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Extravascular Inflammation in Experimental Atherosclerosis

The Role of the Liver and Lungs

Man Chi Wong

Cover: Relationship between liver, heart and lungs according to the Five elements theory

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Extravascular Inflammation in Experimental Atherosclerosis

The Role of the Liver and Lungs

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“Real knowledge is to know the extent of one’s ignorance.”
Confucius, 孔夫子*, 1498-1557*

Aan mijn ouders

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1

General Introduction

Cardiovascular diseases (CVDs) are currently the main cause of mortality and morbidity worldwide and expected to remain the leading cause of death at least till 2030 according to the World Health Organization. CVDs are a group of disorders involving the heart and blood vessels and most people die of coronary heart disease and stroke. The root cause of most cases of CVD is atherosclerosis. Atherosclerosis affects medium and large sized arteries in the body and was primarily thought to be solely caused by accumulation of lipids in the vessel wall. Russell Ross was one of the first researchers to state that inflammatory processes play an important role in the pathogenesis of atherosclerosis.[1] Nowadays, disturbances in lipid levels and increased inflammation are the two established contributors to atherosclerosis development. Since the liver is a key role player in both lipid metabolism and regulation of inflammatory processes, it is a potential interesting organ to study in atherosclerosis development.

In practice, in a single patient CVD is often present in combination with other diseases, such as diabetes, cancer, arthritis and chronic obstructive lung disease (COPD). Some features of these comorbidities, such as poor lung function in COPD, are found to be strong independent predictors of cardiovascular risk.[2] Like CVD, COPD is a major cause of mortality and morbidity globally. It is the only leading cause of death that still has a rising mortality rate, and projected to be ranking as the third leading cause of death by 2020.[3] Even though the respiratory and cardiovascular systems interact closely, not many experimental studies have been performed in which the role of both organ systems in atherosclerosis development have been investigated. Furthermore, next to the elucidation of the pathogenic process, it is important to develop new therapies to control the risk factors and inhibit the initiation and progression of CVD and its comorbidities.

This chapter provides background information on dyslipidemia and increased inflammation in atherogenesis and on a new promising anti-atherogenic drug, resveratrol. Furthermore, effects of modulations in lipid metabolism and inflammation in the liver and lungs on atherosclerosis development are highlighted.

1. Dyslipidemia

Nikolai N. Anichkov demonstrated for the first time in 1913 that cholesterol alone caused the atheromatous changes in the vascular wall.[4] Cholesterol is essential for life, it is required to build and maintain cell membranes, involved in intracellular transport, cell signaling, nerve conduction and it is an important precursor molecule for the synthesis of bile and hormones. As it is insoluble in blood due to its lipophilic characteristics, it is transported through the circulation in lipoproteins.[5] These particles have a lipid-rich core containing triglycerides (TGs) and cholesteryl esters (CEs) and an amphipathic surface consisting of phospholipids (PLs), unesterified cholesterol and proteins. These proteins, called apolipoproteins, bind lipids and solubilize them, and act as coenzymes and ligands for lipoprotein receptors on tissues, like the liver. Lipoproteins are classified based on their density (in the order of increasing density): chylomicrons, very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL).

In the fed state, dietary lipids form chylomicrons in the intestine. Here, the products of fat digestion, TG, PL and CE are combined with apolipoprotein (apo) B48 in the enterocyte.

The lipoproteins formed in this manner are secreted into the lymph (chyle) and are termed chylomicrons. TG in chylomicrons is hydrolyzed by lipoprotein lipase (LPL) and the released fatty acids (FAs) are subsequently taken up by adipose and muscle tissue. An endogenous source for FA from TG is VLDL, which is synthesized by the liver. After processing by LPL, VLDL is converted to IDL and LDL. Remnants of apoB-containing lipoproteins can be taken up by the liver.

HDL is the smallest and only anti-atherogenic lipoprotein.[6] Its most abundant apolipoproteins are apoAI and apoAII. The liver synthesizes HDL as complexes of apolipoproteins and PLs, which are capable of picking up cholesterol from the periphery, *e.g.* atherosclerotic lesions, and transporting it back to the liver, where it can be excreted out of the body through the bile. This process is termed reverse cholesterol transport. HDL also delivers cholesterol to adrenals, ovaries, and testes for the synthesis of steroid hormones.

The process of atherogenesis was considered for many years merely to constitute of progressive accumulation of lipids within the vessel wall. Indeed at the earliest stages, accumulation and modification of LDL was observed and has been recognized as the initiating factor of atherosclerosis development.[7] Healthy endothelium forms a smooth monolayer of elongated endothelial cells, without adhesive capacities. However, especially in areas where the blood flow is disturbed, *e.g.* branched or curved points, the permeability of the endothelium is increased.[8] Endothelial dysfunction and increased permeability can also be caused by elevated levels of LDL, hypertension, hyperglycemia, increased inflammation and oxidative stress, *e.g.* by cigarette smoking.[1] Remnant lipoproteins and mainly the small, dense LDL, can enter the intima of the blood vessel.[9] LDL is retained in the intima by binding of apoB to proteoglycans of the extracellular matrix.[10] Although disturbances in lipid metabolism have long been held responsible for atherosclerosis development, the earliest stage of atherogenesis is characterized by accumulation of (lipid-laden) inflammatory cells, and more specifically macrophages. In recent years, atherosclerosis research has turned its focus more to the inflammatory processes involved.

2. Inflammation

Small, dense LDL particles easily penetrate into the endothelium, become entrapped in the intima, where they become modified by *e.g.* oxidation (Figure 1). This activates the endothelium and resident macrophages to produce adhesion molecules and chemokines, such as monocyte chemoattractant protein 1 (MCP-1) and vascular cell adhesion molecule (VCAM), to attract monocytes (the most numerous of the leukocytes recruited), dendritic cells (DCs), and T cells into the intima.[11] In early atherosclerosis, vascular smooth muscle cells (VSMCs) may contribute to the development of the atheroma through the production of pro-inflammatory mediators and through the synthesis of matrix molecules required for the retention of lipoproteins. After stimulation with macrophage colony-stimulating factor (M-CSF), monocytes differentiate into macrophages in the intima, which take up the modified LDL (mLDL) through their scavenger receptors. As cholesterol accumulates, these macrophages transform into foam cells, the prototypical cell in atherosclerosis, when the LDL particles cannot be mobilized out of the cell to a sufficient extent.

Antigens of mLDL are presented by macrophages and DCs in the intima and trigger the activation of other macrophages and antigen-specific T-cells.[12] Most of the activated

macrophages and T-cells produce cytokines, *e.g.* interferon (IFN)γ, interleukin (IL)-6, tumor necrosis factor (TNF)α, which attract and activate more inflammatory cells, *e.g.* mast cells, natural killer (NK)T-cells, leading to magnification and sustainment of the inflammatory response and the formation of a complex conglomeration of lipids, cholesterol crystals, inflammatory and necrotic cells. In addition, cytokines and growth factors secreted by macrophages and T-cells are important for VSMC migration, proliferation and production of collagen to form a fibrous cap covering the lesion. The fibrous cap maintains stability of the plaque by protecting the thrombogenic mixture of leukocytes, lipid, and debris from the blood stream. Extracellular lipid derived from dead and dying cells can accumulate in the plaque,

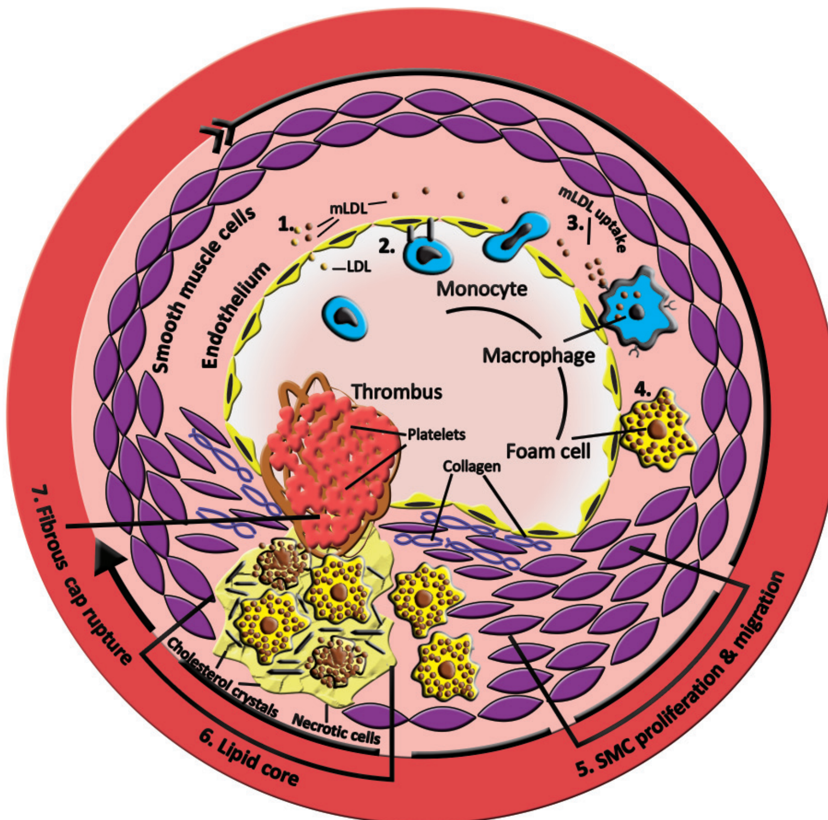

Fig. 1. Stages in atherogenesis. 1) When LDL becomes entrapped in the vessel wall, it becomes modified (mLDL). This mLDL induces an inflammatory response leading to 2) expression of adhesion molecules on the activated endothelium, by which leukocytes, such as monocytes are attracted to migrate into the vessel wall. 3) The monocytes mature into macrophages, take up the mLDL through scavenger receptors and 4) become foam cells. 5) In the progressing atherosclerotic lesion, smooth muscle cells (SMCs) proliferate, migrate and produce collagen to form a fibrous cap covering 6) the lipid-rich core consisting of necrotic and apoptotic foam cells, cholesterol crystals and other debris. 7) If this thrombotic content becomes exposed to the blood stream, due to weakening and rupture of the cap by *e.g.* proteases, a thrombus is formed which can occlude the vessel, causing an infarction.

often denoted the lipid or necrotic core. On the other hand, proteases (*e.g.* metalloproteinases (MMPs)), cytokines and radicals are also produced that can destabilize the lesions and break down the fibrous cap. While clinical complications, *e.g. angina pectoris*, can arise from growing plaques creating flow-limiting stenoses, the most severe adverse events follow the rupture of a plaque, which exposes the prothrombotic material in the plaque to coagulation factors in the blood and causes a sudden occlusion of the artery, which can result in ischemia and infarction of the supplied tissue.[13]

2.1. Nuclear factor-κB (NF-κB)

Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) is a family of five protein products belonging to two classes. Class I consists of NF-κB1 (p50) and NF-κB2 (p52), which are synthesized from the precursors p105 and p100, respectively. Class II includes RelA (p65), RelB and cRel.[14] The most common and best-characterized form of NF-κB is the p65/p50 heterodimer.

In unstimulated cells, the NF-κB p65/p50 dimer is kept inactive in the cytosol bound with its inhibitor, IκBα (Figure 2). IκBα keeps NF-κB inactive by masking the nuclear localization site and sequestered in the cytoplasm.[15] The classical activation of the NF-κB pathway can be initiated by a wide range of extracellular stimuli, including cytokines, such as TNFα and IL-1β, viral products, bacterial components (pathogen-associated molecular patterns (PAMPs)), but also saturated FAs[16] and endogenous stress signals following tissue damage, termed danger-associated molecular patterns (DAMPs)[17] through different receptors, such as pattern recognition receptors (PRRs). This results in the activation of different signal transduction cascades which eventually activate the IκB kinase (IKK) complex, which consists of IKKα, -β and -γ (the latter is also called NF-κB essential modulator (NEMO)). This complex will mediate the phosphorylation of IκBα, resulting in its ubiquitination and degradation, allowing nuclear entry of the liberated NF-κB, where it can induce expression of specific target genes, encoding cytokines, growth factors, immunomodulatory molecules, apoptosis related genes and others.

Under physiological circumstances, NF-κB activation is rapid and short-acting (approximately 30-60 min), and expression of NF-κB-dependent genes is downregulated after a limited period of time. One of the target genes of NF-κB is IκBα, thereby forming a negative feedback loop. In atherosclerosis and many other chronic pathological conditions, however, NF-κB is persistently activated by a combination of numerous factors.

In the initial stages of atherosclerosis development, NF-κB regulates the expression of cytokines, chemokines and adhesion molecules.[18] Later in the progression of the lesion, NF-κB regulates gene expression of M-CSF, which is important for the formation of foam cells.[19] NF-κB is an essential regulator of MMP gene expression, especially MMP-2 and MMP-9, which are critical in plaque rupture.[20] Increased NF-κB activity was found especially in unstable regions of atherosclerotic plaques.[21] Thus, NF-κB regulates the expression of a wide spectrum of factors influencing different stages of atherosclerosis development. Furthermore, many of these factors increase NF-κB activity which propagates a positive feedback loop. Increased NF-κB activity was not only observed locally within the lesion, but also in circulating leukocytes as

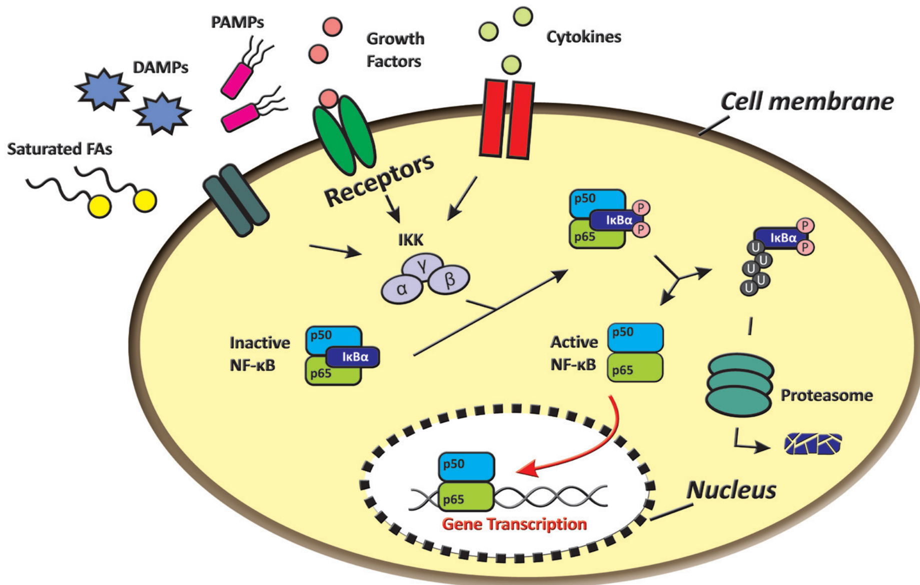

Fig. 2. Activation of the NF-κB pathway. Extracellular and endogenous triggers, such as pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and saturated fatty acids (FAs), bind to pattern recognition receptors (PPRs) and activate the IκB kinase (IKK) complex. IKK phosphorylates the inhibitor of NF-κB (IκBα), resulting in the release and phosphorylation of NF-κB, after which it can translocate to the nucleus to activate the transcription of its target genes.

demonstrated in a study showing highest activity in circulating leukocytes from unstable *angina* patients when compared to stable *angina* patients, and low activity in control patients.[22]

Nowadays, many research projects on the discovery of anti-atherogenic drugs are targeted on the inhibition of NF-κB activity.[23] NF-κB signaling and its interactions with other networks are very complex and hence the inhibition of the whole NF-κB system will be detrimental. It is therefore essential to identify and dissect the crucial signaling connections and in this way develop more specific and safer therapeutic agents.[24]

3. Lipid metabolism and inflammation

The term 'lipemia of sepsis' was coined already in the late 1950s, when patients with cholera were noted to have lipidemic blood and high serum levels of TGs.[25] The functional importance of this cytokine-induced hyperlipidemia lies not only in the mobilization of lipid stores to fuel the host immune system to combat the pathogen, but also in the capacity of TG-rich lipoproteins to bind and neutralize lipopolysaccharide (LPS), the most toxic component of the cell membrane of Gram-negative bacteria.[26] In addition, apolipoproteins, *e.g.* apoAI and apoE have been shown to have strong anti-inflammatory features, next to their more classical role in lipid metabolism.[27]

So, there is ample evidence that lipid metabolism and inflammatory pathways interact with each other. As mentioned above, they are the two key contributors to atherosclerosis development. Thus, studying their crosstalk in atherosclerosis-prone animal models will provide more insight in the involved processes in atherogenesis. For example, hypercholesterolemic mice have a peripheral blood monocytosis that develops over time in both chow-fed and fat-fed *apoe*$^{-/-}$ mice.[28] This monocytosis is mainly attributed to an increase in the pro-inflammatory population of Ly6C^{hi} expressing monocytes compared with the 'patrolling' Ly6C^{lo} expressing subset. The Ly6C^{hi} monocytes are pro-atherogenic because they adhere more strongly to activated endothelium, accumulate in plaques and become lesional macrophages.[28]

4. Animal models for atherosclerosis

Analysis of human atherosclerosis has been predominantly observational, although with the advancement of non-invasive techniques, *e.g.* intravascular ultrasound, atherogenesis in humans in a more experimental setting is increasingly being studied. However, for mechanistic insight into the processes involved, studies in animal models remain to be a necessary resource. One of the main reasons why mice form suitable models is the possibility to generate transgenic, knock-out or knock-in strains to study the role of specific genes and their ensuing products. These advantages of murine models not only account for the field of atherosclerosis research.

Since the plasma cholesterol levels of wild-type mice are low (approximately 2 mM) and mainly confined to HDL, they do not develop atherosclerosis. Therefore, atherosclerosis-prone mouse models are developed through alteration of key components controlling circulating lipoprotein levels, causing hyperlipidemia and/or -cholesterolemia. ApoE and LDL receptor (LDLr) knockout and transgenic *APOE*3-Leiden* (*E3L*) models are most widely used.

ApoE is essential for the uptake of (remnants of) apoB-containing lipoproteins by the liver via LDLr, LDLr-related protein (LRP) and heparan-sulphate proteoglycans (HSPGs). Furthermore, apoE derived from macrophages has been shown to have an important role in cholesterol efflux from foam cells.[29] *Apoe*$^{-/-}$ mice have a pronounced elevation of plasma VLDL-cholesterol level on a normal chow diet (approximately 9 mM) and, as such, develop spontaneously atherosclerotic lesions. In addition, evidence indicates that apoE has anti-atherogenic properties independent of plasma lipoprotein regulation.[30] On chow as well as on a high cholesterol (1.25%) diet, *apoe*$^{-/-}$ mice have higher systemic cytokine levels than wild-type mice.[31] In addition, *apoe*$^{-/-}$ mice have an increased number of circulating monocytes, and upon high fat and high cholesterol diet feeding, these monocytes are skewed to a pro-inflammatory phenotype.[32] A possible mechanism for the immunomodulatory characteristics of apoE is that it can bind LPS directly, redirecting it to bile, and thereby preventing it from binding to its receptor.[33, 34] Another mechanism described, involves apoE binding to cell surface receptors and HSPG and the consequential inhibition of JNK and c-Jun phosphorylation that is required for IL-6, IL-1β, and TNF-α secretion in Toll-like receptor (TLR) signaling.[35] Irrespective of the involved mechanism, it is clear that not only the hyperlipidemia, but also the increased inflammatory state of *apoe*$^{-/-}$ mice accounts for their susceptibility to develop atherosclerosis.

Ldlr$^{-/-}$ mice lack the expression of a functional LDLr due to an insertion of a neomycin resistance cassette into exon 4 of the gene, which leads to the production of an inactive truncated protein.[36] On chow diet, the plasma cholesterol levels are not high enough (approximately 6 mM) for atherosclerosis development. To this end, these mice are fed a cholesterol-rich diet for atherosclerosis studies, resulting in plasma cholesterol levels up to 50 mM, predominantly in the LDL fraction. The elevated levels of LDL as a consequence of the lack of a functional LDLr can augment inflammation. LDL becomes immunogenic when it undergoes modifications such as oxidation and glycation *in vivo*.[37] mLDL not only promotes the transformation of macrophages into foam cells but their recognition by auto-antibodies also triggers the release of pro-inflammatory cytokines by human monocyte-derived macrophages.[38] Thus, *ldlr$^{-/-}$* mice are likely to have an enhanced inflammatory state, at least indirectly, due to higher levels of LDL.

E3L mice carry a construct containing the human *APOE*3-Leiden* gene, a dominant negative mutation of apoE that causes hyperlipidemia, together with apoCI that elevates plasma TG by inhibiting LPL activity. In addition, apoCI also interferes with apoE-mediated uptake of apoB-containing lipoproteins. This results in moderately raised plasma cholesterol and TG levels compared to the *apoe$^{-/-}$* and *ldlr$^{-/-}$* models, which in addition can be modulated by varying the dose of cholesterol in the diet. Furthermore, *E3L* mice are more sensitive to lipid modulating drugs, such as statins, fibrates and nicotinic acids, than *apoe$^{-/-}$* and *ldlr$^{-/-}$* mice.[39, 40]

To further humanize the lipoprotein profile of *E3L* mice, *E3L* mice have been crossbred to mice expressing human cholesteryl ester transfer protein (CETP). This protein is naturally missing in wild-type mice and transfers cholesteryl esters from HDL to (V)LDL in exchange for triglycerides, thereby lowering HDL and increasing VLDL. These *E3L.CETP* mice respond to HDL-modifying drugs similar to humans and are therefore used extensively in studies in which potential anti-atherogenic therapies are investigated,[41, 42] such as described in **chapter 5**.

Crossbreeding of these atherosclerosis-prone mice with mice that carry deletions in genes encoding crucial components of the immune system or bone marrow transplantation has provided important information on the role of the immune system in atherogenesis.[43] Organ-, tissue- or cell-restricted genetic altering of mice is another technique to gain more insight in the role of certain cell populations or organs in the pathogenesis of atherosclerosis, which will be further elaborated in the next section.

5. Extravascular lipid metabolism and inflammation

To study how the interaction of different organ systems plays a role in atherosclerosis development, experimental animals form a suitable model. CVD has been associated with other chronic inflammatory autoimmune diseases, *e.g.* systemic lupus erythematosus and rheumatoid arthritis.[44] Chronic inflammation can also derive from exogenous and/or infectious agents, when they are not eradicated effectively by the acute inflammatory response, as has been shown for *e.g. Porphyromonas gingivalis*, one of most important bacteria implicated in periodontitis[45] and *Chlamydophila pneumoniae*, a gram-negative bacterium that is a frequent cause of low-grade respiratory infection.

Most pathogens enter their host through the mucosa of the lungs and gut by the route of inhalation or ingestion, respectively. Following the gut, the liver is the second line of defense for incoming pathogens. The lungs and liver are therefore equipped with critical immune defense mechanisms. Apart from their role in immunity, the liver is known to be a key regulator of lipid metabolism. In the framework of the two main contributors to atherosclerosis, dyslipidemia and especially, enhanced inflammation, the role of the liver and the lungs in atherosclerosis development are described in the following paragraphs.

5.1. Liver

The liver is the largest internal organ in the human body comprising about 1/50 of the adult body weight. It is the only organ in the body that receives blood from both the systemic circulation (via the hepatic artery, ~20%) and the gastrointestinal tract (via the portal vein, ~80%). In the liver, blood from the portal vein and arterioles derived from the hepatic artery flows to the central vein, while passing the hepatic sinusoids. The hepatic sinusoids are fenestrated and lack a basement membrane, which facilitates the interaction between the contents of both blood supplies with liver sinusoidal endothelial cells, hepatic immune cells in the space of Disse, and hepatocytes, arranged in cords (Figure 3).[46] This blood passing the hepatic sinusoids contains products of digestion, including carbohydrates, peptides, FAs, along with antigens and microbial products that originate from the bacteria in the small and large intestine. Apart from its role in energy metabolism and immunity, which will be discussed more extensively below, the liver, or more specifically, the hepatocytes, carry out various vital functions, such as the production of coagulation factors, break down or modification of toxic substances and drugs, storage of vitamins, production of albumin and synthesis of angiotensinogen.

The liver is composed of many different cell types which are divided into parenchymal cells (hepatocytes) and non-parenchymal cells, *i.e*. sinusoidal endothelial cells (SECs), lymphocytes, Kupffer cells, biliary epithelial cells, hepatic stellate cells (HSCs) and DCs (Figure 3). The hepatocyte population accounts for approximately 80% of all cells in the liver, while non-parenchymal cells constitute 20%, of which about 50% are endothelial cells, 25% lymphocytes and 20% Kupffer cells. Compared with peripheral blood, the liver is enriched with NK and NKT cells.[47]

5.1.1. Lipids

The liver plays a major role in energy metabolism of the three nutrients, carbohydrates, proteins and fat. It synthesizes, stores and releases carbohydrates through gluconeogenesis, glycogenolysis and glycogenesis. In addition, it is responsible for the synthesis and the degradation of proteins and removal of ammonia from the body by synthesis of urea. Excess carbohydrates and proteins are converted into FAs and TGs to be exported to the rest of the body. Because of its prominent role in atherogenesis, the role of the liver in lipid metabolism is described more in detail.

Chylomicrons from the intestine travel through the lacteals to join lymph from other parts of the body, and enter the blood circulation via the thoracic duct. LPL releases TG from the core of the chylomicron by hydrolyzing them to FA and monoglycerides, which are taken up by the

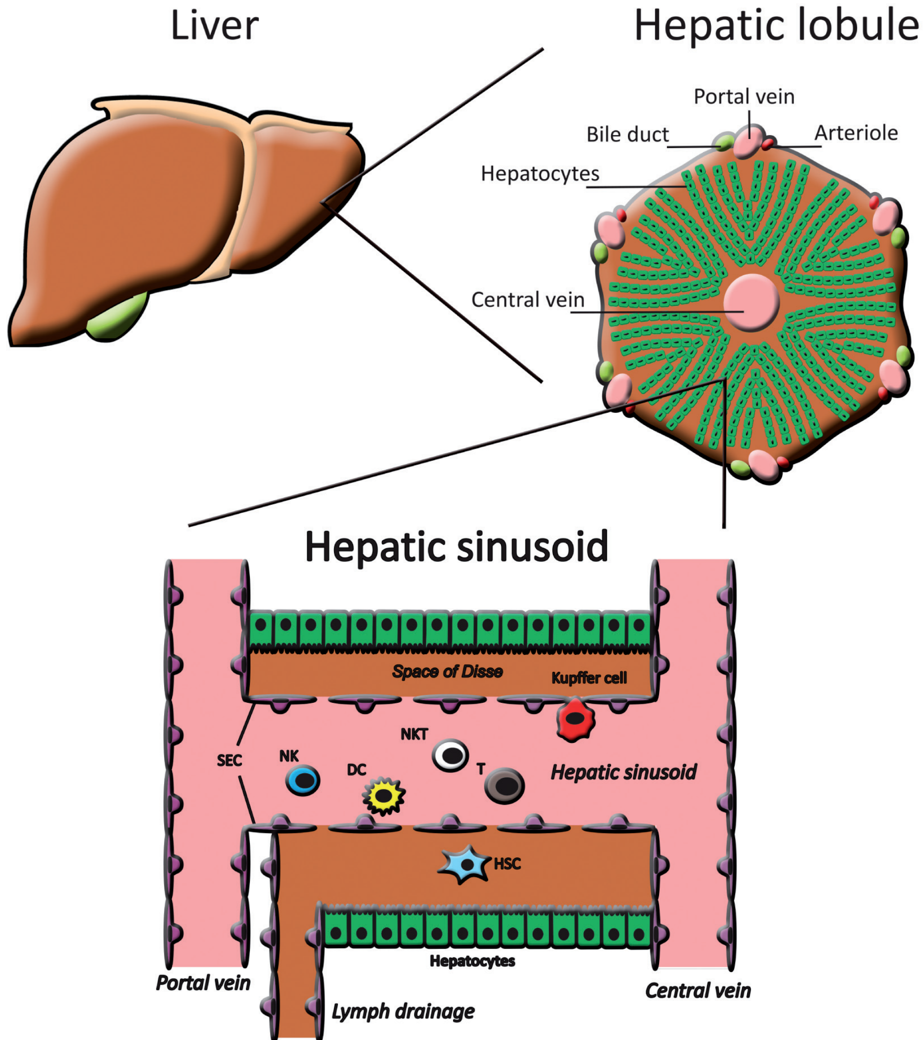

Fig. 3. Anatomical organization of the liver. **Hepatic lobule.** When blood drained from the gastrointestinal system flows from the portal vein to the central vein, it passes cords of hepatocytes through sinusoids. **Hepatic sinusoid.** Sinusoidal endothelial cells (SECs) form the specialized, fenestrated endothelium lining the sinusoids. Hepatic stellate cells (HSCs) are found in the space of Disse, while Kupffer cells, lymphocytes and dendritic cells (DCs) can be found both in the sinusoids and space of Disse.

tissues locally. Remaining chylomicron remnants are removed from the circulation largely by the liver. VLDL is synthesized by the liver and hydrolyzed by LPL resulting in the formation of IDL, which is partly taken up by the liver by apoE. The remainder is further processed by LPL and hepatic lipase to become LDL, which is recognized by the receptors on liver and peripheral tissues. VLDL, IDL and LDL are apoB100-containing particles. Within the hepatocyte, lipidation

of apoB100 requires an adequate supply of lipids and microsomal triglyceride transfer protein (MTTP).[48] The lipids added to apoB100 can originate from the diet or be synthesized *de novo* by hepatocytes. *De novo* lipogenesis is upregulated via insulin-mediated stimulation of sterol response element-binding protein 1c (SREBP-1c), a major lipogenic transcription factor. Fatty acid synthase (FAS) is one of the most important genes involved in *de novo* synthesis of TG, while carnitine palmitoyltransferase 1A (CPT1A) is an enzyme essential for fatty acid oxidation.

High circulating levels of LDL can cause retention of LDL within the vessel wall, which is the first step of atherosclerosis development, as described above. Hepatic synthesis of bile acids accounts for the majority of cholesterol removal out of the body. Bile is excreted in the duodenum and facilitates the digestion and transport of lipids. The largest amount (~95%) of bile acids excreted in the duodenum, are absorbed back into blood within the ileum, where it returns to the sinusoids of the liver through the portal vein (enterohepatic recirculation). Bile acids are then transported across the hepatocytes to be resecreted into bile canaliculi.

5.1.2. Inflammation

Facing the continuous exposure to gut-derived antigens present in portal venous blood, the liver's immune cells exist in a state of tolerance which is also termed 'liver tolerance'.[46] On the one hand, a reasonable speculation is that most of these antigens originate from harmless material in food, on the other hand, however, this makes the liver also susceptible to invasion by pathogens that breach the intestinal mucosa and invade the circulation. Therefore, the liver is an organ with a specialized immune system. Kupffer cells and lymphocytes, including a relative high number of NK cells and NKT-cells, make up the majority of immune cells in the liver. Kupffer cells are specialized macrophages located in the liver and form the body's largest compartment of macrophages. They reside within the lumen of the liver sinusoids; therefore, they are the first cells to be exposed to materials absorbed from the gastrointestinal tract. In addition to their phagocytic capacities, Kupffer cells process and present antigens.[49] Cytokines produced by Kupffer cells induce the expression of acute phase proteins, such as C-reactive protein (CRP), serum amyloid A (SAA), α1-antitrypsin, ceruloplasmin or haptoglobin, partly through NF-κB signaling, in hepatocytes.

NK cells make up as many as 50% of liver lymphocytes and respond both to cytokine activation and to engagement of an excess of activating receptors over inhibitory receptors, *i.e.* recognizing major histocompatibility complex (MHC) class I alleles. NKT cells recognize glycolipid antigens that are conserved features of bacterial walls, thus respond to those derived from the intestinal bacteria.[50] From this perspective, NKT cells can be considered to represent a bridge between lipids and inflammation. Given that lipid accumulation is a hallmark of atherosclerosis and the fact that NKT cells are activated by glycolipid antigens, it is not surprising that NKT cells were hypothesized to play a role in atherogenesis. ApoE on circulating lipoproteins can enhance NKT cell responses to glycolipid antigen presented by human DCs[51] and activation of NKT cells has been demonstrated to accelerate atherogenesis.[52]

The other non-parenchymal cells in the liver, *i.e.* SECs, DCs and biliary epithelial cells all express TLRs and play an immunomodulatory role,[53] but are not further discussed in this introduction.

5.1.3. Lipids and inflammation

Hypercholesterolemic *apoe*$^{-/-}$ mice on chow diet display not only inflammation in atherosclerotic lesions, but also in periadventitial and visceral adipose tissue, liver and pancreatic islets.[31] Dietary cholesterol can induce hepatic inflammation in *ldlr*$^{-/-}$[54] and *E3L* mice.[55, 56] Like atherosclerosis, insulin resistance is commonly associated with both chronic inflammation and a metabolic dyslipidemic profile with increased levels of atherogenic apoB-containing lipoproteins. In an insulin resistant experimental animal model, it was shown that inhibition of NF-κB activity resulted in reduced apoB100 synthesis by primary hamster hepatocytes.[57] This implies that hepatic inflammation is an important factor underlying hepatic atherogenic lipoprotein production observed in insulin resistance and atherosclerosis. Moreover, Luchtefeld *et al*. have shown that hepatocyte-specific deficiency of gp130, a key component of the IL-6 signaling pathway, reduces atherosclerosis development in *apoe*$^{-/-}$ mice.[58] The liver is a prototypical organ in which lipid metabolism and inflammation converge, thus it is not surprising that it plays a central role in atherosclerosis.

5.2. Lungs

The lungs are not only the organ system that is localized nearest to the heart, but also work in close collaboration with the cardiovascular system. Lungs are filled with about 90% air and 10% tissue, which reflect their most important function, *i.e.* gas exchange of oxygen and carbon dioxide. Inhaled air travels from the nose to the trachea, bronchi, bronchioles, terminal bronchioles, respiratory bronchioles and finally the alveoli, which are the functional units of lungs, where most of gas exchange takes place (Figure 4). Oxygen and carbon dioxide are transported to and from the rest of the body respectively, in erythrocytes and dissolved in plasma.

The pulmonary epithelium in the bronchi and bronchioles consists of different cell types: ciliated cells, goblet cells, basal cells, brush cells and Clara cells. The cilia of the ciliated cells beat in concert cranially, effectively moving secreted mucus containing trapped foreign particles to the oropharynx for either expectoration or swallowing to the stomach where the acidic pH helps to neutralize foreign material and micro-organisms. Goblet cells secrete mucus, which acts as a barrier and traps particulate material and pathogens moving through the airway. Basal cells serve as a reserve population by maintaining individual cell replacement in the epithelium. Brush cells have a synaptic contact with an afferent nerve ending at the basal surface and are therefore regarded as receptor cells with microvilli. Clara cells increase in number as the ciliated cells decrease along the length of the bronchiole. They secrete surfactant-related proteins, which are needed to prevent luminal adhesion in case the wall of the airway folds on itself, particularly during expiration.

The most important types of cells surrounding the alveoli are type I pneumocytes, type II pneumocytes and alveolar macrophages (Figure 4). Type I pneumocytes are the major cell type lining the alveolar surfaces, through which gas is exchanged. The type II cells serve as stem cells for themselves and the type I cells. They secrete a fluid which acts as a surfactant by reducing surface tension, and thereby increase pulmonary compliance, prevent atelectasis (collapse of the lung) at the end of expiration and facilitate recruitment of collapsed airways. The alveolar

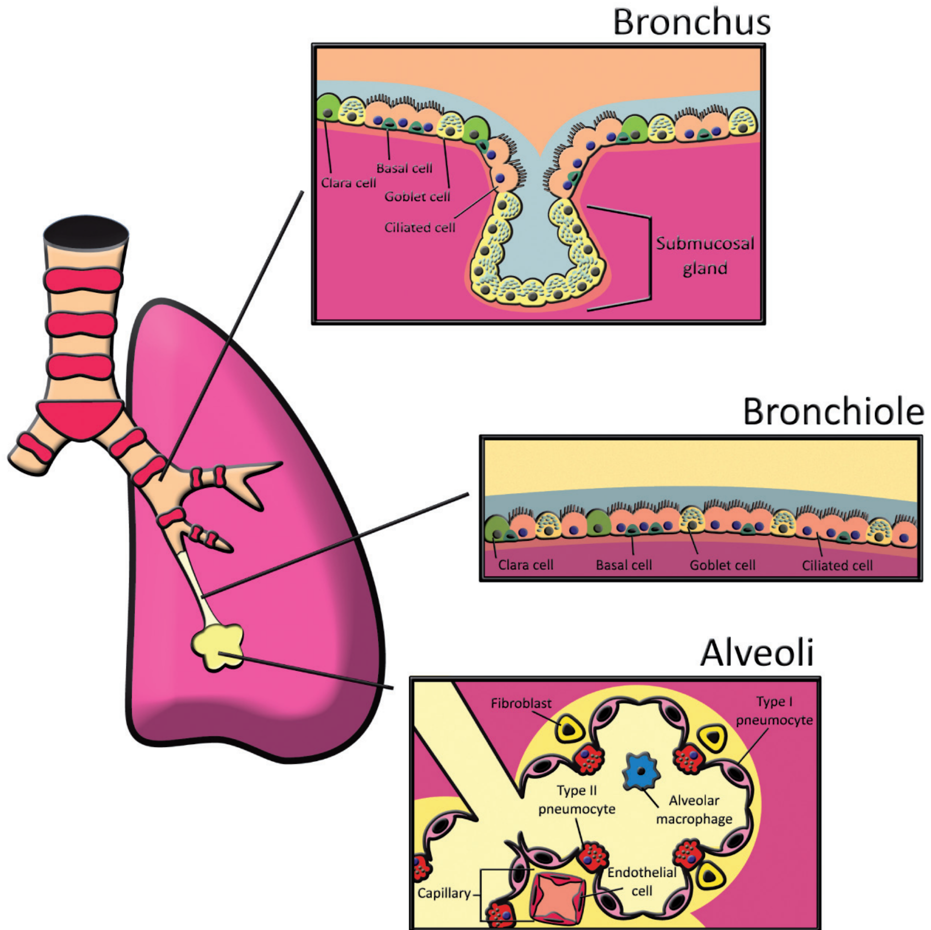

Fig. 4. Epithelium of the bronchial tree and alveoli. Inhaled air first passes the nasal cavities, naso-, oropharynx, larynx, trachea, primary bronchi, before it enters the lungs, where it sequentially travels through the internal bronchi, bronchioles, alveolar ducts, - sacs, and alveoli. **Bronchial epithelium**. Basal cells, ciliated columnar cells, and goblet cells are the principal cell types in the bronchial epithelium. The basal cells, located at the base of the epithelial layer, are the stem cells from which the other cell types arise. The ciliated columnar cells are the most numerous and provide sweeping of the mucus toward the pharynx, thus serving as an important protective mechanism for removing small inhaled particles from the lungs. Subepithelial glands are present in the larger bronchi and are a main source of mucus in the central airways. **Bronchiolar epithelium.** The composition of the epithelium changes as the ducts narrows from bronchi, the larger diameter bronchioles towards smaller ones: cartilage plates and subepithelial glands are not present in bronchioles, the ciliated columnar epithelium transforms to simple cubiodal epithelium, the number of goblet cells decreases, while Clara cells increase in number. Clara cells are nonciliated cells that secrete surfactant-related proteins. **Alveoli.** Alveoli are surrounded and separated from each other by a thin connective tissue layer that contains numerous blood capillaries. The tissue between adjacent alveolar air spaces is called the alveolar septum. The alveolar epithelium is composed of two types of pneumocytes: type I which line most of the surface of the alveoli, and type II that secrete surfactant and are important for host defense. Alveolar macrophages are present both in the connective tissue of the septum and in the air space of alveoli.

macrophages phagocytose the bacteria, dust particles or other debris and move towards the bronchioles, where phagocytosed materials are eliminated by coughing.

