

HMG-CoA Reductase Inhibitors: Effects on Chronic Subacute Inflammation and Onset of Atherosclerosis Induced by Dietary Cholesterol

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Abstract: Besides classical risk factors such as hypercholesterolemia and hypertension, chronic subacute inflammation has recently been recognized as an important force driving the development of atherosclerosis, the most common underlying cause of myocardial infarction and stroke. There is compelling evidence that a disturbance of cholesterol homeostasis contributes to the development of a chronic inflammatory state and that inhibitors of HMG-CoA reductase (statins) may dampen inappropriate inflammatory responses. We review the evidence and suggest mechanisms by which dietary cholesterol can induce an atherogenic inflammatory response in liver and vessel wall, with particular emphasis on the time course of this inflammatory response during atherogenesis and the interplay between these tissues. We discuss how statins interfere in this process, and whether they may reduce chronic subacute inflammation *via* a) their cholesterol-lowering effect, and/or b) their cholesterol-independent (pleiotropic) vasculoprotective activities. Recent studies performed in (humanized) animal models allow us to distinguish the lipid-lowering-dependent from the lipid-lowering-independent functions of statins. Using these data, we discuss the degree to which the lipid-lowering-dependent and lipid-lowering-independent effects of statins contribute to a reduction of inflammation, allowing estimation of the relevance of pleiotropic statin effects for the human situation.

Key Words: Atherosclerosis, biomarkers, C-reactive protein (CRP), cholesterol, inflammation, HMG-CoA reductase inhibitor, liver, pleiotropic effect, statins.

INTRODUCTION

Despite remarkable progress in medical therapeutics and in the understanding of their biology, cardiovascular diseases (CVD) are expected to remain a leading cause of morbidity and mortality in the next decades. CVD such as coronary artery disease, peripheral artery disease and cerebrovascular disease are all common consequences of a chronic degenerative disease, atherosclerosis, and are associated with serious clinical events such as myocardial infarction and stroke. Current therapeutic strategies are generally directed at lowering serum LDL cholesterol levels. Remarkably, half of all cardiovascular events occur in persons with normal or even low plasma lipids [1,2], and the majority of patients suffering from coronary artery disease do so without having elevated cholesterol [3,4]. Recent epidemiological and experimental findings point to subacute chronic inflammation as an important novel risk factor of CVD, and plasma markers which sense elevated levels of inflammation appear to be very strong predictors of cardiovascular events independent of LDL cholesterol [5,6]. On the basis of these and other observations [7-9], the more realistic view emerges that atherosclerosis is not solely a lipid disorder but, at least, also a chronic inflammatory disease.

The notion that atherosclerosis also is an inflammatory disease offers new opportunities for the prevention and treatment of CVD. However, knowledge about factors and mechanisms causing and/or contributing to the chronic inflammatory state underlying the pathogenesis of atherosclerosis is still scarce, and no specific anti-inflammatory drugs are yet available for clinical use (many are in

development) to prevent or treat this inflammatory state. In fact, the ways in which the dietary stressor cholesterol exerts its pro-atherogenic effects and may contribute to inflammation is only partly understood [10,11].

The scope of this review is intended to discuss new insights regarding the pro-inflammatory role of cholesterol and to review the remarkable anti-inflammatory properties of a class of cholesterol-lowering drugs, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins). After an overview of cholesterol with respect to its uptake, biosynthesis and homeostasis, the properties of cholesterol that induce adverse inflammatory effects in liver and aorta will be discussed. The upregulation of vascular cell adhesion molecule-1 (VCAM-1) in the endothelium in response to hypercholesterolemia is well-known, but the initiation of an inflammatory response in the liver is less appreciated. Related to this, the anti-inflammatory properties of cholesterol-lowering statins will be reviewed. Using *in vivo* data regarding statins and clinical trials, the discussion will center on whether statins may reduce cholesterol-induced inflammation in the liver and/or the vessel wall through a) their cholesterol-lowering effect and b) their pleiotropic activities, i.e. their activities independent of lowering cholesterol. Finally, the review will explore the extent to which both statin effects contribute to a reduction of atherogenesis with particular emphasis on data derived from humanized *in vivo* models.

CHOLESTEROL BIOSYNTHESIS AND INHIBITION OF HMG-COA REDUCTASE

Cholesterol Uptake and Intracellular Cholesterol Synthesis

Cholesterol is an essential structural component of cellular membranes and is converted into steroid hormones and bile acids, which fulfill important roles in the mediation

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of cellular signals, the control of gene expression, the absorption of fat from the small intestine and the secretion of liver waste products [12,13]. Mammalian cells either acquire cholesterol *via* the diet, or synthesize cholesterol *de novo* [14-16].

In the intestine, dietary cholesterol and triglycerides are packaged into chylomicrons which enter the circulation *via* the lymph as illustrated in Fig. 1. The capillary vessel wall of peripheral tissues contains lipoprotein lipase, which hydrolyzes triglycerides in the core of chylomicrons into free fatty acids. The resulting chylomicron remnant particles are relatively enriched in cholesterol and are rapidly cleared by the liver *via* apolipoprotein E-mediated binding to specific cell surface receptors, i.e. low density lipoprotein receptor (LDLR) and low density lipoprotein receptor-related protein (LRP).

