. Oxidized LDL seemed to be the most cytotoxic agent to damage endothelial cells. For further experiments, a concentration of 25 μg was used, because of no detrimental cytotoxicity of modified LDL after incubation of HCAEC.

The evidence could be enforced that estrogen protects endothelial cells from stress, because the cytotoxicity decreased dramatically after incubation of HCAEC with 100 μg of modified LDL and E2. Additionally, HDL had a potent impact to lower cytotoxic

effects of modified LDL on HCAEC. Concluding, that incubation with E2 or HDL decreased cytotoxicity to baseline apoptosis in endothelial cells. Recently published data showed, that HDL diminishes the uptake of LDL or minimally modified LDL by downregulation of receptors CD36 and FcγI [Carvalho, 2010]. HDL and estrogen were described to impair oxidative stress and to inhibit the effect of modified LDL on endothelium in vasculature.

By the experiments described, we wanted to discover the insights into the molecular mechanism of endothelial injury due to modified LDL and the role of LDLR family members as LDLR, LRP1, and LRP2.

The expression levels of the LDL receptor family members such as LDLR, LRP1 and LRP2 were elucidated in HCAEC in normal and treated cells with different modified LDL. Furthermore the expression pattern at mRNA level of these receptors should be investigated under chronic shear stress. Cell culture cartidge, Fiber™module, allows to study endothelial cells under shear stress. Endothelial cells grow similar to the situation in a blood vessel and are subjected to low and high shear stress. This module mimics the human physiology of blood flow.

LDLR

The LDLR acts as endocytic receptor for recognition of cholesterol-laden lipoproteins in peripheral tissue, for membrane integrity, and synthesis of steroid hormones [Schneider, 2003, Goldstein, 2001]. The transcription is under tight control of SREBP, whereas sterol accumulation represses the synthesis of the LDLR. The LDLR was defined to be anti-atherogenic. A downregulation of the LDLR was seen in atherosclerotic events.

Cell culture experiments showed that the LDL receptor mRNA level increased upon exposure to oxidized LDL. The reproduceability of in vitro oxidation of LDL is quite low. It depends on various factors, mainly on the blood donor. An increase of LDL retention at the endothelium was described to induce LDLR expression. In case of glycated and carbamylated LDL the expression increased not as dramatically as in oxidized LDL samples. Glycated LDL is more likely to be cleared by scavenger receptors on macrophages and endothelial cells and not by LDLR [Younis, 2009].

The same group also revealed that glycated apoB in LDL influences the recognition by LDLR because of a conformational change.

Interestingly, an excessive upregulation of LDLR could be demonstrated after E2 treatment of HCAEC. LDLR responded to E2 in vasculature. Smith et al, 2004 published the upregulation of LDLR in HepG2 cells after administration of E2. Treatment with E2 and modified LDL under static conditions showed a downregulation of LDLR mRNA expression.

The transcription of LDLR in HCAEC incubated with native LDL under static conditions correlated with the transcript level under LSS. HSS raised LDL receptor mRNA level to a maximum. Blood flow with high velocity was already defined to be protective for vasculature. The effect on LDLR mRNA level after exposure to carbamylated LDL on HCAEC seemed to increase under LSS. LSS was defined as proatherogenic factor. Sites with LSS are more susceptible to atherosclerotic plaque progression.

Concluding the results, LDLR seemed to be downregulated by modified LDL in atherosclerotic events. The impaired uptake of lipoproteins and lipid deposition in the arterial wall could be described because of reduced affinity of LDLR and LDLR-family members to modified lipoproteins.

LRP1

LRP1 is widely expressed in distinct tissues and different cell types, and was defined to be abundantly expressed in the vascular wall, such as vascular smooth muscle cells (VSMC) and macrophages. However, the expression of LRP1 is somewhat controversial, because quantitative results demonstrated no expression of LRP1 in HCAEC in cell culture, and also this group could not detect LRP1 protein in endothelial cells [Lilis, 2008].

Coronary pathologies can be linked to a change in LRP1 gene expression. The protective effect of LRP1 can be described by controlling vascular integrity and SMC [Lilis, 2008; Schulz, 2003]. LRP1 balances the activation of phosphatidyl-inositol-3-kinase (PI3K), which drives the proliferation and SMC migration, seen in smLRP1 -/-mice, which resulted in higher susceptibility to atherosclerosis [Zhou, 2009]. The protein also controls the activation of PDGFRβ and therefore the mitogenic action of

muscle cells [Boucher, 2007] and by means of LRP1 the vascular permeability is provided.

