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Habilitationsschrift

High-density lipoproteins
for the treatment of diabetic cardiomyopathy

zur Erlangung der venia legendi
für das Fach Experimentelle Kardiologie

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von

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TABLE OF CONTENTS

ABBREVIATIONS

-/-	knock out
ABC	ATP-binding cassette transporter
Ad.hapoA-I	$E_1/E_3/E_4$-deleted adenoviral vector containing the *hAAT.gA-I.4xapoE* expression cassette
Ad.Null	$E_1/E_3/E_4$-deleted adenoviral vector containing no expression cassette
AMPK	AMP-activated protein kinase
Ang II	angiotensin II
apo	apolipoprotein
AT1R	angiotensin II type 1 receptor
CETP	cholesterol ester transfer protein
ec	extracellular
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
ERK	extracellular signal-related kinase
hAAT.gA-I.4xapoE	the human *apo A-I* gene driven by the human α*1-antitrypsin* promoter and followed by 4 *apo E* enhancers
HDL	high-density lipoprotein
ICAM	intracellular adhesion molecule
IDL	intermediate low-density lipoprotein
IL	interleukin
LCAT	lecithin:cholesteryl acyltransferase
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LV	left ventricle
MAPK	mitogen activated protein kinase
MyD88	myeloid differentiation factor 88
NO	nitric oxide
NOX	NAD(P)H oxidase
O_2^-	superoxide anion
PAF	platelet activator factor
PAF-AH	PAF-acetyl hydrolase
PI3K	phosphatidylinositol-3-kinase
PON	paraoxonase
ROS	reactive oxygen species
S1P	sphingosine-1-phosphate
SOD	superoxide dismutase
SR-BI	scavenger receptor class B type I
STZ	streptozotocin
TBARS	thiobarbituric acid reactive substances
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRIF	Toll/IL-1R-containing adaptor inducing interferon β
VCAM	vascular cell adhesion molecule
VLDL	very low-density lipoprotein

1. INTRODUCTION

Diabetes mellitus currently affects more than 190 million people and the number of people suffering from diabetes mellitus is expected to increase to 300 million in the year 2025 [1]. Among the plethora of secondary complications associated with diabetes, cardiovascular disorders remain the major cause of death in individuals with diabetes, accounting for nearly 80% of mortality [2]. The development of chronic heart failure in diabetic patients has conventionally been attributed to the concurrent hypertensive and coronary heart disease. Much less appreciated is the notion that diabetes mellitus affects the cardiac structure and function, independent of blood pressure and coronary artery disease, a clinical entity referred to as "diabetic cardiomyopathy", which is characterized by an impaired cardiac ventricle function due to cardiomyocyte hypertrophy, interstitial and perivascular fibrosis, intramyocardial microangiopathy, interstitial inflammation, abnormal intracellular Ca^{2+}-handling, endothelial dysfunction, and a defect in substrate metabolism [3-9].

Epidemiological and clinical studies have consistently demonstrated an inverse correlation between high-density lipoprotein (HDL) cholesterol levels and the incidence of ischemic cardiovascular diseases [10,11]. The primary mechanism for this protective effect of HDL has mainly been attributed to its role in reverse cholesterol transport, i.e. the centripetal transport of excess cholesterol from peripheral tissue towards the liver for excretion into bile or to steroidogenic organs for steroid hormone synthesis. However, HDL also have direct anti-inflammatory [12,13], anti-oxidative [14], anti-apoptotic [15], pro-angiogenic [16,17], and anti-thrombotic [18,19] features, so called "pleiotropic" effects, and metabolic, anti-diabetic [20] properties.

The aim of this cumulative work was to get insights in the potential of HDL to improve diabetic cardiomyopathy and to unravel underlying mechanisms.

1.1. Diabetic cardiomyopathy

Diabetic cardiomyopathy has first been recognized by Rubler *et al.* [21] in 1972 who identified 4 patients who had heart failure without evidence of coronary artery disease, hypertension or other cardiovascular complications. Epidemiological, clinical, and experimental studies during the last three decades subsequently confirmed the existence of diabetic cardiomyopathy. Several lines of evidence indicate that left ventricular diastolic dysfunction represents the earliest preclinical manifestation of diabetic cardiomyopathy, preceding systolic dysfunction, and illustrate that it can progress to symptomatic heart failure [22-24]. The pathogenesis of diabetic cardiomyopathy is multifactorial. Hyperglycemia, hyperinsulinemia, and dyslipidemia, each trigger cellular signaling leading to specific alterations in cardiac structure. Though, hyperglycemia, affecting cardiac inflammation, fibrosis, apoptosis, endothelial function, Ca^{2+} metabolism, and the state of cardiac progenitor cells, via the induction of oxidative stress, has been proposed to be the key determinant in the development of diabetic cardiomyopathy [25,26]. With respect to diastolic dysfunction, hyperglycemia-induced advanced glycation end products produce irreversible cross-links between extracellular matrix proteins [27,28] compromising tissue compliance and causing myocardial stiffness [29], and induce endothelial dysfunction [30], which has been demonstrated to underly diastolic dysfunction [31].

The activation of the angiotensin (Ang) II type 1 receptor (AT1R), which expression is increased under diabetes mellitus [32,33], also plays an important role in both diabetes-associated endothelial dysfunction and diabetic cardiomyopathy. This follows from the findings that AT1R antagonism under diabetes mellitus improves endothelial function [34] and reduces cardiac inflammation and fibrosis, resulting in an improvement in cardiac function [35]. The prevention of hyperglycemia-induced myocardial apoptosis by gene silencing of Toll-like receptor (TLR) 4 [36], the primary receptor for lipopolysaccharide (LPS) present in Gram-negative bacteria, which also recognizes oxidized low-density lipoprotein (LDL) [37] and advanced glycation end products of LDL, also suggests a role of TLR4 in the pathogenesis of diabetic cardiomyopathy.

1.2. Lipoproteins

Lipoproteins are particles composed of lipids and proteins and are responsible for the intercellular transport of cholesterol and lipids through the circulatory system. They consist of a hydrophobic core containing mainly cholesterol esters and triglycerides, surrounded by a surface monolayer of phospholipids, apolipoproteins (apo) and unesterified cholesterol, which is orientated with the polar portions exposed to the surface of the lipoprotein. Based on differences in density, lipoproteins can be divided in 5 classes: chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL, and HDL [38] (**Table 1**). The different protein and lipid compositions determine their density, size, and (patho)physiological activities. Chylomicrons and VLDL are the least dense lipoproteins and contain a core, which is primarily comprised of triglycerides. LDL and HDL are the smallest and most dense lipoproteins and their core mainly consists of cholesterol esters [39].

Table 1: Physical and chemical properties of lipoproteins. VLDL: very low-density lipoproteins; IDL: intermediate-density lipoproteins; LDL: low-density lipoproteins; HDL: high-density lipoproteins.

Lipoproteins	Chylomicrons	VLDL	IDL	LDL	HDL
density (g/ml)	< 0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.210
diameter (nm)	80-500	30-80	25-35	18-28	7-12
apolipoproteins	A-I, A-IV, B-48, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100, E	B-100	A-I, A-II, A-IV, C-I, C-II, C-III, E
lipids	dietary triglycerides	endogeneous triglycerides	cholesterol esters, triglycerides	cholesterol esters	cholesterol esters

1.3. High-density lipoproteins and high-density lipoprotein metabolism

HDL are the smallest and most dense plasma lipoproteins (d = 1.063-1.21 g/ml). The main apo of HDL are apo A-I and apo A-II, which comprise ~ 70% and 20% of the total protein content, respectively. Apo A-I binds to the scavenger receptor (SR) B-I [40], which can lead

to the activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, and to the ATP-binding cassette transporter (ABC) A1 (ABCA1). Minor apolipoproteins of HDL are apo E, apo C-I, apo C-II, apo C-III, and apo A-IV [41]. The lipid fraction of HDL consists of a great variety of compounds including phospholipids, free and esterified fatty acids, and different ceramides and sphingolipids, including the anti-inflammatory agent sphingosine-1-phosphate (S1P) [42], which, besides apo A-I, accounts for many of the pleiotropic effects of HDL. S1P binds to five G-protein coupled receptors: S1P1 to S1P5 [43], leading to the activation of classic G protein (G_i, G_q, $G_{12/13}$) signaling pathways: phospholipase C, extracellular signal-related kinase (ERK), PI3K, Rho, actin rearrangement, and the inhibition of adenylate cyclase [44].