The *de novo* synthesis of cholesterol is depicted in Fig. 2 and can be divided in three major parts: 1) In the cytoplasmic compartment, thiolase combines two acetyl-CoA units to acetoacetyl-CoA which is further combined by

HMG-CoA synthase with a third acetyl-CoA unit to form the six carbon (C6) intermediate HMG-CoA. HMG-CoA is transported to the ER membrane where it is reduced to mevalonate by the membrane-bound enzyme HMG-CoA reductase using NADPH as reductant [17]. HMG-CoA reductase is considered to be the rate-limiting enzyme of cholesterol synthesis and the pharmacological target of all statins. 2) Mevalonate is subsequently decarboxylated to form isoprene, a key C5 intermediate, which is also involved in the synthesis of ubiquinone, vitamin K and carotenoids. Six isoprene units are used to generate the C30 compound squalene. During squalene synthesis, the intermediate farnesyl-pyrophosphate (F-PP) and the side product geranylgeranyl-pyrophosphate (GG-PP) are formed. GG-PP and F-PP are required for the posttranslational modification of proteins and serve as lipid attachments [18-21] (Fig. 2). 3) In the last stage of cholesterol synthesis, squalene is reduced in the presence of molecular oxygen and NADPH to squalene epoxide and cyclized to form lanosterol. Lanosterol is finally reduced and demethylated to form the end product, cholesterol.

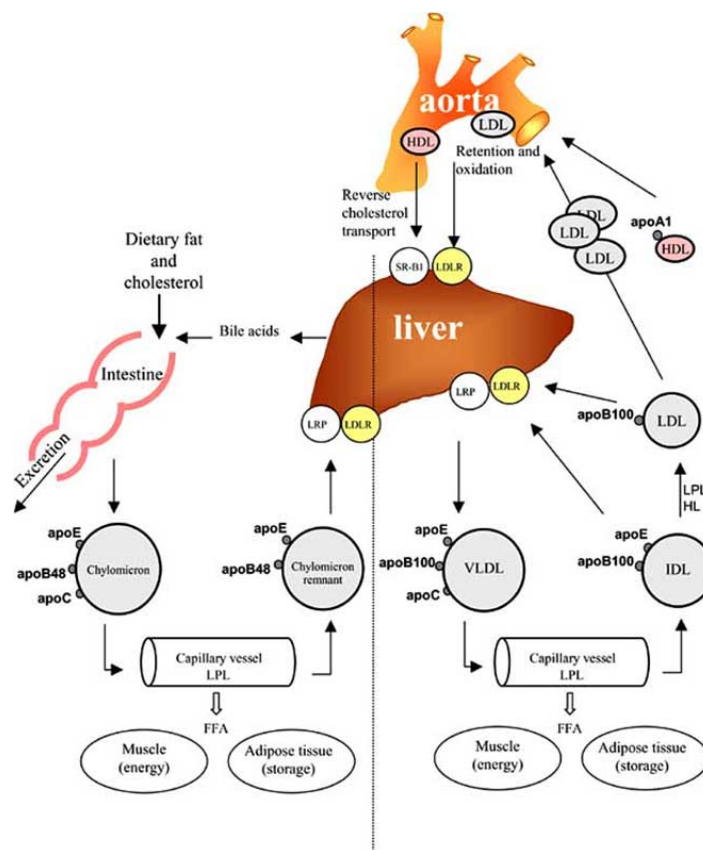


Fig. (1). Extracellular cholesterol homeostasis.

Schematic illustration of processes having a role in the maintenance of extracellular cholesterol homeostasis: uptake of dietary cholesterol in the intestine and chylomicron-dependent transport of cholesterol to the liver; packaging of cholesterol in very low density lipoproteins (VLDL) in the liver; formation of intermediate density lipoproteins (IDL) and low density lipoproteins (LDL) involving lipoprotein lipase (LPL) and hepatic lipase (HL); lipoprotein uptake by the liver; LDL retention and oxidation in the vessel wall. ApoE: apolipoprotein E; apoB100: apolipoprotein B100; apoC: apolipoprotein C; FFA: free fatty acids; LDLR: low density lipoprotein receptor.

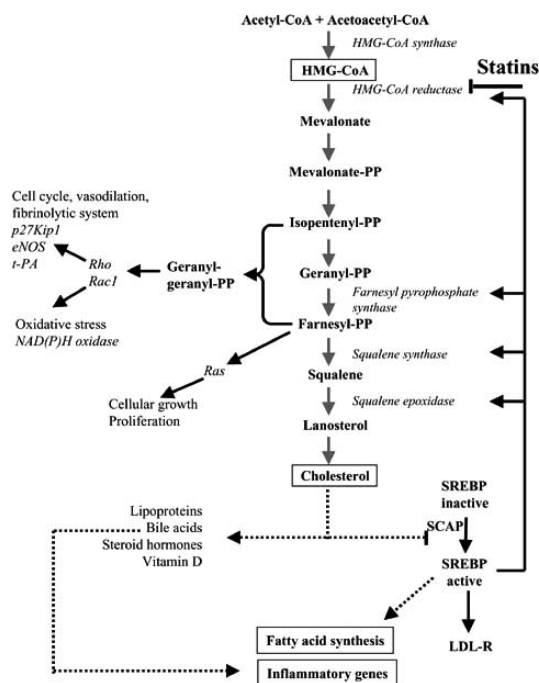


Fig. (2). Cholesterol biosynthesis and biological actions of isoprenoids.

