The interplay between cholesterol and inflammation in the evolution of atherosclerosis

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Chapter 4

Fenofibrate reduces atherogenesis in ApoE*3Leiden mice: evidence for multiple anti-atherogenic effects besides lowering plasma cholesterol

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Arterioscler Thromb Vasc Biol. 2006 Oct;26(10):2322-30

Abstract

Background: To demonstrate, quantify, and mechanistically dissect anti-atherosclerotic effects of fenofibrate besides lowering plasma cholesterol per se.

Results: ApoE*3-Leiden transgenic mice received either a high-cholesterol diet (HC) or HC containing fenofibrate (HC+FF) resulting in 52% plasma cholesterol-lowering. In a separate low-cholesterol diet (LC) control group, plasma cholesterol was adjusted to the level achieved in the HC+FF group. Low plasma cholesterol alone (assessed in LC) resulted in reduced atherosclerosis (lesion area, number and severity) and moderately decreased plasma serum amyloid-A (SAA) concentrations. Compared to LC, fenofibrate additively reduced lesion area, number and severity, and the total aortic plaque load. This additional effect in HC+FF was paralleled by an extra reduction of aortic inflammation (macrophage content; monocyte adhesion; ICAM-1, sVCAM-1, GM-CSF, MCP-1, and NF-κB expression), systemic inflammation (plasma SAA and fibrinogen levels), and by an upregulation of plasma ApoE levels. Also, enhanced expression of ABC-A1 and SR-B1 in aortic macrophages may contribute to the anti-atherosclerotic effect of fenofibrate by promoting cholesterol efflux.

Conclusions: Fenofibrate reduces atherosclerosis more than can be explained by lowering total plasma cholesterol per se. Impaired recruitment of monocytes/macrophages, reduced vascular and systemic inflammation, and stimulation of cholesterol efflux may all contribute to these beneficial effect of fenofibrate.

Introduction

Atherosclerosis is a complex, multifactorial disease of the large arteries and the leading cause of morbidity and mortality in industrialized nations.[1] Hypercholesterolemia is a well-established risk factor for the incidence of atherosclerosis and its pathologic complications. Current therapies for treatment of atherosclerosis are generally directed at lowering cholesterol levels. Statins have been shown to effectively lower circulating cholesterol levels and to reduce cardiovascular causes of death.[1] Yet, many (>50%) patients still experience adverse coronary events, despite statin therapy.[2] Also, the rate at which atherosclerosis progresses can vary considerably at any given plasma cholesterol level, and 20% of MIs occur in individuals with normal plasma cholesterol levels.[3,4] This suggests that factors other than cholesterol also contribute to the development of atherosclerosis.

Accumulating evidence points to inflammation as a driving force of atherogenesis [2,5], and chronically elevated levels of circulating inflammation markers are independently predictive of atherosclerotic risk and mortality in otherwise healthy patients.[6-8] Furthermore, an inverse relationship exists between high-density lipoprotein (HDL) cholesterol and atherosclerosis in the general population[1,5,9], possibly related to the anti-oxidative and anti-inflammatory properties of HDL as well as to its role in reverse cholesterol transport (RCT).[9,10]

Fibrates are potent lipid-lowering drugs to treat hypertriglyceridemia and mixed dyslipidemia.[11] They efficiently lower plasma triglycerides and LDL cholesterol, and in increase HDL cholesterol by upregulating the hepatic gene expression and synthesis of apoAI, the major apolipoprotein of HDL.[11] Fibrates exert their activity via activation of the nuclear receptor peroxisome proliferator-activated receptor α (PPARα). PPARα in conjunction with retinoid X receptor(RXR), positively regulates the transcription of target genes through binding to specific gene promoter response elements. This mode of action is particularly important for the regulation of genes that control lipid and lipoprotein metabolism and may, in large part, explain the normolipidemic action of fibrates.[12] Independent of this mechanism, PPARα can also act as a negative regulator of pro-inflammatory genes via antagonizing the activity of inflammatory transcription factors.[13-15] Together, these properties may result in an overall beneficial action of fibrates on cardiovascular disease.[11,12]. A large part of this effect of fibrates is independent of their effect on the plasma lipoprotein levels *per se*, because the changes in major lipids and lipoproteins could account for less than 25% of the beneficial effects of fibrate therapy.[11,16]