LRP1 expression could be colocalized with aggregated LDL and VSMCs in atherosclerotic plaques [Llorente-Cortés, 2005].

In accordance to the data [Lillis, 2008], LRP1 could not be detected on mRNA level under static conditions only when subjected to shear stress. Under LSS a decrease of LRP1 mRNA level pointed out compared to HSS. Similar to the expression level of LDLR, resulted also in a downregulation after treatment with modified LDL under LSS condition. Interestingly, in the case of glycated LDL the expression was higher in LSS than in HSS samples.

The doubletreatment of HCAEC with modified LDL and E2 under static conditions resulted in a slightly upregulation of LRP1. LRP1 could not be detected on protein level because of its low expression.

By known publications, LRP1 seemed to play an important role in smooth muscle cells and in atherosclerotic events, but in our study it seemed not to be as essentail in endothelial cells, because of low-level transcription.

LRP2

LRP2 is abundantly expressed in renal proximal tubule for renal filtration of vital substances [Christensen & Birn, 2001] and it is characterized as scavenger receptor for binding LDL. Another important function is the response and binding of sex steroids, such as estrogen bound to sex hormone binding globulin (SHBG). Vasculature is highly responsive to estrogen.

LRP2 expression could be detected at low-level under different conditions. HCAEC seemed not to be responding to E2 in vascular endothelium. An approximately 10 fold increase after treatment with modified LDL and E2 could be determined on mRNA level. The mRNA expression of LRP2 increased with glycated LDL, correlating with decreased LDLR mRNA.

The transcription of LRP2 showed no alteration under chronic shear stress as HSS and LSS. In summary, LRP2 seemed to be at low mRNA level in endothelial cells.

The protein level of all LDLR family members should be estimated under the same conditions. LDLR could be detected on protein level exclusively. LRP1 and LRP2

could not be determined by western blotting or Co-Immunoprecipitation with RAP. Determination of receptors on protein level remains to be elucidated under static conditions and under subjection to shear stress.

Summarizing the data, LDLR seemed to be downregulated upon exposure to modified LDL in HCAEC in endothelial dysfunction, because of its reduced affinity to modified LDL. LRP1 and LRP2 were expressed at low mRNA levels under static conditions. LRP1 mRNA level increased under shear stress conditions. Because of low-level expression of LRP1 and LRP2, LDLR seemed to be the abundant receptor for the uptake of LDL in HCAEC. To conclude, receptors such as LDLR and LRP1 participate in physiological functions and change when exposed to shear stress. Shear stress altered gene expression and had a fundamental influence on gene expression pattern of receptors. HSS protects and activates endothelium in vasculature, whereas LSS seemed to be proatherogenic.

Based on these results, more interest should be raised on the gene expression pattern of different endothelial markers in stages of endothelial dysfunction. Performance of microarray analysis should be done to reveal the up- and downregulation of specific genes in HCAEC incubated with different modified LDL. Further, to investigate the role of estrogen and HDL under chronic shear stress. The change in gene regulation could cause the identification of protective molecules, which prevent atherosclerotic genesis.

A second step should be to study the interaction of human coronary endothelial cells and vascular smooth muscle cells in cell culture cartridge, to mimic the in vivo situation of cell contacts between these two layers even better.

A fundamental improvement would be to use blood from patients with diabetes or kidney disease. This will represent a mixture between native, oxidized, and other modified LDLs in vivo and add this blood to HCAEC in contact with VSMC.

These future perspectives are expected to contribute to deeper insights in pathophysiology of atherosclerosis and thus to reveal atheroprotective strategies.

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Abbreviations

A	adenine
APS	ammonium persulfate
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	Celsius
Ca	calcium
carb	carbamylated
cDNA	complementary DNA
C-terminal	carboxy-terminal
ctrl	control
Da	dalton
dd	double deionized
DNA	desoxyribonucleic acid
dNTP	desoxynucleotidetriphosphate
E2	moxistrol (estradiol)
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmatic reticulum
FH	familial hypercholesterolemia
fwd	forward
G	guanine
g	gram or gravity
gly	glycated
h	hour
HCAEC	Human coronary artery endothelial cells
HDL	high-density lipoprotein
HRP	horseradish peroxidase
HSS	high sheer stress
kb	kilobases
kDa	kilodalton
l	liter
LA-repeat	LDL receptor type A repeat
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LDH	lactat dehydrogenase
LRP1	low-density lipoprotein receptor-related protein
LSS	low sheer stress
M	molar
µg	microgram
mg	milligram
min	minute
µl	microliter
ml	milliliter
mM	millimolar
mRNA	messenger RNA

n	native
nm	nanometer
nt	nucleotides
N-terminal	amino-terminal
oN	over night
OD	optical density
ox	oxidized
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate-buffered-saline
PCR	polymerase chain reaction
qPCR	quantitative PCR
rev	reverse
RNA	ribonucleic acid
Rnase	ribonuclease
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcriptase-PCR
s	second
SDS	sodium dodecyl sulfate
T	thymine
TAE	tris-acetate-EDTA
TBS	tris-buffered-saline
TBS-T	tris-buffered-saline + Tween 100
TEMED	N,N,N´,N´tetramethylethylendiamine
Tm	melting temperature
U	unit
UV	ultraviolet
v	volume
V	volt