Diagram of the cholesterol biosynthesis pathway and related processes. HMG-CoA reductase, the pharmacological target of statins, is considered to be rate-limiting for the synthesis of cholesterol from acetyl-CoA. HMG-CoA reductase and many other enzymes involved in cholesterol biosynthesis are regulated by sterol regulatory element binding proteins (SREBPs). SREBPs also control the expression of the low density lipoprotein receptor (LDLR) and genes involved in fatty acid synthesis and inflammation. Inhibition of HMG-CoA reductase by statins results in a decreased intracellular content of isoprenoid intermediates. As a consequence, posttranslational isoprenylation of signaling molecules, such as Ras, Rho and Rac1, is impaired and leads to modulation of cellular and biological processes.

Intracellular Cholesterol and Extracellular Homeostasis

Because an excess of intracellular cholesterol is cytotoxic and lack of cholesterol leads to membrane dysfunction [16,22-24], the cellular level of cholesterol has to be controlled carefully. Sterol regulatory element-binding proteins (SREBPs) are important transcriptional regulators of intracellular cholesterol homeostasis (Fig. 2). When intracellular cholesterol levels decrease, SREBP-1 and SREBP-2, which are located at the endoplasmic reticulum (ER) membrane, are transported from the ER to the Golgi by an SREBP-cleavage activating protein (SCAP), which contains a cholesterol-sensing domain (reviewed in detail in [25]). At the Golgi, two proteases, site 1 protease (S1P) and S2P, sequentially cleave the SREBPs. After the second cleavage, the active SREBPs are released into the cytosol, allowing them to enter the nucleus where they bind to sterol regulatory elements (SRE-1) in the promoter regions of a

genes required for cholesterol biosynthesis, viz. 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase, HMG-CoA reductase, farnesyl pyrophosphate (F-PP) synthase, squalene synthase and squalene epoxidase [25,26], b) genes involved in fatty acid synthesis such as fatty acid synthase and stearoyl-CoA desaturase 1 [27,28] and c) genes mediating inflammatory reactions such as C/EBP β and IL-8 [28,29] (Fig. 2).

Hepatically synthesized cholesterol designated for secretion and transport to other cells in the body is acetylated before it is packaged into very low density lipoprotein (VLDL) transport particles (Fig. 1). VLDL particles also contain triglycerides which are unloaded at target organs (adipocyte, muscle) giving rise to intermediate density lipoprotein (IDL) and low density lipoprotein (LDL) remnant particles. LDL particles constitute the major transporters of cholesterol and are recognized by other cell types by their LDLR and LRP receptors. After endocytosis of LDL particles, cholesterol is extracted in the lysosomal compartment and the receptors are recycled to the cellular surface [16]. The promoter region of the LDL receptor gene also contains an SRE-1 site [30]. Hence, activation of SREBPs not only accelerates *de novo* cholesterol synthesis but also receptor-mediated cholesterol uptake (Fig. 2). When intracellular cholesterol concentrations are sufficient, the movement of SCAP-SREBP complexes to the Golgi and SREBP processing is suppressed which leads to reductions in cholesterol synthesis and LDL uptake [26].

Mode of Action and Pharmacokinetics of Clinically Relevant Statins

Statins are referred to as a very potent class of drugs that reduce serum cholesterol levels and prevent CVD. It is estimated that about thirty million people worldwide are currently using a statin. Statins were originally designed and developed to compete with HMG-CoA for binding at the catalytic site of HMG-CoA reductase and thereby to reduce the synthesis of mevalonate as illustrated in Fig. 2. Simvastatin, cerivastatin, lovastatin, fluvastatin, atorvastatin, rosuvastatin and pravastatin all share an HMG-like moiety (Fig. 3). They inhibit HMG-CoA reductase activity with K_i values in the nanomolar range, whereas the natural substrate HMG-CoA binds only at micromolar concentrations and is therefore displaced by the drugs. The plasma cholesterol-lowering effect of statins results mainly from the enhanced receptor-mediated uptake of LDL in the liver, i.e. the increased clearance from the bloodstream *via* the upregulation of LDLR, and only to a lesser extent from the reduction of cholesterol biosynthesis [26,31]. The newer 'third-generation' statins, such as atorvastatin and rosuvastatin, inhibit HMG-CoA reductase with higher affinity and for longer duration than lovastatin and pravastatin. The third generation statins also appear to be more effective in lowering serum cholesterol levels and reductions of up to 60% have been reported [32-34]. Statins are structurally different and differ in their lipophilicity and tissue permeability which is especially relevant to peripheral (side) effects (Fig. 3). While the very hydrophilic statins, such as rosuvastatin and pravastatin, are predominantly taken up by hepatic cells *via* organic anion transporting polypeptides C (OATP-C) [33,35,36], the more lipophilic statins, such as