Human HDL are heterogeneous and can be divided in subpopulations on the basis of density (HDL_2 and HDL_3), size (in order of decreasing size: HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b}, HDL_{3c}), variations in surface charge (α- versus pre β-migrating HDL) and apolipoprotein composition (Lp A-I containing apo A-I but not apo A-II; Lp A-I:A-II containing both apo A-I and apo A-II) [41]. Large HDL are known to be cardioprotective [45], whereas small HDL are positively associated with the severity of coronary artery disease [46].

Unlike other lipoproteins, HDL are not formed as mature lipoproteins, but appear in plasma as precursor particles. These particles have a discoidal shape and consist of bilayers of phospholipids and unesterified cholesterol that are stabilized by apolipoproteins [47]. This nascent discoidal or pre-β HDL is formed via the transfer of excess of free cholesterol in the peripheral tissues to lipid-poor apo A-I via the ATP-binding cassette transporter ABCA1 [48]. Activation of lecithin:cholesteryl acyltransferase (LCAT) leads to esterification of free cholesterol on the surface of discoidal HDL and subsequent internalization into the hydrophobic core of the particle. By increased cholesterol esterification, HDL becomes increasingly larger and more spherical, forming HDL_3 and then HDL_2. Free cholesterol from peripheral cells can also directly efflux to the more mature spherical HDL particles via the scavenger receptor class B type I (SR-BI) or ABCG1 and ABCG4 [49,50]. The transfer of

cholesterol towards lipid-poor apo A-I or more mature HDL particles, is the first step in the so called reverse cholesterol transport: the transport of cholesterol from the peripheral tissues to the liver for excretion in the bile and to steroidogenic organs for steroid hormone synthesis. This concept was first introduced in 1968 by Glomset *et al.* [51] and has been considered to be the main mechanism responsible for the inverse relationship between plasma HDL cholesterol levels and the risk for cardiovascular diseases [52-54]. During reverse cholesterol transport, a series of enzyme reactions serve to extract cholesterol from macrophages in the subendothelial space and to transport this cholesterol back to the liver for disposal as either biliary cholesterol or as bile acids via the activity of 7α-hydroxylase [55]. Reverse cholesterol transport can be divided into a direct and an indirect reverse cholesterol transport. In direct reverse cholesterol transport, HDL binds via apo A-I to SR-BI receptors on the hepatocyte surface. SR-BI selectively delipidates HDL and then releases the depleted particle back into the circulation [56]. In indirect reverse cholesterol transport, cholesterol ester transfer protein (CETP) mediates the transfer of cholesterol ester from HDL in exchange for triglycerides in apo B100-containing lipoproteins such as VLDL and IDL, which can be converted into LDL by lipoprotein lipase. The apo B-containing lipoproteins are then catabolized in the liver. On the other hand, HDL enriched with triglycerides, becomes a better target for lipolysis by hepatic lipase. As hepatic lipase lipolyzes HDL, HDL become smaller and can become unstable, leading to the dissociation of apo A-I. Apo A-I can then be connected to substances like cubulin and megalin and finally be eliminated in urine. Both, the direct and indirect reverse cholesterol transport, are not completely defined yet and are still under investigation [57].

Under diabetes mellitus, the action of enzymes involved in HDL metabolism/remodeling, including LCAT, CETP, hepatic lipase etc. and the receptor-mediated uptake or efflux of cholesterol/cholesterolesters towards/from HDL is modified, leading to altered HDL remodeling. Besides these changes in remodeling, alterations in the structure of proteins and

the activity of enzymes associated with HDL, including apo A-I, PON, LCAT, and others [58,59] due to glycation and peroxidation, modify the functionality of HDL [60-63] under diabetes mellitus and may transform HDL from an anti-inflammatory towards a pro-inflammatory particle [20].

1.4. Pleiotropic and metabolic effects of high-density lipoproteins

1.4.1. Anti-inflammatory features of high-density lipoproteins

HDL and its components counteract inflammation by modulating the immune cells in their activity and cytokine expression, and by reducing the pro-inflammatory activation of the endothelium. In brief, apo A-I inhibits the production of the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α by blocking contact-mediated activation of monocytes by T lymphocytes [64-66] on the one hand, and apo A-I/HDL induce the expression of the anti-inflammatory cytokine IL-10 in mononuclear cells [67,68] on the other hand. Furthermore, HDL can bind and remove TNF-α [69]. Recently, an inverse correlation between HDL and the pro-inflammatory Th17 subset in diabetes mellitus type II patients has been demonstrated [70]. In contrast, apo A-I has been shown to induce T regulatory cells [71], which can decrease the pro-inflammatory activation of endothelial cells, including the expression of endothelial adhesion molecules and pro-inflammatory cytokines, and the activation of nuclear factor-κB [72]. Besides these immunomodulatory effects, HDL can directly counteract monocyte-endothelial cell interactions. HDL have the potential to reduce TNF-α- and IL-1-induced expression of VCAM-1, ICAM-1, and E-selectin on endothelial cells [12,13] and to decrease the expression of CD11b on monocyte surfaces [73]. HDL also prevent platelet activator factor (PAF)-induced adhesion of leukocytes to the activated endothelium via limiting its production by endothelial cells [74] and enhancing its degradation by the circulating enzymes PAF-acetyl hydrolase (PAF-AH), paraoxonase (PON), and LCAT [75-77]. Beyond their capacity to reduce the adhesiveness of monocytes to the endothelium

[78], HDL can impair the monocyte chemotactic protein-1-induced transendothelial migration [79]. This feature is mainly attributed to the lipid components of HDL [79,80].

1.4.2. Anti-oxidative features of high-density lipoproteins

HDL have the capacity to decrease oxidative stress via different mechanisms. They can 1) scavenge superoxide anions (O_2^-) [81]; 2) reduce the production of reactive oxygen species (ROS) [82,83]; 3) increase the expression of anti-oxidative enzymes including heme oxygenase-1 and superoxide dismutases [83,84]; 4) take up and degrade/hydrolyze oxidized phospholipids. The ability of HDL to hydrolyze these biologically active oxidized phospholipids (on oxidized lipoproteins) has mainly been attributed to several enzymes which are associated with HDL, including PAF-AH, PON, plasma glutathione selenoperoxidase, and LCAT [85]. LCAT can – besides decreasing lipid oxidation for which it requires apo A-I as coactivator [86] – also promote the concentration of PON and PAF-AH [87]. Recent studies could demonstrate that both HDL components apo A-I [88] and S1P [82] have intrinsic anti-oxidative properties. Finally, HDL contain high amounts of ceruloplasmine and transferrin, two metal chelators which inhibit the catalysis of lipid peroxidation [89].

1.4.3. Pro-angiogenic and endothelial-protective features of high-density lipoproteins

HDL restore the endothelium in different ways: they induce endothelial cell proliferation [17,90,91] and migration [16,92] (pro-angiogenic effects), decrease endothelial apoptosis [15,91,93,94], and stimulate the so called "endothelial progenitor cells" (EPCs) [40,95-97] (pro-vasculogenic effects). In all these processes, an involvement of apo A-I and/or S1P has been documented. In an early report of 1986, the mitogenic effect of HDL on endothelial cells has been shown to be attributed to its apolipoprotein moiety involving activation of protein kinase C and subsequent protein phosphorylation [98]. The capacity of HDL to stimulate endothelial cell migration has been reported to be NO-independent involving SR-BI-mediated

activation of Rac GTPase and to be dependent on the activation of Src kinases, PI3K, and p44/42 mitogen activated protein kinase (MAPK) [99]. The importance of apo A-I and of the SR-BI receptor in the re-endothelialization process follows from an *in vivo* study indicating that carotid artery re-endothelialization after perivascular electric injury was impaired in SR-BI$^{-/-}$ mice and blunted in apo A-I$^{-/-}$ mice, and reconstitution of apo A-I expression rescued normal re-endothelialization [99]. An involvement of S1P in the HDL-induced migration process has also been documented, by which the migration is at least partially mediated by the activation of PI3K, p38 MAPK, and Rho kinase over the S1P1 and S1P3 receptor [92].