In line with this, pleiotropic effects of fibrates have been demonstrated: fibrates strongly reduced circulating markers of inflammation and suppressed cytokine-induced acute phase response reactions in mice and man, independent of an effect on plasma cholesterol.[15,17,18]

Based on these findings and the above characteristics of fibrates we hypothesized that fibrates may exert beneficial effects on atherosclerotic lesion development by mechanisms involving both lipid-lowering (i.e. hypocholesterolemic) effects and effects not directly linked to this, e.g. anti-inflammatory and RCT-stimulating effects.

Previous studies directed at evaluating the role of fibrates in atherogenesis in mice, *viz.* in apoE-deficient and LDLR-deficient animals, were hampered by model-inherent drawbacks.[19-21] Here, we used ApoE*3Leiden (E3L) transgenic mice, a well-established mouse atherosclerosis model. E3L mice display a lipoprotein profile comparable to that of patients suffering from dysbetalipoproteinemia, i.e. plasma cholesterol and triglyceride levels are mainly confined to the VLDL/LDL fraction[22], and respond to hypolipidemic drugs in a similar way as humans.[22,23] These characteristics, in combination with the possibility to titrate plasma cholesterol levels of E3L mice to a desired level by adjusting their dietary cholesterol intake, enabled us to quantify the fenofibrate-evoked reduction in atherosclerosis with particular emphasis on its anti-inflammatory properties and its reverse cholesterol transport promoting activities.

Methods

Animals

Female E3L transgenic mice were characterized for expression of human apoE by ELISA. Mice were 12 weeks of age at the beginning of the study. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Scientific Research (TNO) and were in compliance with European Community specifications.

Diets

During a run-in period of 3 weeks, 51 female E3L mice received an atherogenic, 0.50% w/w cholesterol containing Western-type diet[24] further referred to as high cholesterol (HC) diet. Then, mice were subdivided into 3 groups (n=17). In one group, HC diet feeding was continued for another 18 weeks (HC group). The fenofibrate-treated group (HC+FF group) received the same diet as the HC group but supplemented with fenofibrate (Sigma-Aldrich). Based on the food intake in the HC+FF group, the daily dose of fenofibrate was 30 mg/kg bodyweight. The low cholesterol control group (LC group) received the same Western-type diet[24] but containing only 0.05% w/w cholesterol to reach a comparable plasma cholesterol level as the HC+FF group.

Analysis of plasma lipids, lipoproteins and plasma inflammation markers

Total plasma cholesterol and triglyceride levels were measured after 4 hours of fasting, using kits No.1489437 (Roche Diagnostics, Almere, The Netherlands) and No.337-B (Sigma Diagnostics), respectively. For lipoprotein profiles, pooled plasma was fractionated using an ÅKTA FPLC system (Pharmacia, Roosendaal, The Netherlands).[23]

For analysis of apolipoproteins, (fractionated) plasma samples were analyzed according to a described SDS-PAGE/Western blotting procedure[13] using the following antibodies: anti-mouse apoE (Santa Cruz, The Netherlands; sc-6384), anti-mouse apoAI- and apoB-specific antibodies (prepared at TNO-Pharma) and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz; sc-2304 and sc-2305).

The plasma levels of sVCAM-1 (R&D Systems), SAA (Biosource) and fibrinogen (in-house assay) were determined by ELISA as reported.[22]

Atherosclerotic lesion analysis

After 18 weeks of treatment mice were sacrificed to collect hearts and aortas. Hearts were fixed and embedded in paraffin to prepare serial cross sections (5 µm thick) throughout the entire aortic valve area for (immuno)histological analysis. Cross sections were stained with hematoxylin-phloxine-saffron (HPS), and atherosclerosis was analyzed blindly in four cross-sections of each specimen (at intervals of 30 µm) as described.[23] QWin-software (Leica) was used for morphometric computer-assisted analysis of lesion number, lesion area, and lesion severity[23] according to the classification of the American Heart Association.[25] Analysis of atherosclerosis in longitudinally opened oil-red O-stained aortas has been described in detail elsewhere.[23]

Monocytes and macrophages were immunostained in cross sections adjacent to the ones used for quantification of atherosclerosis using AIA31240 (1:3000, Accurate Chemical and Scientific, Brussels, Belgium) to determine the number of monocytes attached to the endothelium, the macrophage-containing lesion area.[22,23] SMC area was determined as described.[23] For immunostaining of ICAM-1, MCP-1 and NF-kB/p65, antibodies CBL1316 (Chemicon International, Heule, Belgium), sc-1784 and sc-8008 (Santa Cruz) were used, respectively.