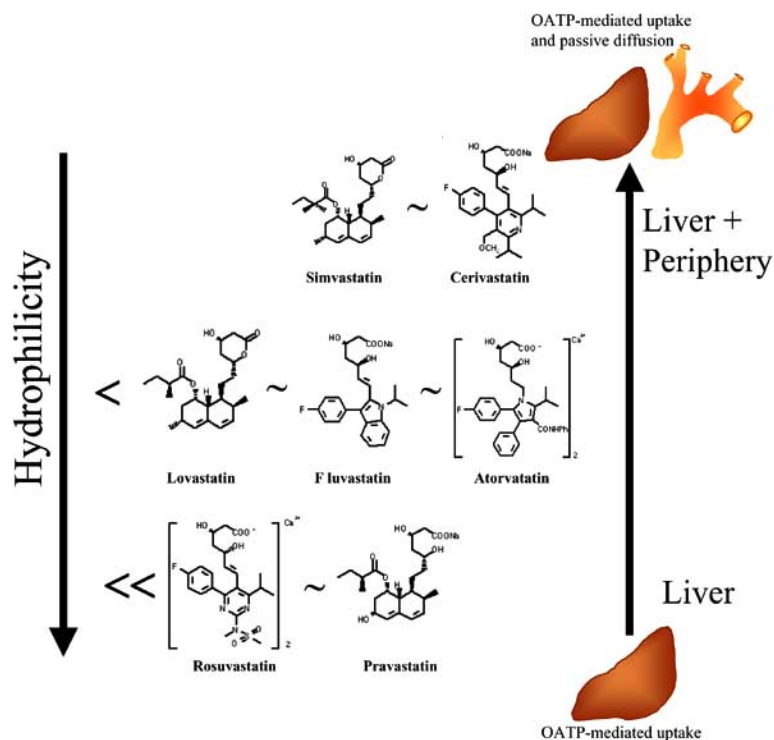


Fig. (3). Hydrophilicity of statins and extrahepatic effects.

Differences in the lipophilicity and tissue permeability of clinically relevant statins. Very hydrophilic statins, such as rosuvastatin and pravastatin, are predominantly taken up by hepatic cells *via* organic anion transporters (OATP). More lipophilic statins have a greater chance to enter endothelial cells by passive diffusion and to exert extrahepatic effects.

atorvastatin, fluvastatin and simvastatin, may also enter endothelial cells by passive diffusion [31,32,37]. Taking into account pharmacokinetic factors such as hepatic extraction [38], the statin concentration achieved in such extrahepatic target tissues is likely to be lower than the concentration achieved in hepatocytes for the hydrophilic statins.

Before discussing whether statins dampen subacute chronic inflammation associated with CVD through their activities in liver and in extrahepatic tissues, we will review evidence and suggest mechanisms by which dietary cholesterol may induce such a chronic inflammatory state.

SUBACUTE CHRONIC INFLAMMATION: MARKERS, INDUCERS AND ROLE OF THE LIVER

Markers of Hepatic and Aortic Inflammation

A state of chronic subacute inflammation that is associated with increased atherosclerotic risk, is reflected by elevated levels of blood inflammation markers among which are C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, von Willebrand factor (vWF), E-selectin, VCAM-1 and intracellular cell adhesion molecule-1 (ICAM-1) [6]. These factors are relatively stable and sense the activation of pro-inflammatory transcription factors such as nuclear factor- κ B (NF- κ B), activator protein-1 (AP-1) or signal transducer and

activator of transcription-3 (STAT3) through positive regulatory elements in their promoter regions. CRP, for example, integrates and stabilizes the inflammatory signals of the relatively unstable cytokines IL-1 β , IL-6, leukemia inhibitory factor (LIF) and tumor necrosis factor- α (TNF α) [39-41]. Because many blood inflammation markers are expressed in a tissue-specific way, i.e. CRP, SAA and fibrinogen in hepatocytes and vWF, E-selectin, VCAM-1 and ICAM-1 in endothelial cells, they can be viewed as indirect measures of the inflammatory status of the liver and the vessel wall, respectively.

There is still debate whether some of the liver-derived inflammatory reactants are merely biomarkers for the atherosclerotic processes or whether they may also directly participate in atherosclerotic lesion development [42-44]. The observations that CRP accelerates diet-induced atherosclerosis [45] and is enriched in atherosclerotic lesions, where it induces monocyte chemoattractant protein-1 (MCP-1) and adhesion molecule expression [46,47], and that SAA functions as an apolipoprotein that enhances cholesterol uptake by vascular smooth muscle cells (SMCs) [48,49] argue that hepatic proteins may also participate in atherosclerotic lesion development. In addition to this, the complement factors (C3, C4, C9, factor B, factor D), coagulant/fibrinolytic factors (fibrinogen, plasminogen),

antiproteases (alpha1-antitrypsin, PAI-1), and the vasoactive precursor angiotensinogen, are established inflammation-induced hepatic acute phase reactants with adverse cardiovascular effects [50-53]. Together, these findings support the view that liver-derived inflammatory proteins may *directly* participate in the atherogenic process and may play key roles in actuating its initiation and progression.

Time Course of Cholesterol-Induced Inflammation

Although inflammation is considered to be a major force driving the development of atherosclerosis, the time course, extent and tissue specificity of inflammation during disease development remains uncertain. To address this issue we measured the plasma levels of hepatic and vascular inflammation markers during diet-induced onset of atherosclerosis in an established mouse atherosclerosis model, ApoE3-Leiden (A3L) mice (Fig. 4A) [54,55]. After 2 weeks on a high cholesterol (HC) diet containing 1% (w/w) cholesterol, the plasma level of (liver-derived) SAA was already strongly elevated, whereas the vascular inflammation marker E-selectin was hardly increased (not shown).