5.2.1. Lipids

The role of lipids in the lungs has not been a subject of extensive study in current literature. Lipids are naturally secreted in the pulmonary surfactant in the lungs by type II pneumocytes and play an important role in the maintenance of airway patency by lowering the surface tension during respiration. Surfactant consists for 90% of lipids (mainly saturated phospholipids) and 10% of protein. It has been demonstrated that deletion of the ATP-binding cassette (ABC) transporter, ATP-binding cassette sub-family G member 1 (ABCG1) in macrophages causes lipid accumulation in the alveolar macrophages.[59] Traditionally, ABCG1 is known for its role in facilitating cellular cholesterol and phospholipid efflux from macrophages to mature HDL. Another ABC transporter, ATP-binding cassette sub-family A member 1 (ABCA1) mediates the efflux of cholesterol and PLs from macrophages. In addition, ABCA1 has been shown to be expressed in the lung and highest expressed in type II pneumocytes,[60] implying a role in surfactant regulation.[61] It controls transport of cholesterol and phospholipids to apoA1 in type II pneumocytes. Defective ABCA1 manifests as Tangier's disease, characterized by severely reduced levels of HDL, and *abc1*$^{-/-}$ mice have pulmonary lesions consisting of foamy type II pneumocytes, lipid-laden alveolar macrophages and cholesterol clefts and display pathophysiologic hallmarks similar to human Tangier's disease.[62] *Abca1*$^{-/-}$/*abcg*$^{-/-}$ mice exhibit extreme lipid accumulation in tissue macrophages of the lung amongst other organs (liver, spleen, Peyer's patches, and lymph nodes).[63] Whether the accumulation of lipid-laden cells in these murine models is also accompanied with increased pulmonary inflammation is likely, but not reported. Together, these data demonstrate that lipids do play an important role in the lungs, which becomes evident when ABC transporters involved in lipid trafficking are affected.

5.2.2. Inflammation

The obstructive lung diseases COPD and asthma are the most frequent causes of respiratory ill health, covering all ages.[64] Both conditions are associated with many comorbidities, including CVD,[65, 66] albeit asthma to a lesser extent than COPD. One of the most obvious reasons for this is that most COPD patients are aging individuals, and therefore are at higher risk to have more comorbidities. It has been proposed that COPD and CVD are entities of the same systemic inflammatory syndrome, of which other diseases, such as Alzheimer's disease, osteoporosis also take part. This syndrome has also been called 'inflamm-aging' by some,[67] with a reduction in adaptive immunity and an increase in innate immunity driven by NF-κB activation.[68] There is abundant evidence of increased systemic inflammation in COPD and CVD, as demonstrated by the presence of activated circulating leukocytes and increased levels of circulating inflammatory mediators.[69] CRP for example, known as a biomarker of systemic inflammation, is not only a marker of increased mortality in COPD,[70] but also a marker of increased cardiovascular risk.[71] Although, it is often hypothesized that inflammation in the systemic compartment is the result of 'spill over' of the inflammatory process locally, in this case from the lungs, evidence from

cross-sectional studies did not point out a correlation between pulmonary and circulatory inflammatory markers in stable COPD.[72] As mentioned before, a poor lung function has been shown to be a strong independent risk factor for CVD-related morbidity and mortality in many epidemiological studies.[2, 65] It is not clear however whether COPD also plays a causative role in this increased risk, or COPD and CVD are part of a 'syndrome' where systemic inflammation is a common denominator.

5.2.3. Lipids and inflammation

In clinical COPD studies, the prevalence of obesity is higher with a lower GOLD stage, *i.e.* less severe COPD, in line with the intriguing finding that a higher body mass index (BMI) (and higher fat-free mass) is associated with a lower mortality rate in COPD patients.[73, 74] On the other hand, obesity can cause low-grade systemic inflammation and as high CRP levels in young healthy adults are associated with a faster decline in lung function, obesity may be a risk factor for COPD. This obesity paradox in COPD can be ascribed to several possible causes. Firstly, the different phenotypes of COPD partly contributed to this paradox. Obesity in COPD is more prevalent in the chronic obstructive phenotype, whereas underweight is more prevalent in the emphysematous phenotype.[75] A reduced respiratory function in COPD impairs physical activity and increases the risk of developing obesity. In addition, there is no plausible reason why adiposity should protect against mortality in COPD.[74] In fact, one study indicates that lean mass index is a better predictor of mortality than BMI in moderate to severe COPD, while fat mass index is not a significant prognostic indicator.[76] Another study demonstrated that excessive visceral fat (independent of total fat mass) contributes to increased plasma IL-6, which, in turn, is strongly associated with all-cause and cardiovascular mortality in patients with obstructive lung disease.[77] These data suggest that the overall physical condition as reflected by a larger lean mass index and/or the fat composition rather than obesity *per se* are important determinants of the severity and inflammatory state of COPD.

Interestingly, feeding *apoe*$^{-/-}$ mice a Western-type diet (high fat 21% and high cholesterol 0.15% content) also induces increased inflammation in the lung.[78] These mice had higher levels of pro-inflammatory cytokines than the control wild-type mice on the same diet. Furthermore, Goldklang *et al*. demonstrated that *apoe*$^{-/-}$ mice fed a Western-type diet (21% fat and 0.21% cholesterol) for 10 weeks compared to chow-fed *apoe*$^{-/-}$ mice exhibited elevated number of macrophages and lymphocytes in the lung, accompanied by an increase in MMP-9 and -12 activity and more importantly, air space enlargement, *i.e.* emphysema, a major feature of COPD.[79] These changes did not occur in *ldlr*$^{-/-}$ mice fed the same diet. In addition, macrophages from the *apoe*$^{-/-}$ mice treated with a TLR4 ligand showed enhanced MMP-9 expression, which was further augmented with the addition of oxidized LDL. These studies provide a possible mechanism linking emphysema and atherosclerosis, where persistent systemic inflammation, from multiple different sources, act through *e.g.* the TLR4 signaling pathway with the resultant inflammatory and protease cascade contributing to the development and disease progression of both COPD and atherosclerosis. Moreover, they support the hypothesis that COPD and CVD may be entities of a shared systemic inflammatory disease, which may be triggered by a Western-type diet.

6. Pharmacological therapies

Among all available anti-atherogenic drugs, *e.g.* fibrates, nicotinic acid, peroxisome proliferator-activated receptor (PPAR)α and -γ agonists, and statins are the most well-studied and proven to be effective in preventing CVD events.[80] Although powerful drugs for atherosclerosis, like statins, are abundantly used in developing countries, CVDs remain to be the leading cause of morbidity and mortality in the Western world. Next to statins, a new multi-target therapeutic agent, resveratrol is discussed below. Resveratrol is a moderate activator of sirtuin (silent mating type information regulation 2 homolog 1, (SIRT1), a nicotinamide adenosine dinucleotide-dependent histone deacetylase with anti-aging and anti-inflammatory properties. The potential benefit of the use of statins and SIRT1 activation has been studied in CVD, but notably, also in COPD.[81, 82]

6.1. Statins

Numerous clinical trials have established that statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, can reduce various atherosclerotic complications.[80] As more than two thirds of the body's total cholesterol is synthesized by the body itself (mainly in the liver) and not consumed through diet, blocking this pathway to decrease serum cholesterol is a logical step to take in an effort to lower cholesterol levels. By blocking HMG-CoA reductase, the rate limiting enzyme in cholesterol synthesis, statins lower the endogenous VLDL production and thereby reduce plasma LDL levels. Some classes of statins also induce an increase in HDL levels by an enhancing apoA1 production up to approximately 15%, primarily in the liver.[83] Statin treatment not only results in a more favorable lipoprotein profile by reducing LDL and increasing HDL levels, but there is evidence that part of the clinical benefit of statins can be ascribed to other effects, *e.g.* anti-inflammatory, antithrombotic, and improvement of endothelial function.[84] Part of the pleiotropic actions of statins is through NF-κB suppression. It has been reported that statins stabilize IκB by inhibiting kinases in monocytes and VSMCs.[85] These off-target beneficial side-effects of statins have expanded the attention of these drugs to non-cardiovascular fields. Simvastatin, for instance, has been demonstrated to be able to reverse or inhibit pulmonary emphysema, a common manifestation of COPD in animal models.[86, 87] Trials are being undertaken to evaluate the potential benefit of statins on morbidity and mortality in COPD.[81] Given that the majority of COPD patients die due to a cardiovascular event, it is also important, but difficult to delineate the beneficial effects of statins on COPD independently from their favorable effects on the cardiovascular system.

6.2. Resveratrol

NF-κB appears to be a tempting therapeutic target, because it is a point of intersection of multiple pathways in atherogenesis. A number of compounds, especially anti-oxidants, proteasome- and IKK inhibitors, have been shown to suppress NF-κB. NF-κB modulation is often part of the pleiotropic actions from pharmaceutical drugs, *e.g.* glucocorticoids and statins.[88, 89] In addition, NF-κB inhibiting compounds have been present in our diet from ancient times, such as curcumin,[90] and resveratrol, a polyphenol found in red wine.[91] Resveratrol is primarily

present in the skins of grapes and thus in red wine. In addition, resveratrol is present in Chinese herbs, peanuts and a large variety of fruits including various berries and jackfruit.

The mechanism of action of resveratrol is not completely understood. As mentioned before, resveratrol is also a moderate activator of SIRT1, which has been shown to physically interact with the p65 subunit of NF-κB and to inhibit its activity by deacetylating p65 at lysine 310.[92] Resveratrol has been shown to potentiate the SIRT1-mediated anti-inflammatory response.[93] Atheroprotective effects of resveratrol are numerous, such as suppression of IL-6 and M-CSF secretion,[94, 95] prevention of oxidation of LDL and uptake into the vascular wall,[96] lowering of blood pressure and glucose levels.[97] Similar to statins, resveratrol is reported to have many pleiotropic effects. Thus, resveratrol is considered a promising candidate for the new generation of anti-atherosclerotic drugs, and as mentioned previously, its therapeutic possibilities are currently also being explored in lung diseases. Whether resveratrol can beat the current leading anti-atherogenic drug statin, is a challenging question which remains to be answered.

7. Outline of thesis

Increased inflammation is a main contributor to atherogenesis. Inflammatory processes within the vessel wall are studied extensively. Less is known about the effects of more distal organs. The studies in this thesis focus on the role of two organs, *i.e.* the liver and lungs, in atherosclerosis development.

The liver is an important organ in the regulation of both lipid metabolism and inflammation, the two key role players in atherosclerosis. In **chapter 2**, we studied the interaction between liver inflammation and lipid metabolism. We investigated whether hepatocyte-specific NF-κB activation by transgenic expression of IKKβ affected VLDL production in *E3L* mice. Increased levels of VLDL are an important risk factor for CVD. So, it is reasonable that any effect of hepatocyte-specific NF-κB activation on lipid metabolism found in chapter 2 would influence atherosclerosis development. By using the same transgenic mouse model the role of transgenic NF-κB activation in hepatocytes on both lipid metabolism and inflammation, with atherosclerosis development as primary outcome was examined in **chapter 3**.

Apart from the classical risk factors such as dyslipidemia and inflammation, poor lung function, most commonly caused by COPD, has been identified as another predictor for CVD.[98] Pulmonary emphysema is a major component of COPD and is characterized by loss of alveolar integrity leading to an alveolar space enlargement. We studied whether alveolar destruction *per se* would have an effect on atherosclerosis development in **chapter 4**.

New therapies are continuously being developed to reduce evolving and existing CVD. One of the most promising therapeutic components which currently receives a lot of attention, not only in the field of CVD, is resveratrol. In **chapter 5** we evaluated the potential anti-atherogenic capacity of resveratrol alone or combined with atorvastatin.

Finally, the major findings of the studies described in this thesis, the clinical implications and the future perspectives are discussed in **chapter 6**.

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Hepatocyte-Specific IKKβ Activation Enhances VLDL-Triglyceride Production in *APOE*3-Leiden* Mice

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Abstract

Low-grade inflammation in different tissues, including activation of the nuclear factor-κB (NF-κB) pathway in liver, is involved in metabolic disorders such as type 2 diabetes and cardiovascular diseases (CVD). In this study we investigated the relation between chronic hepatocyte-specific overexpression of IKKβ and hypertriglyceridemia, an important risk factor for CVD, by evaluating whether activation of IKKβ only in the hepatocyte affects VLDL-triglyceride (TG) metabolism directly. Transgenic overexpression of constitutively active human IkB kinase (IKKβ) specifically in hepatocytes of hyperlipidemic *APOE*3-Leiden* mice clearly induced hypertriglyceridemia. Mechanistic *in vivo* studies revealed that the hypertriglyceridemia was caused by increased hepatic VLDL-TG production, rather than a change in plasma VLDL-TG clearance. Studies in primary hepatocytes showed that IKKβ overexpression also enhances TG secretion *in vitro*, indicating a direct relation between IKKβ activation and TG production within the hepatocyte. Hepatic lipid analysis and hepatic gene expression analysis of pathways involved in lipid metabolism suggested that hepatocyte-specific IKKβ overexpression increases VLDL production not by increased steatosis or decreased FA oxidation, but most likely by ChREBP-mediated upregulation of *Fas* expression. These findings implicate that specific activation of inflammatory pathways exclusively within hepatocytes induces hypertriglyceridemia. Furthermore, we identify the hepatocytic IKKβ pathway as a possible target to treat hypertriglyceridemia.

Introduction

Obesity is associated with diseases such as dyslipidemia, type 2 diabetes and cardiovascular disease (CVD). The accumulation of lipids in numerous tissues is accompanied by increased inflammatory processes, such as macrophage infiltration and production of inflammatory mediators in white adipose tissue. In liver, fat accumulation increases the activity of the pro-inflammatory nuclear factor-κB (NF-κB), and liver-specific activation of NF-κB induces metabolic disturbances.[1,2]

Hypertriglyceridemia is caused by accumulation of VLDL particles in the plasma as a consequence of changes in lipid metabolism that are associated with obesity. Pro-inflammatory cytokines can cause hypertriglyceridemia[3] and, conversely, suppression of inflammation may reduce hypertriglyceridemia[4] suggesting a direct causal role for inflammatory pathways in the development of hypertriglyceridemia. In fact, administration of lipopolysaccharide (LPS), an inflammatory component of the outer membrane of Gram-negative bacteria, increases plasma triglyceride (TG) levels.[5] However, many inflammatory mediators affect multiple tissues, such as muscle, adipose tissue and liver and, moreover, they can act on multiple cell types including macrophages. The specific contribution of hepatocytes in the relation between inflammation and TG metabolism has never been studied.

In the current study we, therefore, aimed to investigate whether activation of the inflammatory NF-κB pathway exclusively in hepatocytes affects VLDL-TG metabolism and, as a consequence, causes hypertriglyceridemia. To this end, we used hepatocyte-specific transgenic IKKβ (*LIKK*) mice, which have been described before.[1] *LIKK* mice have an albumin promoter to drive expression of constitutively active human IκB kinase β (IKKβ), which activates the NF-κB pathway selectively in hepatocytes. To study the effects of the hepatocyte-specific inflammation on VLDL-TG metabolism, we crossbred the *LIKK* mouse with the transgenic *APOE*3-Leiden* (*E3L*) mouse that expresses human *APOE*3-Leiden* (a mutant form of APOE3) and human *APOC1*,[6] both of which attenuate the clearance of apoE-containing TG-rich lipoproteins. Therefore, the *E3L* mouse shows increased plasma TG and cholesterol levels and is a well-established model of human-like lipoprotein metabolism.[7] By using the *E3L.LIKK* mouse, we were able to study the effects of the inflammatory NF-κB pathway in the hepatocyte on TG-rich lipoprotein metabolism directly. Our results show that activation of NF-κB in hepatocytes of *E3L* mice induces hypertriglyceridemia by enhancing VLDL-TG production directly within hepatocytes.

Materials and methods

Animals

LIKK mice, which express constitutively active human IKKβ selectively in hepatocytes under control of the albumin promoter[1] were crossbred with *E3L* mice,[6] expressing both human *APOE*3-Leiden* and human *APOC1*, in our animal facility to obtain heterozygous *E3L.LIKK* mice on a C57Bl/6J background. Male *E3L.LIKK* and *E3L* littermates were housed under standard conditions with a 12-hour light-dark cycle and were fed a standard mouse chow diet with free

access to water. Experiments were performed in 14-week old animals after an overnight fast. All experiments were approved by the institutional ethical committee on animal care and experimentation.

Western blot analysis

Tissues were homogenized by Ultraturrax (22,000 rpm; 2x5 sec) in an ice-cold buffer (pH 7.4) containing 30 mM Tris.HCl, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 0.5% (v/v) Triton X-100, 1% (v/v) SDS and protease inhibitors (Complete, Roche, Mijdrecht, The Netherlands) at a 1:6 (w/v) ratio. Homogenates were centrifuged (16,000 rpm; 15 min, 4°C) and the protein content of the supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL). Proteins (20-50 μg) were separated by 7-10% SDS-PAGE followed by transfer to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked for 1 h at room temperature in Tris-buffered saline with Tween-20 (TBST) with 5% non-fat dry milk followed by an overnight incubation with the following antibodies: p-Ser536 NF-κB p65 (#3031), NF-κB p65 (#3034), p-Ser32/36 IκBα (#9246), IκBα (#9242) (all from Cell Signaling), MTP (#612022) (BD Biosciences, Erembodegem, Belgium) and DGAT1 (#54037) (Abcam, Cambridge, UK). Blots were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence (ECL) and quantified using Image J (NIH).

Plasma lipids and lipoprotein profiles

Blood was collected from the tail vein into chilled paraoxon (Sigma, St Louis, MO)-coated capillaries to prevent ongoing lipolysis.[8] Capillaries were placed on ice, centrifuged and plasma was assayed for TG, total cholesterol (TC), and phospholipids (PL) using commercially available enzymatic kits from Roche Molecular Biochemicals (Indianapolis, IN). Free fatty acids (FFA) were measured using NEFA-C kit from Wako Diagnostics (Instruchemie, Delfzijl, The Netherlands). For the determination of lipid distribution over plasma lipoproteins, 50 μL of pooled plasma was used for fast performance liquid chromatography (FPLC). Plasma was injected onto a Superose 6 column (Äkta System; Amersham Pharmacia Biotech, Piscataway, NJ), and eluted at a constant flow rate of 50 μL/min with PBS pH 7.4. TG and TC were measured as described above in collected fractions of 50 μL.

Liver lipids

Lipids were extracted from livers according to a modified protocol from Bligh and Dyer.[9] Briefly, a small piece of liver was homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1800 μL CH_3OH:$CHCl_3$ (3:1 v/v) to 45 μL homogenate. The $CHCl_3$ phase was dried and dissolved in 2% Triton X-100. Hepatic TG and TC concentrations were measured using commercial kits as described earlier. Liver lipids were expressed per mg protein, which was determined using the BCA protein assay kit.

FA composition of liver TG

Liver samples (100 mg) were homogenized with 0.5 mL saline. Subsequently, 5 mL of chloroform: methanol (2: 1 by volume) was added containing butylated hydroxytoluene (BHT).

In addition, an internal TG standard was added before extraction. Liver lipids were extracted according to the method of Folch *et al.*[10] Total TG were separated by spotting lipid extracts onto silica gel 60 (Merck) thin-layer chromatography plates and running in hexane: diethyl ether: acetic acid (85: 15: 1, v/v/v). Lipid bands were visualized under UV light after spraying with 0.1% ANS (8-anilino-1-naphthalene sulfonic acid), and identified using commercial standards. TG bands were scraped into glass tubes and methylated at 80°C with 1.5% H_2SO_4 in methanol for 2 h. TG-derived FA were eluted into hexane. Separation and quantification of the FA methyl esters (FAMEs) from liver TG was achieved using gas chromatography, on an Agilent 6890 GC (Agilent Technologies, UK) fitted with a 30 m x 0.53 mm (film thickness 1 μm) capillary column (RTX-Wax). Individual FA peaks were identified by a reference containing known FAMEs. FA compositions (mol%) were then determined.

Generation of VLDL-like emulsion particles

VLDL-like TG-rich emulsion particles were prepared and characterized as described previously.[11,12] Lipids (100 mg) at a weight ratio of triolein: egg yolk phosphatidylcholine: lysophosphatidylcholine: cholesteryl oleate: cholesterol of 70: 22.7: 2.3: 3.0: 2.0, supplemented with 200 μCi of glycerol tri[9,10(n)-^{3}H]oleate ([^{3}H]TO) were sonicated at 10 μm output using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK). Density gradient ultracentrifugation was used to obtain 80 nm-sized emulsion particles, which were used for subsequent experiments. TG content of the emulsions was measured as described above. Emulsions were stored at 4°C under argon and used within 7 days.

In vivo clearance of VLDL-like emulsion particles

To study the *in vivo* clearance of the VLDL-like emulsion particles, overnight fasted mice were anesthetized by intraperitoneal injection of acepromazine (6.25 mg/kg Neurotranq, Alfasan International BV, Weesp, The Netherlands), midazolam (6.25 mg/kg Dormicum, Roche Diagnostics, Mijdrecht, The Netherlands), and fentanyl (0.31 mg/kg Janssen Pharmaceuticals, Tilburg, The Netherlands). Mice were injected (t=0) via the tail vein with 200 μL of [^{3}H]TO-labeled emulsion particles at a dose of 100 μg of TG per mouse. Blood samples were taken from the tail vein at 1, 2, 5, 10 and 15 minutes after injection and plasma ^{3}H-activity was counted. Plasma volumes were calculated as 0.04706 x body weight (g) as determined from ^{125}I-BSA clearance studies as described previously.[13] After taking the last blood sample, the liver, heart, spleen, muscle and white adipose tissue (*i.e.* gonadal, subcutaneous and visceral) were collected. Organs were dissolved overnight at 60°C in Tissue Solubilizer (Amersham Biosciences, Roosendaal, The Netherlands) and ^{3}H-activity was counted. Uptake of [^{3}H]TO-derived radioactivity by the organs was calculated from the ^{3}H activity in each organ divided by plasma-specific activity of [^{3}H]TG and expressed per mg wet tissue weight.

In vivo hepatic VLDL-TG and VLDL-apoB production

To measure VLDL production *in vivo*, mice were fasted overnight and anesthetized as described above. Mice were injected intravenously with Tran^{35}S label (150 μCi/mouse; MP Biomedicals, Eindhoven, The Netherlands) to label newly produced apolipoprotein B (apoB). After 30 minutes,

at t=0 min, Triton WR-1339 (Sigma-Aldrich) was injected intravenously (0.5 mg/g body weight, 10% solution in PBS) to block serum VLDL clearance. Blood samples were drawn before (t=0) and 15, 30, 60 and 90 min after injection and used for determination of plasma TG concentration as described above. After 120 min, mice were exsanguinated via the retro-orbital plexus and euthanized by cervical dislocation. VLDL was isolated from serum after density gradient ultracentrifugation at d<1.006 g/mL by aspiration[14] and counted for incorporated ^{35}S-activity.

Isolation of primary mouse hepatocytes

Primary hepatocytes were isolated from mouse livers according to the method of Berry and Friend[15] modified by Groen *et al.*[16] Briefly, the portal vein was cannulated and liver was first perfused with a calcium-free Krebs/bicarbonate buffer, saturated with 95% O_2 and 5% CO_2 at a flow rate of 5 mL/min. Subsequently, perfusion of the liver was continued with calcium-containing Krebs/bicarbonate buffer with 0.0125% collagenase (Roche, Penzberg, Germany) during 10-15 min until cellular dissociation was observed. Cells were gently released and centrifuged four times at 50g for 1 min at 4°C to remove non-parenchymal cells from pelleted hepatocytes. Isolated hepatocytes were washed and suspended in complete Williams' E medium containing insulin (Actrapid), fetal calf serum, dexamethasone and penicillin/streptomycin (P/S). Hepatocytes were isolated with similar yields from livers of *E3L.LIKK* and *E3L* mice, 70-80% viable, as assessed by trypan blue dye exclusion, and 99% free of non-parenchymal cells by visual inspection. No differences with respect to viability were observed between cells isolated from *E3L.LIKK* and *E3L* mice. Cells were seeded into 12-well dishes, pre-coated with collagen at a density of 1.0x106 viable cells/well in 2 mL complete Williams' E medium. After a 2 h adherence period, non-attached cells were removed from the cultures by careful washing.

In vitro measurement of TG secretion by hepatocytes

TG secretion *in vitro* was measured as described previously.[17] After an overnight incubation, cells were washed 2 times and incubated 4 h in fetal calf serum-free and hormone-free (SF-HF) Williams' E medium. To measure rates of secretion of TG, cells were subsequently incubated in SF-HF medium containing 4.4 µCi of [^{3}H]glycerol (Amersham; UK) with or without 0.75 mM oleate (C18:1) complexed with BSA to stimulate lipogenesis. After 1, 2, 4 or 20 h incubation, medium was collected and cells were washed three times and harvested in 2 mL PBS. Lipids were extracted from medium according to a modified protocol from Bligh and Dyer.[9,17] The lipids were dried under nitrogen, dissolved into chloroform with 2 mM tripalmitin added as a carrier and subjected to TLC (Silica gel 60, Merck, Belgium) using hexane: diethylether: acetic acid (80/20/1; v/v/v) as mobile phase. Lipid spots were visualized using iodine vapor, and tripalmitin-positive spots were scraped off, dissolved in 0.5 M acetic acid, and assayed for radioactivity by scintillation counting. Protein content of the cells was determined using the BCA protein assay kit as described earlier. Data are expressed as dpm/mg protein.

Hepatic gene expression analysis

Total RNA was extracted from liver tissues using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. RNA quality of each sample was examined by lab-on-a-chip technology using Experion Std Sens analysis kit (Biorad, Hercules,

CA, USA). One µg of total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad) and the obtained cDNA was purified with Nucleospin Extract II kit (Macherey-Nagel). Real-Time PCR was carried out on the IQ5 PCR machine (Biorad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of cyclophilin (*Cyclo*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Primer sequences are listed in Supplemental Table S1.

Statistical analysis

Data are presented as means ± SD. Statistical differences were calculated using the Mann-Whitney *U* test for two independent samples with SPSS 16.0 (SPSS Inc, Chicago, IL). $P<0.05$ was regarded statistically significant.

Results

LIKK increases liver NF-κB signaling in *E3L* mice

To verify that *LIKK* expression in *E3L* mice increases hepatic NF-κB signaling, livers from *E3L* and *E3L.LIKK* mice were assayed for the presence of phosphorylated over total NF-κB and IκBα using Western blot (Fig. 1). Indeed, expression of *LIKK* increased the ratio of pNF-κB Ser^{536} over NF-κB (1.6 ± 0.4 fold; $P<0.05$) (Fig. 1A, B) as well as that of pIκBα $Ser^{32/36}$ over IκBα (1.9±0.6 fold; $P<0.05$)

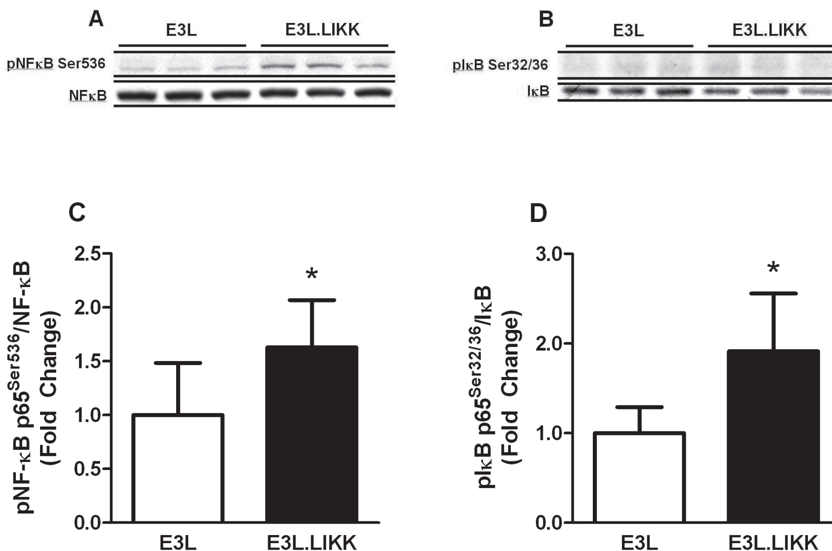

Fig. 1. *LIKK* increases hepatic NF-κB signaling in *E3L* mice. *E3L* and *E3L.LIKK* mice were fed a chow diet and sacrificed at the age of 14 weeks after an overnight fast. NF-κB signaling was measured in liver tissue by phosphorylation of NF-κB (A,C) and IκB (B,D). Representative Western blots of phosphorylated NF-κB (NF-κB Ser^{536}) and total NF-κB (A) and phosphorylated IκBα (pIκBα $Ser^{32/36}$) and total IκBα (B) are shown for 3 mice per group. Ratios of phosphorylated proteins over total proteins were quantified (B,D). Values are means ± SD (n=5-7). *$P<0.05$.

(Fig. 1C, D). The increased ratio of pIκBα Ser[32/36] over IκBα was mainly caused by a decrease of total IκBα (0.8±0.1 fold; $P<0.05$), indicating increased IκBα ubiquitination and degradation by the proteasome, which reflects activation of the NF-κB pathway. These data are in line with the increased NF-κB signaling previously observed in *LIKK* mice as compared to wild-type (WT) mice.[1]

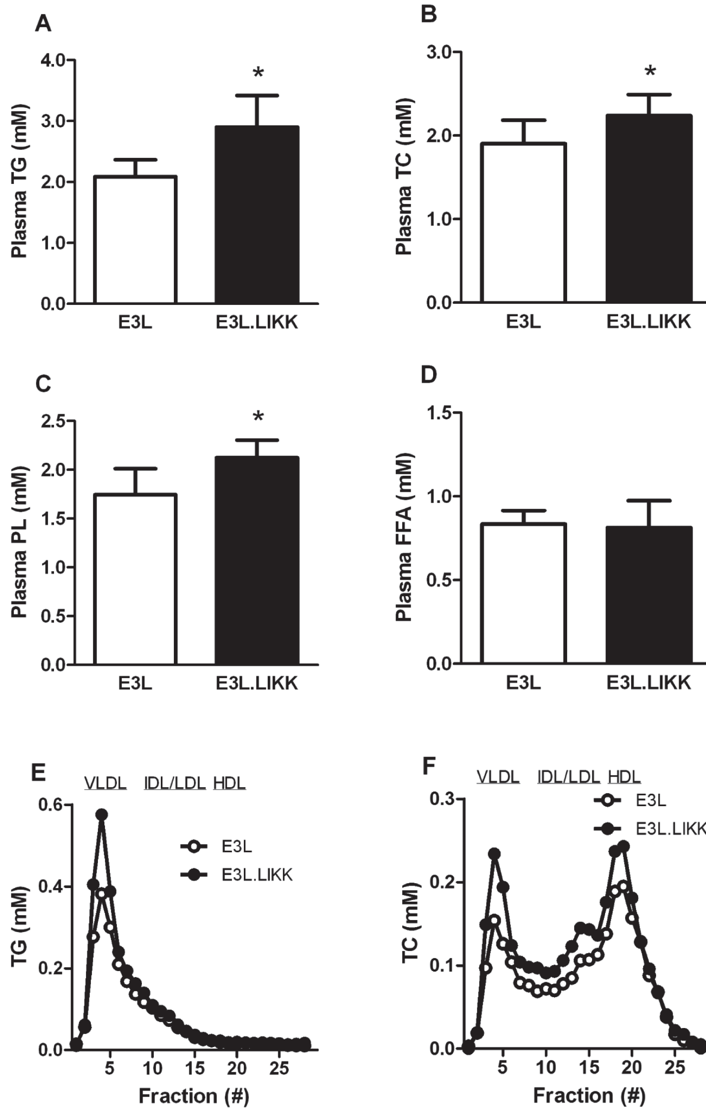

Fig. 2. *LIKK* induces hyperlipidemia in *E3L* mice. Plasma triglycerides (TG) (A), total cholesterol (TC) (B), phospholipids (PL) (C) and free fatty acid (FFA) (D) levels were measured in plasma of overnight fasted *E3L* and *E3L.LIKK* mice. Values are means ± SD (n=5-7). *$P<0.05$. Plasma was collected, pooled per group, and subjected to FPLC to separate lipoproteins. Distribution of TG (E) and TC (F) over lipoproteins was determined.

LIKK induces hypertriglyceridemia in *E3L* mice

To determine whether the hepatocyte-specific inflammation affects plasma lipid levels, TG, TC, PL and FFA levels were measured in plasma of *E3L* and *E3L.LIKK* mice (Fig. 2). *LIKK* expression in *E3L* mice increased TG by +39% (2.90±0.52 vs 2.09±0.28 mmol/L; $P<0.05$; Fig. 2A), TC by +18% (2.24±0.25 vs 1.90±0.28 mmol/L; $P<0.05$; Fig. 2B), and PL by +22% (2.12±0.18 vs 1.74±0.27 mmol/L; $P<0.05$; Fig. 2C). *LIKK* did not affect plasma FFA levels (Fig. 2D). Lipoprotein profiling showed that the *LIKK*-induced increase in plasma TG could be explained by a rise in VLDL-TG (+42%) (Fig. 2E). Likewise, the increase in TC was mainly reflected by an increase in VLDL-C (+54%), LDL-C (+34%) and HDL-C (+25%) (Fig. 2F).

LIKK does not affect clearance of VLDL-like emulsion particle-TG in *E3L* mice

Hypertriglyceridemia is caused by a decrease in VLDL-TG clearance and/or an increase in hepatic VLDL-TG production. To investigate whether *LIKK* inhibits the clearance of VLDL-TG, the plasma clearance and organ distribution of [^{3}H]TO-labeled TG-rich VLDL-like emulsion particles was evaluated in *E3L.LIKK* versus *E3L* mice (Fig. 3). *LIKK* did not affect the plasma half-life of [^{3}H]TO (Fig. 3A), nor the uptake of [^{3}H]TO-derived fatty acids (FA) by the various organs (Fig. 3B), indicating that *LIKK* does not increase plasma TG levels by decreasing TG clearance.

LIKK increases VLDL-TG production in *E3L* mice

As no difference was observed in TG clearance between *E3L.LIKK* and *E3L* mice, it is likely that the *LIKK*-induced increase in plasma TG levels can be explained by an increase of VLDL-TG production. The rate of hepatic VLDL-TG production was measured by determining plasma TG levels after intravenous Triton WR1339 injection (Fig. 4). Indeed, *LIKK* strongly increased the accumulation of plasma TG at all time points (Fig. 4A). The VLDL-TG production rate,

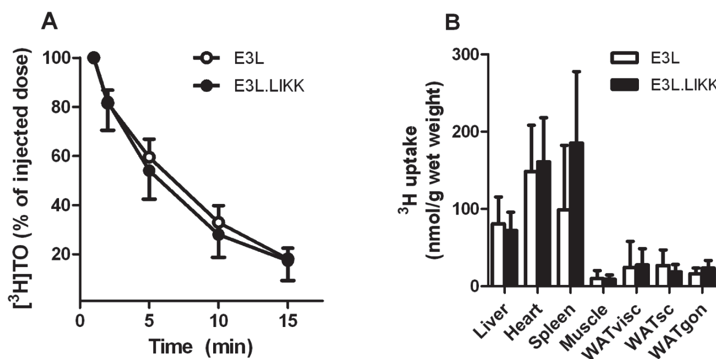

Fig. 3. *LIKK* does not affect clearance of VLDL-like emulsion particle-TG in *E3L* mice. *E3L* and *E3L.LIKK* mice that were fasted overnight were injected with [3H]TO-labeled VLDL-like emulsion particles. Blood was collected at the indicated time points and radioactivity was measured in plasma (A) of *E3L* mice (open circles) and *E3L.LIKK* mice (closed circles). Uptake of [3H]TO-derived activity by various organs was determined, and total FA uptake was calculated from the specific activity of TG in plasma, and expressed as nmol FA per mg wet tissue weight (B). Values are means ± SD (n=8). WAT, white adipose tissue; visc, visceral; sc, subcutaneous; gon, gonadal.

as determined from the slope of the curve from all individual mice, was increased by +48% (3.90 ± 1.01 vs 2.64 ± 0.82 mM/h, *P*<0.05) (Fig. 4B), whereas the rate of VLDL-apoB production did not change significantly (*P*=0.52) (Fig. 4C). Since each VLDL particle contains a single apoB molecule, *LIKK* apparently increases plasma TG levels by enhancing VLDL-TG production without affecting VLDL particle production.