Beyond promoting endothelial cell proliferation and migration, HDL also protect the endothelium via decreasing endothelial cell apoptosis. HDL and its bioactive lysosphingolipids present in HDL particles, sphingosylphosphorylcholine and lysosulfatide have been shown to decrease endothelial apoptosis via activation of Akt, leading to phosphorylation and inactivation of the pro-apoptotic Bad [94]. The anti-apoptotic effects of apo A-I and HDL have further been attributed to their capacity to stimulate cell surface F1-ATPase activity [91] and to block the pathogenic intracellular signaling (culminating in sustained calcium rise) [93].

Different studies document the pro-vasculogenic properties of HDL. Reconstituted HDL has been shown to stimulate EPC differentiation via the PI3K/Akt pathway and to enhance ischemia-induced angiogenesis in a murine ischemic hindlimb model [96]. Attenuation of neointima formation has been demonstrated after human apo A-I gene transfer in a murine model of transplant arteriosclerosis involving an increase in the number of circulating EPCs [97]. Tso *et al.* [95] reported that EPCs are recruited into the aortic endothelial layer of apo E$^{-/-}$ mice, an effect which is increased after HDL supplementation. The impact of HDL on EPC migration/recruitment was further clarified by the finding that HDL induce EPC migration *in vitro* via SR-BI, which is dependent on increased NO production in EPCs [40]. The importance of SR-BI in the HDL-mediated induction of EPC migration was outlined in an *in vivo* study showing that allograft vasculopathy was reduced after human apo A-I transfer in mice with SR-BI$^{+/+}$ bone marrow but not in mice with SR-BI$^{-/-}$ bone marrow [40]. Finally, an

involvement of S1P in the HDL-mediated vasculogenic effect was shown by Walther *et al.* [100], who reported that S1P stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway.

1.4.4. Anti-apoptotic features of high-density lipoproteins

Evidence from *in vitro* studies with cardiomyocytes demonstrate that HDL and its components apo A-I and S1P can directly protect cardiomyocytes under various stresses involving signaling pathways which are unrelated to cholesterol transport [101-104]. An HDL/apoA-I/S1P-mediated reduction in active caspase-3 and poly-ADP-ribose polymerase cleavage in rat neonatal cardiomyocytes deprived of glucose and growth factors was demonstrated by Theilmeier *et al.* [101]. Frias *et al.* [103] reported that native and reconstituted HDL significantly decreased doxorubicin-induced cardiomyocyte apoptosis, essentially due to the S1P component of HDL via the S1P2 receptor, but not the S1P1 or S1P3 receptors. In contrast, Tao *et al.* [104] showed that HDL protects adult cardiomyocytes against hypoxia-reoxygenation injury via the S1P1 and S1P3 receptors.

In addition to these *in vitro* studies showing direct anti-apoptotic effects of HDL on cardiomyocytes, HDL and/or its components have also been demonstrated to reduce cardiac apoptosis *in vivo* in an experimental model of ischemia/reperfusion [69,101].

1.4.5. Anti-fibrotic features of high-density lipoproteins

In contrast to the plethora of studies documenting the anti-inflammatory, anti-oxidative, anti-apoptotic, and pro-angiogenic/endothelial-protective features of HDL and its components, only few studies are available demonstrating the anti-fibrotic potential of HDL. An apo A-I/HDL-mediated reduction in collagen deposition or production follows from apo A-I knock out ($^{-/-}$) mice, which are associated with increased collagen deposition in the lung [105] and from findings with the mimetic apo A-I peptide 4F demonstrating a L-4F-mediated decreased endothelial cell matrix production [106]. Finally, S1P has been shown to decrease collagen

secretion in mouse ventricular fibroblasts [107] and to increase endothelial matrix metalloproteinase-2 production [108].

1.4.6. Metabolic features of high-density lipoproteins

The anti-diabetic properties of HDL are insulin-dependent and -independent. In the pancreas, HDL and its components apo A-I and S1P [109,110] reduce β-cell apoptosis and modulate insulin secretion [111]. This is in contrast to LDL [109,110], oxidized LDL, and VLDL [109], which, in addition to glucose, free fatty acids and inflammatory cytokines derived from adipose tissue (e.g. TNF-α) or the innate immune system (e.g. IL-1 β) [112], contribute to β-cell dysfunction and apoptosis.

In the skeletal muscle, the major site of glucose disposal in the body, HDL, as well as apo A-I and S1P, further modulate plasma glucose levels, independently from insulin, via increasing the glucose disposal [113-115]. Here, HDL [114] and apo A-I [113] stimulate the phosphorylation of the key metabolic regulatory enzyme AMP-activated protein kinase (AMPK) [113], which plays a pivotal role in skeletal muscle glucose disposal and is regulated by nutritional status, exercise, and adipokines. The apo A-I/AMPK axis is not only important for glucose homeostasis in the muscle, but also in the liver and fat. This follows from apo A-I$^{-/-}$ mice, which are characterized by decreased AMPK phosphorylation in skeletal muscle and liver, upregulated expression of gluconeogenic enzymes in liver, increased fat content, and compromised glucose tolerance.

Furthermore, epidemiological [116-122] and intervention [123,124] studies have consistently shown a positive correlation between HDL cholesterol and the adipocyte-derived cytokine adiponectin. This positive link between HDL cholesterol and adiponectin might be explained by the strong negative correlation between adiponectin and the apo A-I fractional catabolic rate, and consequently HDL catabolism [120]. In contrast to the inflammatory cytokines, including TNF-α, IL-6, and leptin, derived from adipose tissue, which contribute to β-cell dysfunction and the development of insulin resistance [125], adiponectin has anti-

inflammatory and anti-diabetic features. Adiponectin stimulates glucose utilization and fatty acid oxidation by activating AMPK and enhances hepatic insulin action [126-128]. In the muscle, adiponectin raises muscle insulin receptor tyrosine phosphorylation [129], an important step in the insulin-signaling cascade, which has been shown to decrease with increasing insulin resistance [130]. The importance of the anti-diabetic effects of adiponectin follow from different epidemiological studies indicating that lower adiponectin levels are associated with a higher incidence of diabetes mellitus and an independent risk factor for the progression of type 2 diabetes [131,132].

1.5. Adenovirus biology and adenoviral vectors

Adenoviruses are nonenveloped, icosahedral, double-stranded DNA viruses of approximately 100 nm in diameter. They account for 2-5 % of respiratory tract infections in humans and 5-10% of gastro-intestinal infections in children [133]. Fifty-one serotypes of human adenoviruses, classified into six subgroups (A-F), have been identified, of which types 2 and 5 of subgroup C have been most extensively studied. These serotypes are nononcogenic in rodents [134] and are the predominant serotypes of adenoviral vectors. Adenoviruses can infect both dividing and non-dividing cells and replicate as episomal elements in the nucleus of the host cell. The viral genome is a linear, double-stranded DNA of approximatly 36 kb. Inverted terminal repeats at the 5' termini, function as origins of replication and a nondispensable packaging sequence at the immediate vicinity of the left inverted terminal repeats is required for packaging of the progeny genomes [133,134].

Expression of adenoviral genes can be divided into three phases: early, intermediate and late, defined by the onset of DNA replication. The 'early' genes, organized in four regions of the adenoviral genome (E_1 to E_4), encode essential regulatory proteins that induce replication of the viral DNA and modulate the functions of the cell to facilitate the replication of the adenoviral DNA and the transcription and translation of the late genes. E_1 (E_{1A} and E_{1B}) encodes transcription factors, which cooperate with the host-cell transcription apparatus to

activate the other early genes. The dependence of viral gene expression and ultimately replication of the viral genome on E_1 gene products is the basis for the construction of replacement vectors in which the endogenous E_1 region is replaced by a foreign gene of interest (E_1-deleted adenoviral vectors) [133]. The intermediate transcription units, IVa2 and IX, are expressed at high levels [135] after the onset of virus replication and mediate the activation of the major late promoter. The 'late' genes (L_1 to L_5), which are transcribed from the major late promoter, encode structural proteins for the capsid and the internal core.