Nucleic acid extraction and gene expression analysis

Total RNA extraction was performed using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads according to the manufacturer's instructions. Then, cDNA was prepared using kit #A3500 (Promega, Leiden, The Netherlands) for real-time polymerase chain reaction (RT-PCR) analysis. The mastermix (Eurogentec, Seraing, Belgium), an ABI-7700 system (PE Biosystems, Nieuwekerk a/d Ijssel, The Netherlands) and established primer/probe sets[18,23,26,27] were used according to the manufacturer's instructions. Cyclophilin (PE Biosystems) was used as a reference.

Statistical methods

Significance of differences was calculated by one-way analysis of variance (ANOVA) test followed by a least significant difference (LSD) post hoc analysis using SPSS 11.5 for Windows (SPSS, Chicago, USA). The level of statistical significance was set at $P<0.05$.

Results

Fenofibrate reduces plasma lipids in E3L mice

The food intake was on average 2.6±0.1 g/day in HC, which is comparable to that in HC+FF (2.7±0.1 g/day) and LC (2.8±0.1 g/day). There was no significant difference in body weight gain between the experimental groups.

Plasma cholesterol levels in HC remained at a constant level (on average 25.5±3.4 mM; Figure 1A). Fenofibrate rapidly, within one week, reduced cholesterol to 8.6±2.1 mM ($P<0.01$), a decrease of 66%. Cholesterol levels of HC+FF remained strongly reduced and were on average 12.4±2.8 mM. The dietary cholesterol intake in LC was adjusted to match the plasma cholesterol levels achieved in HC+FF. With the exception of week 1 and 3, plasma cholesterol levels did not significantly differ between HC+FF and LC. Integrated over the whole treatment period, the total cholesterol exposure of HC was 517±42 mM·weeks (Figure 1B).

The total cholesterol exposure of HC+FF and LC did not significantly differ from each other and was 52% ($P<0.01$) less than that of HC.

Fasting plasma triglyceride levels in HC were on average 1.45±0.50 mM and did not significantly change over time (Figure 1C). Fenofibrate-treatment reduced plasma triglyceride concentrations within one week to 0.25±0.06 mM, a decrease of 83% ($P<0.01$); this low level was maintained during the remainder of the study. Triglyceride concentrations in LC averaged 1.2±0.4 mM, which is comparable to HC and thus markedly higher than in HC+FF.

In HC+FF, the number of lesions was reduced by 89% ($P<0.01$), whereas the cholesterol-matched LC group showed only a 67% ($P<0.01$) reduction, a 22% ($P<0.04$) lesser decrease in number of lesions than in HC+FF.

Measurement of the total cross-sectional lesion area revealed a similar additional effect of fenofibrate (Figure 2B). Whereas HC displayed a cross-sectional lesion area of 291,000±31,700 μm^2, cholesterol-lowering alone (LC) reduced the lesion area by 87% ($P<0.01$) while with fenofibrate a further reduction was seen (89% vs. LC; $P<0.05$).

The total plaque load in longitudinally opened, en face oil-red O-stained aortas of HC was 4.2±1.8 mm^2 (not shown). LC displayed a 67% ($P<0.01$ vs. HC) smaller plaque load, and fenofibrate further reduced this area by an additional 28% ($P\leq0.05$ vs LC). Together, these data indicate that fenofibrate reduces atherosclerosis development more than can be explained by lowering plasma cholesterol alone.