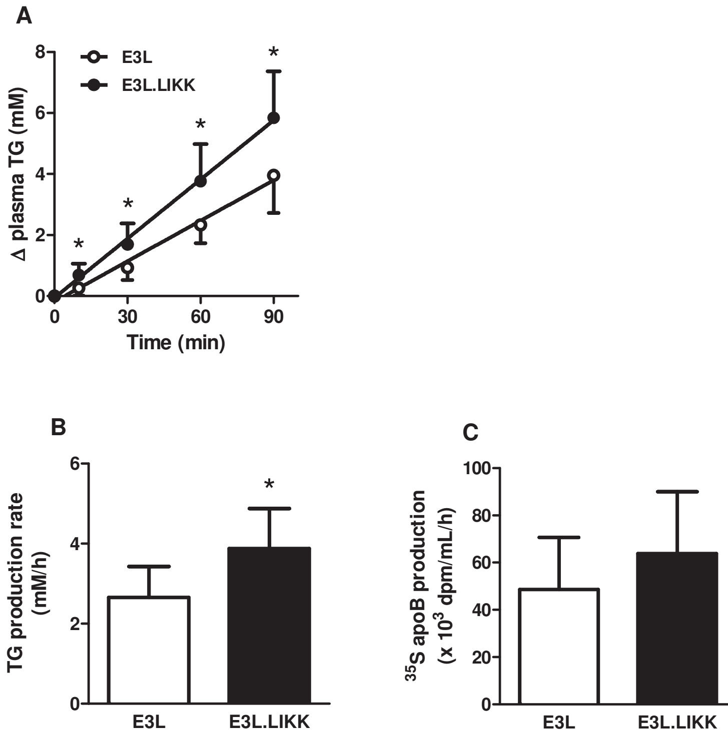

Fig. 4. *LIKK* increases VLDL-TG production in *E3L* mice. *E3L* and *E3L.LIKK* mice were fasted overnight and injected with Trans35S (t=-30 min) and Triton WR1339 (t=0) and blood samples were drawn at the indicated time points. TG concentrations were determined in plasma of *E3L* mice (open circles) and *E3L.LIKK* mice (closed circles), and plotted as the increase in plasma TG relative to t=0 (A). The rate of TG production was calculated from the slopes of the curves from the individual mice (B). After 120 min, VLDL was isolated by ultracentrifugation, 35S-activity was counted, and the production rate of newly synthesized VLDL-35S-apoB was determined (C). Values are means ± SD (n=5-8). *P<0.05.

LIKK does not affect liver lipid levels

To investigate whether the increase in hepatic VLDL-TG production was the result of increased lipid substrate availability in the liver, the effect of *LIKK* on the hepatic lipid content was investigated (Fig. 5). However, *E3L* and *E3L.LIKK* mice did not differ with respect to liver TG levels (Fig. 5A) and TC levels (Fig. 5B). *LIKK* did not influence the FA composition of hepatic TG, apart from a mild increase in the relative abundance of linoleic acid (18:2 n-6) by +19% (*P*<0.05) (Suppl. Fig. S1).

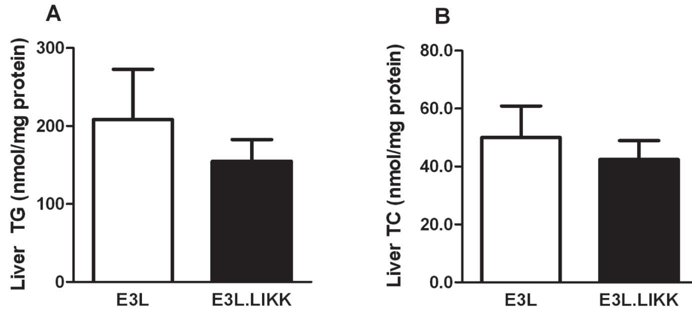

Fig. 5. *LIKK* does not affect liver lipid content in *E3L* mice. Livers were obtained from overnight fasted *E3L* and *E3L.LIKK* mice and lipids were extracted. Triglycerides (TG, A) and total cholesterol (TC, B) concentrations were measured and expressed per mg protein. Values are means ± SD (n=5-7). **P*<0.05.

LIKK directly increases TG secretion in hepatocytes from *E3L* mice

To evaluate whether IKKß overexpression in hepatocytes directly increases VLDL-TG production, we next studied TG secretion from isolated hepatocytes of *E3L* and *E3L.LIKK* mice *in vitro*. We used [^{3}H]glycerol as precursor for TG synthesis, by measuring the accumulation of [^{3}H]TG in the medium (Fig. 6). In the absence of oleate, the [^{3}H]TG secretion was low, but *LIKK* significantly increased the [^{3}H]TG secretion after 20 h of incubation as compared to the [^{3}H]TG secretion from control *E3L* hepatocytes (2.3-fold; *P*<0.05) (Fig. 6A). In the presence of oleate, as a substrate for TG synthesis, [^{3}H]TG secretion was markedly increased, and *LIKK* caused an additional increase in [^{3}H]TG secretion, reaching significance after 20 h of incubation (1.9-fold at 20 h; *P*<0.05) (Fig. 6B).

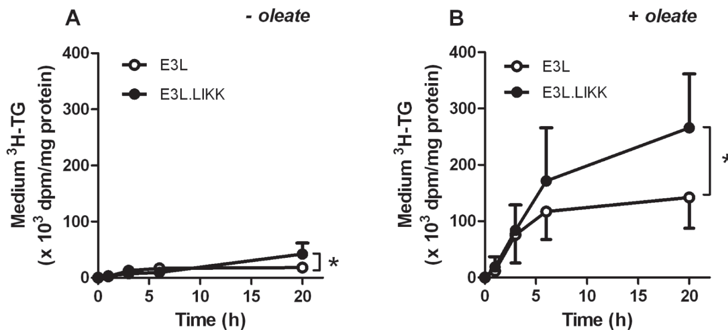

Fig. 6. *LIKK* increases TG secretion in hepatocytes from *E3L* mice. Hepatocytes were isolated from *E3L* (open circles) and *E3L.LIKK* mice (closed circles), cultured overnight, and incubated without or with oleate complexed with bovine serum albumin. [3H]Glycerol was added to quantify newly synthesized triacylglycerols. Medium was collected at the indicated time points, [3H]TG was measured and expressed as dpm per mg cell protein. Values are means ± SD of 3-6 mice per group, *in vitro* experiments were performed in triplicate (n=3-6). **P*<0.05.

LIKK increases hepatic expression of fatty acid synthase, but does not affect protein levels or expression of genes involved in VLDL production

To obtain further insight into the mechanism underlying the effects of IKKß overexpression on VLDL-TG production, we evaluated the hepatic expression of genes involved in VLDL secretion, lipogenesis, FA oxidation, cholesterol metabolism, bile acid metabolism, lipid droplets and

Table 1. Effect of *LIKK* on hepatic expression of genes involved in lipid metabolism in *E3L* mice.

Gene	Protein	*E3L*	*E3L.LIKK*	Change
VLDL secretion				
ApoB	ApoB	1.00 ± 0.35	1.01 ± 0.34	n.s.
Mttp	MTP	1.00 ± 0.46	1.34 ± 0.36	n.s.
Lipogenesis				
Srebp-1c	SREBP1c	1.00 ± 0.39	1.10 ± 0.74	n.s.
Dgat1	DGAT1	1.00 ± 0.32	1.21 ± 0.41	n.s.
Fas	FAS	1.00 ± 0.52	2.43 ± 1.07**	+143%
FA oxidation				
Acox1	ACO	1.00 ± 0.70	1.13 ± 0.20	n.s.
Cpt1a	CPT1a	1.00 ± 0.58	0.95 ± 023	n.s.
Glucose metabolism				
Pklr	L-PK	1.00 ± 0.48	1.72 ± 0.80*	+72%
Cholesterol metabolism				
Abcg5	ABCG5	1.00 ± 0.22	1.05 ± 0.31	n.s.
Abcg8	ABCG6	1.00 ± 0.16	0.93 ± 0.16	n.s.
Hmgcr	HMG-CoA	1.00 ± 0.19	0.98 ± 0.08	n.s.
Bile acid metabolism				
Cyp7a1	CYP7A1	1.00 ± 0.59	1.19 ± 0.55	n.s.
Cyp8b1	CYP8B1	1.00 ± 0.60	1.21 ± 0.27	n.s.
Cyp27a1	CYP27A1	1.00 ± 0.41	1.85 ± 0.51**	+85%
Lipid droplets				
Plin2	PLIN2/ADRP	1.00 ± 0.98	1.04 ± 0.33	n.s.
Plin5	PLIN5/PAT-1	1.00 ± 0.72	1.64 ± 0.55*	+64%
Cidec	CIDE-3/FSP27	1.00 ± 0.98	1.05 ± 0.42	n.s.
Cidea	CIDEA	1.00 ± 0.65	0.85 ± 0.59	n.s.
Transcription factors				
Ppara	PPARα	1.00 ± 0.28	0.89 ± 0.16	n.s.
Ppargc1b	PGC-1β	1.00 ± 0.63	0.77 ± 0.26	n.s.
Nr1h3	LXRα	1.00 ± 0.35	1.09 ± 0.09	n.s.
Nr1h4	FXR	1.00 ± 0.50	1.58 ± 0.43*	+58%

Livers were isolated from overnight fasted *E3L* and *E3L.LIKK* mice. mRNA was isolated and mRNA expression of the indicated genes was quantified by RT-PCR. Data are calculated as fold difference as compared to the control group. Values are means ± SD (n=8). **P*<0.05 and ***P*<0.01 compared to the control group. n.s., not significant. *Abcg5*, ATP-binding cassette sub-family G member 5 ; *Abcg8*, ATP-binding cassette sub-family G member 8; *Acox1*, acyl-coenzyme A oxidase 1; *ApoB*, apolipoprotein B; *Cidea*, cell death activator CIDE-A; *Cidec*, fat-specific protein FSP27; *Cpt1a*, carnitine palmitoyltransferase 1a; *Cyp27a1*, cholesterol 27 hydroxylase; *Cyp7a1*, cholesterol 7 alpha hydroxylase; *Cyp8b1*, sterol 12 alpha-hydrolase; *Dgat1*, diglyceride acyltransferase 1; *Fas*, fatty acid synthase; *Hmgcr*, HMG-CoA reductase; *Mttp*, microsomal triglyceride transfer protein; *Nr1h3*, liver X receptor alpha; *Nr1h4*, farnesoid X activated receptor; *Pklr*, liver-type pyruvate kinase; *Plin2*, perilipin 2; *Plin5*, perilipin 5; *Ppara*, peroxisome proliferator activated receptor alpha; *Ppargc1b*, PPAR-gamma coactivator 1-beta; *Srebp-1c*, sterol-regulatory element binding protein.

nuclear receptors in livers of *E3L* and *E3L.LIKK* mice (Table 1). Even though *LIKK* induced an increase of VLDL-TG production *in vivo* and *in vitro*, *LIKK* did not affect hepatic gene expression or protein level (Suppl. Fig. S2A,B) of microsomal TG transfer protein (*Mttp*), which is involved in the assembly and secretion of VLDL. In addition, *LIKK* did not affect apoB (*ApoB*) expression, in line with the observation that *LIKK* did not increase VLDL-apoB secretion *in vivo*. Also, *LIKK* did not affect expression of sterol regulatory element binding protein 1c (*Srebp-1c*), which regulates genes required for *de novo* lipogenesis, nor did it affect expression or protein levels (Suppl. Fig. S2A,C) of acyl:diacylglycerol transferase 1 (*Dgat1*), which catalyzes the final and only committed step in TG synthesis.

In addition, *LIKK* did not largely affect clusters of genes involved in FA oxidation (acyl-coenzyme A oxidase 1 (*Acox1)* and carnitine palmitoyltransferase 1a *(Cpt1a*), cholesterol metabolism (ATP-binding cassette sub-family G member 5 (*Abcg5),* ATP-binding cassette sub-family G member 8 (*Abcg8) and* HMG-CoA reductase *(Hmgcr*) or bile acid metabolism (cholesterol 7 alpha hydroxylase *(Cyp7a1) and* sterol 12 alpha-hydrolase *(Cyp8b1*)), apart from a 1.9-fold increase in cholesterol 27 hydroxylase *(Cyp27a1)* expression. Additionally, *LIKK* did not affect clusters of genes involved in lipid droplet formation (perilipin 2 (*Plin2),* fat-specific protein FSP27 (*Cidec) and* cell death activator CIDE-A *(Cidea*)),or expression of nuclear receptors (peroxisome proliferator activated receptor alpha *(Ppara),* PPAR-gamma coactivator 1-beta *(Ppargc1) and* liver X receptor alpha *(Nr1h3)*), apart from a 1.6-fold increase in expression of perilipin 5 (*Plin5)* and farnesoid X activated receptor *(Nr1h4)* respectively.

However, *LIKK* did increase expression of FA synthase (*Fas*), which plays a key role in FA synthesis, by 2.4-fold, and of liver-type pyruvate kinase (Pklr) by 1.7-fold, both of which are target genes of ChREBP. Taken together, these data suggest that *LIKK* increases VLDL-TG production by ChREBP-mediated upregulation of Fas expression, suggesting an increase in *de novo* lipogenesis.

Discussion

Obesity leads to an increase in inflammatory processes in numerous organs including the liver.[18] In the current study, we questioned whether increased activation of inflammatory pathways in the liver, specifically in hepatocytes, induces hypertriglyceridemia. Indeed, we show that chronic activation of the inflammatory NF-κB pathway specifically in hepatocytes increases plasma TG, which was caused by an increased VLDL-TG production rather than a decreased clearance of VLDL-TG. Furthermore, we provide evidence that the increased TG production induced by hepatocyte-specific IKKβ overexpression is a direct effect of the transgene expression in the hepatocyte.

The strong relation between inflammation and hypertriglyceridemia has largely been derived from the observed increase in plasma TG during acute infection, which is believed to contribute to the host defense.[19] However, although similar inflammatory pathways are involved, metabolic inflammation is clearly different from acute inflammation with respect to its cause, intensity and duration. The inflammation that is observed in obesity, is a chronic and low-grade inflammation that is caused by a metabolic overload, rather than a pathogen.[20] The NF-κB

activity in the liver of *E3L.LIKK* mice in this study is about 1.5-fold higher compared to control *E3L* mice, which is similar to hepatic NF-κB activation levels seen after HFD feeding and in obesity.[1] The present study shows that this low-grade activation of hepatocyte-specific IKKβ induces an increase in plasma TG levels in *E3L* mice, a model for human-like lipoprotein metabolism, which was due to an increase in plasma VLDL-TG levels. Additional investigation of VLDL-TG metabolism revealed that the increased VLDL-TG levels were not caused by decreased clearance of TG from VLDL-like particles, but rather by increased hepatic production of VLDL-TG. These findings are in line with a study showing that injection of a low dose of LPS increases secretion of VLDL-TG, without affecting its clearance.[5] However, LPS associates with macrophages rather than with hepatocytes,[21] which hampers interpretation which cell type is primarily responsible for the increase in VLDL-TG secretion. In addition to LPS, individual cytokines, that activate various cell types, increase VLDL-TG production.[3,22] Since both LPS and cytokines can activate NF-κB signaling, our findings could suggest that the increase in VLDL secretion caused by LPS and cytokines in these earlier studies has been mediated, at least in part, via direct or indirect activation of NF-κB in the hepatocytes. In the present study, even though *LIKK* clearly increased VLDL-TG secretion, there were no significant effects of *LIKK* on apoB production or hepatic *ApoB* gene expression. This suggests that NF-κB activation increases the intracellular lipidation of apoB, but not the number of VLDL-particles secreted. This is in contrast with a study of Tsai *et al.*,[23] showing that adenoviral-mediated overexpression of IKK did increase apoB secretion in HepG2 cells. This discrepancy could possibly be explained by the level of IKK overexpression, which was higher with adenoviral-mediated IKK overexpression in their *in vitro* HepG2 model than with transgenic overexpression in our *in vivo* study. It is thus reasonable to postulate that low-grade NF-κB activity mainly increases lipidation of the VLDL particles, whereas a higher degree of NF-κB activation could in addition increase the number of secreted VLDL-particles.

It is interesting to speculate about the mechanism why hepatocyte-specific NF-κB activation increases VLDL-TG secretion, as many different factors could theoretically be involved. For example, IKKβ overexpression can cause insulin resistance,[1] which could result in an inability of insulin to suppress VLDL-TG production.[24] Furthermore, Kupffer cells have been suggested to play an important role in hepatic lipid metabolism.[25] Additionally, Kupffer cell products could possibly suppress lipid oxidation in hepatocytes via NF-κB mediated suppression of PPARα activity.[26] Furthermore, although plasma FFA levels were unaltered by *LIKK*, liver-directed FA flux may have been influenced, resulting in altered substrate availability for VLDL-TG production. Therefore, to evaluate the effect of IKKβ overexpression in hepatocytes on VLDL-TG production in absence of these potentially confounding factors, we studied the effect of IKKβ in hepatocytes on TG production *in vitro*. In fact, IKKβ expression in hepatocytes *per se* appeared to directly increase VLDL-TG production. Although additional factors may contribute to the effect of *LIKK* on VLDL-TG production *in vivo*, a direct effect of IKKβ overexpression in hepatocytes thus at least contributes to this phenomenon.

PPARα, LXR and FXR have shown to be activated during inflammation and interact with inflammatory processes[27,28] and could possibly underlie the mechanism by which hepatocyte-specific NF-κB activation increases VLDL-TG secretion directly within the hepatocyte. However, no change was observed in expression of hepatic PPARα and LXR or expression of their target

genes. NF-κB activation did increase FXR expression, but FXR activation has been linked to a lower VLDL-TG secretion,[29] making a causal relationship between FXR activation and the increase in VLDL-TG secretion unlikely. Apparently, chronic hepatocyte-specific activation of NF-κB by IKKβ overexpression does not induce identical changes in lipogenic pathways that are seen in acute inflammation, however, it clearly increases VLDL-TG production and induces hypertriglyceridemia. Increased hepatic lipid availability, by increased lipogenesis and/or decreased lipid oxidation, could also underlie the mechanism by which hepatocyte-specific NF-κB increases VLDL-TG secretion. Acute inflammation has been shown to increase hepatic lipogenesis as measured by incorporation of ^3H_2O into FA *in vivo*.[5,30,31] In our study we measured expression of genes involved in hepatic FA oxidation and *de novo* lipogenesis. Despite the fact that *LIKK* did not decrease the expression of genes involved in FA oxidation, *LIKK* clearly increased expression of *Fas*, which is a key enzyme in the regulation of FA synthesis. Although upregulation of *Fas* could be mediated by the transcription factors LXR, Srebp-1c and ChREBP,[32,33] the observed upregulation of *Pklr* as a main ChREBP target gene suggests that *LIKK* most likely activates ChREBP, thereby increasing *Fas* expression. The fact that activation of NF-κB has been linked to local disturbances in glucose metabolism that could activate ChREBP would underscore this observation.[1,34,35] It is thus conceivable that increased ChREBP mediated *Fas* expression increases hepatic lipogenesis and thereby increases lipid availability for VLDL-TG production.[36] In fact, activation of lipogenesis results in large, but not more, VLDL particles, which is consistent with our findings.[37] The fact that we did not observe an increase in the hepatic TG content or FA oxidation that could have been expected by increased *Fas* expression,[38] can be explained by efficient incorporation of newly synthesized TG into nascent VLDL resulting in the increased hepatic VLDL-TG secretion.

In conclusion, we show that activation of hepatocyte-specific NF-κB through overexpression of IKKß increases TG levels in *E3L* mice by stimulation of VLDL-TG secretion, directly within the hepatocyte, without effects on VLDL-TG clearance. The stimulation of VLDL-TG secretion is not driven by increased steatosis or decreased FA oxidation, but most likely by ChREBP mediated upregulation of *Fas* expression.

Acknowledgements

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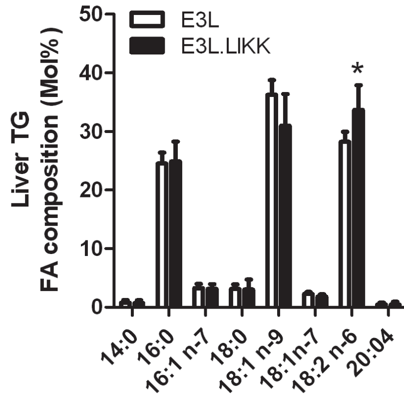
Supplemental data

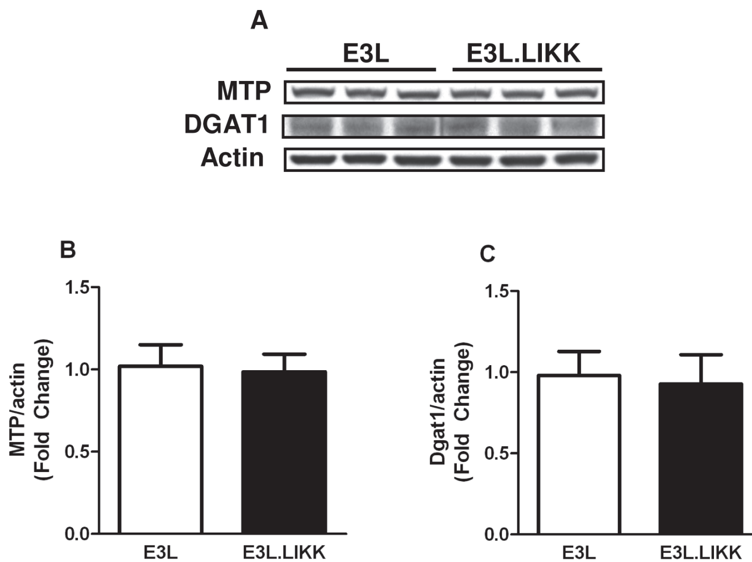
Supplemental Table S1. Primers used for quantatitive real-time PCR analysis.

Gene	Forward primer	Reverse primer
Abcg5	TGTCCTACAGCGTCAGCAACC	GGCCACTCTCGATGTACAAGG
Abcg8	GACAGTTCACAGCCCACAA	GCCTGAAGATGTCAGAGCGA
Acox1	TATGGGATCAGCCAGAAAGG	ACAGAGCCAAGGGTCACATC
ApoB	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
Cidea	CTCGGCTGTCTCAATGTCAA	CCGCATAGACCAGGAACTGT
Cidec	CTGGAGGAAGATGGCACAAT	GGGCCACATCGATCTTCTTA
Cpt1a	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
Cyp27a1	TCTGGCTACCTGCACTTCCT	CTGGATCTCTGGGCTCTTTG
Cyp7a1	CAGGGAGATGCTCTGTGTTCA	AGGCATACATCCCTTCCGTGA
Cyp8b1	GGACAGCCTATCCTTGGTGA	CGGAACTTCCTGAACAGCTC
Dgat1	TCCGTCCAGGGTGGTAGTG	TGAACAAAGAATCTTGCAGACGA
Fasn	TCCTGGGAGGAATGTAAACAGC	CACAAATTCATTCACTGCAGCC
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Hmgcr	CCGGCAACAACAAGATCTGTG	ATGTACAGGATGGCGATGCA
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Nr1h3	CTGCACGCCTACGTCTCCAT	AAGTACGGAGGCTCACCAGCT
Nr1h4	GGCCTCTGGGTACCACTACA	ACATCCCCATCTCTTTGCAC
Pklr	GCAGAACGAGTCACAGCAAT	GTGGAGGCTTCCTTCAAGTG
Plin2	CAGGATGGAGGAAAGACTGC	CTTATCCACCACCCCTGAGA
Plin5	TGTCCAGTGCTTACAACTCGG	CAGGGCACAGGTAGTCACAC
Ppara	ATGCCAGTACTGCCGTTTTC	GGCCTTGACCTTGTTCATGT
Ppargc1b	TTGTAGAGTGCCAGGTGCTG	CCTCCATAGCTCAGGTGGAA
Srebp-1c	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCCAGCATA

Abcg5, ATP-binding cassette sub-family G member 5 ; *Abcg8*, ATP-binding cassette sub-family G member 8; *Acox1*, acyl-coenzyme A oxidase 1; *ApoB*, apolipoprotein B; *Cidea*, cell death activator CIDE-A; *Cidec*, fat-specific protein FSP27; *Cpt1a*, carnitine palmitoyltransferase 1a; *Cyp27a1*, cholesterol 27 hydroxylase; *Cyp7a1*, cholesterol 7 alpha hydroxylase; *Cyp8b1*, sterol 12 alpha-hydrolase; *Dgat1*, diglyceride acyltransferase 1; *Fas*, fatty acid synthase; *Hmgcr*, HMG-CoA reductase; *Mttp*, microsomal triglyceride transfer protein; *Nr1h3*, liver X receptor alpha; *Nr1h4*, farnesoid X activated receptor; *Pklr*, liver-type pyruvate kinase; *Plin2*, perilipin 2; *Plin5*, perilipin 5; *Ppara*, peroxisome proliferator activated receptor alpha; *Ppargc1b*, PPAR-gamma coactivator 1-beta; *Srebp-1c*, sterol-regulatory element binding protein.



Supplemental Fig. S1. Effect of *LIKK* on fatty acid composition of hepatic triglycerides in *E3L* mice. Livers were obtained from overnight fasted *E3L* and *E3L.LIKK* mice and lipids were extracted. TG were isolated by thin-layer chromatography followed by fatty acid separation and quantification using gas chromatography. Fatty acid composition was then determined (in mol%). Values are means ± SD (n=5-7).



Supplemental Fig. S2. *LIKK* does not affect hepatic MTP and DGAT1 protein levels. *E3L* and *E3L.LIKK* mice were fed a chow diet and sacrificed at the age of 14 weeks after an overnight fast. MTP and DGAT1 levels were measured in liver tissue by Western blots and actin was used as an internal control. Representative Western blots are shown for 3 mice per group (A). Ratios of MTP (B) and DGAT1 (C) proteins over actin levels were quantified. Values are means ± SD (n=5-7).

Hepatocyte-Specific IKKβ Expression Aggravates Atherosclerosis Development in *APOE*3-Leiden* Mice

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Abstract

The liver is the key organ involved in systemic inflammation, but the relation between hepatic inflammation and atherogenesis is poorly understood. Since nuclear factor-κB (NF-κB) is a central regulator of inflammatory processes, we hypothesized that chronically enhanced hepatic NF-κB activation, through hepatocyte-specific expression of IκB kinase-β (IKKβ) (*LIKK*), will aggravate atherosclerosis development in *APOE*3-Leiden (E3L*) mice. *E3L.LIKK* and *E3L* control littermates were fed a Western-type diet for 24 weeks. *E3L.LIKK* mice showed a 2.3-fold increased atherosclerotic lesion area and more advanced atherosclerosis in the aortic root with less segments without atherosclerotic lesions (11 *vs*. 42%), and more segments with mild (63% *vs*. 44%) and severe (26% *vs*. 14%) lesions. Expression of *LIKK* did not affect basal levels of inflammatory parameters, but plasma cytokine levels tended to be higher in *E3L.LIKK* mice after lipopolysaccharide (LPS) administration. *E3L.LIKK* mice showed transiently increased plasma cholesterol levels, confined to (V)LDL. This transient character resulted in a mild (+17%) increased cumulative plasma cholesterol exposure. We conclude that selective activation of NF-κB in hepatocytes considerably promotes atherosclerosis development which is (at least partly) explained by an increased sensitivity to pro-inflammatory triggers and transiently increased plasma cholesterol levels.

Introduction

Increased inflammation, in addition to disturbances in lipid metabolism, is the other main contributor to the development of atherosclerosis.[1] Nuclear factor-κB (NF-κB) has been identified as the most important transcription factor in the regulation of inflammatory processes during atherosclerosis development.[2] In unstimulated cells, NF-κB p65/p50 dimer is kept inactive by its inhibitory protein: inhibitor of κB (IκB). A wide range of extracellular stimuli, including cytokines, microbial components, and also free fatty acids, induce activation of the IκB kinase complex, which consists of two kinases (IKKα and -β) and a regulatory subunit, NEMO/IKKγ. This complex mediates the phosphorylation of IκB, resulting in its ubiquitination and degradation, leaving the NF-κB dimer free to translocate to the nucleus and activate its target genes.[2]

While general inhibition of the NF-κB pathway by pharmacological agents reduces atherosclerosis development in mice,[3, 4] the relative contribution of NF-κB may differ at cellular- or tissue-specific level. Suppression of the NF-κB pathway in endothelial cells by ablation of NEMO/IKKγ has been shown to decrease atherosclerosis development.[5] In murine bone marrow transplantation models, inhibition of the NF-κB pathway at distinct levels in hematopoietic cells can have different outcomes, *i.e.* deficiency of the NF-κB p50 subunit resulted in smaller atherosclerotic lesions,[6] whereas deletion of IKKβ increased atherosclerosis development.[7] Surprisingly, the role of the NF-κB pathway in hepatocytes on atherosclerosis development has not been investigated thus far.

The liver plays a central role in both lipid metabolism[8] and inflammation.[9] Disturbances in lipid metabolism and increased inflammation are the two main risk factors for atherogenesis.[1] Hepatocytes form the largest population of cells in the liver and execute most of its important functions. During inflammation, acute phase proteins are mainly synthesized by the hepatocytes.[10] Interestingly, hepatocyte-specific deficiency of gp130, a receptor component of IL-6 signaling which signals independent of the NF-κB pathway, decreases atherosclerosis in *apoe$^{-/-}$* mice,[11] suggesting that reduced hepatic inflammation is associated with less atherosclerosis development.

Despite ample evidence implicating the involvement of NF-κB in atherogenesis, the hepatocyte-specific role of NF-κB in atherosclerosis has not been investigated directly. Therefore, in this study we aimed to investigate whether chronic activation of hepatocyte-specific NF-κB aggravates atherosclerosis development. We used transgenic mice with hepatocyte-specific expression of human IKKβ (Liver-specific IKKβ or *LIKK* mice), resulting in an increase of active NF-κB,[12] crossbred with atherosclerosis-prone *APOE*3-Leiden* (*E3L*) mice. *E3L* mice exhibit a human-like lipoprotein distribution on a cholesterol-rich diet due to transgenic expression of a human mutant of the *APOE3* gene, and are therefore susceptible to atherosclerosis development.[13] Collectively, our results show that hepatocyte-specific NF-κB activation markedly aggravates atherosclerosis development in *E3L* mice.

Methods

Animals and study design

Transgenic *LIKK* mice expressing constitutively active human IKKβ in the hepatocytes by an albumin promoter[12] were crossbred with *E3L* mice[14] to generate heterozygous *E3L.LIKK* and control *E3L* littermates, as described before.[15] Mice were housed under standard conditions with a 12-hour light/dark cycle and had free access to food and water. Female mice of 10-12 weeks of age were fed a Western-type diet containing 15% (w/w) cacao butter supplemented with 0.25% (w/w) cholesterol (Hope Farms, Woerden, The Netherlands). Food intake and body weight were measured weekly. Unless indicated otherwise, blood was drawn every 4 weeks after 4 hours of fasting in EDTA-containing tubes by tail bleeding, and plasma was isolated by centrifugation. All animal experiments were approved by the Institutional Ethical Committee on Animal Care and Experimentation of the Leiden University Medical Center (Leiden, The Netherlands).

mRNA expression analysis

Total RNA from livers of *E3L* and *E3L.LIKK* mice was isolated using an RNA isolation kit according to manufacturer's specifications, including a 15 min. DNAse I treatment (Macherey-Nagel, Düren, Germany). Quality control of the isolated RNA was checked with the lab-on-a-chip technology using Experion Stdsens analysis kit (Bio-Rad, Hercules, CA). One μg of total RNA was converted to cDNA with iScript cDNA Synthesis kit (Bio-Rad) and purified with Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany). Real time PCR (RT-PCR) was carried out on an iQ5 Single-Color real-time PCR detection system (Bio-Rad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). Expression levels were normalized using hypoxanthine-guanine phosphoribosyl transferase (*Hprt*), cyclophilin (*Cyclo*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Primer sequences are listed in Supplemental Table 1.

Western blot analysis

Liver tissue was homogenized by Ultraturrax (22,000 rpm; 2x5 sec) in an ice-cold buffer (pH 7.4) containing 30 mM Tris.HCl, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 0.5% (v/v) Triton X-100, 1% (v/v) SDS and protease inhibitors (Complete, Roche, Mijdrecht, The Netherlands) at a 1:6 (w/v) ratio. Homogenates were centrifuged (16,000 rpm; 15 min., 4°C) and the protein content of the supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL). Proteins (20-50 μg) were separated by 7-10% SDS-PAGE followed by transfer to a polyvinylidene fluoride membrane. Membranes were blocked for 1 hour at room temperature in Tris-buffered saline with Tween-20 with 5% non-fat dry milk followed by an overnight incubation with either IKKβ (ab32135, Abcam, Cambridge, UK), tubulin, pSer536 NF-κB p65 or NF-κB p65 antibodies (#2148, #3031, #3034, resp., Cell Signaling, Danvers, MA). Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Bands were visualized by enhanced chemiluminescence and quantified using Image J software (NIH, USA).

Plasma inflammatory markers

Plasma levels of serum amyloid A (SAA) were determined using the murine Phase Serum Amyloid A Assay kit (Tridelta, County Kildare, Ireland) according to manufacturer's instructions. Plasma levels of inflammatory cytokines and chemokines (IFNγ, IL-12 p70, IL-1β, IL-6, TNFα and IL-10) were measured using a multiplex murine inflammatory cytokine profile immunoassay from Meso Scale Discovery (MSD) on a MSD 2400 plate reader according to the manufacturer's protocol (MSD, Gaithersburg, MD).

Lipopolysaccharide stimulation

Mice were injected i.v. with *Salmonella minnesota* Re595 lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO) (50 mg/kg body weight). Blood was collected 90 min. after injection in heparin-coated capillaries. Plasma was assayed for cytokines as described above.

FACS staining of leukocyte subpopulations in peripheral blood

Nonfasted, whole blood was drawn in EDTA-containing tubes by tail bleeding and incubated with antibodies against Ly6C-FITC (kindly provided by dr P.J. Leenen, Erasmus University, Rotterdam, The Netherlands), Ly6G-PE, CD115-biotin and CD11b-APC (all from Pharmingen, Alphen a/d Rijn, The Netherlands). In between all steps, cells were washed with PBS containing 1% BSA and 0.05% Na-azide.

Binding of anti-CD115-biotin was detected with streptavidin conjugated with PerCP-Cy5.5 (Pharmingen, Alphen a/d Rijn, The Netherlands). Red blood cells were lysed with shock-buffer, cells were fixed with 1% paraformaldehyde and measured with an LSRII (Becton Dickinson, Erembodegem, Belgium).

To obtain leukocyte profiles, the following gating strategy was applied: debris was gated out in a forward (FSC)/side scatter (SSC) plot and leukocytes minus debris were divided according to their CD11b expression (myeloid lineage: CD11b-pos; lymphoid lineage: CD11b-neg). CD11b-pos cells were then selected and using Ly6G expression and SSC, cells were divided in neutrophilic granulocytes (Ly6G-hi/SSC-hi), eosinophilic granulocytes (Ly6G-neg/SSC-hi) and a non-granulocyte population (Ly6G-neg/SSC-low). The non-granulocyte population was then selected and using their CD11b and CD115 expression, monocytes (CD11b-pos/CD115-pos) and NK cells (CD11b-med/CD115-neg) were identified. Next, the monocyte population was selected and using Ly6C expression and FSC, classical monocytes (Ly6C-hi/FSC-low), non-classical monocytes (Ly6C-low/FSC-low) and intermediate monocytes (Ly6C-med/FSC-low) were identified. Going back to the lymphoid lineage (CD11b-neg cell population), using Ly6C-expression, lymphocytes (Ly6C-low/FSC-low) and lymphoblasts (Ly6-ly6C-med/FSC-hi) were identified.

Plasma lipids, lipoprotein profile, hepatic VLDL-TG production and liver lipids analysis

Plasma total cholesterol (TC), triglycerides (TG) and phospholipids (PL) levels were determined using enzymatic kits from Roche Molecular Biochemicals (Woerden, The Netherlands) according to the manufacturer's protocols. We used the following formula to calculate the cumulative plasma TC levels over 24 weeks of Western-type diet feeding which equals the area

under the curve for the plasma TC in time: $4*TC_{t=0} + 0.5*4*(TC_{t=4} - TC_{t=0}) + 4*TC_{t=4} + 0.5*4*(TC_{t=8} - TC_{t=4}) + 4*TC_{t=8} + 0.5*4*(TC_{t=12} - TC_{t=8}) + 4*TC_{t=12} + 0.5*4*(TC_{t=16} - TC_{t=12}) + 4*TC_{t=16} + 0.5*4*(TC_{t=20} - TC_{t=16}) + 4*TC_{t=20} + 0.5*4*(TC_{t=24} - TC_{t=20})$. The correlation between cumulative plasma TC level and atherosclerotic lesion area was assessed for both *E3L* and *E3L.LIKK* mice. For the determination of lipid distribution over plasma lipoproteins, pooled plasma per group was size-fractionated using an ÄKTA fast performance liquid chromatography (FPLC) system (Pharmacia, Roosendaal, The Netherlands). Each sample was injected onto a Superose 6 HR3.2/30 column and eluted at a constant flow rate of 50 μL/min in PBS, pH 7.4. Fractions of 50 μL were collected and assayed for TC as described above.

Mice were fasted for 4 hours prior to the start of the hepatic VLDL-TG production experiment, and, subsequently, sedated with 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag). At timepoint zero, blood was taken via tail bleeding and mice were i.v. injected with 100 μL PBS containing 100 μCi Trans^{35}S label to measure *de novo* total apolipoprotein B (apoB) synthesis. After 30 min., the animals received 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg body weight as a 10% (w/w) solution in sterile saline, to prevent systemic lipolysis of newly secreted hepatic VLDL-TG.[16] Additional blood samples were taken at regular timepoints after tyloxapol injection and used for determination of plasma TG concentration. At timepoint 120 min., the animals were sacrificed and blood was collected by orbital puncture for isolation of VLDL by density gradient ultracentrifugation.[17] ^{35}S-labeled total apoB content was measured in the VLDL fraction after precipitation with isopropanol.[18]

Lipids from liver tissue were extracted according to a protocol adapted from Bligh and Dyer.[19] Hepatic TG, PL concentrations were determined using the enzymatic kits as described previously. TC, free cholesterol (FC) and cholesteryl ester (CE) content were assessed with a cholesterol/cholesteryl ester quantitation enzymatic kit according to manufacturer's specifications (Biovision Research Products, Mountain View, CA). Liver lipids were expressed per mg protein, which was measured using the BCA protein assay kit (Pierce, Rockford, IL).

Atherosclerosis quantification

Mice were euthanized by carbon dioxide inhalation after 24 weeks of diet. Hearts were isolated and fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin, and were cross-sectioned (5 μm) throughout the entire aortic root area. Of each mouse, 4 sections with 50-μm intervals were used for quantification of atherosclerotic lesion area. Characterization of lesion severity was performed separately in each of the 3 segments between the aortic valves in the 4 sections.[20]

Sections were stained with hematoxylin-phloxine-saffron (HPS). Atherosclerotic lesions were categorized for severity by one blinded observer, according to the guidelines of the American Heart Association, adapted for mice. Various types of lesions were distinguished: type 0 (no lesions) (Supplemental Fig. 8A), type I to III (early fatty streak-like lesions containing foam cells) (Supplemental Fig. 8B-D), and type IV to V (advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization, and/or necrosis) (Supplemental Fig. 8E-F).