1.6. Rationale for human apolipoprotein A-I adenoviral gene transfer

1.6.1. Rationale for human apolipoprotein A-I

There exists a strong correlation between apo A-I plasma levels and HDL cholesterol plasma concentrations. Furthermore, as outlined in the above paragraphs, many of the protective effects of HDL are mediated via apo A-I. In brief, besides their role in reverse cholesterol transport [136,137], apo A-I has several anti-inflammatory and anti-oxidative properties. Among others, apo A-I 1) has the ability of inhibiting the production of IL-1β and TNF-α by blocking contact-mediated activation of monocytes by lymphocytes [64]; 2) induces T regulatory cells [71]; 3) is important for optimal PON [138] and LCAT activity [86,87] and 4) has intrinsic anti-oxidative features [88]. Furthermore, apo A-I has anti-fibrotic [105,106], anti-apoptotic [101], and direct endothelial-protective characteristics [135,139]. The anti-inflammatory, anti-oxidative, anti-fibrotic, anti-apoptotic, and endothelial-protective effects of apo A-I/HDL were the reason for investigating the impact of apo A-I transfer on the development of diabetic cardiomyopathy of which cardiac inflammation, oxidative stress, fibrosis, apoptosis, and endothelial dysfunction are major characteristics (**Figure 1**).
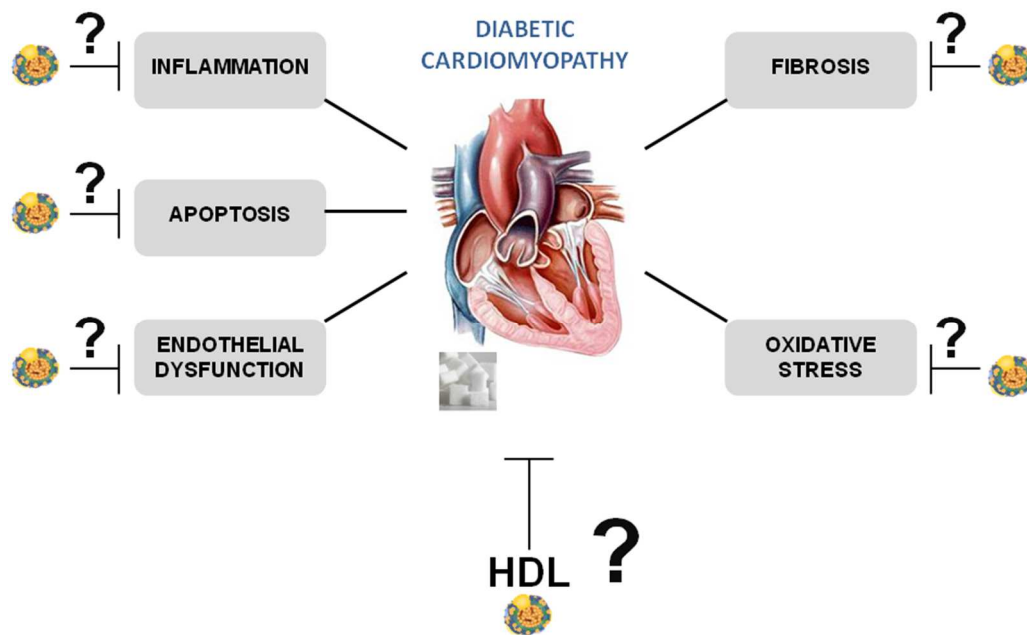
Figure 1. Can HDL reduce the development of diabetic cardiomyopathy? Can HDL via its anti-inflammatory, anti-oxidative, anti-fibrotic, anti-apoptotic, and endothelial-protective effects decrease the development of diabetic cardiomyopathy of which cardiac inflammation, oxidative stress, fibrosis, apoptosis, and endothelial dysfunction are major characteristics?

1.6.2. Rationale for $E_1/E_3/E_4$-deleted adenoviral gene transfer

Several features of adenoviral vectors make them attractive candidates for experimental use as gene transfer system. Adenoviral vectors can be grown at very high titers (10^{11} plaque forming units per ml) [140], can transduce both proliferating and quiescent cells, do not integrate in the host genome, avoiding oncogenesis by insertional mutagenesis, and can accomodate large inserts (minimum 7-8 kb; larger inserts are possible, provided that an equivalent part of the viral genome is deleted) [133,141]. With respect to apo A-I, which is mainly endogenously expressed in hepatocytes, the hepatotropism of adenoviral vectors in mice and rats after intravenous injection [142,143] is another major advantage.

E_1- and E_1/E_3-deleted adenoviral vectors have been among the most efficient vehicles for *in vivo* gene transfer, but their use is hampered by transient transgene expression and hepatotoxicity [144,145]. Studies with $E_1/E_3/E_4$-deleted adenoviral vectors have demonstrated dramatically reduced expression of viral proteins, improved transgene DNA persistence, and reduced hepatotoxicity [146-149]. However, persistent transgene expression was generally

not obtained due to the dependence of the viral promoters used on transactivation by E_4 gene products [146,150]. Based on previous studies in E_1-deleted adenoviral vectors comparing the effect of different expression cassettes, i.e. promoter and/or enhancer combinations, on the expression of the transgene apo A-I, an $E_1/E_3/E_4$-deleted adenoviral vector containing the non-viral expression cassette associated with the highest and most stable apo A-I expression, *hAAT.gA-I.4xapoE* [151,152], was developed [153]. This adenoviral vector (*Ad.hapoA-I*) resulted in persistent apo A-I expression for 1 year in C57BL/6 mice. The sustained expression of apo A-I in the absence of hepatotoxicity was the base to use this vector for the following conducted *in vivo* studies focused at evaluating the potential of an increase of HDL after apo A-I gene transfer at reducing the development of diabetic cardiomyopathy.

1.7. Aims

The global aim of the present cumulative work was to investigate whether and how HDL/apo A-I can attenuate the development of diabetic cardiomyopathy. Therefore, the direct effect of an increase of HDL via human apo A-I gene transfer on diabetic cardiomyopathy and on diabetes-associated endothelial dysfunction was evaluated as well as novel pleiotropic and metabolic features of HDL, of potential relevance with respect to diabetic cardiomyopathy, were explored. Hence, the following studies were conducted:

1) *Prior* to the evaluation of the impact of apo A-I transfer on streptozotocin (STZ)-induced diabetic cardiomyopathy, the first study was performed in view of getting insights in the experimental STZ model with respect to cholesterol metabolism on the one hand, and in the pleiotropic effects of atorvastatin on the other hand;

2) The second study was focused at investigating whether and how an increase of HDL via human apo A-I gene transfer could reduce the development of diabetic cardiomyopathy in an experimental model of STZ-induced diabetes mellitus;

3) The third study was conducted at investigating the effect of an increase of HDL on endothelial dysfunction, a main hallmark of diabetic cardiomyopathy. In this context, also the impact of HDL on the expression of the AT1R, which has been shown to play an important role in diabetes mellitus-associated endothelial dysfunction and diabetic cardiomyopathy, was analyzed in an experimental model of STZ-induced diabetes mellitus;

4) In the fourth study, the impact of HDL on the expression of the cardioprotective adipokine adiponectin, which expression is reduced under extreme inflammation, as well as under diabetes mellitus, was analyzed. Furthermore, the effect of HDL on adipocyte metabolism was investigated;

5) Finally, the effect of an increase of HDL on the expression and signaling of (endothelial) TLR4, which importance in diabetic cardiomyopathy has recently been demonstrated, was examined.

2. MANUSCRIPTS / STUDIES

2.1. Anti-inflammatory effects of atorvastatin improve left ventricular function in experimental diabetic cardiomyopathy

Anti-inflammatory effects of atorvastatin improve left ventricular function in experimental diabetic cardiomyopathy.

Van Linthout S, Riad A, Dhayat N, Spillmann F, Du J, Dhayat S, Westermann D, Hilfiker-Kleiner D, Noutsias M, Laufs U, Schultheiss H-P, Tschöpe C. *Diabetologia*, 2007; 50: 1977-1986.

Emerging evidence suggests that statins exert beneficial effects beyond those predicted by their cholesterol lowering actions. Many of these pleiotropic effects of statins are mediated by antagonism of isoprenoid-mediated activation of small GTP-binding proteins, including Rac1 and RhoA. We investigated whether atorvastatin influences the development of left ventricular (LV) dysfunction, independently of cholesterol-lowering, in an experimental model of STZ-induced diabetic cardiomyopathy. Therefore, STZ-induced diabetic rats were treated with atorvastatin (50 mg/kg daily, orally) or with vehicle for 6 weeks, followed by hemodynamic characterization.