In the liver, HC diet-feeding increased the mRNA expression levels of inflammatory pro-atherogenic factors including acute phase factors (SAA, orosomucoid), complement factors (factor D, C3a), cytokines (IL-1, VEGF, GM-CSF), transcription factors (NF- κ B) and proteases (MMP-12, MMP-19). It is noteworthy that the induction of these atherogenic genes was less pronounced in mice which received a low cholesterol (LC) diet containing only 0.15% w/w cholesterol, suggesting a dose-dependent effect of the dietary stressor cholesterol. We next analyzed whether the hepatic inflammatory response elicited by HC-feeding would again normalize when the cholesterol pressure would be decreased. Indeed, HC-induced plasma SAA levels declined and reached initial values 10 weeks after a switch had been made from a HC to a LC diet (Fig. 4B). Of note, this normalization process is a much slower process than the dietary induction of a hepatic response.

The observation that dietary cholesterol induces a rapid inflammatory response in livers of A3L mice (*cf.* Fig. 4) is in agreement with the findings of other groups using different animal models and different dietary stressors [10,11]. In addition to their results, our data demonstrate that the hepatic inflammatory response precedes inflammation in the vessel wall and the development of first (mild) atherosclerotic lesions in the aortic root (see arrow in Fig. 4A). As the development of atherosclerosis continues, hepatic inflammation further increased, and inflammation associated with atherosclerosis in the vessel wall appeared to intensify the inflammatory response of the liver. A hypothetical model illustrating the initiating role of the liver in early atherogenesis and the subsequent interplay with the vessel wall is depicted in Fig. 5. A role for hepatic inflammatory reactants in early atherogenesis is also supported by the observation that circulating CRP and other hepatic acute phase reactants are among the strongest independent predictors of atherosclerotic risk, both in patients suffering from metabolic diseases *and* in apparently healthy patients [5,7,43,56].

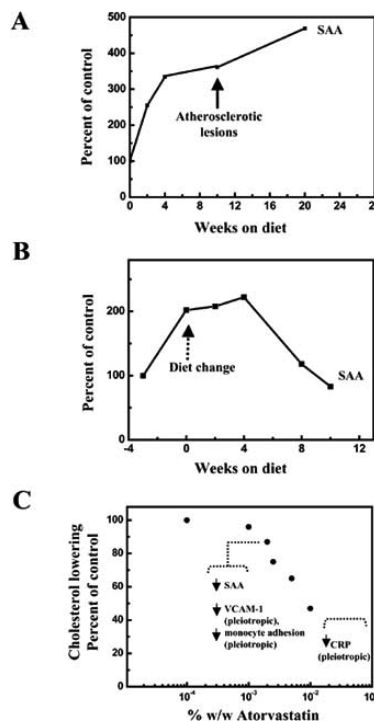


Fig. (4). Time course of cholesterol-induced subacute chronic inflammation.

A. Time course of hepatic inflammation. Female ApoE3-Leiden (A3L) mice were fed a Western type diet containing 1% w/w cholesterol to develop atherosclerotic lesions. Tail blood samples were taken at the time points indicated. The plasma concentrations of serum amyloid A (SAA), reflecting hepatic inflammation, was determined as described [54] and expressed as a percentage of the initial value at $t=0$ ($2.6 \mu\text{g SAA/mL}$). The black arrow indicates the time point at which the first atherosclerotic lesions have been observed in monitor mice. **B.** Effect of lowering dietary cholesterol intake on subacute chronic hepatic inflammation. Female A3L were fed a diet containing 0.25% w/w cholesterol for 4 weeks ($t=-4$ to $t=0$) to induce a hepatic inflammatory response and received then (see arrow) a hypocholesterolemic diet containing only 0.15% w/w cholesterol for 10 additional weeks. Plasma SAA concentrations were measured over time as indicated in A. **C.** Dose-dependency of the cholesterol-lowering effect of atorvastatin in A3L mice on a Western type diet. Data in C are derived from Verschuren *et al.* [85]. The effects of atorvastatin on monocyte adhesion, aortic VCAM-1 expression and plasma SAA have been analyzed in the A3L mouse model. Pleiotropic effects have been determined through comparison of an atorvastatin-treated group to a low cholesterol control group. In this low cholesterol control group the plasma cholesterol level was adjusted to match the level achieved by atorvastatin treatment. Cholesterol-lowering-dependent and cholesterol-lowering-independent (pleiotropic) effects are indicated with arrows. The pleiotropic effect of atorvastatin on CRP has been observed in human CRP transgenic mice on a chow diet as described [86].

the vessel wall *via* the bloodstream and therefore may link early hepatic inflammation to atherogenic processes in the vessel wall.