Immunohistochemistry for determination of adhering monocytes and macrophage-, and smooth muscle cell content in the lesions was performed as described previously.[21, 22] The sections were incubated overnight with antibody M18 (1:100, Santa Cruz Biotechnology, Santa Cruz, Calif) and, subsequently, with biotinylated rabbit anti-goat conjugate (1:400, Brunschwig chemie, Amsterdam, The Netherlands) for quantification of MCP-1. AIA 31240 rabbit antiserum (1:1000, Accurate Chemical and Scientific, Westbury, NY) in combination with biotinylated donkey anti-rabbit conjugate (1:3000, Amersham Pharmacia Biotech) were used for quantification of both the number of monocytes adhering to the endothelium as well as the macrophage area. Antibody M0851 (1:800, Dako, Carpinteria, CA) and biotinylated horse anti-mouse conjugate (1:400, Vector Laboratories, Burlingame, CA) were used to quantify smooth muscle actin. Immunostaining was amplified using Vector Laboratories Elite ABC kit (Vector Laboratories, Burlingame, CA) and the immunoperoxidase complex was visualized with Nova Red (Vector Laboratories, Burlingame, CA). Counterstaining was performed with Mayer's hematoxylin. Sirius red was used to stain for collagen in the lesions (Chroma, Stuttgart, Germany).

Total lesion size, MCP-1-, macrophage-, smooth muscle cell-, and collagen content were quantified using Cell^D image analysis software (Olympus Soft Imaging Solutions, Münster, Germany).

Statistical analysis

Data are presented as means ±SEM. SPSS 17.0 for Windows (SPSS, Chicago, Ill) was used for statistical analysis. Statistical differences were assessed with the Mann-Whitney *U* test. For lesion typing, differences were determined by the χ^2 test. To assess the correlation between cumulative cholesterol exposure and atherosclerotic lesion area, the Pearson correlation test was performed after log transformation of the atherosclerotic lesion area. Differences at $P<0.05$ were regarded as statistically significant.

Results

LIKK causes low-grade inflammation

The overall appearance of *E3L* and *E3L.LIKK* mice during the study was similar. To assess whether expression of *LIKK* affects body weight gain, we measured food intake and body weight weekly. Both were not different between *E3L.LIKK* and *E3L* control mice (Supplemental Fig. 1A-B). The liver- and spleen weight and histological morphology of the liver were also comparable between *E3L.LIKK* and *E3L* mice (data not shown). To gain more insight in the effects of *LIKK* on inflammation, we determined whether *LIKK* expression increased the inflammatory state of the liver and systemic inflammatory markers in *E3L.LIKK* mice on a Western-type diet. We confirmed previous findings[15] showing that the enhanced expression of hepatocyte-specific human IKKβ (Supplemental Fig. 2A) resulted in a 1.4-fold increased hepatic NF-κB activation, as shown by an increase in the phosphorylated p65 subunit (pNF-κB^{Ser536}) (Supplemental Fig. 2B). IKKβ kinase phosphorylates subunit p65 of NF-κB at the position Ser536, which activates the transcriptional activity of NF-κB.[23] The transgenic expression of human IKKβ mRNA was present only in *E3L.LIKK* mice and did not alter murine IKKβ mRNA expression (Supplemental Fig. 2C-D). The enhanced

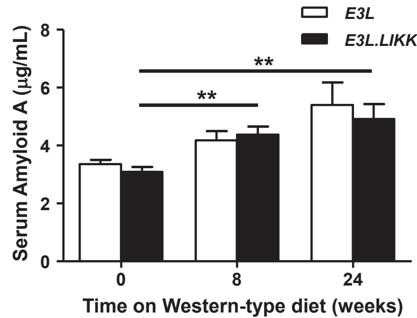

Fig. 1. *LIKK* does not increase plasma SAA levels. SAA levels were determined in plasma from *E3L.LIKK* (black bars) and *E3L* (white bars) mice fed a Western-type diet for 0, 8 and 24 weeks. Values are means ±SEM; n=15/group; $^{**}P<0.01$.

hepatic NF-κB activation in *E3L.LIKK* mice did not result in increased IL-6 expression in whole liver, but did result in a tendency towards increased IL-1β expression ($P=0.085$) and a significant increase in MCP-1 expression (Supplemental Table 2).

To evaluate whether the increased hepatocyte-specific NF-κB activation in *E3L.LIKK* mice enhanced the systemic inflammatory state, we determined the plasma inflammation marker SAA and plasma cytokines under basal conditions. *LIKK* expression did not affect SAA before (3.1 ±0.17 *vs*. 3.4 ±0.15 μg/mL) and after 8 weeks (4.4 ±0.28 *vs*. 4.2 ±0.31 μg/mL) and 24 weeks (4.9 ±0.51 *vs*. 5.4 ±0.78 μg/mL) of Western-type diet feeding (Fig. 1), and neither the determined plasma cytokine levels (Supplemental Fig. 3A-F). SAA levels increased with Western-type diet feeding in both *E3L* and *E3L.LIKK* mice, but this difference only reached statistical significance in *E3L.LIKK* mice (Fig. 1).

Since we did not observe a clear increased systemic pro-inflammatory state under basal conditions, we challenged the mice with LPS to boost the inflammatory response. Interestingly, after injection of LPS, pro-inflammatory cytokines (*e.g.* IL-1β, IFNγ) showed a tendency towards increased plasma levels in *E3L.LIKK* mice as compared to *E3L* mice (Fig. 2A-F). The anti-inflammatory IL-10:IL-1β ratio was significantly lower in *E3L.LIKK* mice (Fig. 2G). Overall, these data indicate that *E3L.LIKK* mice are more sensitive to pro-inflammatory triggers compared to their *E3L* littermates.

To study whether this chronic low-grade inflammation in *E3L.LIKK* mice also resulted in increased inflammatory cell counts in liver and plasma, we determined the hepatic mRNA expression of various cell-type markers of inflammatory cells present in the liver, which are likely to influence atherogenesis,[24] and the number of circulating monocytes. Hepatic mRNA expression of CD68 (Kupffer cells), CD3 ((NK)T cells), and Vα14 (NKT cells) were not different between the genotypes (Supplemental Table 2), neither were the total number of circulating monocytes, the pro-inflammatory Ly6C-hi monocyte subset, the intermediate Ly6C-med monocyte subset and the less inflammatory Ly6C-lo monocyte subset (Supplemental Fig. 4A-D). Together, the above findings indicate that the enhanced hepatocyte-specific NF-κB activation

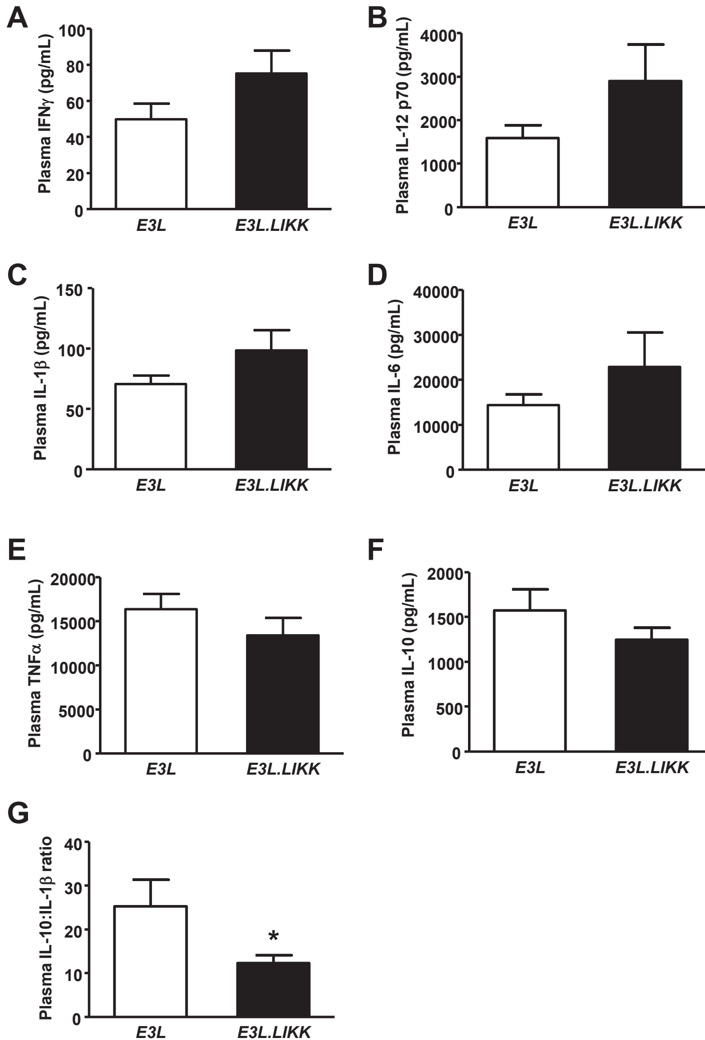

Fig. 2. *LIKK* tends to enhance plasma cytokines after LPS stimulation. LPS was injected intravenously in *E3L.LIKK* (black bars) and *E3L* (white bars) mice. Cytokine levels were measured 90 minutes after LPS injection (A-F). The IL-10:IL-1β ratio was calculated (G). Values are means ±SEM; n=7/group; **P*<0.05.

in *E3L.LIKK* mice results in a tendency towards a mildly enhanced hepatic pro-inflammatory state and an elevated sensitivity to pro-inflammatory stimuli as compared to *E3L* littermates.

LIKK transiently enhances VLDL cholesterol levels

To assess the effect of hepatocyte-specific NF-κB activation on plasma lipid levels, TC, TG and PL concentrations were determined every 4 weeks in *E3L.LIKK* and *E3L* mice. *LIKK* expression caused a transient increase of plasma TC levels only at 8 weeks (+50%; *P*<0.0001) and 12 weeks

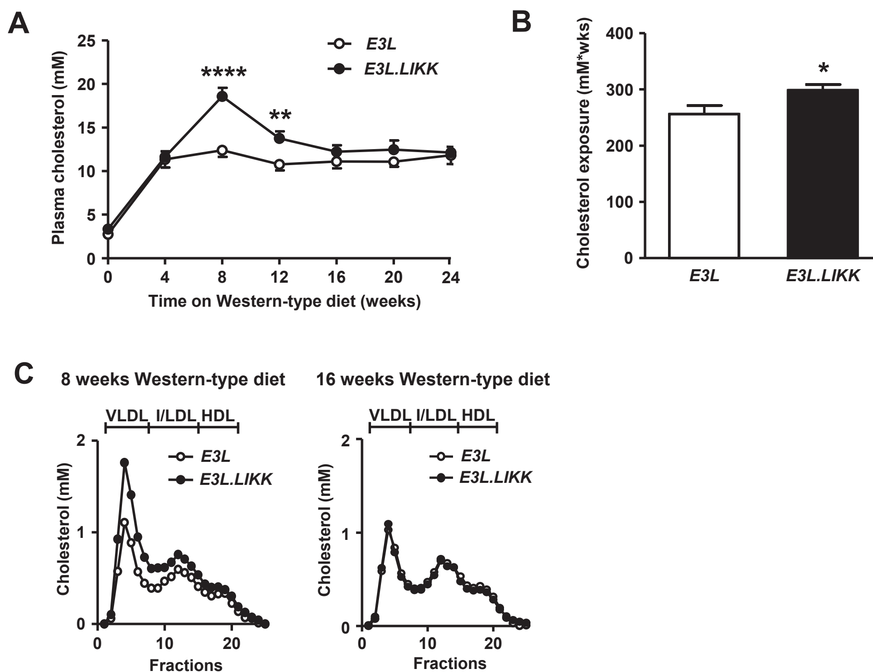

Fig. 3. *LIKK* transiently increases (V)LDL. Plasma cholesterol levels of *E3L.LIKK* (black symbols) and *E3L* (white symbols) mice fed a Western-type diet were assessed (A), and cumulative total cholesterol exposure was calculated (B). Lipoprotein profiles were determined at 8 (left) and 16 (right) weeks (C). Values are means ±SEM; n=15/group; *P<0.05, **P<0.01, ****P<0.0001.

(+28%; P<0.05) of Western-type diet feeding (Fig. 3A). Accordingly, the cumulative total cholesterol exposure was higher in *E3L.LIKK* than in *E3L* mice (+17%; P<0.05; Fig. 3B). A similar transient increase was found for plasma TG and PL levels (Supplemental Fig. 5A-B).

To determine which lipoproteins contribute to the transient elevated plasma TC levels, lipoproteins were size-fractionated by FPLC, and cholesterol was measured in the individual fractions. The transient increase in plasma TC levels at 8 weeks of Western-type diet feeding in *E3L.LIKK* mice was confined to (V)LDL, whereas at 16 weeks the lipoprotein distribution in the *E3L.LIKK* mice was similar to that of the *E3L* mice, in line with the plasma lipid levels (Fig. 3C). Consistent with our previous finding that expression of *LIKK* increased the VLDL production in male mice on chow diet,[15] we found that expression of *LIKK* increased, albeit not significantly, the VLDL-TG production rate (+24%) (Supplemental Fig. 6A), and tended to increase the VLDL-apolipoprotein B (apoB) production rate (+33%) (Supplemental Fig. 6B). No differences were observed in the liver lipid content between *E3L.LIKK* and *E3L* mice (Supplemental Fig. 7A-E). Taken together, these findings indicate that hepatocyte-specific NF-κB activation results in a modest and transient increase in plasma lipid levels in *E3L* mice.

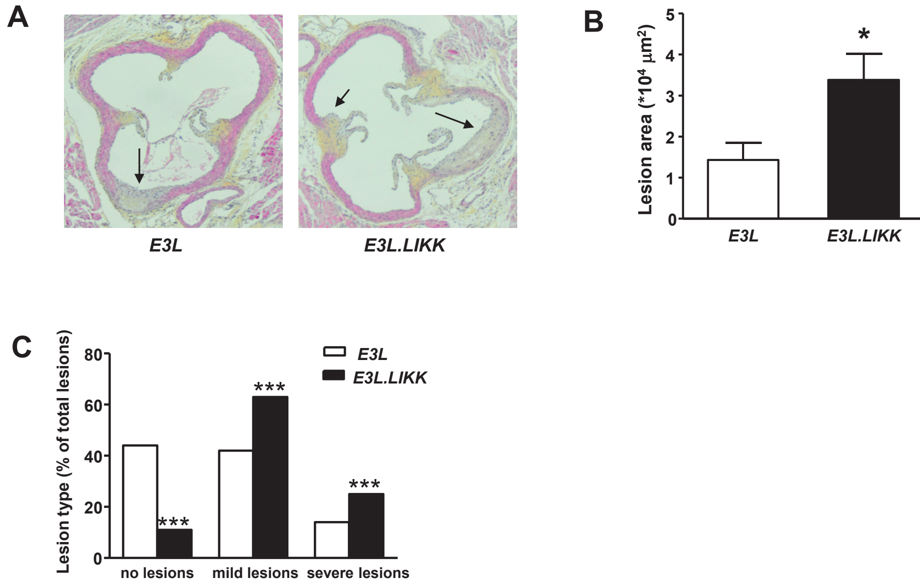

Fig. 4. *LIKK* aggravates atherosclerotic lesion area and severity. After 24 weeks of Western-type diet feeding, *E3L.LIKK* (black bars) and *E3L* (white bars) mice were sacrificed and cross-sections of aortic roots were stained with HPS. Representative pictures are shown. Arrows indicate lesions (A). Total lesion area was assessed in 4 sections of the aortic root (B) and lesion severity was determined separately in each of the 3 segments between the aortic valves of the 4 sections (C). Statistical analysis for lesion area was performed using the Mann-Whitney *U* test, and for lesion severity the χ^2 test. Values are means ±SEM; n=15/group; **P*<0.05, ****P*<0.001.

LIKK enhances atherosclerosis development

To investigate the effect of *LIKK* expression on atherosclerosis development, *E3L.LIKK* and *E3L* mice were sacrificed after 24 weeks of Western-type diet feeding, and lesion size and severity were measured in the aortic root. Representative pictures of both groups are shown in Fig. 4A. *E3L.LIKK* mice developed more than 2-fold larger atherosclerotic lesions (+131%; *P*<0.05; Fig. 4B) as compared to their *E3L* littermates. This increased lesion area coincided with more advanced lesion progression, since we found markedly fewer segments without atherosclerotic lesions (11% *vs*. 42%; *P*<0.001) and more segments with mild (63% *vs*. 44%; *P*<0.001) and severe lesions (26% *vs*. 14%; *P*<0.001) as compared to *E3L* mice (Fig. 4C). Examples of mild and severe lesions are shown in Supplemental Figure 8. These data indicate that chronic hepatocyte-specific NF-κB activation severely augments atherosclerosis development in *E3L* mice.

LIKK aggravates atherosclerotic lesion composition

We next evaluated whether *LIKK* expression would affect monocyte adherence and recruitment to the vascular wall, as well as the composition of the atherosclerotic lesions with respect to the macrophage, smooth muscle cell, and collagen content of the lesions. Adherence of monocytes to the vessel wall and the content of the chemokine monocyte chemoattractant protein-1

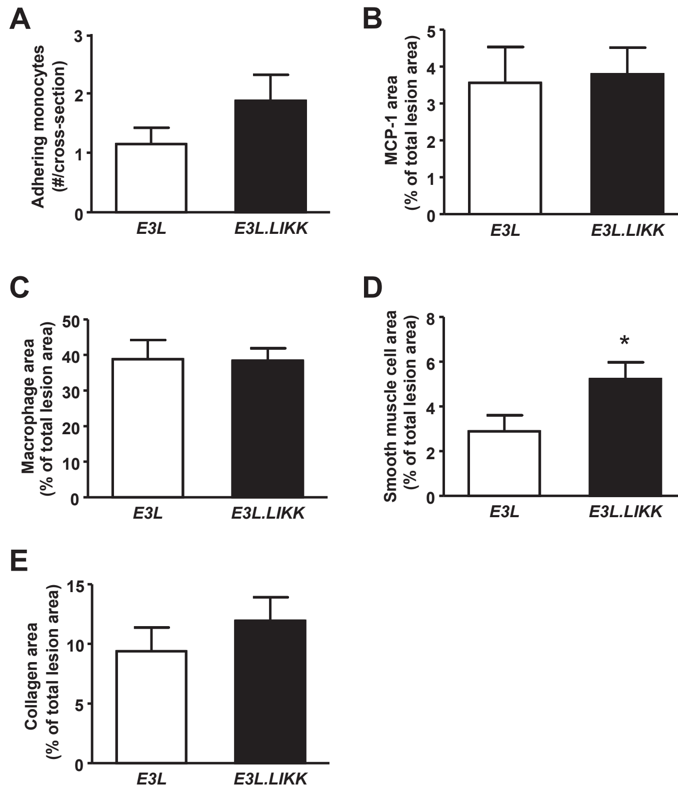

Fig. 5. *LIKK* induces more advanced atherosclerotic lesions. In the sections obtained as described in Fig. 4, the number of adhering monocytes (A), monocyte chemoattractant protein-1 (MCP-1) content (B), macrophage content (C), smooth muscle content (D) and collagen content (E) was determined. Values are means ±SEM; n=15/group; **P*<0.05, ***P*<0.01.

(MCP-1) of the atherosclerotic lesions were not significantly enhanced in *E3L.LIKK* mice as compared to *E3L* mice (Fig. 5A-B). *LIKK* expression did not affect the relative macrophage and collagen content of the lesions (Fig. 5C+E), but did result in an increased smooth muscle cell content of the lesions (+79%, *P*<0.05; Fig. 5D).

Aggravated atherosclerosis development in *E3L.LIKK* mice does not solely depend on the transient increase in plasma cholesterol levels

In *E3L* mice on Western-type diet, the cumulative plasma cholesterol exposure is highly predictive for the atherosclerotic lesion area (unpublished data, J.F.P. Berbée, P.C.N. Rensen). To verify if the transient increase in plasma TC levels (Fig. 3) alone could account for the aggravation in atherosclerosis development observed in *E3L.LIKK* mice, or whether additional mechanism(s) could contribute, including the low-grade systemic inflammation, we assessed the correlation between the cumulative plasma total cholesterol exposure and the atherosclerotic lesion area of the *E3L.LIKK* and *E3L* mice. As expected, there was a significant positive logarithmic correlation

between the atherosclerotic lesion area and the cumulative plasma cholesterol exposure in the control *E3L* mice (Supplemental Fig. 9A; r^2=0.757, *P*=0.002). However, we did not observe such a correlation in the *E3L.LIKK* mice (Supplemental Fig. 9B; r^2=-0.250, *P*=0.369), indicating that in addition to the transient increase in plasma TC levels in *E3L.LIKK* mice, additional mechanism(s), most likely the increased sensitivity to pro-inflammatory stimuli, contributed to the aggravated atherosclerosis development in these mice.

Discussion

NF-κB is regarded as a potential therapeutic target in atherosclerosis[3, 4] and studying tissue- and cell-specific effects of NF-κB in atherogenesis will expand our knowledge in the comprehensive actions of NF-κB on atherosclerosis development. The present study demonstrates for the first time that chronic, hepatocyte-specific expression of IKKβ (*LIKK*) and subsequent activation of NF-κB aggravates atherosclerosis development in *E3L* mice. In addition, the atherosclerotic lesion composition with respect to the macrophage and collagen content was not affected by *LIKK*, but in line with the presence of more advanced lesions, the smooth muscle cell content was increased. Expression of *LIKK* resulted in transiently increased plasma cholesterol levels and an enhanced sensitivity to pro-inflammatory triggers, which both are likely to have contributed to the increased atherosclerotic lesion size and severity. Since lesion size and severity are often correlated in atherosclerosis studies with different murine models,[13, 25] the increased lesion severity in *E3L.LIKK* mice is likely to be mainly attributed to the larger size of the lesions.

Expression of *LIKK* in *E3L* mice increased the activation of the NF-κB pathway in the liver, in line with our previous report.[15] In addition, hepatic mRNA expression of inflammatory parameters was increased or tended to be increased in *E3L.LIKK* mice, indicating that inflammatory mediators at local tissue level were enhanced in *E3L.LIKK* mice. This enhanced activation of hepatocyte-specific NF-κB in *E3L.LIKK* mice, however, did not result in a significant increased systemic pro-inflammatory state under basal conditions as compared to their *E3L* littermates. Importantly, Cai *et al.*[12] demonstrated that in *LIKK* mice on a wild-type background, systemic levels of IL-6 were only mildly elevated, while IL-1β and TNFα levels were similar as in wild-type mice. Our results show that *LIKK* expression on an *E3L* background resulted in a less pronounced hepatic inflammatory state as compared to *LIKK* expression on a wild-type background as described by Cai *et al.*,[12] as reflected in a smaller increase in active NF-κB (1.4- *vs.* 2.2-fold) and mRNA levels of pro-inflammatory cytokines levels in the liver. Furthermore, under basal conditions *E3L* mice have lower levels of active NF-κB present in the liver as compared to wild-type mice (unpublished data, J.A. van Diepen, M.C. Wong, P.J. Voshol). This implies that *E3L* mice have a lower chronic inflammatory state than wild-type mice, which could interfere with the pro-inflammatory effects caused by expression of *LIKK* in the present study. Also, in comparison with other murine atherosclerosis models, *e.g.* the $apoe^{-/-}$ and $ldlr^{-/-}$ mice, *E3L* mice display a milder phenotype with respect to hyperlipidemia and increased inflammation.[26, 27] In the current study, basal circulating levels of some cytokines were at borderline of the detection limit of current assays (Supplemental Fig. 3) and, as expected, the cytokine levels increased approximately 5- to 3700-fold after LPS injection (Fig. 2). Furthermore, after stimulation with

LPS, *E3L.LIKK* mice showed a tendency towards a higher systemic inflammatory state than *E3L* mice.

There is a strong interaction between inflammation and lipid metabolism.[28] For example, lowering inflammation using salicylate did not only reduced NF-κB activation, but concomitantly also reduced circulating cholesterol levels in *E3L* mice.[29] In line with this observation, in the present study we found higher plasma lipid levels at 8 weeks of Western-type diet feeding in female *E3L.LIKK* compared to *E3L* mice, which were confined to (V)LDL. We hypothesize that the increased lipid levels at this time point are accompanied by increased systemic inflammation, which is in line with previous findings showing that lipid metabolism and inflammation strongly influence each other.[28] A possible cause for the increased plasma lipid levels at 8 weeks of diet is therefore a more enhanced inflammation in the liver, possibly due to an increased activation of the NF-κB pathway in the liver.

We recently reported that male *E3L.LIKK* mice on chow diet also showed enhanced (V)LDL levels as a result of an increased hepatic VLDL-TG production rate,[15] and found in the current study a trend towards an enhanced VLDL-apoB production in female *E3L.LIKK* mice on Western-type diet, with a similar effect-size. Possible reasons for the less apparent increase of VLDL-TG production in females compared to males are differences in gender and/or diet. Although the increase in VLDL-TG production is more apparent in male *E3L.LIKK* mice, we used female mice in the present study. The main reason for this is that female *E3L* mice are more susceptible to develop atherosclerosis. In order for male *E3L* mice to become similarly atherosclerosis-prone they need to be fed Western-type diets not only with higher percentages of cholesterol, but also containing cholate. In addition, fructose was added to the drinking water to further raise their (V)LDL-cholesterol levels.[30] The increase in (V)LDL levels in females in the current study was only transient at 8 weeks of Western-type diet feeding and disappeared at 16 weeks. Since no differences in plasma lipid levels and hepatic mRNA expression of genes involved in lipid metabolism were detected between both groups at 24 weeks of diet, the increased VLDL-TG production at 8 weeks of diet is likely to be transient. At present, we cannot explain the transient nature of this increase in (V)LDL levels, but it may be the result of a progressive negative feedback mechanism to reduce the hepatic VLDL production which takes place during long-term Western-type diet feeding.

Dyslipidemia is regarded as the classical risk factor for atherosclerosis development. The transiently enhanced total cholesterol levels, resulting in a modest increase (+17%) in cumulative total cholesterol exposure upon *LIKK* expression, thus likely contributed to the enhanced atherosclerosis development. Previous diet-induced atherosclerosis studies in *E3L* mice have consistently demonstrated that there is a positive logarithmic relation between the cumulative cholesterol exposure during the study and the atherosclerotic lesion area (J.F.P. Berbée, P.C.N. Rensen, unpublished data). In agreement with these previous observations, we did observe such a significant logarithmic relation in *E3L* mice but not in *E3L.LIKK* mice. This suggests that the increase in atherosclerotic lesion area in *E3L.LIKK* mice can only partly be attributed to the transiently enhanced plasma cholesterol levels and that additional mechanisms are involved.

Inflammation is the second main risk factor for atherosclerosis. Enhanced extravascular or systemic inflammation, by the periodontal pathogen *Porphyromonas gingivalis*[31] or by repeated

administration of LPS,[21] respectively, promotes atherosclerosis development. In addition, in humans, low-grade systemic inflammation is associated with enhanced risk of coronary artery disease.[32,33] It is thus likely that, as discussed above, the increased sensitivity for pro-inflammatory triggers such as LPS in *E3L.LIKK* mice also directly contributed to the enhanced atherosclerotic lesion formation.

We excluded higher circulating levels of pro-inflammatory Ly6C-hi monocytes as being another possible contributor to the aggravated atherosclerosis development in *E3L.LIKK* mice. Adhesion of monocytes to endothelial cells and subsequent migration into the vessel wall is one of the crucial steps in atherosclerotic lesion formation. Ly6C-hi monocytes are more prone to adhere to activated endothelium than Ly6C-lo monocytes and are, therefore, associated with enhanced atherosclerosis development.[34] We found that *E3L.LIKK* mice had similar levels of circulating subsets of monocytes as compared to their *E3L* controls, which is consistent with the observed similar number of adhering monocytes to the vascular wall.

In line with the enhanced atherosclerosis development that we observed in *E3L.LIKK* mice, Luchtefeld *et al*.[11] have reported that gp130-deficient mice with defective IL-6 signaling specifically in hepatocytes, develop less atherosclerosis, indicating that modulation of hepatic inflammation can have profound effects on atherogenesis. These studies also underscore that enhanced inflammation in the liver, *e.g.* due to viral hepatitis or steatohepatitis, may augment atherosclerosis development. Indeed, in several clinical studies, such hepatic pathological conditions are associated with an elevated occurrence of CVD.[35, 36, 37] Even after adjustment for classical risk factors for CVD, such as LDL cholesterol levels, chronic hepatitis C infection was still significantly associated with increased atherosclerosis in a cross-sectional study.[37] Together, these findings suggest that there is a direct effect of hepatic inflammation on atherosclerosis development, independent of systemic lipid levels. Moreover, they suggest that in addition to the currently used lipid-targeted drugs such as statins, reducing NF-κB activity in the liver may be a promising additive therapeutic strategy against atherosclerosis development.

In conclusion, we have shown that hepatocyte-specific activation of NF-κB leads to larger and more advanced atherosclerotic lesions. Our studies furthermore suggest that both the transient elevated (V)LDL cholesterol levels as well as the increased sensitivity to pro-inflammatory stimuli are most likely responsible for this aggravating effect on atherosclerosis. These findings contribute to the present understanding of the role of the liver, and more specifically the role of hepatic NF-κB, in atherosclerosis development and may help to develop new innovative anti-atherosclerotic strategies.

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Supplemental data

Supplemental Table 1. Primers used for RT-PCR.

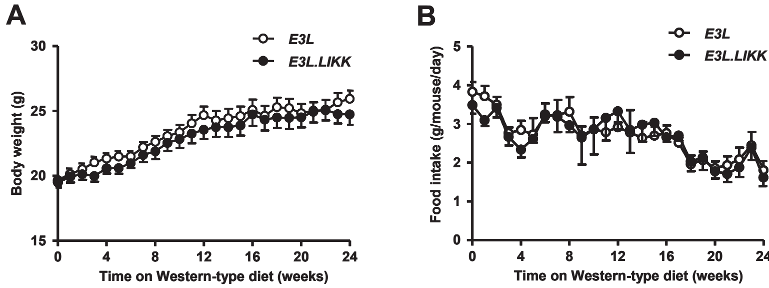
Human target	Forward primer	Reverse primer
IKKβ	GGAGCTCTGTGGGCGGGAGA	GGCCCATGGGGCTCCTCTGT
Murine target	**Forward primer**	**Reverse primer**
ApoB	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
CD3	CTGTCTAGAGGGCACGTCAA	GATGCGGTGGAACACTTTCT
CD68	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTTGA
Cpt1a	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
Fas	TCCTGGGAGGAATGTAAACAGC	CACAAATTCATTCACTGCAGCC
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Hmgcr	CCGGCAACAACAAGATCTGTG	ATGTACAGGATGGCGATGCA
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
IKKβ	GCCCTCTGCTCCCGGCTAGA	CCAGTCTAGAGTCGTGAAGCTTCTGT
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	CCGGAGAGGAGACTTCACAG	TTCTGCAAGTGCATCATCGT
MCP-1	GCATCTGCCCTAAGGTCTTCA	TTCACTGTCACACTGGTCACTCCTA
MTTP	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Vα14	GTGGGTGGCTGGCAAGAC	TCTCCCTGACGCACAACCA
Srebp-1c	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT

IKKβ, IκB kinase-β; *Apob*, apolipoprotein B; *Cd3*, marker for (NK)T cells; *CD68*, marker for macrophages (Kupffer cells); *Cpt1a*, carnitine palmitoyltransferase 1a; *Cyclo*, cyclophilin; *Fas*, fatty acid synthase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Hmgcr*, HMG-CoA reductase; *Hprt*, hypoxanthine-guanine phosphoribosyl transferase; *IL-1β*, interleukin-1β; *IL-6*, interleukin-6; *MCP-1*, monocyte chemoattractant protein-1; *Mttp*, microsomal triglyceride transfer protein; *Srebp-1c*, sterol-regulatory element binding protein; *Vα14*, marker for NKT cells.

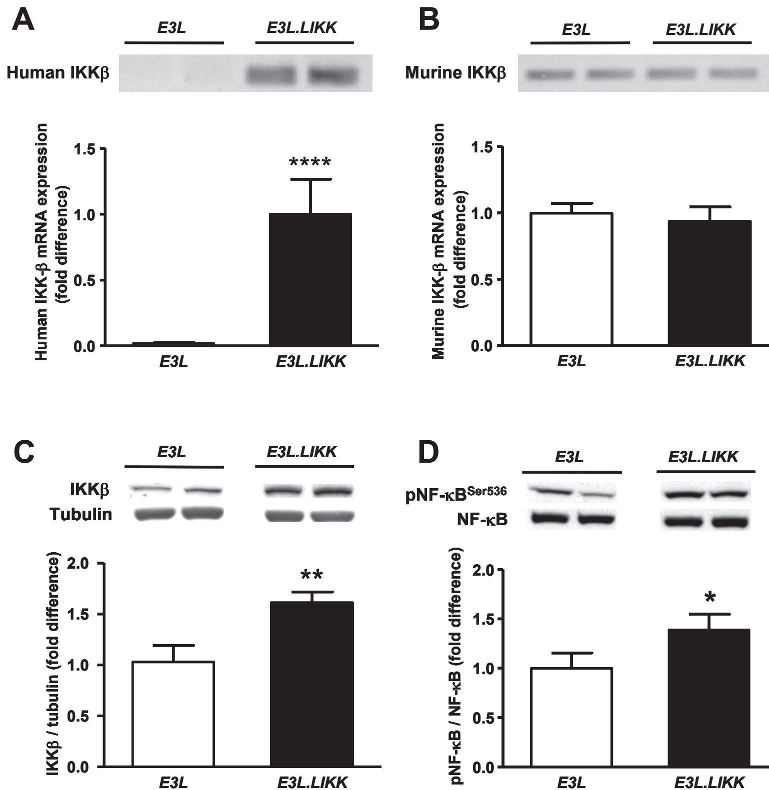
Supplemental Table 2. Expression of *LIKK* increases hepatic MCP-1 expression.

Target	***E3L***	***E3L.LIKK***	**Significance**
Cytokines and chemokines			
*IL-1*β	1.00 ± 0.05	1.24 ± 0.14	*P* = 0.085
IL-6	1.00 ± 0.13	1.24 ± 0.20	n.s.
MCP-1	1.00 ± 0.27	1.39 ± 0.32*	*P* = 0.049
Inflammatory cells			
CD68	1.00 ± 0.29	1.15 ± 0.14	n.s.
CD3	1.00 ± 0.14	0.69 ± 0.30	n.s.
Vα14	1.00 ± 0.08	0.94 ± 0.21	n.s.
VLDL secretion			
ApoB	1.00 ± 0.24	1.00 ± 0.33	n.s.
MTTP	1.00 ± 0.13	1.15 ± 0.28	n.s.
Lipogenesis			
Srebp-1c	1.00 ± 0.19	1.46 ± 0.49	n.s.
Fas	1.00 ± 0.21	0.83 ± 0.19	n.s.

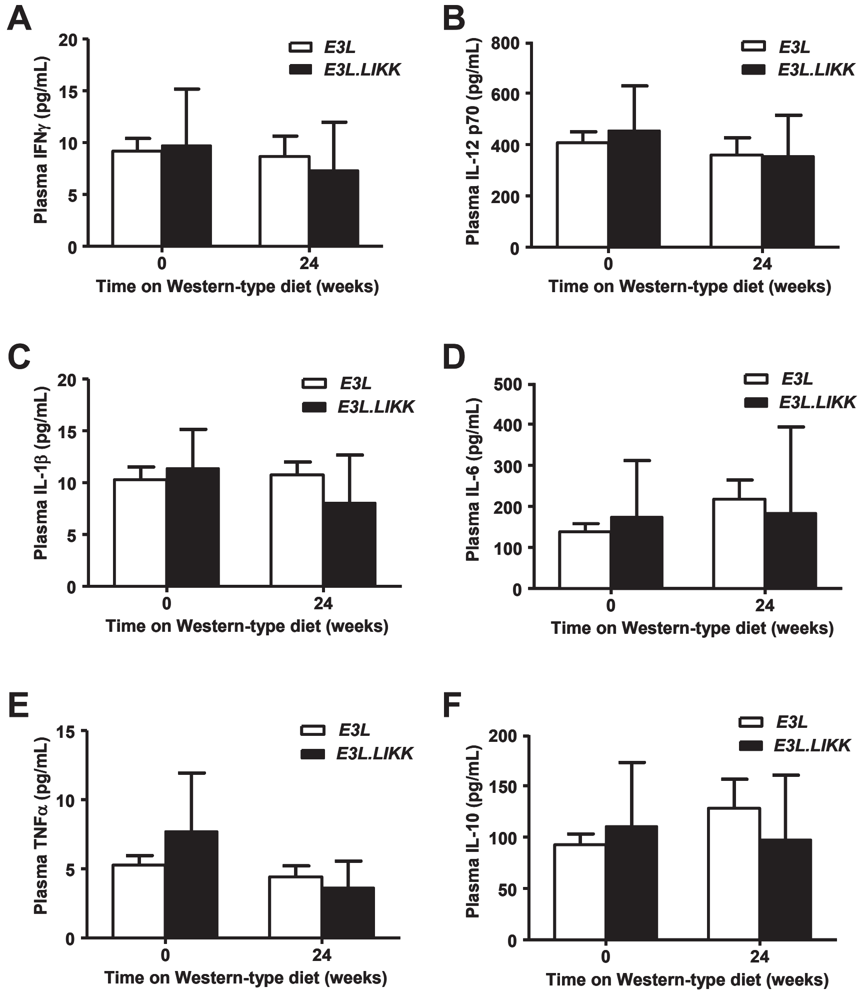
After 24 weeks of Western-type diet, *E3L.LIKK* and *E3L* mice were sacrificed, livers were isolated and mRNA expression of indicated targets were quantified by RT-PCR. Data are calculated as fold difference as compared to the control group. Values are means ±SEM; n=6-15/group; *$P<0.05$ compared to the control group; n.s., not significant. *IKKβ*, IκB kinase-β; *Apob*, apolipoprotein B; *Cd3*, marker for (NK)T cells; *CD68*, marker for macrophages (Kupffer cells); *Cpt1a*, carnitine palmitoyltransferase 1a; *Cyclo*, cyclophilin; *Fas*, fatty acid synthase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Hmgcr*, HMG-CoA reductase; *Hprt*, hypoxanthine-guanine phosphoribosyl transferase; *IL-1β*, interleukin-1β; *IL-6*, interleukin-6; *MCP-1*, monocyte chemoattractant protein-1; *Mttp*, microsomal triglyceride transfer protein; *Srebp-1c*, sterol-regulatory element binding protein; *Vα14*, marker for NKT cells.