Danksagung

Das Studium neigt sich dem Ende zu und im Rückblick denke ich an eine spannende Zeit, die mein Leben mit enormem Wissen bereichert hat.

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Danke!

Curriculum Vitae

Eva Theres Gensberger

Personal details

Date of birth:	1st November 1985
Citizenship:	Austrian

Work experience

August 2009 to present

Diploma thesis on work done in the lab of Assoc.Prof. Marcela Hermann at the Laboratory of Vienna Biocenter - Department of medical biochemistry, Division of Molecular Genetics, Max F. Perutz Laboratories and cooperation with Vienna General Hospital

- Title `Role of low-density-lipoproteins receptor (LDLR) gene family members in human coronary artery endothelial cells to modified LDL
- Insights in the field of atherosclerosis in coronary arteries and impact of modified lipoproteins to regulation of LDL receptor family members (LDLR, LRP1, LRP2)
- Sex specific difference, influence of estrogen on endothelium
- Observation of human cell culture (HCAEC) and mouse heart
- Working with chronic sheer stress module-cell culture cartridges for simulation of in vivo blood flow
- Co-working on another project-establishment of a primary chicken kidney cell-culture

April 2009 to May 2009 **Practical work at the Laboratory of Vienna Biocenter - Department of medical biochemistry, Division of Molecular Genetics, Max F. Perutz Laboratories**

- Gained work experience in the lab of Assoc.Prof. N. Erwin Ivessa: Synthesis, folding, transport, and degradation of proteins in the early secretory pathway, received training in pulse chase labelling and introduction in confocal fluorescence microscopy

- Experience gained by working in the laboratory of Assoc.Prof. Marcela Hermann: Role of LDL receptor gene family member- LRP2 in chicken, working skills with animal model like chicken and standard techniques as western blotting, immunohistochemistry, real time PCR

February 2009 to March 2009 **Practical work at Vienna General Hospital (AKH) Institute of pathophysiology**

- Gained work experience in the lab of Assoc.Prof. Heimo Breiteneder: Characterisation and classification of food allergens, investigation of peanut allergen Ara h 2, screening an ETH-2 phage display library and performing Elisa assays, training in general microbiological laboratory methods

July 2008 to August 2008 **Internship at Böhringer Ingelheim Austria, Research of oncology/pharmacokinetics**

- Insights of angiogenesis and tumor cell signaling, research in cell signaling of cancer cell-lines and proliferation versus apoptosis of cancer cells after

treatment with BIBF 1120 and practice in mouse tumor biology

July 2005   **Educational work in a turkey retail and wholesale company ´Pöttelsdorfer   Putenspezialitäten`**

- During high school graduation, two months of practical work had to be done in a chemistry laboratory
- Learn about quality control systems (ISO certification) in food companies and analysation of food samples for retail

July 2003   **Educational work at Sanochemia Austria, Laboratory of analytic chemistry**

- During high school graduation, two months of practical work had to be done in a chemistry laboratory
- Performing of analytical diagnostics of pharmaceutical products

Education

2005 to current   **University of Vienna, AUSTRIA**

Master of Science in molecular biology, focus on cellbiology, molecular medicine, immunology and microbiology

First diploma examination in `Molecular biology`: September 2009

Expected graduation: October 2010

2000 – 2005   **Rosensteingasse High School of Chemistry** `Höhere Bundeslehr- und Versuchsanstalt für chemische Industrie` in Vienna

Three years work experience qualifiy as an engineer of chemistry

<u>Training and Qualifications</u>

Computer: Microsoft office, system software of laboratory

equipment

Language: German (native), English (certificate of abroad English course with EFA)

Knowledge of general molecuar-biological techniques:

Skilled in standard laboratory methods such as PCR, real time PCR, cloning, Western/ Northern/Southern blotting, immunological assays, immunohistochemistry, fluorescence microscopy, proliferation assays, apoptose assays, cytotoxicity assays. Basic techniques in cell culture and working with phages and bacteria
Certificate in animal handling