In the absence of LDL-cholesterol lowering, atorvastatin reduced both intramyocardial inflammation and myocardial fibrosis, resulting in improved LV function. This effect was paralleled with a normalization of diabetes-induced activity of Rac1 and RhoA, which are suggested mediators of inflammation. In addition, atorvastatin decreased the diabetes-induced cardiac oxidative stress as indicated by 1.3-fold ($p<0.05$) and 3.2-fold ($p<0.0005$) lower cardiac lipid peroxide levels and phosphorylation of the stress signaling kinase p38 MAPK, respectively. Furthermore, atorvastatin treatment normalized the diabetes-reduced LV eNOS expression to levels of non-diabetic controls.

2.2. Human apolipoprotein A-I gene transfer reduces the development of experimental diabetic cardiomyopathy

Human apolipoprotein A-I gene transfer reduces the development of experimental diabetic cardiomyopathy.

Van Linthout S, Spillmann F, Riad A, Trimpert C, Lievens J, Meloni M, Escher F, Filenberg E, Demir O, Li J, Shakibaei M, Schimke I, Staudt A, Felix SB, Schultheiss H-P, De Geest B, Tschöpe C. *Circulation*, 2008; 117: 1563-1573.

Hallmarks of diabetic cardiomyopathy are cardiac oxidative stress, intramyocardial inflammation, cardiac fibrosis, and cardiac apoptosis. Given the anti-oxidative, anti-inflammatory, and anti-apoptotic potential of HDL, we evaluated the hypothesis that increased HDL via gene transfer with human apo A-I may reduce the development of diabetic cardiomyopathy. Therefore, 3×10^{12} particles/kg of the $E_1/E_3/E_4$-deleted adenoviral vector *Ad.hapoA-I*, expressing human apo A-I, or of *Ad.Null*, containing no expression cassette, was intravenously injected in a STZ-induced experimental model of type 1 diabetes mellitus associated with extreme hyperglycemia. Six weeks after apo A-I gene transfer, HDL cholesterol levels were increased by 1.6-fold ($p<0.001$) compared to diabetic controls injected with the *Ad.Null* vector (STZ-*Ad.Null*) without affecting glucose or LDL cholesterol levels. This was associated with a reduction in systemic oxidative stress as indicated by a decrease in thiobarbituric acid reactive substance (TBARS) levels compared to STZ-*Ad.Null* rats and with a decline in cardiac oxidative stress obviated by a reduction in the left ventricular (LV) phosphorylation state of the stress-activated MAPK p38 and by an increase in the LV mRNA expression of the anti-oxidative enzymes superoxide dismutase (SOD) 2 and extracellular (ec)-SOD. Furthermore, apo A-I gene transfer resulted in less cardiac inflammation (less *TNF-α*, *ICAM-1*, *VCAM-1* mRNA expression), fibrosis, and glycogen accumulation in STZ rats. LV caspase 3/7 activity was decreased and the ratio of the anti-apoptotic Bcl-2 versus Bax up-regulated, translating in reduced total number of cardiomyocytes with apoptotic characteristics and reduced damaged endothelial cells

compared to STZ-*Ad.Null* rats. The cardiac-protective effects of apo A-I gene transfer were paralleled by an increase in the diabetes-downregulated phosphorylation state of LV Akt, endothelial nitric oxide synthase (eNOS), and glycogen synthase kinase, and resulted in an improvement of LV function. In agreement with the increased phosphorylation state of Akt and eNOS in STZ-*Ad.hapoA-I* compared to STZ-*Ad.Null* rat hearts, supporting a HDL-Akt-eNOS pathway, HDL supplementation *ex vivo* on cardiomyocytes in hyperglycemia reduced apoptosis and improved cardiomyocyte contractility in a PI3K- and NO-dependent manner.

The importance of the reduction in LV p38 MAPK activity and *TNF-α* mRNA expression after *Ad.hapoA-I* transfer in STZ rats is underscored by previous findings demonstrating the prominence of p38 MAPK and TNF-α in the development of diabetic cardiomyopathy.

Westermann D, Rutschow S, **Van Linthout S**, Linderer A, Riad A, Pauschinger M, Schultheiss H-P, Tschöpe C. p38 MAP Kinase Inhibition attenuates left ventricular dysfunction by mediating pro-inflammatory cardiac cytokine levels in an animal model of diabetes mellitus. *Diabetologia* 2006; 49:2507-2513.

Westermann D, **Van Linthout S**, Dhayat S, Dhayat N, Schmidt A, Noutsias M, Song XY, Spillmann F, Riad A, Schultheiss HP, Tschope C. Tumor necrosis factor-alpha antagonism protects from myocardial inflammation and fibrosis in experimental diabetic cardiomyopathy. *Basic Res Cardiol.* 2007; 102:500-507.

2.3. Vascular-protective effects of high-density lipoprotein include the downregulation of the angiotensin II type 1 receptor

Vascular-protective effects of high-density lipoprotein include the downregulation of the angiotensin II type 1 receptor.

Van Linthout S*, Spillmann F*, Lorenz M, Meloni M, Jacobs F, Egorova M, Stangl V, De Geest B, Schultheiss H-P, Tschöpe C. *Hypertension*, 2009; 53: 682-687.
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There is growing evidence that a crosstalk exists between the renin-angiotensin system and lipoproteins. We investigated the role of HDL on AT1R regulation and subsequent Ang II-mediated signaling under diabetic conditions. To investigate the effect of HDL on AT1R expression *in vivo*, apo A-I gene transfer was performed 5 days after STZ injection. Six weeks after apo A-I gene transfer, the 1.9-fold (p=0.001) increase of HDL cholesterol was associated with a 4.7-fold (p<0.05) reduction in diabetes-induced aortic AT1R expression. Concomitantly, diabetes-induced NAD(P)H oxidase (NOX) activity and mRNA expression of the NOX family members *NOX 4* and *p22^phox* was decreased, whereas eNOS dimerisation was increased. Apo A-I transfer improved NO bioavailability as indicated by ameliorated acetylcholine-dependent vasodilation in the STZ-*Ad.hapoA-I* group compared to STZ-induced diabetes mellitus. *In vitro,* HDL reduced the hyperglycemia-induced up-regulation of the AT1R in human aortic endothelial cells. This was associated with a 1.3-fold and 2.2-fold decrease in the production of ROS and in NOX activity, respectively (p<0.05). Finally, HDL reduced the responsiveness to Ang II, as shown by decreased oxidative stress in human aortic endothelial cells cultured under hyperglycemia in the presence of HDL preceding Ang II supplementation compared to endothelial cells cultured under hyperglycemia without HDL before Ang II supplementation.

2.4. Impact of HDL on adipose tissue metabolism and adiponectin expression

Impact of HDL on adipose tissue metabolism and adiponectin expression.

Van Linthout S, Foryst-Ludwig A, Spillmann F, Peng J, Feng Y, Meloni M, Van Craeyveld E, Kintscher U, Schultheiss H-P, De Geest B, Tschöpe C. *Atherosclerosis*, 2010; 210: 438-44.

HDL and the adipocyte-derived cytokine adiponectin, are both known for their cardiovascular protective effects. Epidemiological studies have demonstrated a positive correlation between adiponectin and HDL cholesterol levels. The impact of adiponectin and adipose tissue on HDL metabolism is well-established. However, it is unknown whether HDL exerts reciprocal effects on adiponectin expression and adipocyte metabolism. The finding that adipocytes possess HDL binding sites supported us to investigate this hypothesis. Therefore, we increased HDL *in vivo* by human *apo A-I* (*Ad.hapoA-I*) gene transfer and supplemented HDL *in vitro* on partially differentiated adipocytes. These experiments were performed under basal conditions and in the presence of the TLR4 ligand LPS, which affects adipose triglyceride metabolism and reduces adiponectin expression. Apo A-I transfer resulted in a significant increase of HDL cholesterol in control and LPS-injected C57BL/6 mice, which was paralleled by an increase in plasma adiponectin levels and adiponectin expression in abdominal fat. Triglyceride and free fatty acids levels after LPS administration were significantly lower in *Ad.hapoA-I*-LPS than in *Ad.Null*-LPS mice. In parallel, apo A-I transfer decreased the LPS-induced mRNA expression in abdominal fat of the enzyme *hormone sensitive lipase*, which hydrolyzes stored triglycerides to free fatty acids. On the other hand, apo A-I transfer abrogated the LPS-mediated reduction in mRNA expression of *lipin-1*, a key molecule in triglyceride synthesis, and of the class B scavenger receptor *CD36,* which is crucial for fatty acid uptake. Concomitantly, the phosphorylation state of Akt in abdominal fat was 2.0-fold ($p<0.05$) increased in the *Ad.hapoA-I*-LPS compared to the *Ad.Null*-LPS group. In agreement with the increased adiponectin levels and the induced ratio of phosphorylated to total Akt after apo A-I gene transfer in control and LPS mice, pre-incubation of partly differentiated adipocytes with HDL increased adiponectin expression under basal conditions and could

abrogate the LPS-induced down-regulation of adiponectin, both in a PI3K-dependent manner.