REDUCTION OF CHRONIC SUBACUTE INFLAMMATION BY STATINS

Cholesterol-Lowering Effect of Statins and Beyond

The data shown in Fig. 4B demonstrate that hepatic inflammation is reduced when the dietary cholesterol intake is lowered suggesting that plasma cholesterol-lowering by statins may have a similar protective effect. We have reviewed animal studies which addressed this issue and which a) reported a cholesterol-lowering effect by statins and b) have measured hepatic inflammatory reactants during treatment with statins (Table 1). In a mouse atherosclerosis study, atorvastatin lowered plasma cholesterol levels by 53% and reduced plasma SAA and fibrinogen levels by 67% and 22%, respectively when compared with placebo [84]. In A3L mice with established mild atherosclerotic lesions and elevated plasma SAA levels, atorvastatin decreased plasma LDL cholesterol and, in parallel, SAA levels (Fig. 4C) [85]. The decrease in SAA could be fully explained by the cholesterol-lowering effect of atorvastatin. Another study analyzed the effect of statins on basal and IL-1 β -induced inflammation, i.e. in the absence of atherosclerosis or dietary stress, using human CRP transgenic mice [86]. In this study, anti-inflammatory CRP-reducing effects of atorvastatin and simvastatin were found at statin concentrations that were higher than required for cholesterol-lowering *per se*. This study also provides a molecular rationale for the suppression of CRP by statins at high doses: statins have anti-inflammatory activity through the reduction of p50-NF- κ B translocation into the nucleus *via* upregulation of I κ B- α , the cytosolic inhibitor of NF- κ B. This results in a diminished amount of nuclear p50-NF- κ B~C/EBP β complexes which are rate-limiting for CRP transcription [39,39].

To summarize, these observations demonstrate a) that statins reduce dietary stress-induced inflammation through their cholesterol-lowering effect, and b) that statins also have the potential to suppress the formation of hepatic inflammatory reactants independent of their cholesterol-lowering effect. The clinical relevance of the latter notion has recently been demonstrated: patients who have low CRP levels after statin therapy have better clinical outcomes than those with higher CRP levels, regardless of the resultant level of LDL cholesterol [87]. In the following paragraph we discuss how statins may suppress hepatic/aortic inflammation through their pleiotropic anti-inflammatory activities, i.e. independently of lowering serum cholesterol levels.

Statin Effects Independent of Cholesterol-Lowering

A pleiotropic effect of a drug is defined as an effect for which the drug is not specifically developed and which is not directly related to its primary mechanism of action [88], i.e. inhibition of HMG-CoA reductase and lowering cholesterol in the case of statins. Although the clinical benefit of statin therapy is primarily attributed to its LDL-lowering effect, various subgroup analyses of clinical trials have demonstrated that statins reduce cardiovascular risk, more than was expected, by LDL lowering [89,90]. For example, in the West of Scotland Coronary Prevention Study (WOSCOPS),

the event rates between placebo and pravastatin groups with the same LDL cholesterol level were compared, and CV risk was found to be lower in the pravastatin-treated group [91].

In addition to these clinical observations, a large number of early *in vitro* studies in which statins were employed, albeit at relatively high concentrations [18,92-95], demonstrated that statins have the potential to exert effects independently of lowering cholesterol. Many of these pleiotropic effects observed *in vitro* are related to the protection of the vascular endothelium. The enhancement of endothelial nitric oxide synthase (eNOS) activity and, as a result of this, the increased bioavailability of nitric oxide (NO) is a good example to illustrate the diversity of underlying molecular mechanisms: statins have been shown to stabilize eNOS mRNA [96]; to decrease the production of reactive oxygen species that inactivate NO [97]; to activate serine/threonine kinase Akt and enhance eNOS phosphorylation; and to block the function of Rho as an endogenous inhibitor of endothelial NO generation [18,94] (compare Fig. 2). Rho is a guanosine triphosphate (GTP)-binding protein which cycles between the inactive GDP-bound state and the active GTP-bound state, and translocates from the cytoplasm to the plasma membrane. Posttranslational geranylgeranylation of Rho is a requirement for its function as a signal transducer. Statins reduce the intracellular content of GG-PP isoprenoids as shown in Fig. 2 and thereby inhibit Rho-dependent effects [18,94]. A similar inhibitory mechanism applies to Ras, the translocation of which is dependent on farnesylation, and to other Ras-like proteins, such as Rab, Rac1, Ral or Rap.

In addition to the statin-mediated effects on NO synthesis, the pleiotropic vasculoprotective *in vitro* effects of statins also include beneficial effects on cell adhesion, migration, proliferation, matrix degradation, thrombosis and apoptosis as reviewed by others (for details see [26,98-100]). Important examples of pleiotropic *in vitro* effects which are already observed at physiological relevant concentrations are the inhibition of endothelial cell proliferation [101]; the inhibition of E-selectin expression [102]; the inhibition of cytokine-stimulated CD40 expression in human endothelial cells [103]; the increase of fibrinolytic activity in human peritoneal mesothelial cells [19,20]; and the downregulation of inflammatory transcription factors in human endothelial and smooth muscle cells [104].

The clinical relevance of the pleiotropic effects of statins, however, is discussed widely because many *in vitro* studies demonstrating pleiotropic effects employ relatively high statin doses which are not achievable *in vivo*. Furthermore, there is a lack of a) direct clinical evidence that distinguishes the lipid-lowering-dependent from the lipid-lowering-independent functions of statins [98] and b) information about the *degree* to which the lipid-lowering-dependent and lipid-lowering-independent functions of statins contribute to a particular *in vivo* effect. These facts, together with our poor understanding of the molecular basis of the pleiotropic effects, emphasize the need to analyze the cholesterol-independent effects of statins in more human-like experimental settings (i.e. human primary cells, humanized animal models) using relevant drug doses which only moderately lower plasma cholesterol.