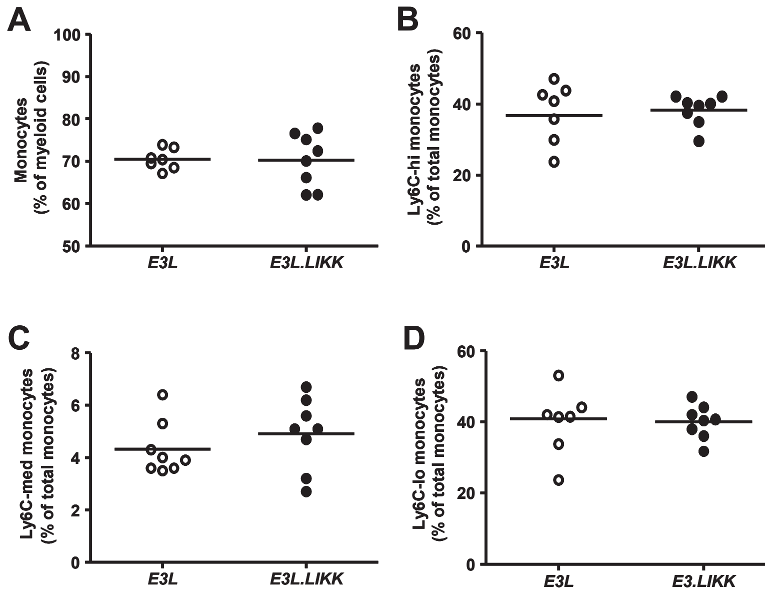
Supplemental Fig. 1. Expression of *LIKK* does not affect body weight and food intake. *E3L.LIKK* (black symbols) and *E3L* mice (white symbols) were fed a Western-type diet for 24 weeks. Body weight (A) and food intake (B) were measured weekly. Values are means ±SEM; n=15/group.



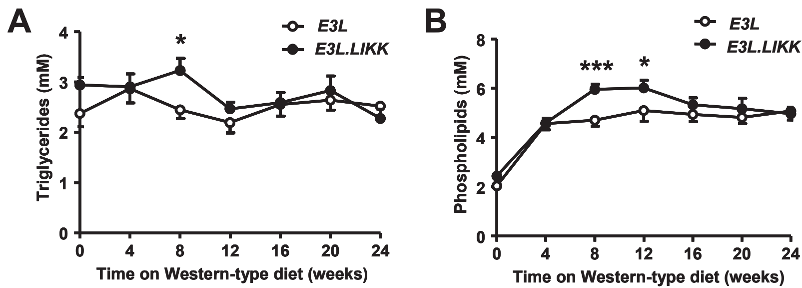
Supplemental Fig. 2. Expression of *LIKK* increases hepatic IKKβ mRNA and protein expression, and NF-κB protein activity. After 24 weeks of Western-type diet, *E3L.LIKK* (black bars) and *E3L* mice (white bars) were sacrificed, livers were isolated and hepatic mRNA expression of human IKKβ (A) and murine IKKβ (B) was quantified by RT-PCR. Human IKKβ is expressed as fold difference compared to *E3L.LIKK* mice and murine IKKβ to *E3L* mice. The inserts show representative bands of two mice per group of the qPCR product run on a gel. Values are mean ±SEM; n=14-15/group; ****P<0.0001. Hepatic protein expression of IKKβ normalized to tubulin (C) and phosphorylated NF-κB p65Ser536 normalized to total NF-κB (D) was assessed in *E3L.LIKK* (black bars) and *E3L* mice (white bars). Values are mean ±SEM; n=6-7/group; *P<0.05, **P<0.01.



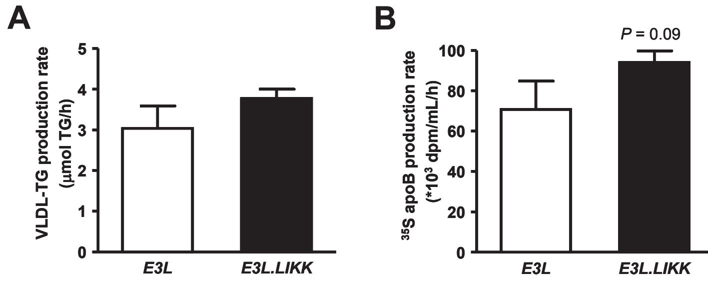
Supplemental Fig. 3. *LIKK* does not affect plasma cytokines after 0 and 24 weeks of diet. *E3L.LIKK* (black bars) and *E3L* (white bars) were fed 24 weeks of Western-type diet. Plasma levels of the indicated cytokines were measured at t=0 and 24 weeks of diet. Values are means ±SEM; n=15/group.



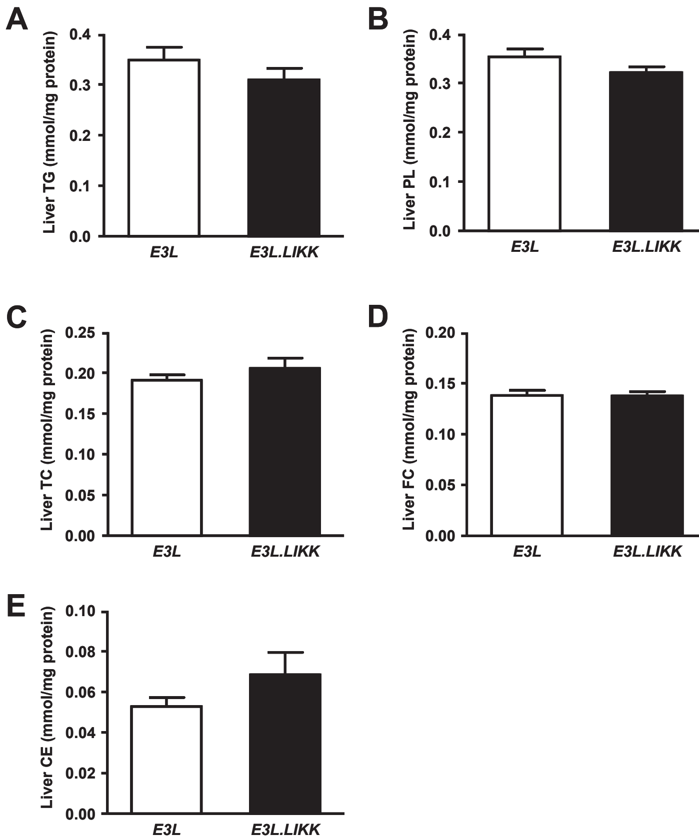
Supplemental Fig. 4. *LIKK* does not affect circulating subsets of monocytes. Blood was drawn from *E3L.LIKK* (black circles) and *E3L* (open circles) mice fed a Western-type diet for 8 weeks. The number of monocytes (A) and Ly6C-hi (B), Ly6C-med (C) and Ly6C-lo (D) expressing subsets were determined by FACS analysis. n=7-8/group.



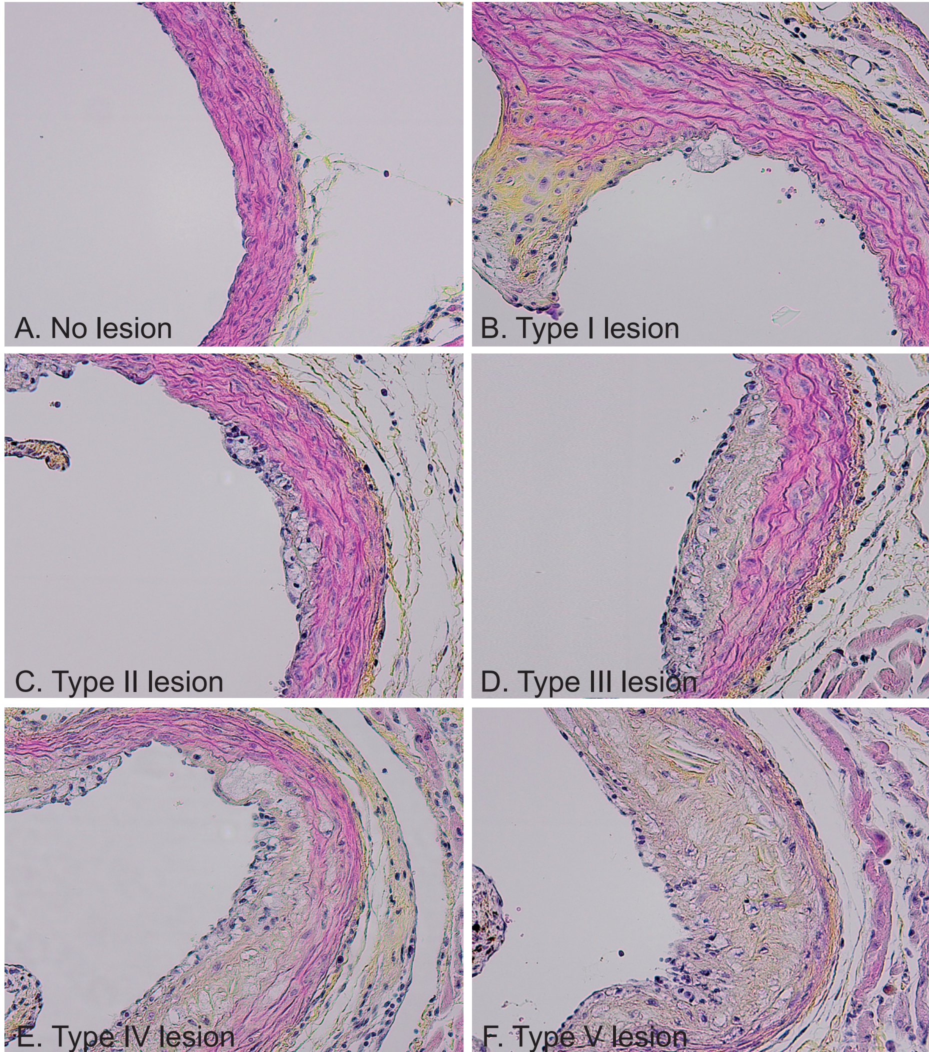
Supplemental Fig. 5. *LIKK* transiently increases plasma triglycerides and phospholipids. *E3L.LIKK* (black symbols) and *E3L* mice (white symbols) were fed a Western-type diet for 24 weeks. Plasma was obtained every 4 weeks to determine triglycerides (TG) (A) and phospholipids (PL) (B) concentration over time. Values are means ±SEM; n=15/group; *$P<0.05$, ***$P<0.001$.



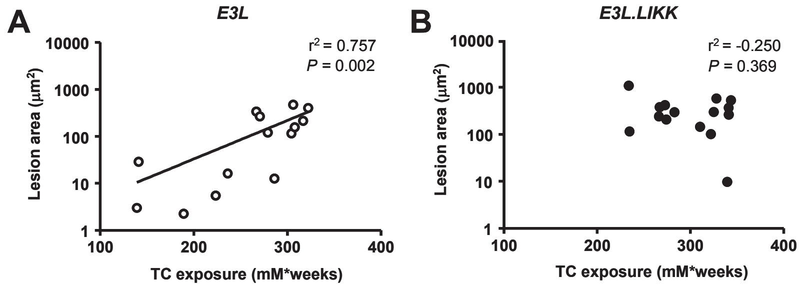
Supplemental Fig. 6. Expression of *LIKK* tends to increase VLDL-apoB production. After 8 weeks of Western-type diet, *E3L.LIKK* (black bars) and *E3L* mice (white bars) were fasted 4 hours and injected with Trans35S and tyloxapol, and blood samples were drawn after tyloxapol injection. The rate of TG production was calculated from the slopes of the curves from the individual mice (A). After 120 min, the total VLDL-fraction was isolated by ultracentrifugation, ^{35}S-activity was counted, and the production rate of newly synthesized VLDL-^{35}S-apoB was determined (B). Values are means ±SEM; n=5-11/group.



Supplemental Fig. 7. *LIKK* does not affect liver lipid content. After 24 weeks of Western-type diet, the livers of *E3L.LIKK* (black bars) and *E3L* mice (white bars) were isolated and triglyceride (TG) (A), phospholipid (PL) (B), total cholesterol (TC) (C), free cholesterol (FE) (D), cholesteryl ester (CE) (E) content was determined. Values are means ±SEM; n=14-15/group.



Supplemental Fig. 8. Representative pictures of HPS-stained segments classified in different severities. Atherosclerotic lesions are categorized into mild (type I-III) and severe (type IV-V) phenotypes. Magnification 200x. (A). No lesion. (B). Type I, early fatty streak: per section up to 10 foam cells present in the intima. (C). Type II, regular fatty streak: more than 10 foam cells present in the intima. (D). Type III, mild plaque: extension of foam cells into the media and covered by a fibrotic cap. (E). Type IV, moderate plaque: a more progressive lesion infiltrating into the media, fibrosis in the media, without loss of architecture. (F). Type V, severe plaque: the media is severely damaged, elastic lamina are broken, presence of cholesterol clefts, mineralization and/or necrosis.



Supplemental Fig. 9. Correlation between cumulative plasma TC exposure and atherosclerotic lesion area. The correlation between the cumulative plasma TC exposure and atherosclerotic lesion area, after log transformation, in *E3L* (open circles) (A) and the *E3L.LIKK* mice (closed circles) (B) was determined using the Pearson correlation test. n=15/group.

Elastase-Induced Emphysema Does not Affect Atherosclerosis Development in *APOE*3-Leiden* Mice

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Submitted

Abstract

Chronic obstructive pulmonary disease (COPD) is a strong risk factor for cardiovascular diseases (CVD), independent of common risk factors such as smoking. The pathophysiological mechanism underlying the link between COPD and CVD, however, is incompletely understood. The aim of the present study was to investigate whether established emphysema in the absence of chronic pulmonary inflammation affects atherosclerosis development in mice. To address this, hypercholesterolemic *APOE*3-Leiden* transgenic mice were intratracheally instilled with 15 or 30 µg of porcine pancreatic elastase (PPE) to induce emphysema, or vehicle as control. After 3 weeks of recovery, mice were fed a diet with 0.4% (w/w) cholesterol to induce atherosclerosis. Blood was drawn to assess plasma lipid and inflammatory parameters. After 20 weeks of diet, mice were sacrificed to analyze the lungs, hearts, and atherosclerosis in the aortic root. Our results showed that intratracheal PPE instillation dose-dependently induced emphysema and right ventricular hypertrophy development. PPE instillation did not affect plasma levels of lipids and acute-phase proteins, but did result in a dose-dependent decrease in numbers of circulating monocytes and neutrophils. There was no difference in atherosclerotic lesion area, but the atherosclerotic lesion severity was dose-dependently decreased after PPE instillation. These data show that PPE-induced emphysema did not affect atherosclerotic lesion area and even reduced atherosclerosis severity in hypercholesterolemic *APOE*3-Leiden* transgenic mice. The findings suggest that emphysema, in the absence of (smoking-induced) inflammation, does not increase atherosclerotic CVD.

Introduction

Clinical epidemiological studies have demonstrated a strong positive correlation between chronic obstructive pulmonary disease (COPD) and cardiovascular disease (CVD).[1] This increased risk of CVD is not entirely explained by traditional shared risk factors, *e.g.* smoking, suggesting that COPD by itself may play a causal role in the pathophysiological mechanisms involved in CVD.[2] Markers of systemic inflammation are increased in many patients with COPD, especially in those with more severe disease and/or exacerbations.[3] This systemic inflammation, which may in part result from overspill of inflammatory mediators from the inflamed lung, could explain this increased risk for CVD.[2, 4]

Although this enhanced systemic inflammation appears to be a plausible link between CVD and COPD, little is known about the origin of these circulating inflammatory mediators. Moreover, the evidence for a prominent role of systemic inflammation in the association between COPD and extrapulmonary manifestations of COPD such as CVD is circumstantial.[3] Emphysema severity is associated with arterial stiffness independent of C-reactive protein (CRP).[5] Anti-inflammatory therapies in COPD, *e.g.* inhaled corticosteroids and tiotropium, have not been consistently shown to modify serum levels of CRP or IL-6, nor the long-term cardiovascular complications.[6,7] This suggests that while local delivery of anti-inflammatory drugs to the lungs does have a limited effect on lung inflammation, it does not reduce systemic inflammation. Furthermore, levels of inflammatory mediators (*i.e.* TNFα, IL-6 and IL-8) in induced sputum do not correlate with the values in blood in COPD patients.[8] Therefore, it is likely that other mechanisms than inflammation are also involved in the interaction between COPD and CVD.

Pulmonary emphysema is a major component of COPD and defined as abnormal airspace enlargement distal to the terminal bronchioles.[9] Recently, it was demonstrated in a study with current, ex- and never-smokers with or without COPD that the occurrence of atherosclerotic plaques in the internal carotid arteries is positively associated with two function parameters that are associated with pulmonary emphysema, *i.e.* a low diffusing capacity for carbon monoxide and a high residual volume.[10] These associations still remained significant after correction for established risk factors for atherosclerosis, such as older age, blood pressure, total cholesterol and smoking. Interestingly, after multivariate analysis, the extent of atherosclerosis was not associated with variables such as forced expiratory volume in one second (FEV_1) and CRP. These results suggest that in addition to airflow obstruction and low-grade systemic inflammation, other features of COPD (*i.e.* reduced diffusing capacity and increased residual volume) may contribute to the association between reduced lung function and CVD.

Intratracheal administration of porcine pancreatic elastase (PPE) in animals reproduces key features of emphysema.[11] When compared to other emphysema models, *e.g.* chronic smoke exposure or intrapulmonary lipopolysaccharide instillation,[11] the PPE model is not characterized by chronic inflammation. This allows a separate analysis of the contribution of emphysema in absence of chronic inflammation alone to atherosclerosis development. This is important since it is unknown whether the main characteristic of emphysema, *i.e.* alveolar destruction,[12] in the absence of pulmonary inflammation can enhance atherosclerosis.[13] Intratracheal PPE

instillation induces a transient acute inflammatory response in the lung and, subsequently, alveolar destruction.[14] PPE-induced emphysema is thus a suitable model to investigate the role of alveolar destruction, in the absence of smoke-induced chronic inflammation, on atherosclerosis development.

The aim of this study was to assess whether emphysema *per se*, without concomitant pulmonary inflammation, enhances atherosclerosis development in atherosclerosis-prone *APOE*3-Leiden* (*E3L*) mice.[15] We found that mice with PPE-induced emphysema had similar plasma levels of lipids and inflammatory markers, but lower circulating monocyte and neutrophil levels. In line with these findings, their atherosclerotic lesion size was not different, while their atherosclerosis severity was reduced compared to the control mice without emphysema.

Materials and methods

Animals

Female *APOE*3-Leiden* (*E3L*) mice[15] of 10-12 weeks of age were housed under standard conditions with a 12-hour light/dark cycle and had free access to food and water. *E3L* mice represent a well-established model for human-like lipoprotein metabolism and develop atherosclerosis when fed a Western-type diet. All mice were fed a synthetic diet containing 15% (w/w) cacao butter (diet T, Hope Farms, Woerden, The Netherlands) for 3 weeks. Subsequently, mice were divided into 3 groups after matching for plasma cholesterol levels, age and body weight. To induce pulmonary emphysema, porcine pancreatic elastase (PPE) (E7885, Sigma-Aldrich, Schnelldorff, Germany) was administered intratracheally (15 µg or 30 µg in 40 µL PBS).[16, 17] Control mice received 40 µL sterile PBS (vehicle). After 3 weeks of recovery, all mice were fed a Western-type diet (*i.e.* diet T supplemented with 0.4% (w/w) cholesterol) to induce atherosclerosis development. All mice were weighed weekly and food intake was assessed for one week at 12 weeks after instillation of vehicle or PPE.

At baseline (before intratracheal PPE instillation) and every 4 weeks thereafter, blood was drawn in EDTA-coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding after 4 hours of fasting, and plasma was isolated by centrifugation. All animal experiments were approved by the Institutional Ethical Committee on Animal Care and Experimentation of the Leiden University Medical Center (Leiden, The Netherlands).

Pulmonary function measurements

Total respiratory amplitude and respiratory rate were assessed 13 weeks after vehicle or PPE instillation with non-invasive whole body plethysmography (RM-80, Columbus Instruments, Columbus, OH, USA) as described previously.[18] The total respiratory amplitude was calculated from the measured peak-to-peak signals and reflects the tidal volume. Flow derived parameters of breath amplitude and frequency were collected for and averaged over 2 minutes per mouse. The signal was digitized using a Digidata 1440A interface (Axon Instruments/Molecular Devices, Union City, CA, USA) and analyzed with the event detection feature of Clampfit 9.2 (Axon Instruments/Molecular Devices).

Plasma lipids and inflammatory markers

Plasma total cholesterol, triglyceride and phospholipid levels were determined using enzymatic kits from Roche Molecular Biochemicals (Woerden, The Netherlands) according to the manufacturer's protocols. The cholesterol distribution over plasma lipoproteins was determined after size-fractionation of pooled plasma samples using an ÄKTA fast performance liquid chromatography (FPLC) system (Pharmacia, Roosendaal, The Netherlands).[19]

Plasma levels of serum amyloid A (SAA) and soluble E-selectin (sE-selectin) were determined at 24 weeks after intratracheal PPE instillation using the murine SAA assay kit (Tridelta, County Kildare, Ireland) and murine E-selectin ELISA kit (R&D, Minneapolis, MN), respectively, according to manufacturer's instructions.

Blood count analysis

Eight weeks after intratracheal vehicle or PPE instillation, unfasted blood was collected in EDTA-coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding of mice. Complete blood cell count and hematological analysis was performed in whole blood using a Sysmex XT-2000iV veterinary hematology analyzer (Sysmex Corporation, Kobe, Japan), as described previously.[20] The XT-2000iV employs a fluorescent flow cytometry method using a fluorescent dye to stain cellular DNA and RNA and a semiconductor laser to detect forward-, side-scattered, and fluorescent light.

Arterial blood and tissue collection

Mice were anesthetized by intraperitoneal injection of 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.31 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands) 24 weeks after intratracheal PPE instillation. Blood was obtained by cardiac puncture. Subsequently, the mice were sacrificed by cervical dislocation and the pulmonary and systemic circulation was rinsed with ice-cold PBS. The lungs were fixated *in situ* by gentle infusion of fixative (phosphate-buffered 4% formaldehyde) by a continuous-release pump under constant pressure (12 mL/hour; 8 min) through a tracheal cannula. After excision, the lungs and heart were immersed in fresh fixative for a period of 24 hours at 4°C.

Histological analysis of the lungs

Lungs were processed for paraffin embedding and cut in 5 µm coronal sections. Tissue samples were stained with hematoxylin-eosin (HE). To assess air space enlargement, the mean linear intercept (MLI) and air/tissue ratio was quantified by one observer in a blinded fashion by superimposing a line grid with 21 lines and 42 points on the images of lung sections at a magnification of 200x as described previously.[21] To calculate the MLI, the number of intersections between the lines of the grid and the alveolar walls was quantified for each mouse in 10 non-overlapping fields. To determine the air/tissue ratio, the number of points in alveolar space was counted.

For immunohistochemical staining of macrophages and neutrophils in the lungs, rat anti-mouse MAC-3 antibody (1:50, BD Pharmingen, Breda, The Netherlands)[22] and anti-

myeloperoxidase (MPO) (1:1500, Thermo Fisher scientific, Runcorn, United Kingdom)[21] were used as described previously. Results of MAC-3- and MPO-positive cells are represented as the average count from 10 non-overlapping fields per mouse (400x magnification) corrected for tissue density by calculating the ratio between the number of cells and the average area of tissue per field. To determine the average area of tissue, the number of points superimposed on alveolar tissue was counted (400x magnification), using the same line grid for assessment of air space enlargement, as described above.

Histological analysis of the heart and aorta

Hearts were isolated and fixed in phosphate-buffered 4% formaldehyde, dehydrated and embedded in paraffin. A 5 μm transversal section of the heart halfway in the long axis was stained with HE. Thickness of the right and left ventricular free walls was assessed at a 40x magnification by averaging 6 measurements per structure with the NIH Image J program.

For quantification and classification of atherosclerosis, the hearts were cross-sectioned (5 μm) throughout the entire aortic root area. Per mouse, 4 sections with 40-μm intervals were used for quantification of atherosclerotic lesion area and characterization of lesion severity. Sections were stained with hematoxylin-phloxine-saffron. According to the guidelines of the American Heart Association, adapted for mice,[23, 24] atherosclerotic lesions were categorized for severity as follows:

- Type I: early fatty streak: per section up to 10 foam cells present in the intima.
- Type II: regular fatty streak: more than 10 foam cells present in the intima.
- Type III: mild plaque: extension of foam cells into the media and covered by a fibrotic cap.
- Type IV: moderate plaque: a more progressive lesion infiltrating into the media, fibrosis in the media, without loss of architecture.
- Type V: severe plaque: the media is severely damaged, elastic lamina are broken, presence of cholesterol clefts, mineralization and/or necrosis.

All segments were categorized into: 1) no lesions (undiseased) 2) mild (type I-III) and 3) severe (type IV-V) lesions. The percentage of lesion-free segments and the percentages of lesions belonging to the respective lesion categories were calculated.

AIA31240 rabbit antiserum (1:1000, Accurate Chemical and Scientific, Westbury, NY) was used for determination of adhering monocytes and macrophage- and mouse monoclonal antibody M0851 (1:800, Dako, Carpinteria, CA) for quantification of smooth muscle cell content in the lesions, described previously.[25] Sirius red (Chroma, Stuttgart, Germany) was used to stain for collagen in the lesions.

Total lesion area, macrophage -, smooth muscle cell - and collagen content were quantified using Cell^D image analysis software (Olympus Soft Imaging Solutions, Münster, Germany).

Statistical analysis

Statistical differences were assessed with one-way ANOVA analysis, followed by post-hoc analysis using Fisher's LSD multiple comparison test. For lesion typing, differences were

assessed by the χ^2 test. SPSS 16.0 for Windows (SPSS, Chicago, Ill) was used for statistical analysis. Differences at $P<0.05$ were regarded as statistically significant. Data are presented as mean ± SEM.

Results

Intratracheal administration of PPE induces emphysema and increases the total respiratory amplitude

To investigate whether PPE-induced emphysema results in changes in respiration pattern, we used non-invasive whole body plethysmography. PPE instillation dose-dependently increased the total respiratory amplitude of the measured signal, reflecting an increased tidal volume (Fig. 1A). The respiration rate was not different between the groups (Fig. 1B).

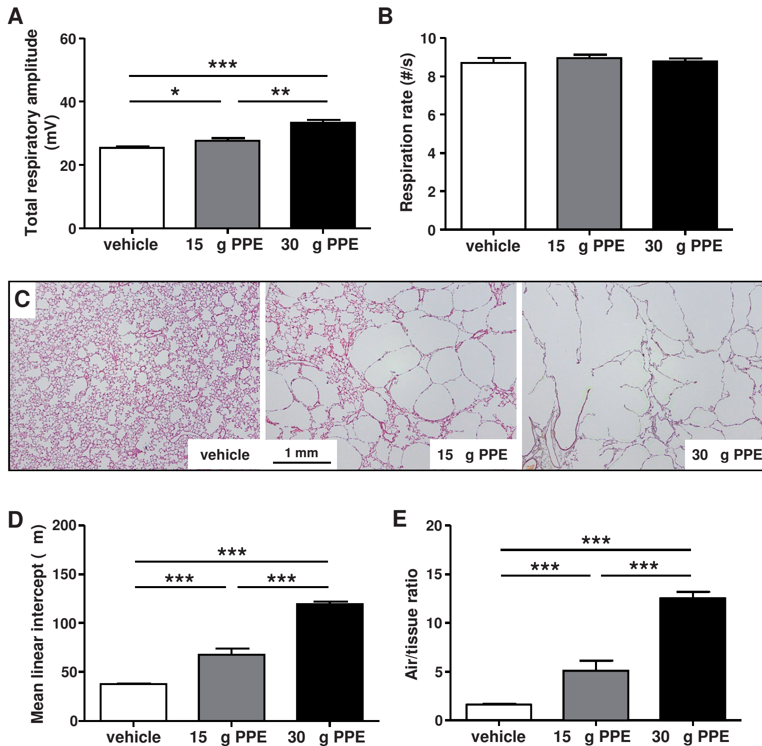

Fig. 1. Intratracheal PPE instillation dose-dependently increases total respiratory amplitude, mean linear intercept (MLI) and air/tissue ratio. Thirteen weeks after instillation of vehicle or PPE, total respiratory amplitude (A) and respiration rate (B) were analyzed by non-invasive whole body plethysmography. Twenty-four weeks after instillation *E3L* mice were sacrificed. The lungs were isolated and sections were HE-stained (C) to determine MLI (D) and air/tissue ratio (E). Values are means ± SEM; n=13-18; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

To determine the extent of emphysema development after intratracheal PPE instillation, the lungs were perfused under continuous pressure at 24 weeks after PPE instillation and analyzed by morphometry (Fig. 1C). PPE instillation caused a significant dose-dependent increase in mean linear intercept (MLI) (Fig. 1D) and air/tissue ratio (Fig. 1E) as compared to instillation of vehicle, indicating destruction and enlargement of alveolar space. To assess whether PPE instillation generates prolonged inflammatory cell influx into the lungs, we also quantified the number of macrophages and neutrophils by immunohistochemistry. No differences in the number of macrophages and neutrophils in lung tissue were observed (Fig. 2A-B).

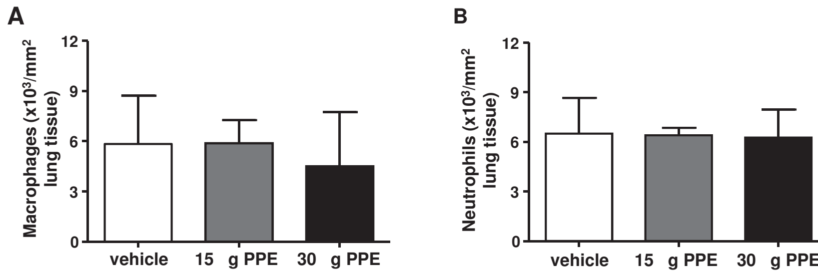

Fig. 2. Intratracheal instillation of PPE does not affect number of macrophages and neutrophils in the lungs. Twenty-four weeks after instillation of vehicle or PPE, *E3L* mice were sacrificed and the lungs were isolated to determine the number of macrophages (A) and neutrophils (B) by immunohistochemistry. Values are means ± SEM; n=13-16.

Intratracheal administration of PPE induces right ventricular hypertrophy

In a previous study in which mice were treated repetitively with PPE, right ventricular hypertrophy was observed.[17] Therefore, we determined whether the development of emphysema also affected the pulmonary circulation and the heart in this study. We found a dose-dependent increase in right ventricular hypertrophy with a single dose of PPE (Table 1 and Fig. 3A-B), which was also reflected by a significantly higher total heart weight/body weight ratio in the mice treated with the highest dose of PPE (Table 1).

Table 1. Intratracheal PPE instillation increases heart weight and induces right ventricular hypertrophy.

Group	RV free wall thickness (mm)	LV free wall thickness (mm)	Heart weight / body weight ($x10^{-3}$)
Vehicle	0.21 ± 0.009	0.96 ± 0.02	4.9 ± 0.1
15 µg PPE	0.25 ± 0.020**	0.97 ± 0.04	4.8 ± 0.1
30 µg PPE	0.30 ± 0.008***$^{\Delta\Delta}$	1.00 ± 0.03	5.1 ± 0.1$^{\Delta}$

Twenty-four weeks after intratracheal vehicle or PPE instillation, mice were weighed and sacrificed. The hearts were isolated, weighed and processed for histological analysis. Values are means ± SEM; n=11-18; ***P*<0.01, ****P*<0.001 compared to vehicle, $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ compared to 15 µg PPE.

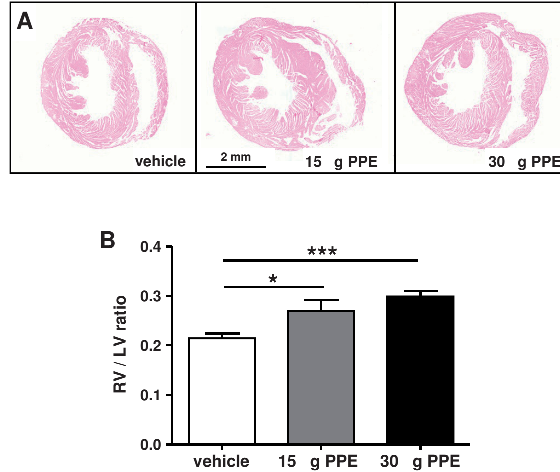

Fig. 3. Intratracheal PPE instillation dose-dependently induces right ventricular hypertrophy. Twenty-four weeks after intratracheal instillation of vehicle or PPE, *E3L* mice were sacrificed and the hearts were isolated. Transversal sections of the hearts were HE-stained (A). Right ventricular (RV) and left ventricular (LV) wall thickness was determined and the RV/LV was calculated (B). Values are means ± SEM; n=11-18; $^{*}P<0.05$, $^{***}P<0.001$.

Intratracheal administration of PPE reduces circulating monocytes and neutrophils

To evaluate whether PPE instillation affected systemic inflammation, we measured leukocytes concentration in whole blood and plasma levels of the acute-phase proteins SAA and sE-selectin. Circulating levels of monocytes and of neutrophils were dose-dependently decreased in the PPE-treated groups. Despite this decrease, the concentration of total leukocytes was similar between the groups, since the majority (86%) of leukocytes consisted of lymphocytes, which was unaffected by PPE treatment (Fig. 4A-D). Plasma levels of SAA and sE-selectin were not different between groups (not shown).

As a measure for chronic hypoxia, erythrocyte count, hemoglobin and hematocrit were determined. The total number of erythrocytes, the hemoglobin concentration and hematocrit value in blood were increased in the 30 µg PPE group (Table 2).

Table 2. Intratracheal PPE instillation increases number of erythrocytes, hemoglobin concentration and hematocrit.

Group	Erythrocytes (x10^{6}/µL)	Hemoglobin (mmol/L)	Hematocrit (vol%)
Vehicle	8.7 ± 0.5	8.2 ± 0.4	40.1 ± 2.1
15 µg PPE	8.5 ± 0.6	8.4 ± 0.4	39.4 ± 2.5
30 µg PPE	9.9 ± 0.3$^{\Delta}$	9.2 ± 0.1*	45.2 ± 1.0$^{*\Delta}$

Eight weeks after intratracheal vehicle or PPE instillation, blood was collected to determine the number of erythrocytes, hemoglobin concentration and hematocrit. Values are means ± SEM; n=13-18; $^{*}P<0.05$ compared to vehicle, $^{\Delta}P<0.05$ compared to 15 µg PPE.

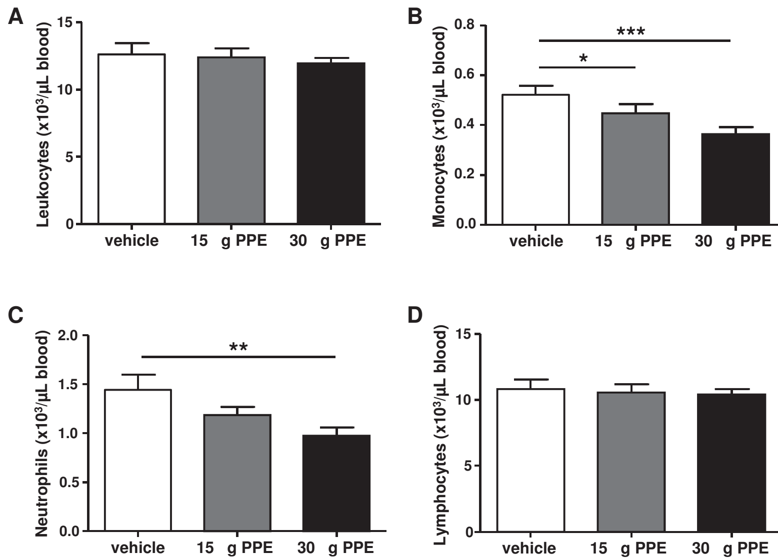

Fig. 4. PPE-induced emphysema dose-dependently reduces circulating monocytes and neutrophils. Eight weeks after intratracheal instillation of vehicle or PPE, blood was drawn to determine total leukocyte count (A), monocytes (B), neutrophils (C) and lymphocytes (D) using an automated veterinary hematology analyzer. Values are means ± SEM; n=13-16; **P*<0.05, ***P*<0.01, ****P*<0.001.

Intratracheal administration of PPE does not affect plasma lipids

PPE treatment did not affect body weight gain (Table 3) and food intake (2.8 ± 0.15 g/mouse/day for vehicle; 2.7 ± 0.11 for 15 µg PPE; 2.3 ± 0.07 for 30 µg PPE). Since dyslipidemia is a major contributor to atherosclerosis development, we next assessed the possibility that PPE-induced emphysema affects plasma lipid levels. After PPE or vehicle instillation, mice had a recovery period of 3 weeks before the mice were fed the Western-type diet (containing 0.4% cholesterol, w/w). By using this design, PPE activity and the accompanying inflammatory response had resolved[26,27] before atherosclerosis development was initiated. Plasma total cholesterol levels (Fig. 5A), as well as triglycerides and phospholipids levels (not shown) were similar in all groups throughout the study. In addition, the plasma lipoprotein distribution was not different (not shown).

Table 3. Intratracheal PPE instillation does not affect body weight.

	Body weight		
Group	**Before instillation (t=0 wks)**	**Before start of diet (t=4 wks)**	**At end of study (t=24 wks)**
Vehicle	20.1 ± 0.3	21.3 ± 0.28	36.7 ± 0.44
15 µg PPE	20.2 ± 0.4	21.5 ± 0.40	37.3 ± 0.58
30 µg PPE	20.3 ± 0.3	21.6 ± 0.23	37.2 ± 0.52

Values are means ± SEM; n=13-18.