2.5. Apolipoprotein A-I gene transfer reduces endothelial Toll-like receptor 4 signalling

Down-regulation of endothelial TLR4 signalling after apo A-I gene transfer contributes to improved survival in an experimental model of lipopolysaccharide-induced inflammation

Van Linthout S, Spillmann F, Graiani G, Miteva K, Peng J, Van Craeyveld E, Meloni M, Tölle M, Escher F, Subasigüller A, Doehner W, Quaini F, De Geest B, Schultheiss H-P, Tschöpe C. *J Mol Med*, 2011; 89: 151-160.

Notwithstanding the well-documented anti-inflammatory effects of HDL and its components during LPS-induced endotoxemia, a potential direct role of HDL or apo A-I in innate immunity regulation under LPS-induced inflammation has not yet been investigated. Innate immunity is characterized by a natural selection of germline-encoded receptors, which focus the host response to highly conserved pathogen-associated molecular patterns (PAMPS) shared by many microorganisms. Of these PAMPS, TLR4 is the main receptor involved in recognizing LPS of Gram negative bacteria, as well as of oxidized LDL, and is of importance in the pathogenesis of different cardiovascular disorders. Recently, it has been demonstrated that endothelial TLR4 rather than leukocyte TLR4 is the key player in LPS-induced neutrophil sequestration into lungs and subsequent lung oedema. This study was performed to investigate whether an effect of HDL on TLR4 expression and signalling may contribute to its endothelial-protective effects and to improved outcome of LPS-induced inflammation and lethality. To explore this hypothesis, we investigated the effect of HDL on TLR4 regulation and TLR4-mediated signalling *in vivo*, following human apo A-I gene transfer in an experimental model of LPS-induced inflammation and lethality. Apo A-I gene transfer decreased lung endothelial TLR4 expression in the absence of LPS and attenuated lung *TLR4* and *Myeloid differentiation factor 88 (MyD88)* mRNA expression, reflecting TLR4 signalling, following LPS administration. Concomitantly, LPS-induced lung neutrophil infiltration, lung oedema and mortality were significantly attenuated following apo A-I transfer. *In vitro*, supplementation of HDL or apo A-I to human microvascular endothelial cells-1, twenty-four hours before LPS administration, reduced TLR4 expression, as assessed by

fluorescent-activated cell sorting, and decreased the LPS-induced *MyD88* mRNA expression

and NF-κB activity, independently of LPS binding.

3. DISCUSSION

3.1. Human apo A-I gene transfer in streptozotocin-induced diabetic cardiomyopathy

To increase HDL *in vivo,* we chose a gene transfer strategy, since currently available drugs such as fibrates, nicotinacids, and statins only moderately and not exclusively increase HDL [154]. On the other hand, the use of CETP inhibitors, which are known to profoundly increase HDL [155], were not an HDL-raising option for our experimental studies in mice and rats, since they intrinsically lack CETP. In STZ rats, gene transfer with *Ad.hapoA-I* resulted in sustained expression of human apo A-I for the entire duration of the experiment, 6 weeks, and increased HDL cholesterol levels by 60% at the day of sacrifice (day 42), compared to diabetic controls.

3.2. Effect of human apo A-I gene transfer on metabolic parameters in streptozotocin-induced diabetic cardiomyopathy

The STZ-induced diabetes mellitus model is characterized by severe hyperglycemia due to a remaining insulin production below 1% [156] and by triglyceridemia which is partly a consequence of activated lipolysis in adipose tissue [157]. Furthermore, the STZ model is, in contrast to other diabetic animal models, not associated with reduced HDL cholesterol levels [158,159]. Though, under diabetes mellitus, HDL loses its protective features [60,62,160,161] and/or becomes even pro-inflammatory [162]. Therefore, further investigation is required to unravel whether apo A-I gene transfer also affects the quality and not only the quantity of HDL. This could be demonstrated for extended release niacin therapy in diabetic patients whereby not only HDL levels were increased, but also the lipid oxidation of HDL was reduced [62]. The increase in HDL cholesterol after apo A-I transfer was paralleled with a significant decrease in VLDL cholesterol, IDL cholesterol, and triglycerides, whereas LDL cholesterol and blood glucose levels were not significantly changed. The decline in triglycerides and the triglyceride-rich lipoproteins VLDL and IDL after apo A-I transfer suggests an HDL-mediated

decrease in lipolysis of adipose tissue, leading to less free fatty acids in the circulation, less triglyceride synthesis in the liver, and subsequent less VLDL and IDL synthesis [163]. This hypothesis is supported by our findings in a model of LPS-induced inflammation, showing that apo A-I gene transfer i) reduces the expression of hormone sensitive lipase, the rate-limiting enzyme in adipocyte lipoysis, in abdominal fat [157] and ii) increases the LPS-downregulated expression of lipin-1 [164], which is a key molecule in triglyceride synthesis and is required for the expression of key adipogenic genes [165], in abdominal fat. In parallel, apo A-I gene transfer enhanced the phosphorylation of the PI3K downstream target Akt in abdominal fat, which is involved in the anti-lipolytic and lipogenic effects of insulin in adipose tissue [166] and in the regulation of the expression of the adipokine adiponectin [167,168], which is known to improve insulin sensitivity under diabetes [169]. In agreement, apo A-I transfer in the model of LPS-induced inflammation increased adiponectin plasma levels and adiponectin expression in abdominal fat. Furthermore, we demonstrated that HDL increases adiponectin expression in partly differentiated adipocytes in a PI3K-dependent manner. However, we cannot exclude that in addition to the HDL-mediated effects on adipocyte metabolism, the changes in triglyceride levels after apo A-I transfer (in STZ rats) can also be due to HDL-mediated changes in hepatic expression of genes involved in triglyceride metabolism. Furthermore, the decrease in cardiac glycogen content and the reduction in plasma triglycerides suggests that apo A-I transfer in STZ rats partly restored the cardiac metabolism under diabetes mellitus switching back to carbohydrates as main energy source instead of free fatty acids [170]. The finding that apo A-I gene transfer did not affect LDL cholesterol in STZ rats, can be explained by the specific cholesterol metabolism under STZ conditions [159], namely low cholesterol synthesis and high cholesterol absorption, mainly due to polyphagia and to a lesser extent to increased fractional absorption. Our finding that atorvastatin, which is well known to reduce LDL cholesterol and triglycerides, and to increase HDL cholesterol [171], did not decrease LDL cholesterol, nor triglycerides, and did not induce HDL cholesterol, at a dose of 50 mg/kg daily, orally in STZ rats, might also be explained by this specific cholesterol metabolism. In fact, a potential explanation for the unaltered LDL

cholesterol levels after atorvastatin treatment in STZ rats is that an excess of exogenous cholesterol due to polyphagia desensitized the reaction to intracellular cholesterol depletion [172] induced by HMG CoA reductase inhibitors, which normally leads to increased LDL receptor production and increased LDL catabolism.