Table 1. Anti-Inflammatory Pleiotropic Effects of Statins in Animal Models

Statin (Dose) Animal model	Effect on plasma cholesterol	Protective effect
Simvastatin (20 mg/kg/d) Cynomolgus monkeys [109]	(↑)	Plaque stabilization: macrophage and collagen content (↓); TF, VCAM-1, IL-1 expression (↓)
Simvastatin (50 mg/kg/day) ApoE ^{-/-} mice [111]	(↑)	Tissue factor expression in atherosclerotic lesions (↓)
Simvastatin (50 mg/kg/day) ApoE ^{-/-} mice [112]	(↑)	Plaque stabilization: calcification (↓)
Simvastatin (100 mg/kg/day) ApoE ^{-/-} mice [113]	(-)	Aortic cholesterol content (↓)
Simvastatin (120 mg/kg/day) Transgenic huCRP C57BL/6J mice [86]	(↓)	Chronic subacute and acute inflammation (↓); basal and IL-1β-induced CRP levels (↓)
Cerivastatin (0.6 mg/kg/day) WHHL rabbits {Shiomi, 1999 141 /id	(↓)	Plaque stabilization: macrophage content (↓)
Cerivastatin (2.5 mg/kg/day) C57/BL6 mice [114]	(NR)	Tumor growth and vascularization (↓)
Lovastatin (2 and 5 mg/kg/d) SJL/J mice [115]	(NR)	Inflammatory state (NF-κB) (↓) in experimental autoimmune encephalomyelitis (EAE)
Fluvastatin (1 mg/kg/day) C57BL/6J mice [116]	(-)	Neointima formation (↓); smooth muscle cell proliferation (↓)
Fluvastatin (5 mg/kg/day) New Zealand White rabbits [117]	(-)	Macrophage lesion content (↓)
Atorvastatin (2 mg/kg/day) ApoE*3-Leiden mice [85]	(↓)	Progression of established lesions (↓); monocyte adhesion (↓); VCAM-1 expression (↓)
Atorvastatin (10 mg/kg/day) 129/SV mice [118]	(-)	Cerebral ischemia (↓); platelet activation (↓)
Atorvastatin (10 mg/kg/day) ApoE*3-Leiden mice [84]	(↓)	SAA, fibrinogen (↓); lesion area and lesion calcification (↓)
Atorvastatin (120 mg/kg/day) Transgenic huCRP C57BL/6J mice [86]	(↓)	Chronic subacute and acute inflammation (↓); basal and IL-1β-induced CRP levels (↓)
Rosuvastatin (5 mg/kg/day) ApoE*3-Leiden mice [54]	(↓)	Lesion formation (↓); monocyte adhesion, macrophage content (↓); MCP-1 and TNFα expression (↓)
Rosuvastatin (1 and 10 mg/kg/day) ApoE ^{-/-} mice [119]	(-)	Neointima formation (↓)
Pravastatin (10 mg/kg/day) LDLR ^{-/-} mice [120]	(-)	Plaque stabilization: atherosclerotic lesion area (↓); collagen content (↓)
Pravastatin (40 mg/kg/day) Cynomolgus monkeys [109]	(-)	Plaque stabilization: macrophage and collagen content (↓); TF, VCAM-1, IL-1 expression (↓)

In vivo studies with statins in which pleiotropic effects on parameters of vascular and/or hepatic inflammation have been described. The various statins are listed according to their degree of hydrophilicity and the dose used. (↑) indicates upregulated; (↓) indicates downregulated; NR: not reported.

Pleiotropic Effects in (Humanized) Animal Models

The ApoE*3-Leiden (A3L) mouse model is to date one of the very few atherosclerosis models in which both plasma

cholesterol-dependent and plasma cholesterol-independent effects of statins can be evaluated and distinguished [54]. The model offers the opportunity to titrate plasma

cholesterol levels to a desired level by adjusting the dietary cholesterol intake [55,105]. This enables the investigator to compare a drug-treated group to a control group which receives a milder hypercholesterolemic diet resulting in the same plasma cholesterol level [54,105]. Furthermore, A3L mice display a human-like lipoprotein profile, i.e. a lipoprotein profile in which elevated plasma cholesterol and triglyceride levels are mainly confined to the VLDL/LDL-sized lipoprotein fraction and develop lesions that are comparable to their human counterparts with respect to morphological, histological and immunohistochemical characteristics [55,106]. So far, three statins, rosuvastatin, atorvastatin and pravastatin, have been tested in this model to estimate the degree by which the cholesterol-lowering-independent effects of statins may contribute to a further reduction of onset of lesion formation [54] and to a further reduction of lesion progression [85] (Table 1).

Treatment of mildly hyperlipidemic A3L mice with rosuvastatin (5 mg/kg/day), resulted in a (submaximal) 25% reduction of plasma cholesterol [54], i.e. a plasma cholesterol reduction which is similar to or lower than the reduction commonly observed in statin-treated humans [8,91,107,108]. Under these rather modest cholesterol-modulating conditions in A3L mice, which mimic the treatment regime in humans, rosuvastatin reduced the formation of new atherosclerotic lesion beyond and independently of its plasma cholesterol-lowering effect [54]. Subsequent analysis of both vascular (MCP-1 and TNF α) and plasma (SAA and fibrinogen) inflammation markers indicated that the pleiotropic anti-inflammatory effects of rosuvastatin contributed directly to a further reduction of atherogenesis. This observation is also consistent with several *in vivo* studies that have demonstrated anti-atherogenic effects of statins on lesion formation in the absence of lipid-lowering but with an anti-inflammatory effect as a common denominator (Table 1). For example, simvastatin and pravastatin reduced the vascular expression of VCAM-1, IL-1 and tissue factor (TF) in cynomolgus monkeys [109]. Both statins also increased the intimal SMC content of abdominal aorta and the interstitial collagen content of atherosclerotic plaques. Similar plaque-stabilizing effects were reported for cerivastatin, fluvastatin, atorvastatin and rosuvastatin, i.e. for lipophilic and hydrophilic statins. While pleiotropic effects related to vasculoprotection appear to be unrelated to the chemical properties of statins, beneficial effects on tumor growth or experimental autoimmune encephalomyelitis have so far predominantly been reported for the more lipophilic statins. An effect of the more hydrophilic statins in extrahepatic compartments cannot be excluded because pravastatin has been detected in the cerebral cortex and has been shown to modulate the expression of genes involved in cell growth and signaling and trafficking in the brain [110].