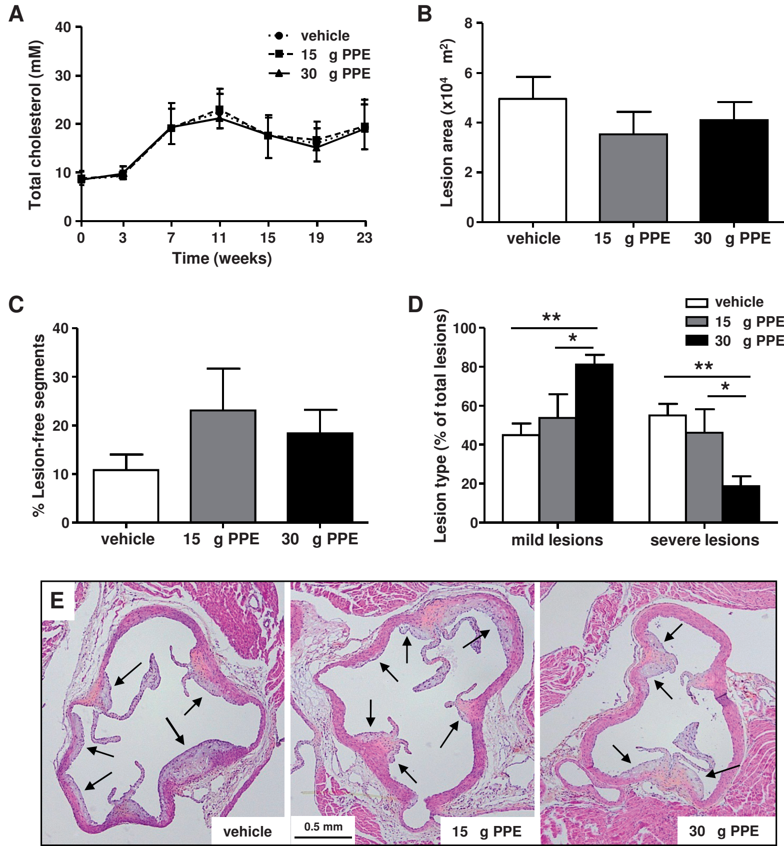

Fig. 5. Intratracheal PPE instillation does not affect atherosclerosis lesion area, but reduces lesion severity. Three weeks after intratracheal vehicle or PPE instillation, mice were fed a Western-type diet. Every 4 weeks thereafter, blood was drawn to assess plasma cholesterol levels (A). After 20 weeks of diet, the mice were sacrificed and the total lesion area was determined in the segments between the three aortic valves (B). In addition, the percentages of lesion-free segments (C), and with mild and severe lesions (D) were assessed. Representative pictures of each group are shown (E). Arrows indicate lesions. Values are means ± SEM, n=12-18; *P<0.05, **P<0.01.

Intratracheal administration of PPE reduces atherosclerotic lesion severity

To study whether PPE-induced emphysema enhanced atherosclerosis development in the absence of chronic pulmonal inflammation, we determined the atherosclerotic plaque area and plaque severity in the aortic root area of the heart. Total lesion area, lesion severity and lesion composition were scored in the segments between the three aortic valves. PPE instillation did not affect atherosclerotic lesion size and percentage of lesion-free segments as compared to vehicle-treated control mice (Fig. 5B-C). However, classification of the segments that contained lesions showed a dose-dependent decrease in severity in the PPE-treated groups, as is demonstrated by an increased percentage of mild lesions and a decreased percentage of

severe lesions (Fig. 5D-E). With respect to the lesion composition, no differences in adhering monocytes to the vessel wall and lesion content of macrophages and smooth muscle actin was observed (Fig. 6A-C). The group treated with 15 μg of PPE had a significantly higher collagen content in the lesions (Fig. 6D) compared to the control group, but no difference was observed between the 30 μg PPE group and the control or 15 μg PPE groups.

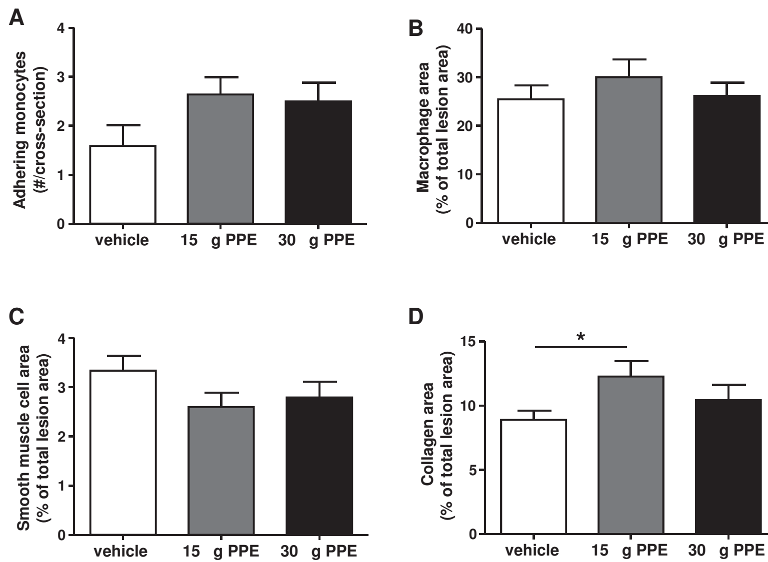

Fig. 6. Intratracheal PPE instillation does not affect adhering monocytes and tends to increase collagen content. Twenty-four weeks after intratracheal vehicle or PPE instillation, mice were sacrificed and the number of adhering monocytes (A), macrophage content (B), smooth muscle content (C) and collagen content (D) in the atherosclerotic lesions in the aortic root was determined. Values are means ± SEM; n=12-18; *$P<0.05$.

Discussion

There is ample clinical evidence that a poor lung function, most commonly caused by COPD, is an independent predictor of CVD.[28,29,30] Since smoking is the leading cause of COPD, smoke models have been utilized to investigate the relationship between pulmonary emphysema and atherosclerosis.[31, 32] Smoking itself, however, has a direct effect on atherogenesis, *i.e.* through induction of a systemic inflammatory response and dyslipidemia, and therefore interferes with the elucidation of the role of the different aspects of COPD in atherosclerosis development. Furthermore, although the majority of COPD patients are current or former smokers (~90%), the disease also occurs in patients who have never smoked.[33] Emphysema, characterized by alveolar tissue destruction, is a common manifestation of COPD, but its role in atherosclerosis development independent of systemic inflammation has never been studied. Therefore, in the present study we investigated whether emphysema in the absence of smoke-induced pulmonary inflammation would affect atherosclerosis development.

For the first time we show that PPE-induced emphysema *per se* does not worsen atherosclerosis development. Atherosclerotic lesion size and composition were not different between the groups. Thus, our results indicate that alveolar destruction alone cannot explain the increased incidence of CVD in COPD patients. Systemic inflammation has been regarded as a possible mediator between COPD and CVD,[4,34] although the evidence for this link is circumstantial.[3] Nevertheless, because of the known role of systemic inflammation in aggravating CVD, the absence of enhanced systemic inflammation in the current study may explain why atherosclerosis was not affected by the presence of emphysema in our study. Our results are in line with a study showing that allergic airways inflammation induced by *Aspergillus fumigatus*, resulted in marked increase in pulmonary cytokine levels and inflammatory cell influx and mild increased plasma SAA levels, but did not affect atherosclerosis development in *apoe*$^{-/-}$ mice.[35] This indicates that an increase in pulmonary (and subsequent systemic) inflammation does not necessarily affect atherosclerosis development. In our study, emphysema was developed before the induction of atherosclerosis, whereas in humans these diseases often develop concomitantly. The reason for this study design is that we aimed to study the effect of alveolar destruction separate from the effect of lung inflammation and spill over in the systemic circulation. Therefore, the study was designed to make sure that during development of atherosclerosis, which was started 3 weeks after PPE instillation to induce emphysema, systemic inflammation due to a spillover from the lung is no longer a contributing factor. Future studies using a different study design and other experimental COPD models should therefore focus on whether pulmonary inflammation by itself, or synergistically with alveolar destruction affects atherosclerosis development.

Unexpectedly, we found a dose-dependent decrease in lesion severity in the PPE-treated groups. We therefore considered the possibility that PPE treatment affected mechanisms involved in atherosclerosis development. First, dyslipidemia is one of the main contributors to atherosclerosis development. We found however, that intratracheal PPE instillation did not affect plasma lipid levels nor the lipoprotein profile. Therefore, the moderating effect of PPE-induced emphysema on atherosclerosis severity in our mouse model cannot be explained by a reduction in lipid levels. Second, inflammation is another well-known player in atherogenesis and may be decreased in experimental emphysema. A reduction of inflammation in the lungs and accompanying decrease in systemic inflammation in the PPE-treated mice may explain the observed decrease in atherosclerosis severity. However, at the same time point of determination of atherosclerosis development, *i.e.* 24 weeks after intratracheal PPE instillation, no differences in the number of macrophages and neutrophils in the lungs were found, making this mechanism unlikely. On the other hand, we found a reduction in circulating monocytes and neutrophils after intratracheal PPE instillation, which may have contributed to the reduced atherosclerotic lesion severity in these mice.

How do we explain the observed decrease in the number of circulating monocytes and neutrophils? Recruitment of monocytes and neutrophils towards the lung during the acute phase of inflammation due to PPE-induced damage could be a mechanism.[36] This is however an unlikely explanation, since the acute inflammatory reaction after intratracheal administration of PPE to rodents is resolved within 3 weeks,[37,38] and we found that the number of macrophages

and neutrophils in lung tissue at the end of the study was not affected by PPE treatment. A second potential mechanism for the decrease in circulating monocytes and neutrophils in the PPE-treated animals is that it is part of a resolution process. Resolution after inflammation is programmed within the normal inflammatory response itself after an acute injury to minimize tissue and organ damage.[39] Possibly, this resolution of inflammation reduces systemic inflammation involved in atherosclerosis. Failure of effective resolution has been suggested to be a contributor to the dysregulated inflammatory process in atherosclerosis development.[40] In addition, the decrease in circulating monocytes and neutrophils may be the result of a progressive negative feedback mechanism for the production of inflammatory cells, after the acute inflammation induced by PPE instillation. In view of the time span of 8 weeks between the intratracheal PPE instillation and the observed reduction in circulating monocytes and neutrophils, this possibility is not presumable. It is likely that there are other causes for this reduction which are currently unknown to us.

Another potential mechanism linking COPD to enhanced CVD is chronic hypoxia.[2] PPE-treated mice also showed signs of chronic hypoxia, indicated by a significantly higher number of circulating erythrocytes, hemoglobin and hematocrit levels. As it is known that hypoxia can promote atherogenesis,[41] it seems contradictory that the mice treated with PPE in our study showed less severe atherosclerotic lesions. However, the magnitude of hypoxia in the PPE-treated mice was likely compensated by several mechanisms. First, the respiration amplitude was increased, probably as a compensation mechanism for the reduction in diffusion area caused by PPE instillation. This compensating mechanism may have sufficed to prevent hypoxia to some degree, since the respiratory rate did not differ between the groups. In addition, hypoxia was probably at least partly compensated by the increase in the number of circulating erythrocytes. Moreover, the development of right ventricular hypertrophy as observed in the present study, and reported in COPD patients by others[42] may also have acted as a compensation mechanism. Therefore, in the PPE-treated mice several compensatory mechanisms are likely to have developed in order to minimize the level of hypoxia. Finally, the possibility that hypoxia does increase atherosclerosis only in presence of nicotine needs to be considered. Previous studies have shown that nicotine increases growth of atherosclerotic lesions in mice.[43] Since this effect was subsequently shown to be mediated by the α7-non-neuronal nicotinic acetylcholine receptor, and because the endothelial expression of this receptor is increased by hypoxia,[44] which may have resulted from emphysema in the present study, it is possible that nicotine-mediated effects on atherosclerotic plaque growth are increased in PPE-treated mice.

Finally, physical inactivity and obesity are risk factors for the development of atherosclerosis.[45] In the present study, PPE treatment did not affect body weight gain and food intake. Therefore, it is unlikely that these factors have influenced atherosclerosis development. Although we did not measure the physical activity of the mice, there was no evident difference in appearance and behavior between the groups, but we cannot formally exclude this.

In summary, our results indicate that in atherosclerosis-prone *E3L* mice, emphysema *per se*, in the absence of inflammation, does not aggravate the development of atherosclerosis size and even diminishes its severity. Thereby, this study has provided more mechanistic insight into the development of the major comorbidity CVD in COPD patients.

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Resveratrol Protects against Atherosclerosis, but Does not Add to the Anti-Atherogenic Effect of Atorvastatin, in *APOE*3-Leiden.CETP* Mice

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Abstract

Resveratrol is a major constituent of traditional Asian medicinal herbs and red wine and is suggested to be a potential anti-atherosclerotic drug, due to its proposed hypolipidemic, anti-inflammatory and anti-oxidative properties. The aim of this study was to evaluate whether resveratrol protects against atherosclerosis development in *APOE*3-Leiden.CETP* (*E3L.CETP*) mice, and adds to the anti-atherogenic effect of mild statin treatment, currently the most widely used anti-atherogenic therapy. *E3L.CETP* mice were fed a cholesterol-rich diet without (control) or with resveratrol (0.01% w/w), atorvastatin (0.0027% w/w), or both for 14 weeks. During the study plasma lipid, inflammatory and oxidative stress parameters were determined. Resveratrol reduced atherosclerotic lesion area (-52%) in the aortic root, comparable to atorvastatin (-40%) and the combination of both drugs (-47%). The collagen/macrophage ratio in the atherosclerotic lesion, a marker of plaque stability, was increased by resveratrol (+108%), atorvastatin (+124%) and the combination (+154%). Resveratrol decreased plasma cholesterol levels (-19%) comparable to atorvastatin (-19%) and the combination (-22%), which was completely confined to (V)LDL-cholesterol levels in all groups. Posthoc analyses showed that the anti-atherogenic effect of atorvastatin could be explained by cholesterol lowering, while the anti-atherosclerotic effect of resveratrol could be attributed to factors additional to cholesterol lowering. Markers of inflammation and oxidative stress were not different, but resveratrol improved macrophage function. We conclude that resveratrol potently reduces atherosclerosis development and induces a more stable lesion phenotype in *E3L.CETP* mice. However, under the experimental conditions tested, resveratrol does not add to the anti-atherogenic effect of atorvastatin.

Introduction

Dyslipidemia, characterized by high plasma levels of VLDL and LDL, and low plasma levels of HDL, is a well recognized risk factor for atherosclerosis, the main cause for cardiovascular events. Current treatment of atherogenic dyslipidemia mainly aims at reducing plasma (V)LDL-cholesterol levels, for example by using statins, which effectively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. However, statin use prevents only 15-30% of all cardiovascular events.[1]

Atherosclerosis is considered to be a multifactorial inflammatory disease and optimal therapeutic treatment of atherosclerosis should thus encompass different approaches. In addition to dyslipidemia, oxidative stress, inflammation and macrophage foam cell formation are crucial processes in the development of atherosclerotic plaques.[2] (V)LDL particles are oxidized locally within the vascular wall, but also circulating oxidized (V)LDL can enter the vascular wall, and can trigger an inflammatory reaction by resident macrophages. This inflammatory reaction includes the production of cytokines, chemokines and reactive oxygen species (ROS), and results in upregulation of adhesion receptors on endothelial cells and recruitment of monocytes from the circulation.

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a phytoalexin produced in plants in response to stress, and is present in large amounts in some nuts, *Polygonum cuspidatum* (Japanese knotweed) and in the skin of red grapes. Consequently, resveratrol is a major constituent of traditional Asian medicinal herbs and red wine. Resveratrol has been shown to extend the life span of yeast[3] and mice,[4] to improve insulin sensitivity[4] and to prevent cancer development[5] in experimental models. Importantly, resveratrol has also been recognized to possess various anti-atherosclerotic activities, including hypolipidemic,[6-8], anti-oxidative[9-12] and anti-inflammatory[9,11,13,14] properties.

Despite these promising and diverse anti-atherosclerotic actions, studies addressing the effect of resveratrol on atherosclerosis are scarce. Recent preliminary reports suggest that resveratrol indeed reduces atherosclerosis development.[6,15,16] In the current study we investigated whether resveratrol protects against atherosclerosis development in *APOE*3-Leiden.CETP* (*E3L.CETP*) transgenic mice and whether resveratrol adds to mild atorvastatin treatment because of its proposed diverse anti-atherosclerotic properties. To address this, we used a dose of resveratrol (*i.e.* 11 mg/kg/day) similar to dosages in other experimental models (*i.e.* 2-20 mg/kg/day) in which resveratrol effectively reduced atherosclerosis.[6,15,16] To study a potential modulating effect of resveratrol on top of atorvastatin treatment, we used a mild atorvastatin dose (*i.e.* 2 mg/kg/day) aiming at a reduction in plasma cholesterol of 25%, which is approximately half of the maximal cholesterol-lowering effect of atorvastatin in this mouse model,[17,18] and similar to the reduction of plasma cholesterol in men. By using this strategy a (potentially) relevant additive effect of resveratrol could be better detected. *E3L.CETP* mice represent a unique murine atherosclerosis model for human-like lipoprotein metabolism that shows a similar response to lipid-lowering drugs including statins[19] and HDL-raising drugs[19,20] as humans. In contrast, classical atherosclerosis models have no human-like response to statins with respect to cholesterol lowering (*apoe*$^{-/-}$) or a variable response with respect to cholesterol

lowering and atherosclerosis development (*ldlr*$^{-/-}$).[21] In addition, *ldlr*$^{-/-}$ mice are unable to upregulate the LDLr after statin treatment, an important additional cholesterol-lowering characteristic of statin treatment. Therefore, the *E3L.CETP* model is thus a more suitable model than these classical models to study whether resveratrol is atheroprotective on top of atorvastatin treatment. Our results show that resveratrol markedly reduces atherosclerosis development, but does not add to the anti-atherogenic effect of atorvastatin.

Methods and materials

Animals

E3L mice were crossbred with mice expressing human cholesteryl ester transfer protein (CETP) under control of its natural flanking regions to generate heterozygous *E3L.CETP* mice.[19] Female *E3L.CETP* mice of 10-12 weeks of age were used for all studies. Mice were housed under standard conditions with a 12-h light/dark cycle and had free access to food and water. They were fed regular chow (Ssniff, Soest, Germany) or a Western-type diet (WTD) containing 15% (w/w) cacao butter (diet T, HopeFarms, Woerden, The Netherlands) supplemented with 0.15% (w/w) cholesterol (Sigma-Aldrich, Zwijndrecht, The Netherlands) with or without resveratrol (Sigma) and/or atorvastatin ([R-(R*,R*)]-2-(4-fluorophenyl)-ß,D-dihydroxy-5-(1-methylethyl)-3-phenyl-4[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid ($C_{33}H_{24}FN_2O_5$) (Lipitor®, Pfizer, Capelle a/d IJssel, The Netherlands). Body weight and food intake were monitored during the study. Food intake was monitored twice per week by determining the amount of diet consumed by the mice of each cage (each cage housed 3-5 mice), divided by the number of mice per cage. Unless indicated otherwise, blood was drawn after a 4-h fast in EDTA-containing cups by tail bleeding, and plasma was isolated by centrifugation and stored frozen at -80°C until further analyses. All animal experiments were approved by the Institutional Ethics Committee on Animal Care and Experimentation.

Atherosclerosis study and atherosclerosis quantification

During a run-in period of 5 weeks, all female *E3L.CETP* mice received the WTD (containing 0.15% w/w cholesterol). After matching into 4 groups based on age, body weight, and plasma cholesterol and triglyceride levels, the mice received the WTD either alone (control) or supplemented with resveratrol (0.01% w/w; 11 mg/kg/day), atorvastatin (0.0018% w/w; 2 mg/kg/day) or both, as described above. Since after 4 weeks of drug intervention the dose of atorvastatin did not result in an anticipated 25% reduction in plasma cholesterol levels, the dose of atorvastatin was adjusted to 0.0027% (w/w; 3 mg/kg/day) in the atorvastatin only group and in the combination group.

After 14 weeks of drug intervention, mice were killed by CO_2 inhalation. Blood was drawn via cardiac puncture for serum isolation and hearts were collected. Hearts were fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin, and perpendicular to the axis of the aorta cross-sectioned (5 μm) throughout the aortic root area starting from the appearance of open aortic valve leaflets. Per mouse, 4 sections with 50 μm intervals were used for atherosclerosis

measurements. Sections were stained with hematoxylin-phloxin-saffron for histological analysis. Lesions were categorized for severity according to the guidelines of the American Heart Association adapted for mice.[22] Various types of lesions were discerned; no lesions, mild lesions (types 1-3) and severe lesions (types 4-5). Lesion area was determined using Cell D imaging software (Olympus Soft Imaging Solutions, Münster, Germany). Immunohistochemistry for determination of lesion composition was performed as described previously.[22] Rabbit anti-mouse antibody AIA 31240 (1:3000; Accurate Chemical and Scientific, Westbury, NY) was used to quantify macrophage area and the number of monocytes adhering to the lesions.[23] Monoclonal mouse antibody M0851 (1:800; Dako, Heverlee, The Netherlands) against smooth muscle cell actin was used to quantify smooth muscle cell area. Sirius Red was used to quantify collagen area.

Plasma lipid, lipoprotein, and inflammatory marker analysis

Plasma was assayed for total cholesterol (TC) and triglycerides (TG) using commercially available enzymatic kits according to the manufacturer's protocols (236691 and 1488872, Roche Molecular Biochemicals, Indianapolis, IN). The cholesterol distribution over plasma lipoproteins was determined by fast performance liquid chromatography using pooled plasma (14-15 mice per pool).[24]

Plasma cytokine levels (*i.e.* IL-1ß, IL-6, IL-10, IL-12p70, TNFα, CXCL1) were assessed using a multiplex murine inflammatory cytokine profile immunoassay from Meso Scale Discovery (MSD) on a MSD 2400 plate reader according to the manufacturer's protocol (MSD, Gaithersburg, MD). Plasma levels of soluble E-selectin (sE-selectin; R&D systems, Abingdon, United Kingdom) and monocyte chemoattractant protein-1 (MCP-1; R&D systems) were determined according the manufacturers' instructions. Plasma levels of MCP-1 were determined in pooled plasma (7 pools per group with 2 mice per pool).

Hepatic and macrophage gene expression analysis

Total RNA from livers or from peritoneal macrophages was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. RNA quality of each sample was checked with the lab-on-a-chip technology using Experion Std Sens analysis kit (Biorad, Hercules, CA). One microgram of total RNA was converted to cDNA with iScript cDNA Synthesis kit (Biorad) and purified with Nucleospin Extract II kit (Macherey-Nagel). Real-Time PCR (RT-PCR) was conducted on the IQ5 PCR machine (Biorad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of hypoxanthine phosphoribosyltransferase (*hprt*) and cyclophilin (*cyclo*). Primer sequences are listed in Supplemental Table S1.

Analyses of oxidative stress markers

Serum levels of IgG1, IgG2a and IgM against oxidized LDL (oxLDL) were determined using the Mouse MonoAb ID kit (HRP) (Zymed Laboratories Inc, San Francisco, CA). Oxidized human LDL (5 µg/ml) dissolved in a $NaHCO_3$ buffer (pH 9.0) was coated overnight onto a flat-bottom 96-well high binding plate (Corning, NY). Subsequently, levels of IgG1, IgG2a and IgM in serum (dilution 1:3) were detected according to the manufacturer's instructions.

Urine from spontaneous urination upon handling of mice was collected in Eppendorf tubes and stored at -20°C until further analyses. The urinary excretion of 15(*S*)-8-*iso*-prostaglandin $F_2\alpha$ (8-*iso*-$PGF_2\alpha$) was determined in pooled urine samples (7 pools per group with 2 mice per pool) by GC-MS/MS after immunoaffinity column chromatography from Cayman Chemicals (Ann Arbor, MI) as described previously.[25] Values were corrected for urinary creatinine excretion, which was measured by GC-MS as described.[25]

Analyses of peritoneal macrophage function *in vitro*

Thioglycollate-elicited peritoneal macrophages from *E3L.CETP* mice were isolated and cholesterol efflux assay was performed as described previously.[26] Macrophages were loaded with [^{3}H]cholesterol-labeled acetylated LDL (AcLDL; 50 μg/ml) for 32 h and subsequently incubated overnight without or with the indicated concentrations of resveratrol. The next day these cholesterol-laden macrophages were incubated with lipid-free apoAI (10 μg protein/ml) or HDL (50 μg/ml) for 6 h and radioactivity was determined in the medium and cell lysates to determine the percentage of cholesterol efflux.

To study the effect of resveratrol on the uptake of AcLDL isolated peritoneal macrophages were incubated overnight without or with the indicated concentrations of resveratrol. The next day, the macrophages were incubated with [^{3}H]cholesteryl oleoyl ether-labeled AcLDL (10 μg protein/ml) for 6 h and radioactivity was measured in the cell lysates to determined the percentage of AcLDL uptake.

To determine the lipopolysaccharide (LPS)-induced TNFα response[27] peritoneal macrophages were incubated overnight with resveratrol (10 μM) and, subsequently, incubated for 4 h with the indicated concentrations of LPS (*Salmonella minnesota* Re595 LPS; Sigma). TNFα was determined in the medium by ELISA (OptEIA™ ELISA, BD Biosciences Pharmingen). For the macrophage assays, values were corrected for the cellular protein content.

Statistical analysis

Data are presented as mean ±SEM unless indicated otherwise. Differences were assessed by one-way ANOVA test followed by the Bonferroni posthoc test. Differences in plasma cholesterol levels were tested using the one-way ANOVA test followed by Dunnett's posthoc test. Two-way ANCOVA was performed to test for differences on atherosclerotic lesion area after controlling for the cholesterol-lowering capacity of the different treatments. The square root was taken of the atherosclerotic lesion area to linearize the relationship with plasma cholesterol exposure. A probability level (*P*) of 0.05 was considered significant. The Student's T-test was used to evaluate differences in the *in vitro* macrophage studies. SPSS 17.0 for Windows (SPSS, Chicago, IL) was used for statistical analysis.

Results

Resveratrol attenuates atherosclerosis development to a similar extent as atorvastatin

To study the effect of resveratrol on atherosclerosis development, *E3L.CETP* mice were fed a Western-type diet (WTD) without or with resveratrol, atorvastatin, or the combination of both

drugs. Neither of the treatments affected food intake and body weight during the study (not shown). Mice were sacrificed after 14 weeks of treatment and lesion size and lesion severity were determined in the valve area of the aortic root. Treatment with resveratrol reduced total atherosclerotic lesion area by -52% (P<0.01), whereas atorvastatin reduced this by -40% (P<0.05) as compared to control treated mice (Figure 1A). The combination of resveratrol and atorvastatin did not further reduce atherosclerotic lesion area (-47% as compared to the control group; P<0.01) as compared to single treatments. This reduction in lesion area was similar over all 4 sections of the sampled valve area of the aortic root (Figure 1B). Neither of the treatments significantly affected lesion severity as classified as mild (type 1-3) and severe (type 4-5) lesions (Figure 1C).

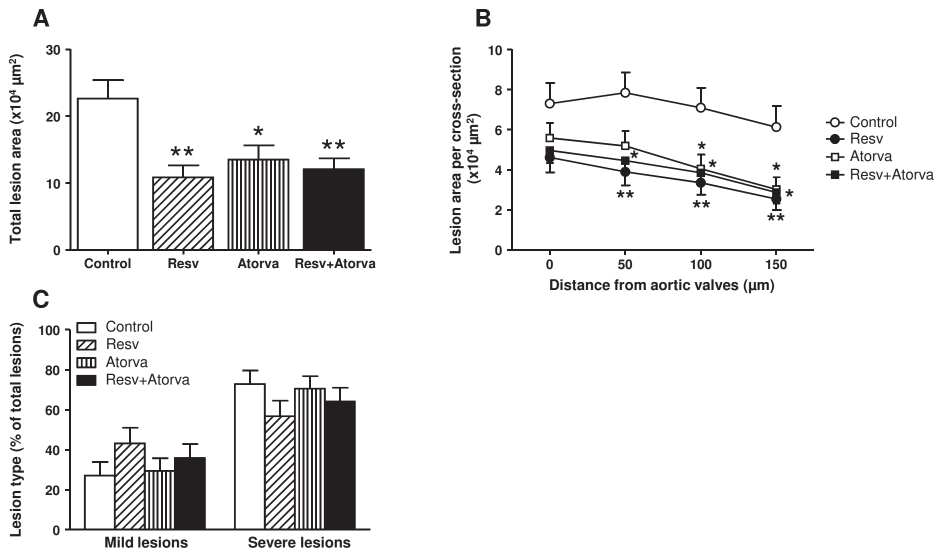

Fig. 1. Resveratrol, atorvastatin and the combination comparably reduce atherosclerosis development. Slides of the valve area of the aortic root were stained with hematoxylin-phloxin-saffron and total lesion area was calculated of 4 sections per mouse starting from the appearance of open aortic valve leaflets as described in *Methods and Materials* (A). Lesion area as a function of distance was determined by calculating the lesion area of each cross-section from A (B). The same 4 sections per mouse were categorized according to lesion severity (C). Values are means ±SEM (n=14-15). *P<0.05, **P<0.01 *vs* control group.

Resveratrol and atorvastatin induce stable lesions

Subsequently, we assessed whether the different treatments affected monocyte recruitment and lesion composition. With respect to lesion composition we characterized macrophage content, a destabilizing component in the lesions, and collagen and smooth muscle cell content, both stabilizing components in the lesions.[2] As shown in Figure 2, neither of the treatments affected the number of adhering monocytes to the endothelium. However, resveratrol tended to decrease macrophage content, whereas atorvastatin significantly decreased it (Figure 2B). Resveratrol enhanced smooth muscle cell and collagen content to a similar extent as

atorvastatin (Figure 2C+D). Both resveratrol and atorvastatin thus induced more stable lesions as indicated by an enhanced collagen/macrophage ratio (2.2-fold and 2.4-fold, respectively) (Figure 2E). The combination treatment did not have a significant additional effect on lesion composition as compared to the single treatments.

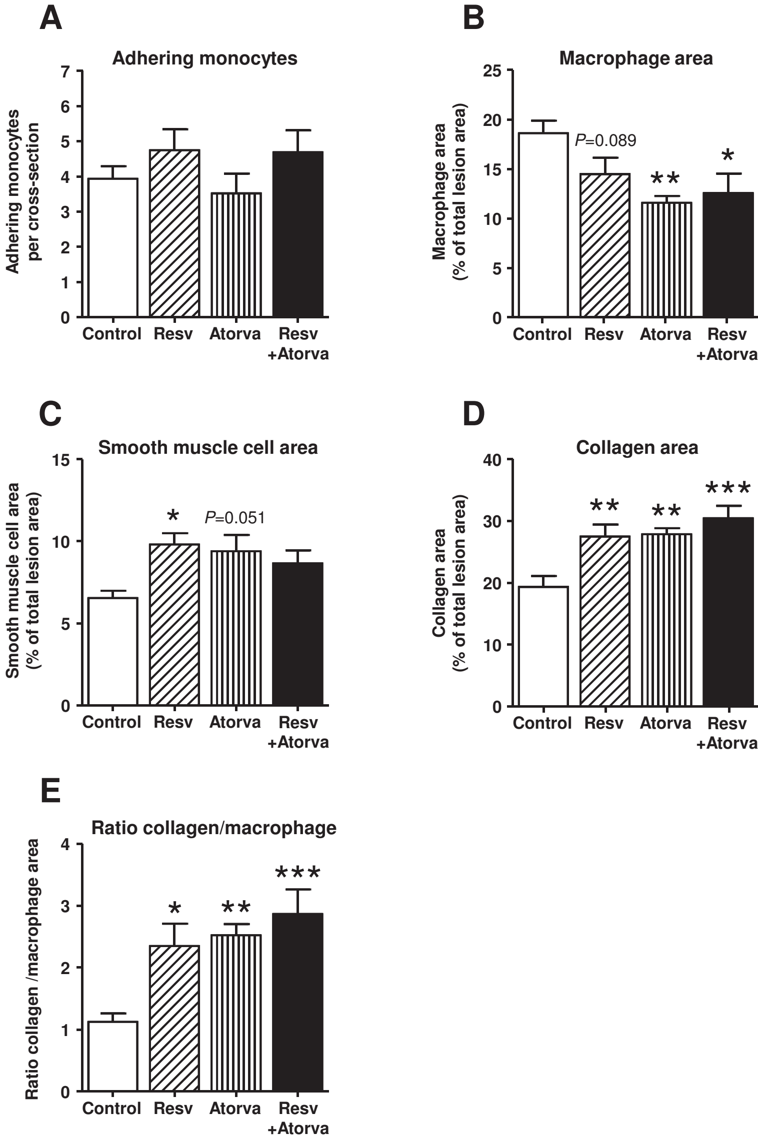

Fig. 2. Resveratrol, atorvastatin and the combination induce more stable lesions. The adhesion of monocytes to the lesions in the aortic root was determined (A) as were the macrophage content (B), collagen content (C), smooth muscle cell (SMC) content (D) and collagen/macrophage ratio (E) of the lesions. Values are means ±SEM (n=14-15). *P<0.05, **P<0.01, ***P<0.001 *vs* control group.

Resveratrol and atorvastatin reduce (V)LDL levels to a similar extent

To study whether the anti-atherogenic effect of the different treatments could be the result of hypolipidemic, anti-inflammatory and/or anti-oxidant properties of resveratrol and atorvastatin, we first determined the effect of the different treatments on plasma lipid levels. Plasma cholesterol levels were similarly reduced by resveratrol (-19%; *P*=0.077) and atorvastatin (-22%; *P*<0.05) as compared to the control group (Figure 3A). The combination treatment did not further reduce plasma cholesterol levels (-21% as compared to control-treated mice; *P*<0.05). Lipoprotein fractionation showed that for all treatments the reduction in plasma cholesterol levels was confined to the (V)LDL fraction, while HDL-cholesterol levels were not affected (Figure 3B).

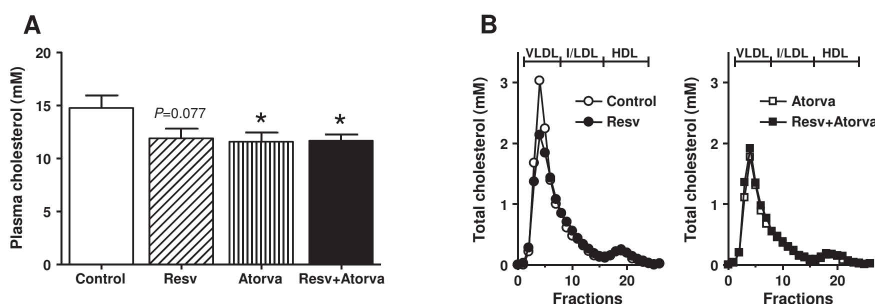

Fig. 3. Resveratrol, atorvastatin and the combination similarly lower plasma (V)LDL-cholesterol levels. Plasma cholesterol was determined after 8 weeks of treatment (A). The cholesterol distribution over lipoproteins was determined in pooled plasma (B). Values are means ±SEM (n=14-15). **P*<0.05 *vs* control group.

To investigate whether this reduction in (V)LDL could be caused by reduced hepatic production of VLDL and/or increased clearance of (V)LDL from plasma, we subsequently determined the expression of hepatic genes involved in cholesterol and TG metabolism (Supplemental Table S2). Resveratrol treatment did not affect hepatic mRNA expression of genes involved in cholesterol metabolism (*i.e. ldlr*, *sr-b1*, *abca1* and *abcg1*) and VLDL metabolism (*i.e. apob, mttp*, *fasn* and *lpl*). Treatment with atorvastatin and/or the combination altered the expression of genes involved in cholesterol metabolism (*i.e.* increased: *ldlr*; decreased: *abca1, abcg1* and *sr-b1*), but not those involved in VLDL metabolism, with the exception of a decreased expression of *lpl*.

Unlike for atorvastatin, the anti-atherogenic effect of resveratrol may not be solely explained by its plasma cholesterol-lowering capacity

Since our collective data suggest that the anti-atherogenic effect of resveratrol and atorvastatin may mainly depend on their ability to reduce plasma cholesterol, we performed 2-way ANCOVA analyses in which we controlled for the cholesterol-lowering effect of the treatments by using the cumulative plasma cholesterol exposure during the study (*i.e.* area under the

curve). The effect of resveratrol on atherosclerotic lesion area reduction remained significant (*F* = 5.34; *P*<0.05), whereas for atorvastatin treatment significance was lost (*F* = 0.88; *P*=0.36). This indicates that the reduction in atherosclerosis development upon atorvastatin treatment can be explained by its cholesterol lowering effect, while additional mechanism(s) could be involved in the anti-atherogenic effect of resveratrol.

Resveratrol and atorvastatin do not affect inflammatory parameters

To elucidate by which additional mechanism(s) resveratrol may be atheroprotective, we assessed the effect of the different treatments on inflammatory parameters in liver and plasma. Neither of the treatments affected hepatic mRNA levels of *il-1ß* and *il-6* (Supplemental Table S2) or a panel of plasma cytokine levels (*i.e.* IL-1ß, IL-6, IL-10, IL-12p70, TNFα and CXCL1, the murine homologue of IL-8), with the exception that the combination treatment reduced CXCL1 levels by -33% (*P*<0.05; not shown). Similarly, resveratrol and atorvastatin did not affect plasma levels of the acute-phase proteins soluble E-selectin (sE-selectin) and MCP-1, but the combination reduced plasma MCP-1 by -30% (*P*<0.05; not shown). These findings indicate that both resveratrol and atorvastatin do not substantially affect the hepatic and systemic inflammatory status of *E3L.CETP* mice.

Resveratrol and atorvastatin do not affect oxidative stress parameters

We next investigated the effect of resveratrol, atorvastatin and their combination on markers of oxidative stress. Resveratrol did not affect hepatic mRNA expression of enzymes involved in oxidative stress (*i.e. mnsod, pon1*, *cox1*, *cox2*, *lox1*) (Supplemental Table S2). Treatment with atorvastatin and/or the combination decreased the expression of *cox2*, *lox1* and *mnsod*, but did not affect expression of *pon1* and *cox1*. Furthermore, circulating levels of IgG1, IgG2a and IgM directed against oxLDL were not different between the four groups (not shown). Likewise, neither of the treatments affected urinary excretion of the isoprostane 15(*S*)-8-*iso*-prostaglandin $F_2\alpha$ (8-*iso*-$PGF_2\alpha$) (not shown), a biomarker of lipid peroxidation.[28]

Resveratrol beneficially influences macrophage function

Since macrophages are the most prominent inflammatory cells in the atherosclerotic lesion and involved in both the initiation, progression and regression of the atherosclerotic lesion,[2] we assessed the effect of resveratrol on macrophage function, *i.e.* cholesterol efflux and release of proinflammatory mediators. Resveratrol enhanced the cholesterol efflux from primary macrophages from *E3L.CETP* mice to apoAI in a dose-dependent manner up to +243% (*P*<0.05), but not to HDL (Figure 4). Moreover, resveratrol reduced the uptake of AcLDL up to -24% (*P*<0.05) as compared to vehicle-treated primary macrophages (Figure 5), without affecting the expression of genes involved in cholesterol efflux and/or uptake (*i.e. abca1, abcg1, sr-b1, sr-a1, cd36, lpl*) (not shown). In addition, resveratrol reduced the lipopolysaccharide (LPS)-induced TNFα secretion up to -63% (*P*<0.05) as compared to untreated primary macrophages (Figure 6). These data show that resveratrol beneficially influences macrophage function, indicating that resveratrol may have local anti-atherosclerotic effects in the vessel wall, which may explain its cholesterol-independent effect on atherosclerosis.