3.3. Effect of human apo A-I gene transfer on cardiac inflammation in streptozotocin-induced diabetic cardiomyopathy

Intramyocardial inflammation is an important hallmark of diabetic cardiomyopathy [8,9]. Glucose [173,174], oxidized LDL [175], and Ang II [176,177], which levels are all induced under diabetes mellitus, upregulate the expression of VCAM-1/ICAM-1 and subsequent monocyte-endothelial cell adhesion via the induction of NF-κB. After monocyte-endothelial cell adhesion, subsequent transendothelial migration of inflammatory cells takes place. The inflammatory cells promote the local expression of cytokines like TNF-α and hereby further enhance the intramyocardial inflammatory reaction by providing an additional stimulus for sustained endothelial CAMs expression. In agreement with the direct anti-inflammatory properties of HDL [13,64], apo A-I gene transfer resulted in a significant decrease in diabetes-induced LV *ICAM-1*, *VCAM-1*, and *TNF-α* mRNA expression to levels not significantly different from non-diabetic *Ad.Null* controls. The reduction in diabetes-induced VCAM-1 expression after apo A-I transfer could later be confirmed by immunohistology [20]. Besides the well described direct anti-inflammatory properties of HDL, the reduction in cardiac inflammation after apo A-I gene transfer in STZ rats can be potentially explained by other HDL-mediated anti-inflammatory mechanisms, which require further investigation in the setting of the diabetic heart. i) We could show that HDL and its components apo A-I and S1P reduce endothelial TLR4 expression and the subsequent activation of NF-κB [178,179] after LPS supplementation [180]. TLR4 is expressed on the cell surface of cardiac cells, including cardiomyocytes, smooth muscle cells, and endothelial cells and a role for TLR4 in the development of diabetic cardiomyopathy has recently been suggested [181]. Since oxidized LDL, which levels are increased under STZ-induced diabetes mellitus [182,183], are TLR4

agonists, one can speculate that the reduction in cardiac inflammation after apo A-I transfer is partly mediated via a decrease in endothelial TLR4 expression, limiting oxidized LDL - endothelial TLR4 interactions and subsequent activation of NF-κB. Furthermore, the reduced levels of TBARS (see supra), which are also retrieved on oxidized LDL, in STZ-*Ad.hapoA-I* compared to STZ-*Ad.Null* rats suggest that apo A-I transfer decreases oxidized LDL and consequently attenuates endothelial TLR4 activation and subsequent cardiac inflammation. ii) We could demonstrate that apo A-I gene transfer reduces aortic AT1R expression in STZ-induced diabetic rats and that HDL decrease the hyperglycemia-induced AT1R expression in endothelial cells [184]. Since the expression levels of the AT1R define the biological efficacy of Ang II and Ang II is known to induce VCAM-1/ICAM-1 expression and subsequent monocyte-endothelial cell adhesion [176,177], it is tempting to hypothesize that apo A-I gene transfer resulted in a reduction of endothelial AT1R in the heart, further contributing to a decrease in cardiac inflammation; iii) Levels of the anti-inflammatory adipokine, adiponectin [185-187], are reduced in diabetic patients and in STZ rats [188] and have been suggested to contribute to a chronic inflammatory phenotype in these subjects/rats. We could demonstrate that apo A-I gene transfer increases the expression of adiponectin in an experimental model of extreme inflammation [189]. Therefore, we hypothesize that the effects of HDL on adiponectin expression may have contributed to its anti-inflammatory effects and to the attenuation of cardiac inflammation in STZ-induced diabetic rats. iv) Finally, apo A-I has recently been shown to induce T regulatory cells [71], which protect the pro-inflammatory status of endothelial cells [72]. An induction in T regulatory cells could therefore be another mechanism by which apo A-I transfer resulted in reduced inflammation in the STZ rat heart.

3.4. Effect of human apo A-I gene transfer on oxidative stress, endothelial function, and cardiac fibrosis in streptozotocin-induced diabetic cardiomyopathy

Hyperglycemia increases oxidative stress by inducing the generation of ROS on the one hand and by attenuating the production/activity of antioxidant enzymes, like SODs,

glutathione peroxidase, and catalase, on the other hand [190]. ROS can induce lipid peroxidation, alter cellular proteins, and initiate diverse stress-signaling pathways including Erk, JNK, and p38 MAPK. In the vasculature, ROS are predominantly produced by vascular NOX [191], whereas "uncoupled" eNOS – when eNOS produces O_2^- rather than NO – is another important source of ROS in diseased, including diabetic, blood vessels [192]. In diabetic cardiomyocytes, ROS are mainly generated by mitochondria, due to mitochondrial oxidation of fatty acids [170], and by NOX [193]. Apo A-I transfer decreased the systemic oxidative stress in STZ rats indicated by the decline in STZ-induced serum levels of TBARS [6], a marker of lipid peroxidation. This is in agreement with Mackness *et al.* [194] who demonstrated that HDL decrease the formation of TBARS on oxidized LDL. This effect is likely mediated by an increase in activity of PON or PAF-AH, 2 enzymes with anti-oxidative features, associated with HDL and known to be increased after apo A-I gene transfer [195]. Apo A-I transfer also resulted in a decrease of vascular oxidative stress in STZ-induced diabetic rats following the reduction in diabetes-enhanced aortic AT1R expression [33,196], NOX activity, and eNOS uncoupling. We postulate that the down-regulation in AT1R after apo A-I transfer is the predominant mediator of reduced NOX activity and eNOS uncoupling, which is consistent with the previously demonstrated role of the AT1R in mediating increased NOX activity and eNOS uncoupling in diabetes [197]. This is further corroborated by our *in vitro* findings showing that the HDL-mediated down-regulation of the AT1R in human aortic endothelial cells was associated with a decrease in hyperglycemia-induced oxidative stress, and a reduced responsiveness to Ang II. These observations support the recent finding of Tölle *et al.* [82], who demonstrated that HDL decreases NOX-dependent ROS generation via inhibition of the activation of Rac1, which is a downstream AT1R-dependent mediator of Ang II [198]. The exact mechanism by which HDL affects AT1R regulation under diabetes mellitus requires further fundamental studies. Since oxidized LDL [199] and ROS [200] play a role in the induction of the AT1R in human aortic endothelial cells, it is tentative to postulate that HDL via intrinsic anti-oxidative features (cfr. infra, via PON and PAF-AH) may contribute to the down-regulation of the AT1R under diabetes mellitus, which results in less NOX activity

and ROS formation and in turn may reduce AT1R expression. The recent finding that S1P and sphingosylphosphorylcholine, two lipid components of HDL without anti-oxidative properties, mimicked the capacity of HDL to reduce ROS generation [82], suggest that also other intrinsic features of HDL may contribute to the down-regulation of the AT1R.

The decrease in vascular oxidative stress after apo A-I transfer in STZ rats was associated with a reduction in endothelial dysfunction, which is a hallmark of diabetic cardiomyopathy. We suggest that reduced peroxynitrite formation as a result of lower NOX activity [201] following apo A-I transfer, decreased eNOS uncoupling and improved NO bioavailability. Furthermore, the increased eNOS dimer:monomer ratio, as a consequence of reduced NOX activity [202], may also have contributed to enhanced NO bioavailability since oxygen reduction is always uncoupled from NO formation in monomers.

Besides the reduced systemic and vascular oxidative stress after apo A-I transfer in STZ rats, also an attenuation of cardiac oxidative stress could be observed. An indication herefore follows from the decline in the activated phosphorylation state of the stress-activated p38 MAPK, which pathological importance in the diabetic heart we could recently elucidate in a study showing that p38 MAPK inhibition reduces cardiac inflammation and improves LV dysfunction in STZ-induced diabetic mice [203]. Furthermore, we could show that the anti-oxidative effects of atorvastatin in STZ rats also include the decrease in the cardiac phosphorylation state of p38 MAPK [159]. Since p38 MAPK is known to activate nuclear factor-κB, which regulates on his turn the expression of pro-inflammatory cytokines, CAMs and others, the reduced activation of p38 MAPK may therefore also have contributed to the decreased cardiac inflammation observed after apo A-I gene transfer. A reduction in cardiac oxidative stress after apo A-I gene transfer in STZ rats was further supported by the impact of apo A-I gene transfer on the cardiac expression of the 3 forms of the anti-oxidant enzyme SOD, SOD-1 (intracellular), SOD-2 (mitochondrial), and ec-SOD (extracellular), which convert O_2^- anions into molecular oxygen and hydrogen peroxide. Their importance for the heart has been outlined in transgenic and knock out animal models [204], and recently for SOD-2 in a diabetic setting [205]. Apo A-I gene transfer led to an increase of diabetes-

reduced SOD-2 expression and normalized the diabetes-downregulated ec-SOD expression to levels found in non-diabetic controls. These findings together with the unaltered SOD-1 mRNA expression in the STZ diabetic heart are in line with the observations of Kruger *et al.* [84], which found an increase of ec-SOD in the aorta of STZ-diabetic rats after administration of the apo A-I mimetic peptide D-4F and no regulation of SOD-1. In the heart, overexpression of SOD-2 has been shown to protect mitochondrial respiratory function and to block apoptosis induction [206], whereas overexpression of ec-SOD has been demonstrated to decrease macrophage infiltration and fibrosis and to improve LV dysfunction [204]. These studies suggest that the reduction in cardiac fibrosis after apo A-I gene transfer in STZ rats can be partly explained by the decreased oxidative stress and inflammation, including downregulated expression of pro-fibrotic cytokines like TNF-α [6] (inflammatory fibrosis) as well as by the reduction in cardiac apoptosis (see supra) and subsequent replacement fibrosis, resulting in improved LV function. The importance of cardiac NOX in the development of diabetic cardiomyopathy [207,208] including cardiac remodeling [193] and the potential of HDL to decrease NOX activity [184], finally also suggest a potential contribution of decreased cardiac NOX following apo A-I transfer in the reduction of cardiac oxidative stress and subsequent fibrosis.