Undoubtedly, the above animal studies have validated the concept of anti-inflammatory pleiotropic actions of statins *in vivo*. The design of most of these studies however deviates from the clinical norm because the statin treatment of the animals was started simultaneously with the onset of the experimentally controlled disease, i.e. for example, long before the first atherosclerotic lesions were formed. There is

a clear need therefore to generate reliable data demonstrating that pleiotropic effects are also beneficial at an advanced stage of the disease process under conditions mimicking current medical practice. Our group recently analyzed whether atorvastatin, when administered at a low dosage, would exert anti-inflammatory effects and would retard the progression of *existing* atherosclerotic lesions beyond its moderate cholesterol-lowering effect [85]. Mildly hyperlipidemic A3L mice were treated with a low-dose atorvastatin (2 mg/kg/day) resulting in an only 19% reduction of plasma cholesterol (compare Fig. 4C). This was sufficient to block the progression of lesions in the aortic arch independently and beyond atorvastatin's cholesterol-lowering effect. Atorvastatin also reduced particular aspects of vascular inflammation, such as monocyte adhesion and VCAM-1 expression (Fig. 4C), independently of its cholesterol-lowering effect. The observation that statins downregulate the activation of the transcription factors NF- κ B and AP-1, both of which are required for VCAM-1 expression, provides a molecular rationale for this suppressive effect [20,104].

CONCLUSION

In conclusion, recent experimental evidence demonstrates that the dietary stressor cholesterol exerts part of its adverse effects by eliciting a rapid inflammatory response in the liver. This hepatic inflammatory response precedes the development of atherosclerotic lesions in the vessel wall and is characterized by an increased expression of inflammatory reactants with potential pro-atherogenic effects. Among the hepatic inflammatory reactants formed are acute phase proteins, cytokines and complement factors, that may reach the vessel wall *via* the bloodstream. A state of subacute inflammation will become a chronic one if cholesterol homeostasis is disturbed continuously and for a longer period of time. Thus, dietary constituents may lead to a state of chronic subacute inflammation which drives the development of atherosclerosis and other metabolic diseases.

One of the strategies for therapy of CVD is to dampen inappropriate inflammatory responses using pharmaceuticals which combine lipid-lowering with anti-inflammatory activities. Statins may exert such a dual effect: they lower serum cholesterol levels and reduce the plasma level of specific hepatic (CRP, fibrinogen) and vascular inflammation markers (VCAM-1, E-selectin). The reduction of inflammation markers by statins is partly a consequence of their cholesterol-lowering effect (i.e. the removal of the stressor) and partly due to anti-inflammatory pleiotropic effects, i.e. above-and-beyond their cholesterol-lowering effect.

Over the last 5 years, a growing number of *in vivo* studies have provided convincing evidence that statins, in particular when applied at high doses, protect the vessel wall more than

can be expected from their cholesterol-lowering effect alone. These animal studies should encourage further investigation of whether the pleiotropic actions of statins may additionally retard the progression of advanced atherosclerotic lesions or potentially contribute to the regression of the disease. The physiological and clinical relevance of such future regression

studies will depend on the statin doses employed and the degree of cholesterol reduction achieved. Therefore, experimental settings which mimic the treatment regime in humans and clinical circumstances should be preferred. Such future regression studies may also provide us with valuable information on when to use a single drug with pleiotropic anti-inflammatory activities and when to combine a lipid-lowering drug with, for example, an anti-inflammatory drug.

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ABBREVIATIONS

A3L	= Apolipoprotein E3-Leiden
AP-1	= Activator protein-1
CVD	= Cardiovascular disease
CRP	= C-reactive protein
eNOS	= Endothelial nitric oxide synthase
hAPR	= Hepatic acute phase reactants
HMG-CoA	= 3-hydroxy-3-methylglutaryl coenzyme A
ICAM-1	= Intracellular adhesion molecule-1
LDL	= Low-density lipoprotein
MCP-1	= Monocyte chemoattractant protein-1
NADPH	= Nicotinamide adenine dinucleotide phosphate (reduced form)
NF- κ B	= Nuclear factor kappa B
NO	= Nitric oxide
SAA	= Serum amyloid A
SCAP	= SREBP cleavage activating protein
SP-1	= Site 1 protease
SMC	= Smooth muscle cells
SRE	= Sterol regulatory element
SREBP	= Sterol regulatory element binding protein
STAT	= Signal transducer and activator of transcription
TF	= Tissue factor

TNF- α	= Tumor necrosis factor- α
VCAM-1	= Vascular cell adhesion molecule-1
VLDL	= Very low density lipoprotein
vWF	= Von Willebrand factor

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