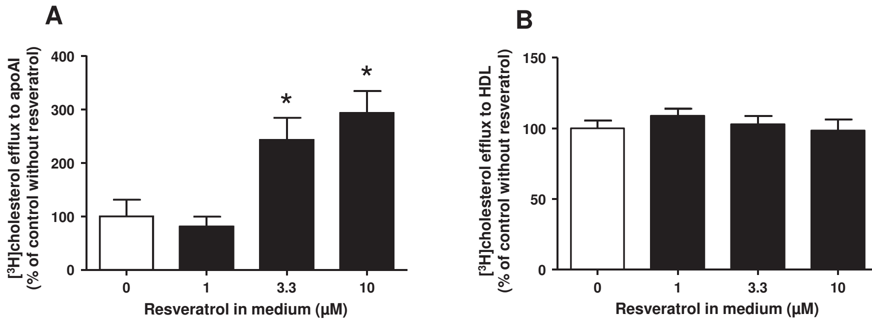

Fig. 4. Resveratrol enhances the cholesterol efflux from macrophages. [^{3}H]cholesterol-laden peritoneal macrophages from *E3L.CETP* mice were equilibrated overnight without or with the indicated concentrations resveratrol. The cholesterol efflux was determined after incubation without or with lipid-free apoAI (10 µg/ml) (A) and HDL (50 µg protein/ml) (B) as percentage of ^{3}H-activity released into the medium per µg cell protein. The data of 2 separate experiments were combined and expressed as percentage relative to the control without addition of resveratrol (white bar) ±SEM (n=8). *P<0.05 *vs* control.

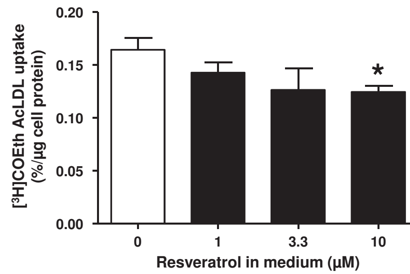

Fig. 5. Resveratrol reduces the uptake of AcLDL by macrophages. Peritoneal macrophages from *E3L.CETP* mice were equilibrated overnight without or with the indicated concentrations of resveratrol. The uptake of [^{3}H]cholesteryl oleoyl ether-labeled AcLDL ([^{3}H]COEth AcLDL; 10 µg protein/ml) was determined after 6 h. Values are expressed as the mean percentage of ^{3}H-activity per µg cell protein ±SEM (n=4). *P<0.05 *vs* control.

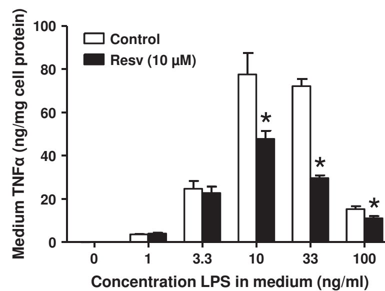

Fig. 6. Resveratrol reduces the LPS-induced inflammatory response of macrophages. Peritoneal macrophages from *E3L.CETP* mice were equilibrated overnight without or with resveratrol (10 µM). Subsequently, cells were incubated for 4 h with the indicated concentrations LPS, and TNFα secretion into the medium was determined. Values are expressed as means per mg cell protein ±SEM (n=4). *P<0.05 *vs* without resveratrol.

Discussion

In the present study we assessed the anti-atherosclerotic potential of resveratrol, either alone or in combination with atorvastatin. We showed that resveratrol reduces atherosclerosis development and improves plaque stability to a similar extent as atorvastatin in *E3L.CETP* mice. Whereas the anti-atherogenic effect of atorvastatin could be explained by its cholesterol-lowering effect, additional mechanisms may be involved in the anti-atherogenic effect of resveratrol, which may involve beneficial modulation of macrophage function. However, the combination of resveratrol and atorvastatin did not additively reduce atherosclerosis.

Our finding that resveratrol efficiently reduces atherosclerotic lesion development in *E3L.CETP* mice is in accordance with preliminary reports in literature. The first report suggested only qualitatively, that resveratrol reduced atherosclerosis development in the aortas of apoE-deficient mice.[6] The second report used a very limited number of mice (*i.e.* n=4-7), but nevertheless showed a significant reduction in atherosclerosis development in apoE/LDLr-double deficient mice.[15] The most recent report observed that resveratrol reduced the intima/media area ratio in New Zealand rabbits fed a 1% cholesterol diet.[16] The fact that resveratrol reduces atherosclerosis supports the hypothesis that resveratrol contributes to the "French Paradox", *i.e.* the notion that moderate and prolonged consumption of red wine is associated with decreased cardiovascular morbidity and mortality in the French population despite high dietary saturated fat consumption.[29] Resveratrol is a major constituent of the skin of red grapes and, therefore, abundantly present in red wine with concentrations ranging from 1-12 mg/L (*i.e.* 0.2-2 mg/glass).[30]

To assess the potential anti-atherosclerotic actions of resveratrol responsible for the protection against atherosclerosis, plasma lipid levels as well as inflammatory and oxidative stress parameters were studied. We found no relevant effect of resveratrol treatment on hepatic and systemic inflammation as indicated by hepatic mRNA expression of cytokines and circulating cytokine and acute-phase protein levels, respectively. Similarly, there was no effect of resveratrol on oxidative stress, as evidenced by unaltered circulating antibodies against oxLDL as well as unaltered urinary excretion of the isoprostane 8-*iso*-$PGF_2\alpha$. The latter is a biomarker of lipid peroxidation[28] and as such has been associated with coronary heart disease.[31] The fact that in our studies resveratrol treatment did not affect systemic inflammatory and oxidative stress parameters may be the result of the mild treatment strategy as compared to most other studies in which a reducing effect of resveratrol on these parameters was observed. We treated the mice chronically with 0.01% (w/w) resveratrol in the diet, comparable to 11 mg/kg/day, whereas most other acute or chronic experimental studies used at least 2.5- to 10-fold higher dosages.[6,9,10,13] We chose for this mild treatment strategy since comparable dosages (2-20 mg/kg/day) effectively reduced atherosclerosis development in the above mentioned preliminary reports.[6,15,16] Furthermore, this dose is similar to the recommended dosage of resveratrol supplementation in humans (ranging from 1-20 mg/kg/day; *e.g.* www.resveratrol.info; www.resveratrolreference.com). The anti-inflammatory and anti-oxidative effect of resveratrol *in vivo* may in particular be apparent after challenging the mice with inflammatory stimuli, since various reports describing the anti-inflammatory effect of resveratrol *in vivo* used such a

experimental set-up, *e.g.* by lipopolysaccharide (LPS) injection[13] or by inducing experimental colitis.[11]

Resveratrol treatment reduced plasma (V)LDL-cholesterol levels similarly as atorvastatin treatment. This hypolipidemic effect of resveratrol is in accordance with previous reports,[6-8] and can result from several mechanisms. First, resveratrol could reduce hepatic VLDL secretion, since resveratrol reduced apoB secretion in human HepG2 cells.[32] However, we did not observe differences in expression of several hepatic genes involved in VLDL biogenesis (*i.e.* *fasn*, *mttp*, *apob*), suggesting that resveratrol did not lower plasma lipids by reducing hepatic VLDL production in the present study. Nevertheless, one has to keep in mind that this does not exclude a potential effect of resveratrol on posttranslational modification of the proteins involved in VLDL biogenesis. Second, resveratrol treatment could enhance VLDL catabolism and/or uptake. However, the lack of an effect of resveratrol treatment on key hepatic genes involved in VLDL clearance (*i.e.* *ldlr*, *lpl*), the lack of a reduction in plasma TG levels (not shown) and the lack of studies in literature, so far do not provide further support for this mechanism. Third, resveratrol may inhibit HMG-CoA reductase, possibly via enhancing cytochrome P450 27-hydroxylase (CYP27A1) activity which syntheses the HMG-CoA reductase-inhibitor 27-hydroxycholesterol.[33] Resveratrol was shown to attenuate the expression of HMG-CoA reductase in hamsters[34] and reduce HMG-CoA reductase activity in mice.[6] Although this could contribute to the hypocholesterolemic effect in the present study, it is unlikely to be the prevailing mechanism. Resveratrol treatment did not alter the hepatic cholesterol content (*i.e.* free cholesterol and cholesteryl esters; Supplemental Figure S1), while treatment with the HMG-CoA reductase inhibitor atorvastatin did result in the expected reduction of hepatic cholesterol. Finally, we speculate that resveratrol treatment may reduce intestinal cholesterol absorption, but this has not been studied so far. Therefore, further studies are necessary to reveal the prevailing mechanism by which resveratrol lowers plasma cholesterol levels. Resveratrol was also suggested to increase plasma HDL-cholesterol levels in apoE-deficient mice.[6] However, it had no HDL-raising effect in our *E3L.CETP* model, which is otherwise highly responsive to HDL-cholesterol increasing drugs.[19,20]

The above considerations show that resveratrol mainly reduces atherosclerosis development as a result of lowering plasma (V)LDL-cholesterol levels. However, controlling for this cholesterol-lowering effect of resveratrol in the statistical analyses showed that additional anti-atherogenic mechanism(s) could be involved. If so, it is likely that resveratrol exerts these additional mechanism(s) locally in the vessel wall by beneficially altering macrophage function. We found that resveratrol improved at least three of the most important characteristics of macrophages related to atherosclerosis, *i.e.* potency to efflux cholesterol, formation of foam cells and the release of proinflammatory mediators. These anti-atherogenic actions of resveratrol in primary macrophages of *E3L.CETP* mice together indicate a potential local effect of resveratrol at the level of macrophages in the vessel wall in addition to its hypolipidemic effect. Our finding that resveratrol enhances the cholesterol efflux from primary macrophages is in line with previous findings by others.[33,35,36] They reported that resveratrol enhanced cholesterol efflux from macrophages via upregulation of the gene and protein expression of ABCA1 and

ABCG1. Upregulation of ABCG1 was not consistent in different cell types, which could explain the lack of an effect of resveratrol on cholesterol efflux towards HDL in our studies. Moreover, Voloshyna *et al.*[33] found that these effects were PPARγ- and adenosine 2A receptor-dependent. However, we were unable to observe an increase in ABCA1 expression. Thus, it is likely that resveratrol upregulated ABCA1 protein posttranscriptionally. Alternatively, resveratrol could have increased CYP27A1 activity,[33] which stimulates the efflux of cholesterol to apoAI by a yet undefined ABCA1-independent pathway.[37]

Resveratrol effectively protected against atherosclerosis development. Despite the suggested differences in underlying mechanisms, treatment with resveratrol did not add to atorvastatin treatment with respect to reducing atherosclerosis in our model. Since resveratrol may act on atherosclerosis by mechanism(s) in addition to cholesterol-lowering, these mechanistic pathways may become more prominent at higher dosage.

In summary, we have demonstrated that resveratrol potently reduces atherosclerosis development and results in more stable lesions in *E3L.CETP* mice, comparable to atorvastatin treatment. This anti-atherogenic effect of resveratrol is mainly explained by its plasma (V)LDL-cholesterol-lowering capacity, but may also include local anti-atherogenic effects in the vessel wall such as on macrophage function. Nonetheless, in the current experimental set-up resveratrol did not add to the effects of atorvastatin with respect to protection against atherosclerosis development and underlying atherosclerotic risk factors.

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Supplemental data

Supplemental Table S1. Primers used for quantitative RT-PCR analysis.

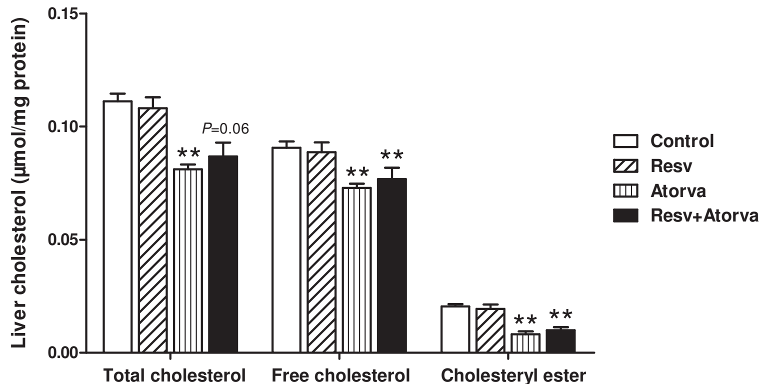
Gene	Forward primer	Reverse primer
Abca1	CCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
Abcg1	GGTCATCATCACTTTGGTCCTTG	TCTCTCGAAGTGAATGAAATTTATCG
Apob	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
Cd36	GCAAAGAACAGCAGCAAATC	CAGTGAAGGCTCAAAGATGG
Cox1	TGGGGTGCCCTCACCAGTCAA	TGGGGCCTGAGTAGCCCGTG
Cox2	GGCCATGGAGTGGACTTAAA	ACTGCAGGTTCTCAGGGATG
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
Fasn	TCCTGGGAGGAATGTAAACAGC	CACAAATTCATTCACTGCAGCC
Hprt	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Il-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Il-6	CCGGAGAGGAGACTTCACAG	TTCTGCAAGTGCATCATCGT
Ldlr	GCATCAGCTTGGACAAGGTGT	GGGAACAGCCACCATTGTTG
Lox1	CGGCAGACCTGCCAATCTTTGG	GGCCAGGCTTCTTCCGATGCA
Lpl	TTTTCTGGGACTGAGGATGG	GTCAGGCCAGCTGAAGTAGG
Mnsod	TCGCTGTGTCCTTGCGGACG	GCCTCCTGCCCGTGCTGC
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Pon1	TCCTCCCGGCTCAGAGGTGC	GTGGTGCCTTGCAGGACGGT
Sr-a1	CAGTCCAGGAACATGGGAAT	ACGTGCGCTTGTTCTTCTTT
Sr-b1	GTTGGTCACCATGGGCCA	CGTAGCCCCACAGGATCTCA

Abca1/abcg1, ATP-binding cassette transporter a1/g1; *Apob*, apolipoprotein B; *Cox1 / 2*, cyclooxygenase-1 / -2; *Cyclo*, cyclophilin; *Fasn*, fatty acid synthase; *Hprt*, hypoxanthine phosphoribosyl-transferase; *Il-1β / -6*, interleukin-1β / -6; *Ldlr*, low-density lipoprotein receptor; *Lox-1*, lectin-like oxidized low-density lipoprotein receptor-1; *Lpl*, lipoprotein lipase; *Mnsod*, manganese superoxide dismutase; *Mttp*, microsomal triglyceride transfer protein; *Pon1*, paraoxonase-1; *Sr-a1 / -b1*, scavenger receptor-A1 / -B1.

Supplemental Table S2. Effect of resveratrol and atorvastatin on hepatic gene expression.

Gene	Control	Resv	Atorva	Resv + Atorva
Lipid metabolism				
Abca1	1.00 ± 0.21	0.93 ± 0.19	0.69 ± 0.15*	0.76 ± 0.19
Abcg1	1.00 ± 0.22	0.81 ± 0.24	0.54 ± 0.19**	0.62 ± 0.05**
Apob	1.00 ± 0.28	0.94 ± 0.35	0.92 ± 0.29	1.00 ± 0.23
Fasn	1.00 ± 0.24	1.73 ± 1.14	1.59 ± 0.49	1.49 ± 0.91
Ldlr	1.00 ± 0.24	0.87 ± 0.37	1.36 ± 0.49	1.74 ± 0.55*
Lpl	1.00 ± 0.35	0.83 ± 0.16	0.54 ± 0.09**	0.55 ± 0.24**
Mttp	1.00 ± 0.18	0.94 ± 0.29	1.10 ± 0.26	1.05 ± 0.16
Sr-b1	1.00 ± 0.25	0.92 ± 0.29	0.62 ± 0.18*	0.73 ± 0.14
Inflammation				
IL-1β	1.00 ± 0.37	0.61 ± 0.24	0.56 ± 0.24	0.76 ± 0.51
IL-6	1.00 ± 0.34	0.81 ± 0.35	1.04 ± 0.55	1.36 ± 0.47
Oxidative stress				
Cox1	1.00 ± 0.32	1.01 ± 0.35	0.78 ± 0.21	0.80 ± 0.40
Cox2	1.00 ± 0.95	0.57 ± 0.45	0.37 ± 0.24	0.38 ± 0.16*
Lox1	1.00 ± 0.72	0.56 ± 0.30	0.42 ± 0.17**	0.36 ± 0.10**
Mnsod	1.00 ± 0.22	0.92 ± 0.26	0.77 ± 0.22	0.67 ± 0.24*
Pon1	1.00 ± 0.15	0.94 ± 0.10	1.21 ± 0.32	1.08 ± 0.32

See the legend of Supplemental Table S1 for explanation of abbreviations. Statistical differences were assessed with one-way ANOVA followed by Bonferroni posthoc test. *P<0.05, **P<0.01 *vs* control group.



Supplemental Fig. S1. Resveratrol does not reduce the hepatic cholesterol content. The hepatic total cholesterol, free cholesterol and cholesteryl ester content were determined according to Wong *et al.* [Wong MC 2012 Atherosclerosis] in livers of *E3L.CETP* mice that were treated with either no drug (control), resveratrol (Resv), atorvastatin (Atorva) or the combination (Resv+Atorva) for 14 weeks. Values are means ± SEM (n=14-15). Statistical differences were assessed with one-way ANOVA followed by Bonferroni posthoc test. **P<0.01 *vs* control group.

General Discussion and Future Perspectives

(Extra)vascular inflammation and lipid metabolism in atherogenesis

Dyslipidemia has been mainly associated with metabolic diseases, and classically regarded as the most prominent underlying cause of atherosclerosis. As a consequence, modulation of lipids gained most interest in the treatment of cardiovascular diseases (CVDs). Although lipid-modulating drugs have been studied extensively and applied on large scale in the clinic, two thirds of cardiovascular events cannot be prevented by current lipid-modifying strategies, and CVD remains to be the leading cause of mortality in the Westernized world.[1] While in recent decades inflammatory processes have been recognized to contribute largely to atherosclerosis development as well, the therapeutic potential of anti-inflammatory drugs in atherosclerosis development is less studied and well-defined.

This disparity can be partly explained by the large role of inflammation in many physiological and pathophysiological processes other than CVD. Interference with physiological inflammatory processes can impair human host defense and result in life-threatening infections. While a healthy immune response includes sporadic bouts of acute inflammation to fight harmful stimuli, chronic low-grade inflammation is associated with the development of major diseases such as cancer, diabetes, asthma, rheumatoid arthritis, and atherosclerosis.[2]

So far, many traditional anti-inflammatory therapies do not improve cardiovascular outcomes, and some may even aggravate cardiovascular events. These observations are usually derived from post-hoc analyses of clinical studies which may reflect off-target actions of the drugs studied, *e.g.* glucocorticoids, non-steroidal anti-inflammatory drugs, or tumor necrosis factor (TNF) inhibitors.[3] While general suppression of inflammation is undesirable, selective regulators of inflammation that are able to normalize the enhanced inflammation or skew the inflammatory response towards the anti-inflammatory side or resolution phase may be beneficial.

In the past two decades atherosclerosis research has focused primarily on local vascular inflammation.[4] The crosstalk between dyslipidemia and inflammatory processes within and close to the arterial wall has been shown to be the primary cause of atherogenesis. Not only in the vessel wall, but also within other organs or tissues there is interaction between lipid metabolism and inflammation. This is for example demonstrated in **chapter 2**, in which increased activity of hepatocyte-specific nuclear factor-κB (NF-κB), primarily known as a central regulator of inflammatory processes, was shown to increase VLDL production by these hepatocytes. In addition, we found that enhanced activation of NF-κB in hepatocytes results in aggravated atherosclerosis development in **chapter 3**. This latter study exemplifies the increasing interest in the interaction between different organs and tissues with the vascular wall in the elucidation of the underlying processes involved in atherosclerosis development (Figure 1). While changes in certain tissues or organs can affect atherogenesis, it is also conceivable that the enhanced arterial inflammation and lipid accumulation which is coupled to atherosclerosis development, can act on other organs, *e.g.* by increase of systemic inflammatory mediators.

This thesis addresses the interaction between lipid metabolism and inflammation and the role of two extravascular organs, *i.e.* the liver and lungs, in atherosclerosis development. In this chapter, implications of our findings and future perspectives are discussed, with special

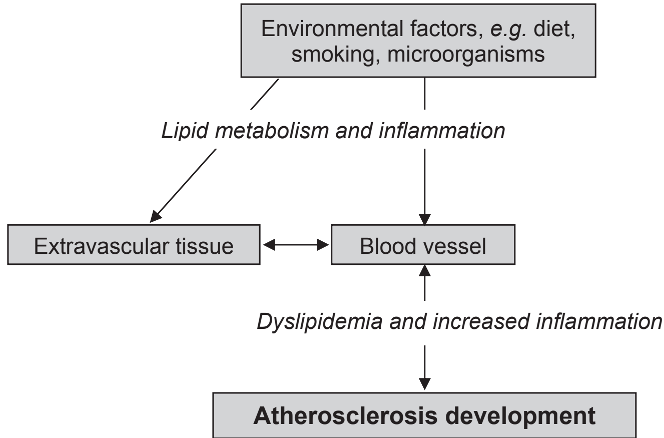


Fig. 1. The relationship between dysregulation of lipid metabolism and inflammatory processes in extravascular tissue and atherosclerosis. In addition to endogenous, *e.g.* hereditary characteristics, environmental factors can affect lipid metabolism and the inflammatory state of different organs and tissues. Disturbances in lipid metabolism and inflammation in these organs can 'spill over' to the systemic compartment and result in lipid accumulation and increased inflammation in the arterial wall, contributing to atherosclerosis development. The inflamed artery in itself also constitutes a source of inflammatory mediators for the systemic circulation.

emphasis on inflammation, since the possibilities of anti-inflammatory therapy in atherosclerosis development have been explored to a lesser extent than lipid-modulating drugs. Furthermore, the role of the lungs in atherosclerosis is discussed. Worldwide, chronic obstructive pulmonary disease (COPD) is the only major cause of death that still has a rising mortality, and it has been estimated that by the year 2020, COPD will be the third leading cause of death. Given the fact that COPD patients are at 2-3 times greater risk for CVD,[5] a better understanding of the interaction between COPD and CVD may help to decrease the burden of these two major killers.

Extravascular inflammation I: liver

Since NF-κB is one of the most important regulators of inflammation, it is an interesting target for the development of new anti-atherogenic agents. However, just like inhibiting all inflammatory processes would have adverse effects, *e.g.* on host defense, full suppression of NF-κB would be harmful. Almost all danger-sensing receptors of the innate and adaptive immune system activate NF-κB to mediate effector function. In addition, complete NF-κB inhibition is undesirable, as NF-κB is involved in many more processes than inflammation, including cell proliferation, differentiation, survival and death, and as we show in **chapter 2**, lipid metabolism. This raised the interest for tissue- or cell-specific interference of NF-κB activity in atherogenesis.[6] The tissue, *i.e.* endothelium,[7] or cells, *i.e.* macrophages[8, 9] that were firstly being investigated were obvious choices because of their prominent role in atherosclerosis development. These studies demonstrate that dependent on the level and tissue, NF-κB activation is not only pro-atherogenic,[7, 9] but can also be anti-atherogenic.[6, 8]

Going more distant from the endothelium and bearing in mind the two key processes involved in atherogenesis, *i.e.* lipid metabolism and inflammation, our interest was drawn towards the role of the liver in atherosclerosis development. Different factors, *e.g.* dietary cholesterol and saturated fatty acids (FAs) can activate the NF-κB pathway in the liver.[10] By utilizing a hepatocyte-specific transgenic murine model, we found that increased hepatocyte-specific NF-κB activity increases very-low-density lipoprotein (VLDL) production (**chapter 2**) and aggravates atherosclerosis development (**chapter 3**) (Figure 2). High concentrations of apolipoprotein (apo) B-containing lipoproteins result in elevated levels of triglycerides (TGs), which can accumulate in the liver, leading to nonalcoholic fatty liver disease (NAFLD).[11] NAFLD affects 20-30% of the general population and is associated with an increased risk for CVD. The term NAFLD spans a spectrum of conditions ranging from accumulation of fat in the liver, *i.e.* steatosis, to progressive nonalcoholic steatohepatitis (NASH), when the liver also exhibits increased inflammation. Patients with NASH display increased hepatic NF-κB activation[12] and are more prone to develop CVD than patients with simple hepatosteatosis.[13] In line with this, our findings that an increased activity of hepatocyte-specific NF-κB aggravates atherosclerosis (**chapter 3**), provide a pathophysiological explanation for the observed association between NASH and CVD. Apart from giving molecular insight into the role of hepatic inflammation in atherosclerosis development, these results also implicate that targeting the NF-κB in the liver would be an interesting anti-atherogenic therapeutic approach avoiding the major obstacle of adverse effects that may arise with broad-spectrum anti-NF-κB therapy. In addition, it is likely that not only patients with liver steatosis, but also hepatitis, due to endogenous or exogenous agents, are at higher risk for increased atherosclerosis. Treatment of these hepatic disorders may therefore not only improve the condition of the liver, but may also slow down the development of CVD.

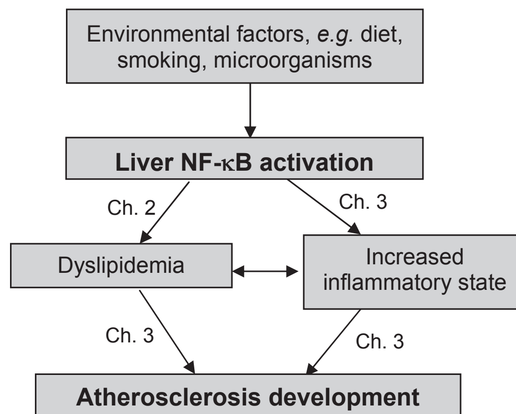


Fig. 2. The role of hepatic NF-kB activation in atherosclerosis. Various environmental factors such as a high dietary intake of cholesterol and saturated fatty acids can activate the NF-κB pathway in the liver. Increased activation of this pathway in hepatocytes enhances VLDL production (**chapter 2**) and aggravates atherosclerosis development (**chapter 3**) in *APOE*3-Leiden* (*E3L*) mice.

Interestingly, smoking increases the risk of developing NAFLD through oxidative stress and the unfavorable metabolic action of tobacco, *e.g.* causing dyslipidemia.[14, 15] It also directly promotes insulin resistance.[16] It is thus likely that smoking increases the hepatic NF-κB activity (Figure 2). In view of our results of **chapter 3**, in which we found that an increased hepatic NF-κB activity aggravates atherosclerosis development, another way by which smoking promotes atherogenesis may be through the liver. Alternatively, smoking may be a contributor to NAFLD and atherosclerosis development independently. In COPD, the NF-κB pathway is activated and cigarette smoking is a strong activator of the pathway.[17] Since an exaggerated inflammatory response to inhaled stimuli (in the Westernized world mainly cigarette smoking) is thought to be central to the pathogenesis of COPD, this may also provide clues to help understand why COPD patients are at increased risk for developing CVD compared to smokers without COPD. In the following part, we will focus on the role of COPD in atherosclerosis development.

Extravascular inflammation II: lungs

The respiratory system is anatomically as well as functionally closely related to the cardiovascular system. Although many epidemiological studies demonstrate that COPD is a strong risk factor for the development of CVD, the causal link and underlying mechanisms are unclear.[18] These two diseases share a couple of risk factors, such as smoking, aging and increased inflammation, which can explain the observed relation. However, studies also indicate that COPD is associated with CVD, independent of these risk factors.[19, 20]

As alveolar destruction is a prevalent manifestation in COPD and the major characteristic of emphysema, the effects of alveolar destruction without the presence of pulmonary inflammation in atherosclerosis development was studied in **chapter 4**. We found that elastase-induced emphysema did not enhance atherosclerosis, and even reduced atherosclerosis severity. This implies that other aspects of COPD than alveolar destruction are involved in the increased risk of atherosclerosis. Notably, consequences of COPD, such as hypoxia and physical inactivity also predispose to atherosclerosis development (Figure 3). It has been shown that after correction for physical activity, COPD remains an independent risk factor for CVD.[5] In experimental animal models, hypoxia, which occurs in COPD, is likely to contribute to atherosclerosis development.[21] In our study, hypoxia and physical inactivity resulting from elastase-induced emphysema were not likely to have played a significant role on atherosclerosis development. The duration and level of hypoxia was at least partly compensated by an increase in respiration amplitude, right ventricular hypertrophy and an increase in number of erythrocytes.

Numerous mechanisms have been proposed to explain the observed link between COPD and CVD,[22] including systemic oxidative stress, hypoxia, physical (in)activity, activation of the sympathetic nervous system, vascular dysfunction, accelerated aging, microbial airway colonization and infections, and probably the most advocated one: increased (low-grade) systemic inflammation (Figure 3). COPD is characterized by pulmonary inflammation, and systemic inflammation has been established as a major pathophysiological factor not only for CVD, but also for COPD.[23] It is thus likely that the presence of increased inflammation explains (part of) the pathophysiological link between COPD and CVD. As mentioned before, increased inflammation in any organ can 'spill over' to the systemic compartment, thereby affecting the

vasculature. To dissect the contributions of two main features of COPD, alveolar destruction and pulmonary inflammation on atherosclerosis development, these two aspects have to be investigated in one study. To address this research question, an appropriate COPD model has to be chosen. In the following section, three commonly used COPD models and their advantages and limitations are described.

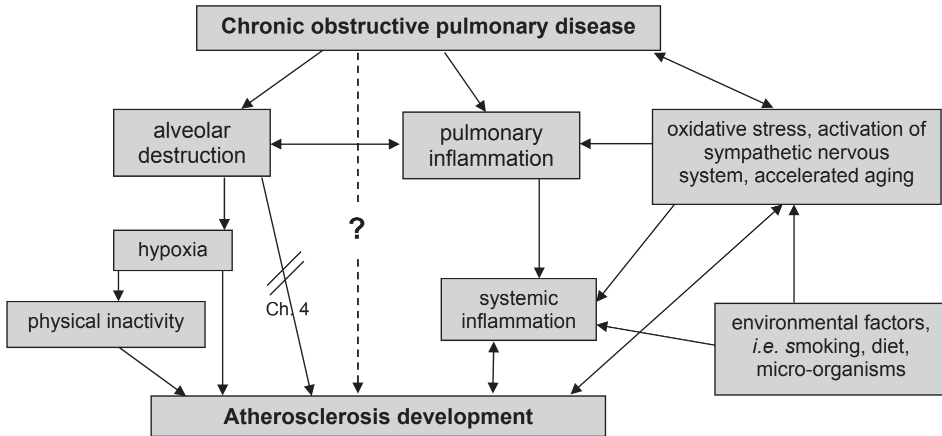


Fig. 3. Potential pathophysiological mechanisms for increased atherosclerosis development in patients with COPD. COPD is characterized by alveolar destruction and increased airway and parenchymal inflammation in the lungs. Possible mechanisms linking the increased CVD risk in COPD are sequelae of several of these features, *e.g.* systemic inflammation, hypoxia. It is hypothesized that the enhanced production of inflammatory mediators in the lungs spills over into the systemic compartment and thereby contributes to atherosclerosis development. The hallmark of emphysema, a major hallmark of COPD, is alveolar destruction and we found that this factor in itself does not explain the increased risk of atherosclerosis development observed in COPD (**chapter 4**).

Experimental COPD models

As smoking is the predominant cause of COPD, smoke exposure systems offer the ability to use the primary disease-causing agent to model several key features of the disease in small animals. Although these models mimic the human COPD development more closely than any other *in vivo* model, an operational smoke exposure system is more laborious to set-up and perform experiments with than most other COPD models. Many aspects have to be kept in mind and can be crucial in succeeding in setting up a smoke exposure model for animals. Factors concerning the choice of smoke exposure machine, set up, dose, frequency, duration, differences in branch and batch of cigarettes used, suppliers, strains and gender of mice, techniques to quantify emphysema and pulmonary inflammation, can all contribute to variations not only between different laboratories, but even within the same laboratory. In addition, because these models require a long time, *i.e.* ~4–6 months, to generate pathologies and functional changes consistent to those observed in merely mild COPD, practical application of these smoking models for assessing potential therapeutic interventions is more time- and labor-consuming

than other COPD models. Smoke exposure induces emphysema in combination with increased pulmonary inflammation. It also affects lipid metabolism unfavorably[15] and increases systemic inflammation,[24] which are known to contribute to atherosclerosis development. These concurrent effects of smoke hinder the differentiation of the impact of the two major features of COPD, *i.e.* alveolar destruction and pulmonary inflammation, independently on atherosclerosis development. However, as smoke exposure models best resemble the natural development of COPD, these models are essential, when the pathophysiological processes involved in the development of COPD are the subject of investigation.

Instillation of elastase in the lungs initially causes edema, hemorrhage and inflammation, consisting of infiltration by mainly neutrophils and monocytes.[25] Once this acute inflammatory response has disappeared, destruction of alveolar walls becomes evident which is consistent with the anatomical lesions in the lung observed in patients with emphysema.[26] One of the major drawbacks of this model to use for studying pathophysiology of COPD is the fact that inflammation is transient, resolves within a week of elastase administration, and does not reflect the progressive, slowly resolving inflammation associated with human COPD. However, the main advantages of the elastase models over other *in vivo* models for COPD are the possibility to easily titrate the severity of emphysema development with the dosage of elastase instilled, and the rapid onset of the emphysematous destruction of the lung. For this reason, the elastase model is ideal for testing therapeutic approaches aimed at reversing or repairing emphysematous damage to the lung. Another advantage of this model is that it is suitable to study the effects of alveolar destruction without the presence of chronic pulmonary inflammation on the development of other diseases, such as comorbidities of COPD (**chapter 4**).

Lipopolysaccharide (LPS) is a bacterial endotoxin that is present in cigarette smoke and, when instilled into the lung, can elicit a pronounced neutrophilic inflammatory response.[27] Because neutrophils are the largest source of neutrophil elastase, it was regarded as the central player driving the pathologies associated with COPD for many years. Similar to elastase instillation, the dosage of intrapulmonary LPS is more easily controlled than *e.g.* cigarette smoke exposure. In consideration of the research question whether pulmonary inflammation can affect atherosclerosis development, intrapulmonary LPS instillation can be adjusted in such way that the amount of systemic inflammation is minimized and the effects of pulmonary inflammation *per se* on atherosclerosis development can be investigated. However, the dosage of LPS needs to be carefully chosen, as a sufficient high dosage and duration of administration, can induce emphysema.[28] This may interfere with the distinction of the role of alveolar destruction and pulmonary inflammation in atherosclerosis development. In addition, if the LPS dose instilled is too high, systemic inflammation may be induced, which directly affects atherosclerosis development.

Taken together, the best approach to investigate the effects of alveolar destruction and pulmonary inflammation on atherosclerosis development, independently and together, seems to be the combination of the elastase-induced emphysema model (chapter 4) with chronic administration of a low dose of LPS in the lungs. Since smoking by itself induces various pro-atherogenic triggers, *e.g.* dyslipidemia, pulmonary and systemic inflammation and oxidative stress, a smoke exposure model is less suitable to discern the role of alveolar destruction and pulmonary inflammation on atherosclerosis development without the interference by confounding factors.

Therapeutical agents for COPD and CVD

COPD is increasingly being recognized as a complex disorder, characterized not only by local pulmonary inflammation, but also by systemic inflammation that may have an adverse impact on various extrapulmonary organs, such as the blood vessels and the heart among others.[29] CVD is one of the most important causes of death in COPD patients. Although smoking cessation is the cheapest, safest and most effective strategy to treat COPD and CVD, it is a hard task to accomplish for the patient and the inflammatory response in many COPD patients persists after smoking cessation.[30] Therefore, new and more effective therapies that deal with not only COPD, but preferably also CVD are needed. Many existing therapeutic options used to treat COPD and CVD appear to have other beneficial properties apart from their classical actions. This raises the idea whether drugs currently applied for COPD could also be beneficial to treat CVD (Table 1), and *vice versa* (Table 2).

Application of COPD therapies in CVD

As inhaled corticosteroids (ICSs) are widely prescribed in COPD patients and CVD is a prevalent comorbidity of COPD, the effects of ICS on CVD are an interesting subject to explore. Glucocorticoids are potent inhibitors of NF-κB activation,[31] and thus may be a promising anti-inflammatory agent for both COPD and CVD. However, not all studies with COPD patients have demonstrated a clear anti-inflammatory and beneficial effect of ICS[32], and such effects may be restricted to subgroups of COPD patients.[33] In a retrospective study it was demonstrated that very low doses of ICSs (50-200 μg/day) were associated with a reduced risk of acute myocardial infarction.[34] However, with higher doses of ICSs, the risk returned to baseline. This lack of benefit at higher doses might be due to counterbalancing adverse effects of other risk factors, or the fact that patients with more severe disease, which in itself is linked to CVD morbidity, were prescribed the higher doses. In line with the latter finding, randomized controlled trials also fail to show any significant effect of ICSs on myocardial infarction and cardiovascular mortality.[35, 36] A controlled trial of high-dose ICS with or without a long acting β-agonist showed no reduction in systemic inflammation in COPD patients, as measured by circulating interleukin (IL)-6 and C-reactive protein (CRP) concentrations, indicating corticosteroid resistance of systemic, as well as local inflammation in patients with COPD.[37] Overall, these data suggest that ICSs do not have a significant beneficial effect on CVD.

Table 1. Possible application of COPD therapies for CVD.