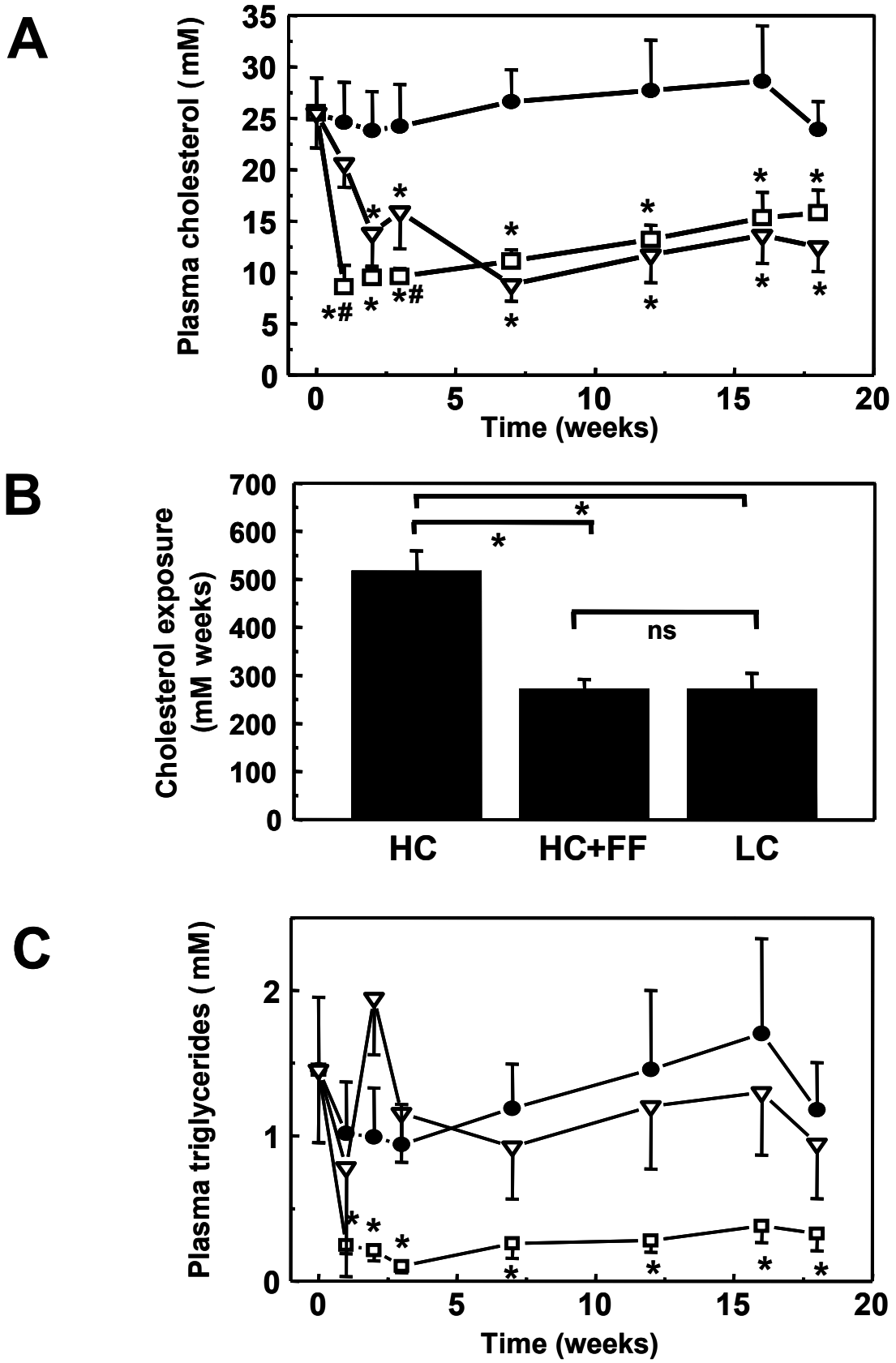

Figure 1. Effect of fenofibrate on plasma cholesterol. A, Plasma cholesterol concentration in E3L mice over time **B,** Total cholesterol exposure after 18 weeks of treatment. **C,** Plasma triglycerides over time during treatment period. Means ± SD (n=17). *P<0.01 compared with HC. #P<0.01 compared with LC. HC is indicated by solid circles, HC+FF by open squares and LC by open triangles.