3.5. Effect of human apo A-I gene transfer on cardiac apoptosis in streptozotocin-induced diabetic cardiomyopathy

Cardiac apoptosis is another hallmark of diabetic cardiomyopathy. The incidence of apoptosis increases in the heart of diabetic patients [209] and STZ-induced diabetic animals [210] and is directly linked to hyperglycemia-induced oxidative stress [210]. Mitochondria play an important role in oxidative stress-induced apoptosis and caspase 3 and 7 are essential mediators in the mitochondrial processes of apoptosis [211]. Apo A-I gene transfer reduced the upregulated caspase 3/7 activity in STZ rats. This was paralleled with an increase in the ratio of the anti-apoptotic Bcl-2, a "guardian" against mitochondrial initiation of

caspase activation [212], towards the pro-apoptotic Bax. Besides the raise in Bcl-2 to Bax ratio, which is a marker of increased cardiomyocyte survival probability [213], apo A-I transfer normalized the diabetes-reduced phosphorylation/activation state of the anti-apoptotic protein kinase B Akt [214,215], and of its effector eNOS to levels found in non-diabetic hearts. Immunofluorescence staining illustrated the presence of activated Akt in cardiomyocytes, as well as in cardiac endothelial cells. On ultrastructural level, the anti-apoptotic effects of apo A-I gene transfer were translated in a reduced number of cardiomyocytes with swollen mitochondria and apoptotic bodies and a more intact endothelium and basement membrane. These findings suggest that the reduction in cardiomyocyte apoptosis as well as the improvement in endothelial integrity found in the hearts of STZ rats which underwent apo A-I transfer are mediated via the activation of Akt. This hypothesis is further corroborated *ex vivo*, showing that HDL supplementation on cardiomyocytes in hyperglycemia reduces apoptosis in a PI3K- and NO-dependent manner. The ameliorated endothelial integrity suggests a potential restoration of the microvascular homeostasis which has been shown to reduce cardiomyocyte apoptosis and to result in the recovery of cardiac function in diabetic cardiomyopathy [216]. Given the importance of the cardiac endothelium on the contractile state and Ca^{2+} handling of subjacent cardiomyocytes [217-219], it is furthermore attempting to speculate that part of the HDL-mediated improvement in LV function in STZ-induced diabetic rats was indirectly due to their protective effect on the cardiac endothelium. This hypothesis is further supported by the improvement in endothelial function after human apo A-I gene transfer in STZ rats [184]. The decrease in cardiac apoptosis after apo A-I gene transfer in STZ rats can besides the direct HDL-mediated cardiomyocyte-protective effect and the endothelial-protecting features also be attributed to several other in parallel triggered processes: (i) HDL may act as biological buffers capable of rapidly removing active TNF-α from the heart. (ii) They have the potential to increase [220], stabilize and activate prostaglandins [221]. This enhanced prostanoid availability/activity may contribute to the HDL-mediated cardioprotection, by acting directly on cardiomyocytes [222], and/or by inhibiting cardiac TNF-α production [223]; iii) TLR 4 has

recently been shown to play an important role in cardiac apoptosis in diabetic cardiomyopathy [181]. Since HDL can reduce TLR4 expression [180], it is intriguing to suggest that part of the anti-apoptotic effects of HDL in the diabetic heart can be attributed to a HDL-mediated decrease in TLR4 expression [180]. However, further studies are necessary to clarify this hypothesis.

Besides the reduction in cardiomyocyte apoptosis and improvement in endothelial integrity, apo A-I gene transfer led to an improved connected structure of the sarcomere (actin-myosin filaments), sharper intercalated discs, less cardiac fibrosis, and reduced glycogen accumulation. In addition, HDL supplementation on isolated cardiomyocytes improved their contractility under hyperglycemia-induced stress in a PI3K- and NO-dependent manner. This suggests that beyond beneficial vascular/cardiac-protective long-term effects, direct myocardial effects of HDL may have contributed to the improvement of cardiac function under severe STZ-induced stress.

In conclusion, this cumulative work demonstrates the capacity of apo A-I gene transfer to reduce the development of experimental diabetic cardiomyopathy via its anti-inflammatory, anti-oxidative, anti-fibrotic, and anti-apoptotic actions. Further studies are requested in investigating the potential of apo A-I gene transfer in improving established diabetic cardiomyopathy, especially in the context of type 2 diabetes mellitus, and in ameliorating the quality of HDL, which is impaired under diabetic conditions.

4. SUMMARY

More than 190 million people worldwide currently suffer from diabetes and its incidence is estimated to almost double by the year 2030. Cardiovascular disorders, including diabetic cardiomyopathy, are the main secondary complications associated with diabetes mellitus. Epidemiological studies have consistently shown that low HDL cholesterol is an independent cardiovascular risk factor. Apo A-I is the main apolipoprotein of HDL and there exists a strong correlation between apo A-I plasma levels and HDL concentrations. The focus of the present cumulative work was to unravel novel pleiotropic and metabolic features of HDL and to evaluate the potential of HDL to decrease the development of diabetic cardiomyopathy.

In STZ rats, which are characterized by severe hyperglycemia, oxidative stress, prominent cardiac inflammation and increased LDL and HDL cholesterol levels as shown in the first study, we could demonstrate, in the second study, that adenoviral apo A-I gene transfer resulted in a reduction in the development of experimental diabetic cardiomyopathy as indicated by a decrease in cardiac inflammation, fibrosis, glycogen accumulation, and apoptosis, and an improvement in endothelial integrity and LV function, involving the activation of the protein kinase Akt and downstream eNOS. Evidence from *ex vivo* studies with isolated cardiomyocytes under hyperglycemia suggests that besides beneficial vascular/cardiac-protective long-term effects, direct effects of HDL on cardiomyocyte contractility may also have contributed to the improvement of cardiac function under severe STZ-induced stress. Attenuation of endothelial dysfunction, another hallmark of diabetic cardiomyopathy was demonstrated after apo A-I transfer in STZ rats in the third study. Here we postulated that the reduction in AT1R expression and subsequent NOX activity and eNOS uncoupling following apo A-I transfer underlied the improvement in endothelial function in STZ rats. In the fourth study, apo A-I transfer increased plasma adiponectin levels and attenuated adipocyte lipolysis in LPS-induced inflammation. This finding supports the metabolic alterations after apo A-I transfer in the STZ model of diabetic cardiomyopathy and further suggests that the anti-inflammatory effects after apo A-I transfer are partly mediated

via adiponectin, which is also known for its anti-inflammatory features. Finally, down-regulation of (endothelial) TLR4 expression after apo A-I transfer was demonstrated in an experimental model of severe inflammation. The recently documented role of TLR4 in the development of diabetic cardiomyopathy corroborates a potential contribution of the decrease in endothelial TLR4 expression in the reduction of cardiac inflammation following apo A-I transfer in STZ rats.

In conclusion, this cumulative study supports the potential of HDL-raising strategies to decrease the development of diabetic cardiomyopathy in type 1 diabetic patients, by which the onset of diastolic dysfunction, the first clinical manifestation of diabetic cardiomyopathy is well-defined. However, further studies are required to elucidate the potential of HDL-raising pharmaca for the treatment of established diabetic cardiomyopathy, especially in the context of type 2 diabetes mellitus.

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6. ACKNOWLEDGEMENTS

7. ERKLÄRUNG

§ 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, daß

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde.

- die vorgelegte Habilitationsschrift ohne fremde Hilfe verfasst, die beschriebenen Ergebnisse selbst gewonnen sowie die verwendeten Hilfsmittel, die Zusammenarbeit mit anderen Wissenschaftlern/Wissenschaftlerinnen und mit technischen Hilfskräften sowie die verwendete Literatur vollständig in der Habilitationsschrift angegeben wurden.

- mir die geltende Habilitationsordnung bekannt ist.

12.03.2013   Dr. S. Van Linthout