Therapy	Main mechanism in COPD	Beneficial effect on CVD?
Inhaled corticosteroids	Anti-inflammatory?	Probably not
β_2-agonists	Bronchodilatory	Probably not
Anti-cholinergics	Bronchodilatory	Caution in patients with high risk for cardiovascular event
Theophylline	Anti-inflammatory	Possibly
Phosphodiesterase 4 inhibitors	Anti-inflammatory	Probably yes
Supplemental oxygen	Restore normal oxygen levels	Probably yes
Lung volume reduction surgery	Improved lung breathing mechanics	If successful probably yes Surgical risk

Bronchodilators, *i.e.* long-acting β_2-agonists and anticholinergics are useful in COPD, but are not known to have marked anti-inflammatory or other anti-atherogenic effects. One trial demonstrated that tiotropium, an anticholinergic drug, had no effect on inflammatory markers in sputum or in the circulation of COPD patients.[38] Whether inhaled or oral β_2-agonists by themselves have any beneficial effects on the systemic inflammatory state of COPD patients has not yet been clarified. There has been concern that long-term use of inhaled bronchodilators may increase the risk of cardiovascular complications.[39] However, in the large Towards a Revolution in COPD Health (TORCH) trial, the three-year risk of cardiovascular adverse events of the use of salmeterol (a β_2-agonist), fluticasone (an ICS), both medications combined, or placebo in COPD patients was similar in all groups.[36] In addition, results from a meta-analysis on the occurrence of cardiovascular events and the use of anticholinergic agents to treat COPD in trials show that there is no increased risk.[40] It is not clear, however, whether this also accounts for patients with an increased risk for cardiovascular events, such as those with coronary artery disease, heart failure and cardiac arrhythmia, because they are excluded from participation for obvious ethical reasons. Furthermore, a poor lung function which is inherent to COPD patients, is a marked risk factor for CVD.[41] Taken together, although bronchodilators are valuable in the treatment of COPD, care must be taken for cardiovascular complications, especially in high-risk patients.

Theophylline seems to be a more promising candidate as a concurrent treatment for inflammation in COPD and CVD. It has been shown to reduce neutrophilic inflammation in patients with COPD[42] and also has the potential to reverse corticosteroid resistance in COPD.[43] However, the molecular mechanism for the anti-inflammatory action of theophylline is currently unknown and deserves at least the same priority of exploring its potential as therapeutic agent for CVD in COPD patients.

Roflumilast, a phosphodiesterase 4 (PDE4) inhibitor, has recently been registered as a novel therapy for COPD and thought to be effective through its anti-inflammatory properties.[44, 45] One of the major anti-inflammatory effects of PDE4 inhibitors is their ability to reduce TNFα release,[46] which supports the potential of these agents for treating systemic inflammation. PDE4 inhibitors increase levels of cyclic adenosine monophosphate (cAMP) through inhibition of its metabolism. The resulting increase in protein kinase A activation stimulates increased protein phosphorylation, with subsequent inhibition of pro-inflammatory cells and mediators. Because of their anti-inflammatory properties, PDE4 inhibitors may be beneficial in treating CVD. This has not been investigated thus far, but several reports support a protective role of cAMP in atherosclerosis.[47] Therefore, based on their anti-inflammatory effects, PDE4 inhibitors would be an interesting subject for future studies on CVD.

COPD patients are subject to intermittent hypoxia and at a more severe stage of disease to sustained hypoxia. Hypoxia can induce increased inflammation and oxidative stress, which contribute to atherosclerosis development. Mice subjected to chronic intermittent hypoxia have increased atherosclerosis development.[21] Long-term use of supplemental oxygen improves survival in patients with COPD and severe resting hypoxemia.[48] Whether oxygen therapy is beneficial in patients suffering from atherosclerosis with a normal lung function has

never been investigated, but based on the findings above, oxygen therapy is likely to reduce the risk of atherosclerosis in COPD patients, in addition to the relieve of pulmonary symptoms.

Lung volume reduction surgery in the treatment of properly selected patients with COPD, *i.e.* with severe and predominantly upper-lobe emphysema and low-exercise capacity, improves survival and quality of life, including exercise tolerance, dyspnea, oxygen requirement and functional status.[49] However, this invasive procedure is mostly offered to severely impaired emphysema patients as one of the last resorts and accompanied with a high risk on cardiopulmonary morbidity (up to 58.7% in the National Emphysema Treatment Trial (NETT)). In the same study, cardiovascular morbidity, *i.e.* myocardial infarction, pulmonary embolus, or cardiac arrhythmia requiring treatment within 30 days of surgery occurred in 20% of patients.[50] Although this kind of therapy has proven to be effective in a subgroup of severe emphysema, the surgical risk of the procedure is a major barrier withstanding broad application for patients with CVD.

Application of CVD therapies in COPD

The discovery of novel effective treatments for COPD, other than bronchodilators and ICS has proven difficult.[51] Thus, it is worthwhile to explore whether drugs commonly used in CVD might also have beneficial effects in COPD.

Promising candidates are statins which are primarily prescribed for patients with CVD, but nowadays seem to have potential additional benefits in many other diseases, including COPD.[52] It was shown in a retrospective study that the use of statins was associated with a reduced mortality in COPD patients, independent of sex, age, smoking, pulmonary function and comorbidities.[53] In addition, ICS appeared to increase the survival benefit associated with statin use. Several possible mechanisms for the beneficial effects of statins have been proposed, including their anti-inflammatory property. This feature of statins makes them an attractive candidate in the treatment of COPD in which inflammation has a fundamental pathophysiological role. In experimental COPD animal models, simvastatin inhibited cigarette smoke-induced emphysema, which was associated with a decrease in pulmonary inflammation.[54, 55] Moreover, in a murine model in which emphysema was already established by elastase instillation in the lungs and the acute inflammation after elastase instillation has resolved, simvastatin was even able to reverse emphysema.[56] Interestingly, in this study the therapeutic effect of simvastatin was not ascribed to anti-inflammatory effects, but to a tendency towards an increase of vascular endothelial growth factor (VEGF) in bronchoalveolar lavage fluid. A study in *apoe*$^{-/-}$ mice demonstrated that a Western-type diet high in fat and cholesterol content not only induced increased systemic, but also pulmonary inflammation in these mice.[57] As an enhanced pulmonary inflammation is one of the most important pathophysiological causes of COPD, these data imply that a Western-type diet can stimulate the development with COPD. The lipid-lowering action of statins thus may be another mechanism through which they can be useful in the treatment of COPD. Taken together, statins seem to be effective in both COPD and CVD, which also indicates that COPD and CVD may have a common pathophysiological cause.

Next to statins, angiotensin converting enzyme (ACE) inhibitors and the more specifically acting angiotensin II receptor blockers (ARBs), classically prescribed to treat hypertension,

have been shown to reduce the risk of COPD hospitalization, and cardiovascular events and death in COPD patients.[58] ACE inhibitors reduce pulmonary hypertension, but may have other beneficial effects in COPD, *e.g.* by inhibiting angiotensin II that has pro-inflammatory properties.[59] Angiotensin II receptors are shown to be expressed in the lung and more highly in lungs of COPD patients.[60] In mice, treatment with the ARB irbesertan after induction of emphysema with elastase, significantly improved the exercise capacity and reduced the development of morphological emphysema.[61] Therefore, the main action of ACE inhibitors and ARBs, *i.e.* inhibition of the pro-inflammatory angiotensin II, makes them potential valuable therapeutic agents in COPD.

Both activators of peroxisome proliferator-activated receptor (PPAR)α and -γ have demonstrated anti-inflammatory properties and other anti-atherogenic effects in human and mice.[62] In fact, part of the anti-inflammatory effects of statins may be explained by activation of PPARα and -γ.[63] PPARα and -γ agonists have been shown to exert beneficial effects mostly in experimental asthma or acute pulmonary inflammation models.[64] So far, no studies are published on the role of PPARs in animal models of COPD. However, PPARα and -γ inhibit airway neutrophil and macrophage influx, as well as cytokine and chemokine production induced by LPS in the mouse,[65, 66] suggesting that activators of these PPAR subtypes may have a beneficial effect on the inflammatory response associated with COPD.

Accelerated aging may be a characteristic common to COPD and CVD.[67] The concept of ‘inflamm-aging’ is now gaining attention, with a reduction in adaptive immunity and an increase in innate immunity driven by NF-κB activation.[68] This suggests that anti-aging drugs may be beneficial in CVD and COPD. One of the key players that has gained much interest in this field is sirtuin 1 (SIRT1). SIRT1 is an enzyme which deacetylates proteins that contribute to cellular regulation, thereby playing an important role in determining lifespan of all organisms.[69] We demonstrated that resveratrol, a moderate SIRT1 activator, protects against atherosclerosis (**chapter 5**). While resveratrol is known to have many pleiotropic effects, we did not find many other systemic anti-atherogenic effects, *e.g.* anti-inflammatory, anti-oxidative, than lipid lowering. This can be attributed to the use of a relatively low dose of resveratrol (0.01% (w/w) in the diet, compared to other studies.[70, 71] Nevertheless, the reported pleiotropic effects of resveratol may have therapeutic potential in COPD.[72] In fact, resveratrol was found to inhibit cigarette smoke extract-mediated pro-inflammatory cytokine release in a human monocyte-macrophage cell line.[73] Furthermore, resveratrol was shown to protect against cigarette smoke-mediated oxidative stress in human lung epithelial cells by inducing glutathione synthesis.[74] Additional studies are required to be able to extrapolate the dosages and effects of resveratrol of these *in vitro* studies and our *in vivo* study with mice to (clinical) human use for CVD and COPD. To address this, experimental studies are needed in which the effects of resveratrol on pulmonary damage induced by cigarette smoke are studied in hyperlipidemic atherosclerosis models and compared to studies in which pulmonary damage is induced by other agents, *e.g.* intrapulmonary elastase-instillation, which do not have the diverse direct effects of smoke on atherosclerosis. Moreover, as resveratrol is just a moderate SIRT1 activator, other more potent SIRT1 activators may have even more beneficial effects, and are possibly more effective than resveratrol in the treatment of COPD. In addition, there is clinical evidence

that SIRT1 may be an interesting therapeutic target, as its expression is decreased in lungs of patients with COPD.[72]

Table 2. Possible application of CVD therapies for COPD.

Therapy	Mechanism in CVD	Beneficial effect on COPD?
Statins	Hypolipidemic Anti-inflammatory Anti-oxidative	Probably yes
Angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs)	Anti-hypertensive Anti-inflammatory	Probably yes
PPARα agonists	Hypolipidemic Anti-inflammatory	Possibly; in experimental phase
PPARγ agonists	Hypoglycemic Anti-inflammatory	Possibly; in experimental phase
Resveratrol	Hypolipidemic Anti-inflammatory Anti-oxidative	Possibly; in experimental phase

In summary, COPD has been demonstrated to be an independent predictor of cardiovascular death.[75] The value of existing drugs that treat both CVD and COPD simultaneously is currently extensively being studied. Despite some promising findings, we still do not know whether treatment of lung inflammation decreases, for example the progression of atherosclerosis, or the risk of acute cardiac events. Alternatively, it is also unclear whether treatment of heart disease can reduce the progression of lung disease. There are data suggesting that a Western-type diet can induce pulmonary inflammation, which is the most important causal factor in COPD development.[57] Minimizing dyslipidemia thus may be another target to inhibit not only CVD but also COPD. In addition, initial data seem to indicate that drugs, originally prescribed for CVD such as statins, ARBs and PPAR agonists, also have the potential to benefit COPD patients. It can even be hypothesized that one of the ways by which these drugs can inhibit CVD is by slowing down progression of the lung disease in COPD patients. Furthermore, since CVD is a major cause of mortality in COPD patients and the presence of a poor lung function is proven to be an important risk factor for CVD, screening for the presence and treatment of CVD in COPD patients is recommended.

Treating infections in CVD and COPD

The clinical course of COPD is punctuated by recurrent episodes of acute increase in both airway and systemic inflammation, otherwise known as exacerbations, with a major negative impact on the patient's quality of life, hospital admission and lung function. The increase in systemic inflammation and oxidative stress during exacerbations are likely to contribute to the inflammatory process underlying atherosclerosis. Furthermore, the peaks of inflammatory

activity accompanying COPD exacerbations could precipitate acute exacerbations of the atherosclerotic process with increased risk of plaque rupture and thrombotic occlusion.[76] Infection is considered the main cause of acute COPD exacerbations and the standard treatment for exacerbations is usually corticosteroids and/or antibiotics.[77]

Chlamydophila pneumoniae (*C. pneumoniae*) has been implicated as an infectious trigger for acute exacerbations of COPD[78] and postulated to contribute to inflammation in atherogenesis,[79] making it an interesting candidate in linking the pathophysiology of CVD and COPD. Several infectious agents have been associated with an increased risk CVD, but for the Gram-negative bacteria *C. pneumoniae* and *Porphorymonas gingivalis* (*P. gingivalis*) most compelling evidence is found.[80] Many studies demonstrated both an associative and causal relation for *C. pneumoniae* with regard to atherosclerosis formation. *C. pneumoniae* is an obligate intracellular pathogen that infects both epithelial cells and macrophages within the lungs and may disseminate outside the lungs through infected monocytes and macrophages.[81] It was the first infectious organism to be found in macrophages and smooth muscle cells of human atherosclerotic plaques but rarely within normal (adjacent) arterial cells.[82] In addition, a number of studies in experimental models showed an acceleration of atherosclerotic lesion development following respiratory infection with *C. pneumoniae*.[79] Macrolides are one of the first choice antibiotics to treat infections with *C. pneumoniae* and reported to have anti-inflammatory properties apart from their antimicrobial activities, making them interesting agents to treat sustained infection and low-grade inflammatory states as encountered in COPD and CVD.[83] Recently, it was demonstrated that erythromycin ameliorates cigarette-smoke-induced pulmonary inflammation and emphysema in rats.[84] Similar to *C. pneumoniae*, *P. gingivalis*, which colonizes the gingival plaque where it can cause periodontitis, has been shown to increase systemic cytokines and acute-phase proteins and increase atherosclerotic lesion development in experimental models.[85] Although a number of studies have shown that treatment of chronic periodontitis results in a reduction in systemic inflammation,[86] a direct causal link between *P. gingivalis* with CVD has not been demonstrated so far.

As mentioned before, although a large body of evidence for the role of infection as risk factor for atherosclerosis exists, intervention trials with antibiotics until now have ended up with disappointing results.[80] A few considerations should be taken into account in the interpretation of the failed trials. First, it is not clear whether pathogens were effectively cleared by antibiotic treatment or chronic (low-grade) infection persisted. This accounts especially for *C. pneumoniae* that replicates intracellularly and exists in a metabolically inactive form, which is not susceptible for antibiotics. Second, the participants of the trials had advanced atherosclerosis, and events being measured were likely due to plaque destabilization and rupture rather than progression of occlusive disease, while the pathophysiological and experimental studies performed so far were focused on the initiating process of atherogenesis. Studies focusing on the effects of antibiotics in patients with early stage atherosclerosis have not been carried out, and are intuitively difficult to design. Furthermore, the beneficial effects of antibiotics in experimental studies with *C. pneumoniae* were only observed when given shortly, *i.e.* within days rather than weeks, after infection,[87] and the acute *C. pneumoniae* infection in humans frequently passes clinically unnoticed. Third, there is an emerging concept that not one organism but an

aggregate of multiple organisms, the infectious or pathogen burden as a whole, is responsible for the effects of infection on atherosclerosis development. This is for example supported by a study in which it was found that an increased pathogen burden was significantly associated with increased coronary artery disease, even after adjustment for traditional cardiovascular risk factors.[88] The antibiotic treatment in the secondary prevention trials performed thus might be ineffective due to the infectious burden of unsusceptible pathogens, allowing these organisms to still contribute to the progression of atherosclerosis.

The failure of antibiotic trials in CVD should therefore not lead to dismissal of the potential role of infectious agents in the pathogenesis of atherosclerosis, although considerations to be taken into account, such as persistent infection despite antibiotic treatment, emergence of antibiotic resistance and requirement of multiple antimicrobial therapies to treat the pathogen burden, are not easily overcome. In conclusion, microbial pathogens may form a bridge between COPD and CVD in acute exacerbations as well as chronic infections.

Concluding remarks

The understanding of the pathophysiological processes involved in the local environment where atherogenesis takes place has resulted in effective treatment strategies to fight CVD. However, the endothelium does not stand by itself, as it is part of a complicated network with intricate connections and interactions with other cells, organs and tissues. Therefore, apart from investigating the local processes, we have to keep in mind that more distal effectors and reactors are present and should broaden our attention when studying the atherosclerotic process.

In this thesis, the role of inflammation in the liver and lungs in atherosclerosis development was addressed. Models combining various conditions that are often observed concomitantly in clinical practice, such as NASH and CVD or COPD and CVD, represent an exciting new approach in order to understand the mechanisms underlying the pathophysiology of these diseases. Investigating the interaction between vascular and extravascular changes may expand the number of therapeutic options and lead to novel approaches that can be used to help manage CVD and associated diseases.

When considering a role of extravascular inflammation in atherosclerosis, conventional therapies used in certain diseases which also seem to be linked to each other, such as CVD and COPD, may be applicable for more domains and may even have synergistic beneficial effects. Given the complex nature of both COPD and CVD, it seems likely that no single cause of the association will be found. However, further studies on the interaction between COPD and CVD are expected to improve early identification of patients likely to develop both diseases to prompt early intervention, as well as promote stratified medicine and tailored therapy in which patients achieve the best possible medicinal care dependent on their individual needs.

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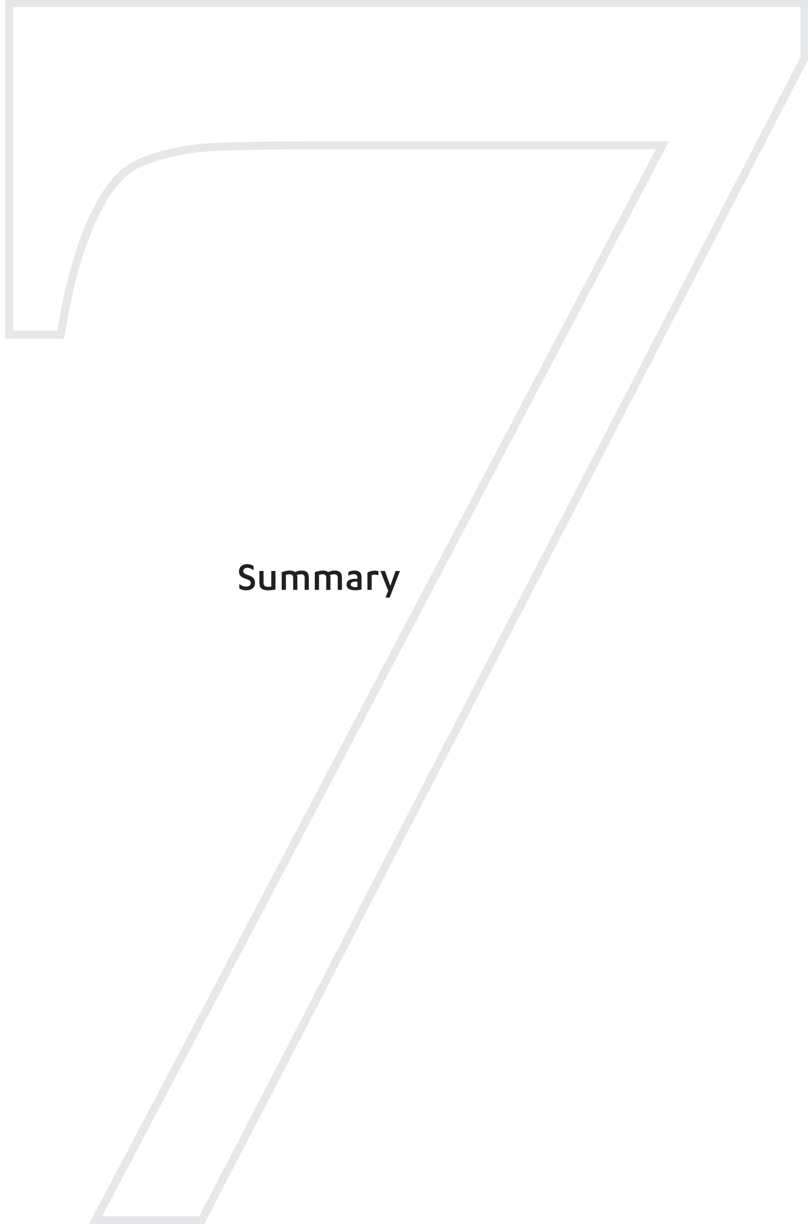

Summary

Cardiovascular diseases (CVDs) are the most important cause of death in the Western world and are predominantly caused by atherosclerosis. Dysregulation of lipid metabolism and inflammation are the two main risk factors of atherosclerosis development. High levels of (very-) low-density lipoprotein ((V)LDL) in the blood result in retention of LDL in the intima of the arteries, where the particles are modified. Leukocytes such as monocytes are attracted, differentiate into macrophages to take up the modified LDL and transform into foam cells. Recruitment of more inflammatory cells and production of inflammatory mediators enhance the formation of atherosclerotic lesions with a lipid-rich necrotic core, which can be covered by a firm fibrous cap that may (partly) obstruct the blood flow in the artery. In a more unfavorable situation an unstable lesion is evolved that is prone to rupture which can result in a complete occlusion of the blood flow, causing infarction of the supplied tissue. Apart from the local processes in the vessel wall, there is also a marked contribution of other organ systems to atherogenesis. In this thesis, the role of the liver and lungs in atherosclerosis development were studied.

The liver plays an important role in lipid metabolism and the inflammatory response and integrates both processes. In **chapter 2** the effects of hepatic activation of the transcription factor nuclear factor-κB (NF-κB), as a central regulator of inflammation, on lipid metabolism were investigated. To this end, mice expressing hepatocyte-specific human IKKβ (Liver-specific IKKβ or *LIKK* mice), which have continuous hepatocyte-specific activation of NF-κB, were crossbred with *APOE*3-Leiden* (*E3L*) mice to generate *E3L.LIKK* mice. These mice had increased plasma triglyceride levels compared with their *E3L* littermates. Mechanistic *in vitro* and *in vivo* experiments demonstrated that this hypertriglyceridemia was not caused by a decrease in VLDL clearance, but resulted from an increased VLDL-production by the hepatocytes. As dyslipidemia is a well-known contributor to atherosclerosis development and NF-κB activation promotes the inflammatory response, we hypothesized that *E3L.LIKK* mice will have aggravated atherosclerosis development as compared to *E3L* littermates. To study this, we fed these mice a Western-type diet for 24 weeks and found that the total atherosclerotic lesion area as well as the severity of atherosclerosis was increased in *E3L.LIKK* mice (**chapter 3**). This was at least partly explained by a transient increase in plasma VLDL-cholesterol levels at 8 weeks after the start of the diet. Hepatic expression of monocyte chemoattractant protein 1 (MCP-1) was increased in *E3L.LIKK* mice. While expression of *LIKK* did not affect basal systemic inflammatory markers, the levels of pro-inflammatory cytokines tended to be higher after intravenous lipopolysaccharide (LPS) injection. This indicates that *E3L.LIKK* mice have a higher inflammatory state than *E3L* mice, which likely has contributed to the aggravated atherosclerosis development.

In addition to the liver, we studied the role of the lungs in atherosclerosis development. A considerable amount of epidemiological data indicates that a poor lung function, most commonly caused by chronic obstructive pulmonary disease (COPD), is a major risk factor for CVD. The exact mechanism underlying this association is unknown. Increased systemic inflammation has been postulated as a common risk factor, but has never been proven to be the missing link. Enlargement of alveoli due to destruction is a fundamental characteristic of emphysema, a major component of COPD. We investigated in **chapter 4** whether alveolar wall destruction by elastase instillation in the lungs, as a model of emphysema without cigarette smoke-induced inflammation, would worsen atherosclerosis development in *E3L* mice. Two

doses, *i.e.* 15 and 30 μg pancreatic porcine elastase, were used next to the control group to detect dose-dependent effects. After feeding the mice an atherogenic Western-type diet for 20 weeks, we did not observe a difference in the atherosclerotic lesion size between the groups, but did find a dose-dependent reduction in lesion severity with elastase treatment. This is likely in part due to a dose-dependent decrease in circulating monocytes and neutrophils. The number of pulmonary macrophages and neutrophils, as well as the systemic lipid levels were similar in all groups. These results indicate that alveolar destruction *per se* is not responsible for the observed increased risk for atherosclerosis in COPD patients. An increased state of systemic inflammation has a pathophysiological role in both COPD and atherosclerosis development. Thus, increased inflammation may be the missing link explaining the association between these diseases. It has been hypothesized that atherosclerosis development is promoted by means of a 'spill over' of pulmonary inflammatory mediators towards the systemic compartment in COPD patients. This concept has yet to be confirmed by future experimental studies.

The purpose of this kind of experimental studies is to gain more insight in the pathophysiological processes involved in order to develop new strategies to fight CVD. One of the promising therapeutic candidates is resveratrol, a compound that is naturally present in some Asian herbs, nuts and the skin of red grapes and consequently in red wine. It has been shown to have various anti-atherogenic, including lipid lowering, anti-inflammatory, and anti-oxidative properties. In **chapter 5** we studied whether resveratrol can protect against atherosclerosis development by itself and secondly, whether it is able to add to the anti-atherogenic effects of atorvastatin in *E3L.CETP* mice, which respond to high-density lipoprotein (HDL)-modifying drugs similar to humans, and are therefore used extensively in studies in which potential anti-atherogenic therapies are investigated. *E3L.CETP* mice were fed a Western-type diet supplemented without (control) or with resveratrol, atorvastatin, or both for 14 weeks. Both size and inflammatory state of the atherosclerotic lesions were reduced to a similar extent by resveratrol, atorvastatin and the combination treatment. This is in part due to a significant, but comparable lowering of plasma cholesterol levels by the three treatments. Inflammatory parameters and markers for oxidative stress were unaffected by the treatments. Resveratrol thus protects against atherosclerosis development, but does not add to the anti-atherogenic effects of atorvastatin. Interestingly, the beneficial effects of resveratrol and statins extend beyond the blood vessel, and are also under investigation as a potential treatment for other diseases such as COPD.

Taken together, disturbances in organs such as the liver and the lungs can affect atherosclerosis development. The clinical implications and future perspectives of these results are discussed in **chapter 6**. While the local processes involved in atherosclerosis development have been the primary subject of interest in past decades, the focus is shifting from the artery towards the potential role of other (distal) organs, tissues and cells in atherogenesis. This exciting trend will shed light on the mechanisms underlying the interactions between different organs, and contributes to the discovery of additional effects of existing drugs and new treatments, that may not only be applicable to single diseases, but also their comorbidities.

Samenvatting

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Samenvatting

Slagaderverkalking of atherosclerose is de belangrijkste onderliggende oorzaak van hart- en vaatziekten. Verstoringen in het vetmetabolisme en ontstekingsprocessen zijn de twee voornaamste risicofactoren voor de ontwikkeling van atherosclerose. Hoge spiegels van zeer-lage-dichtheids lipoproteïne (VLDL) partikels in het bloed leiden tot stapeling van lage-dichtheids lipoproteïne partikels (LDL) in slagaders, waar het LDL chemische veranderingen ondergaat. Afweercellen zoals monocyten worden hierdoor aangetrokken en ontwikkelen zich tot macrofagen die het chemisch veranderde LDL opnemen. Hierdoor ontwikkelen deze macrofagen zich tot zogenaamde schuimcellen. De aantrekking van meer afweercellen en de productie van inflammatoire mediatoren stimuleren de groei van atherosclerotische plaques. Naarmate deze groter worden, kunnen ze een deel van de bloedvoorziening blokkeren. Verdere ontwikkeling van atherosclerose leidt tot zogenaamde laesies met een kern die voornamelijk vet, cholesterol en vergane cellen bevat. De kern wordt van de bloedstroom gescheiden door een schil van bindweefsel, dat in het meest gunstige geval stevig en dik is. Enzymen die bij ontsteking worden geproduceerd kunnen echter bindweefsel afbreken wat kan resulteren in een onderbreking van deze beschermende laag. Dit kan leiden tot de vorming van een stolsel dat de volledige bloedvoorziening blokkeert, waardoor het achterliggende weefsel afsterft als de bloedvoorziening niet tijdig wordt hersteld.

Om atherosclerose te bestuderen zijn diverse muismodellen ontwikkeld, zoals het *APOE*3-Leiden* (*E3L*) muismodel. Muizen ontwikkelen van nature geen atherosclerose, omdat ze ten opzichte van de mens een veel gunstiger vetmetabolisme hebben. Door de aanwezigheid van het *E3L* eiwit in dit muismodel wordt het vetmetabolisme verstoord, waardoor deze meer op die van mensen lijkt met als gevolg dat deze *E3L* muizen atherosclerose kunnen ontwikkelen.

In de afgelopen jaren is gebleken dat niet alleen verstoring van deze processen lokaal in de slagaders, maar ook stoornissen in andere organen kunnen bijdragen aan atherosclerose ontwikkeling. In dit proefschrift wordt de rol van de lever en de longen in de ontwikkeling van atherosclerose bestudeerd.

De lever speelt een prominente rol in zowel het vetmetabolisme als bij ontsteking. In **hoofdstuk 2** worden de effecten van een centrale regulator van ontstekingsreacties, nuclear factor-κB (NF-κB), op het vetmetabolisme onderzocht. Hiervoor werden muizen gebruikt waarbij alleen in de levercellen NF-κB overactief was, het zogenaamde *LIKK* muismodel. Deze muizen werden gekruist met *E3L* muizen, wat resulteerde in *E3L.LIKK* muizen. Deze muizen hadden een toegenomen bloedspiegel van triglyceriden vergeleken met de controle *E3L* muizen (zonder *LIKK*). In het lichaam wordt vet opgeslagen in de vorm van triglyceriden. Uit nader onderzoek bleek dat de toegenomen triglyceriden spiegel in de *E3L.LIKK* muizen het gevolg was een verhoogde productie van de VLDL partikels. VLDL partikels transporteren cholesterol en triglyceriden door het lichaam en verhoogde spiegels van VLDL in het bloed leidt tot de vorming van atherosclerose. Om direct het effect van *LIKK* op atherosclerose-ontwikkeling te onderzoeken werden *E3L.LIKK* en *E3L* muizen op een cholesterolrijk dieet gezet en werd de mate en ernst van atherosclerose bepaald na 24 weken (**hoofdstuk 3**). *E3L.LIKK* muizen bleken

meer en ernstigere atherosclerose te ontwikkelen, wat deels verklaard werd door een tijdelijke verhoging in VLDL spiegels. De ontstekingsparameters in het bloed waren niet verschillend tussen de twee groepen muizen, maar na stimulatie met lipopolysaccharide, een component van bacteriën die vaak wordt gebruikt om een ontstekingrespons teweeg te brengen, waren de spiegels van verschillende ontstekingsmediatoren in het bloed sterker verhoogd in de *E3L.LIKK* muizen vergeleken met de *E3L* muizen. Deze resultaten laten zien dat een verhoogde activiteit van NF-κB in levercellen leidt tot meer atherosclerose ontwikkeling door een toename in VLDL en ontstekingsmediatoren in het bloed.

Naast de bijdrage van de lever, hebben we de rol van de longen in atherosclerose ontwikkeling onderzocht. Roken is een gemeenschappelijke risicofactor voor atherosclerose en chronische obstructieve pulmonaire ziekten (COPD). COPD gaat gepaard met een achteruitgang in longfunctie en staat nu nog op de zesde plaats van doodsoorzaken, maar zal naar verwachting de derde belangrijkste doodsoorzaak wereldwijd worden in 2020 (alleen overtroffen door hart- en vaatziekten en beroerte). Uit veel humane studies is gebleken dat COPD patiënten een twee tot drie keer verhoogde kans hebben op hart- en vaatziekten dan 'gezonde rokers'. Het mechanisme dat hieraan ten grondslag ligt is onduidelijk. De term COPD omvat drie aandoeningen die vaak gecombineerd voorkomen in patiënten: aandoening van de kleine luchtwegen, chronische bronchitis en longemfyseem. Ontsteking speelt een grote rol bij alledrie de aandoeningen. Chronische bronchitis wordt gekenmerkt door chronisch hoesten en sputum (slijm uit de diepe luchtwegen) opgeven. Bij longemfyseem is er verlies van longweefsel door afbraak van longblaasjes. In **hoofdstuk 4** werd onderzocht wat de effecten van longemfyseem, oftewel destructie van longblaasjes onafhankelijk van roken, zijn op atherosclerose ontwikkeling. Hiervoor werd in de longen van *E3L* muizen éénmalig elastase toegediend. Elastase is een enzym dat elastine, een belangrijk component van longblaasjes, afbreekt. Naast de controlegroep waarbij fysiologisch zout werd toegediend, werden twee verschillende doses elastase, *i.e.* 15 en 30 μg, gebruikt om dosisafhankelijke effecten te kunnen bestuderen. Na 20 weken cholesterolrijk dieet werd de mate van atherosclerose in de drie groepen muizen bepaald. Er werd geen verschil in de hoeveelheid atherosclerose gevonden, maar wel een dosisafhankelijke afname in de ernst van atherosclerose in de groepen behandeld met elastase. Dit kan deels verklaard worden door een dosisafhankelijke afname van ontstekingscellen in het bloed van de muizen die elastase toegediend hebben gekregen. De lipidenniveaus in het bloed waren gelijk tussen alle groepen. Deze resultaten wijzen erop dat alleen destructie van longblaasjes geen verklaring is voor het verhoogde risico op atherosclerose in COPD patiënten. Een verhoogde staat van ontsteking in het lichaam draagt bij aan de ontwikkeling van zowel atherosclerose als COPD en kan dus de missende (verklarende) link zijn tussen beide aandoeningen. Mogelijk leidt de toegenomen aanwezigheid van ontstekingsmediatoren in de longen bij COPD tot lekkage van deze mediatoren in de circulatie wat de ontwikkeling van atherosclerose kan stimuleren.

Door meer inzicht te krijgen in de processen die betrokken zijn bij de vorming van atherosclerose kunnen nieuwe strategieën worden ontwikkeld om hart- en vaatziekten tegen te gaan. Een veelbelovend middel hiervoor is resveratrol, een bestanddeel dat van nature aanwezig is in veel Aziatische kruiden, noten, de schil van rode druiven en in rode wijn. Uit verschillende

studies blijkt resveratrol anti-atherosclerotische werkingen te hebben, zoals het verlagen van lipiden en het verminderen van ontstekingsreacties. Statines zijn cholesterolverlagende middelen die reeds veelvuldig gebruikt worden om de vorming van atherosclerose te remmen in patiënten en hebben ook meerdere anti-atherosclerotische werkingen, waaronder het remmen van ontstekingsprocessen. In **hoofdstuk 5** werd onderzocht of resveratrol de vorming van atherosclerose kan tegengaan in muizen én of dit effect van toevoegende waarde is naast een statine, *i.e.* atorvastatine. Hiervoor werden *E3L* muizen gebruikt die ook het cholesteryl ester transfer proteïne (CETP) gen tot expressie brengen, waardoor het vetmetabolisme van de mens nog sterker benaderd wordt. Muizen hebben van nature namelijk geen CETP die nodig is voor uitwisseling van cholesteryl esters en triglyceriden tussen verschillende vetdeeltjes. *E3L.CETP* muizen werden verdeeld over 4 groepen die elk een ander middel in het dieet kregen: 1. zonder toevoeging (controlegroep); 2. met resveratrol; 3. met atorvastatine; en 4. met de combinatie resveratrol en atorvastatine. Atherosclerose was in dezelfde mate verlaagd in alle groepen met toevoeging van resveratrol en/of atorvastatine, wat voornamelijk verklaard kan worden door een gelijke verlaging in de VLDL-spiegels in de drie behandelingsgroepen ten opzichte van de controlegroep. De niveaus van ontstekingsmediatoren in het bloed was niet verschillend tussen de groepen. Resveratrol heeft dus anti-atherosclerotische potenties, maar biedt geen aanvullende bescherming naast de werking van atorvastatine. Gezien de gunstige effecten van resveratrol en statines op ontstekingsprocessen wordt de effectiviteit van deze middelen ook buiten het gebied van de hart- en vaatziekten onderzocht, waaronder voor COPD.

In **hoofdstuk 6** worden de implicaties en het toekomstperspectief van de verkregen resultaten besproken. Op het gebied van onderzoek naar hart- en vaatziekten is de focus van de slagaders, *i.e.* lokaal, aan het verschuiven naar de bijdrage van distale organen zoals de lever en de longen. Aandoeningen van deze organen moeten daarom meegenomen worden om het risico op hart- en vaatziekten in kaart te brengen en een behandelplan op te stellen. Door meer inzicht te krijgen in de wisselwerking tussen verschillende organen wordt de ontwikkeling van nieuwe middelen gestimuleerd en het toepassingsgebied van bestaande therapieën uitgebreid. Dit leidt tot meer mogelijkheden om niet alleen hart- en vaatziekten, maar ook andere geassocieerde aandoeningen zoals COPD tegen te gaan.

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Curriculum Vitae

Man Chi Wong werd geboren op 14 juni 1981 in Den Haag. In 1999 behaalde zij haar gymnasium diploma aan het Gymnasium Haganum in Den Haag. In datzelfde jaar begon zij met haar studie Biomedische Wetenschappen aan de Universiteit Leiden. Na het behalen van haar propedeuse *cum laude* in 2000 begon ze daarnaast aan de studie Geneeskunde. De eerste onderzoeksstage voor Biomedische Wetenschappen voerde zij uit bij de afdeling Nierziekten van het Leids Universitair Medisch Centrum onder begeleiding van dr. Y. W. J. Sijpens. Deze stage richtte zich op epidemiologische, klinische en histologische kenmerken van chronische transplantaat glomerulopathie. Haar tweede stage voerde ze in het kader van de Excellente Studenten Traject uit bij de afdelingen Oogheelkunde en Dermatologie van het Leids Universitair Medisch Centrum onder begeleiding van dr. W. Zuidervaart en dr. P. J. Hensbergen. Tijdens deze stage deed zij onderzoek naar nieuwe biomarkers voor tumorprogressie en –metastasering van uvea melanoom met behulp van proteomics. Zij behaalde in 2004 haar doctoraal diploma Biomedische Wetenschappen *cum laude*. Na het behalen van haar artsenbul in 2006, werkte zij een jaar als arts-assistent Interne geneeskunde in het MCH St. Antoniushove in Leidschendam. In mei 2007 begon zij haar promotieonderzoek op een samenwerkingsproject tussen de afdelingen Longziekten en Endocrinologie en Metabole ziekten van het Leids Universitair Medisch Centrum onder begeleiding van prof. dr. P. S. Hiemstra, prof. dr. ir. L. M. Havekes en dr. J.F.P. Berbée. Het promotieonderzoek, waarvan de resultaten zijn beschreven in dit proefschrift, werd afgerond in juni 2011. Van september 2007 volgende ze de studies Foundations of Chinese Medicine en Acupuncture aan Shenzhou Univerisity in Amsterdam, waar ze in juli 2010 *cum laude* voor slaagde. Vanaf juli 2011 is zij aangesteld als arts-assistent in opleiding tot medisch microbioloog aan het Leids Universitair Medisch Centrum.

Dankwoord

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Man-Chi