Fenofibrate reduces atherosclerosis beyond the effect attributable to lowering total plasma cholesterol *per se*

After 18 weeks of experimental treatment, atherosclerosis was analyzed in cross-sections of the aortic valve area. The average lesion number per mouse was 10.2±3.3 in HC (Figure 2A).

To assess differences in lesion severity, cross-sections of the aortic root were analyzed and lesions were graded[25] (Figure 2C). In HC, no lesion-free sections were detectable; the analyzed cross-sections contained either mild type I-III (31% of all cross-sections) or severe type IV-V (69% of all cross-sections) atherosclerotic lesions. In LC, 63% of all cross-sections were lesion-free, 25% contained mild and 13% contained severe lesions. In HC+FF, 90% of all cross-sections were lesion-free and only 10% contained mild lesions; severe lesions were not observed.

Together, these data demonstrate that fenofibrate strongly reduces initiation and progression of atherosclerotic lesion formation and more than can be explained by lowering plasma cholesterol alone.

Fenofibrate reduces inflammatory processes in the arterial wall

To evaluate whether the above described additional effects of HC+FF vs. LC are paralleled by a reduced inflammatory state of the aorta, we evaluated various inflammation-related parameters, with particular emphasis on the contribution of macrophages.

In HC, the absolute macrophage-containing lesion area was 69,500±9,400 μm^2, which was reduced by 82% ($P<0.01$) in LC ($P<0.01$) and by 99% ($P<0.01$) in HC+FF (Figure 3A). The additional 17% reduction in HC+FF was significantly ($P<0.01$) different from LC.

The macrophage density, i.e. the macrophage area expressed as *percentage of the total cross-sectional lesion area*, did not significantly differ between HC (10±2%) and LC (10±3%), but fenofibrate strongly (by 86%) and significantly ($P<0.01$) lowered the macrophage density (Figure 3B).

To further explore the additional effects of fenofibrate, we analyzed whether fenofibrate inhibits the recruitment of macrophage precursor cells, blood monocytes. Whereas monocyte adhesion in LC was not significantly different from that in HC, HC+FF indeed showed a strongly and significantly reduced monocyte adhesion, equal to the level observed in chow-fed E3L mice (Figure 3C).

These cell-related effects were paralleled by a strongly diminished endothelial expression of the intercellular adhesion molecule ICAM-1 and a lower plasma level of sVCAM-1 in HC+FF (Figures 3D,E,G). Furthermore, the total number of cells per cross-section with positive p65-NFκB-staining (in cytosol and/or nucleus) was strongly reduced in HC+FF (Figure 3F).

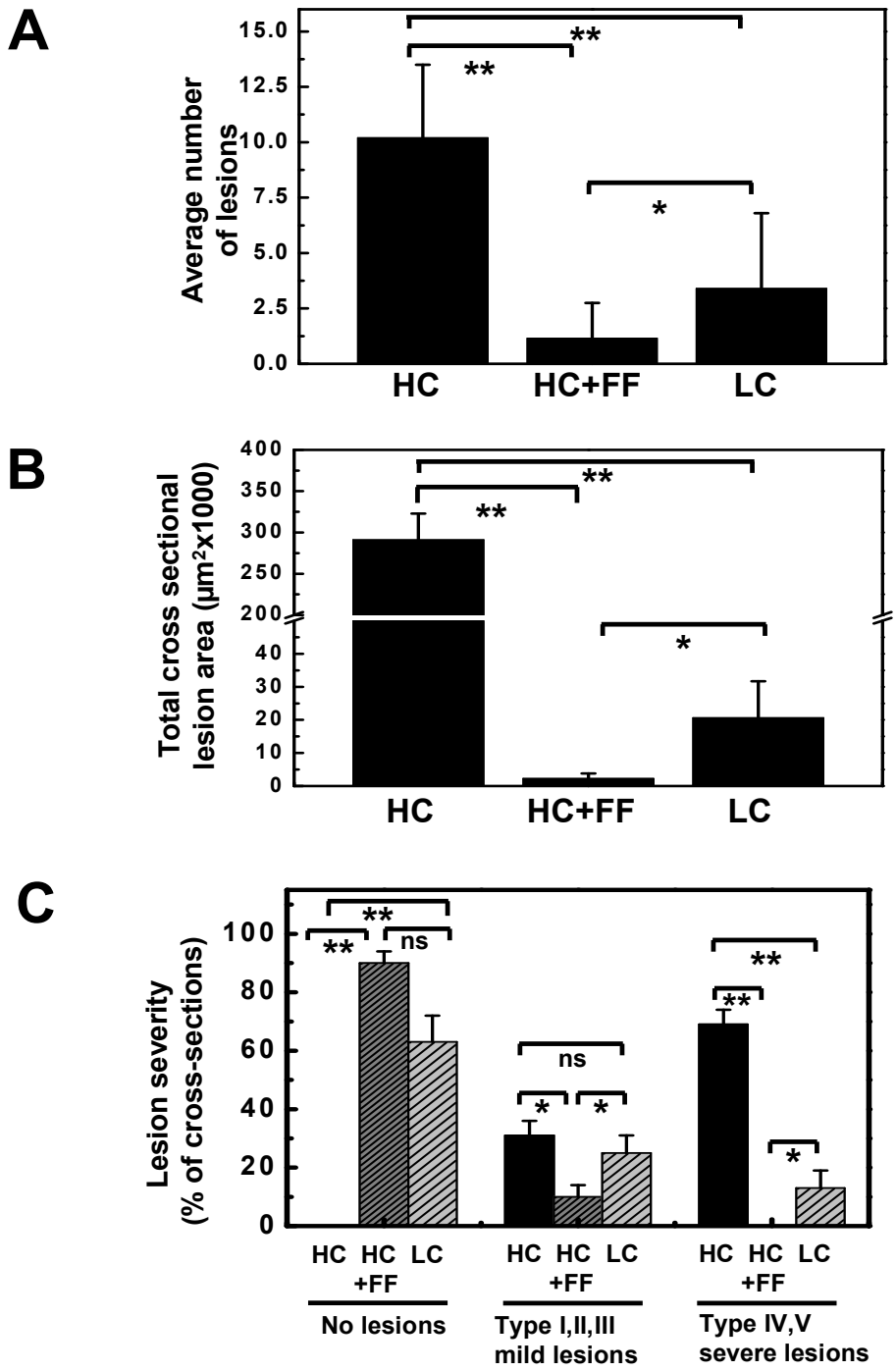

Figure 2. Effect of fenofibrate on atherosclerosis in the aortic root. **A,** Effect of fenofibrate on the lesion number per mouse. Means ± SD. **B,** Effect of fenofibrate on the total cross-sectional lesion area. **C,** Effect of fenofibrate on lesion severity. Data are presented as percentage of cross-sections analyzed. Means ± SEM (n=17 mice). *P<0.05, **P<0.01.

Assessment of p65-NFκB-stained endothelial cells (ECs) per cross-section revealed a comparable number of p65-NFκB-positive ECs in HC (17.9±5.6%) and LC (13.6±9.6%), but a strong reduction in HC+FF (0.8±1.2%; P<0.05 vs. HC and LC). Also, the number of ECs showing active (i.e. nucleus-associated) p65-NFκB immunoreactivity in HC+FF (0.1±0.3%) was strongly and significantly (P<0.05) reduced compared to HC (2.3±5.6%) and LC (0.8±1.4%). These results indicate that fenofibrate not only reduces the number of p65-NFκB- positive (endothelial) cells, but, most importantly, also the number of active p65-NFκB containing ECs, thus providing a molecular explanation for the reduced monocyte adhesion in HC+FF.

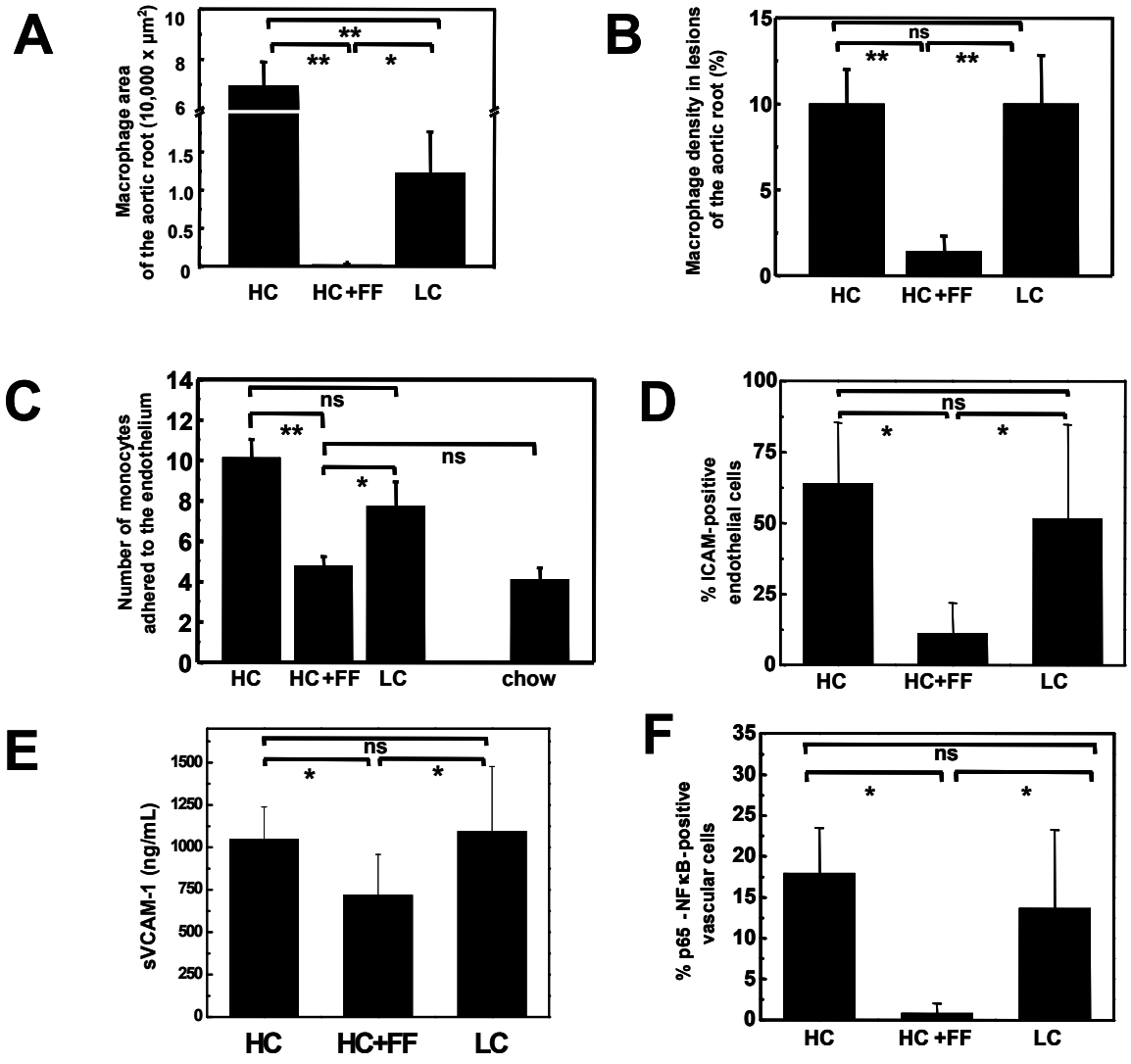

Figure 3. Effect of fenofibrate on the vascular inflammatory state. A, Absolute macrophage area in the aortic sinus. **B,** Macrophage density, i.e. macrophage area expressed as a percentage of the total lesion area (right). **C,** Effect of fenofibrate on the number of monocytes attached to the endothelium. Data A-C represent means ± SEM (n≥10 mice). **D,** Percentage of ICAM-1-positive endothelial cells. **E,** Plasma levels of soluble VCAM-1. **F,** Percentage of p65-NFkB-positive vascular cells (n=7). Data D-F represent means ± SD. (Figure 3 is continued on next page)

The vascular expression of the pro-inflammatory chemokine MCP-1 and the monocyte/macrophage differentiation factor GM-CSF also was strongly reduced in HC+FF but not in LC (Figures 3H and 3I), suggesting that fenofibrate not merely impairs adhesion, but also recruitment and maturation of monocytes/macrophages.

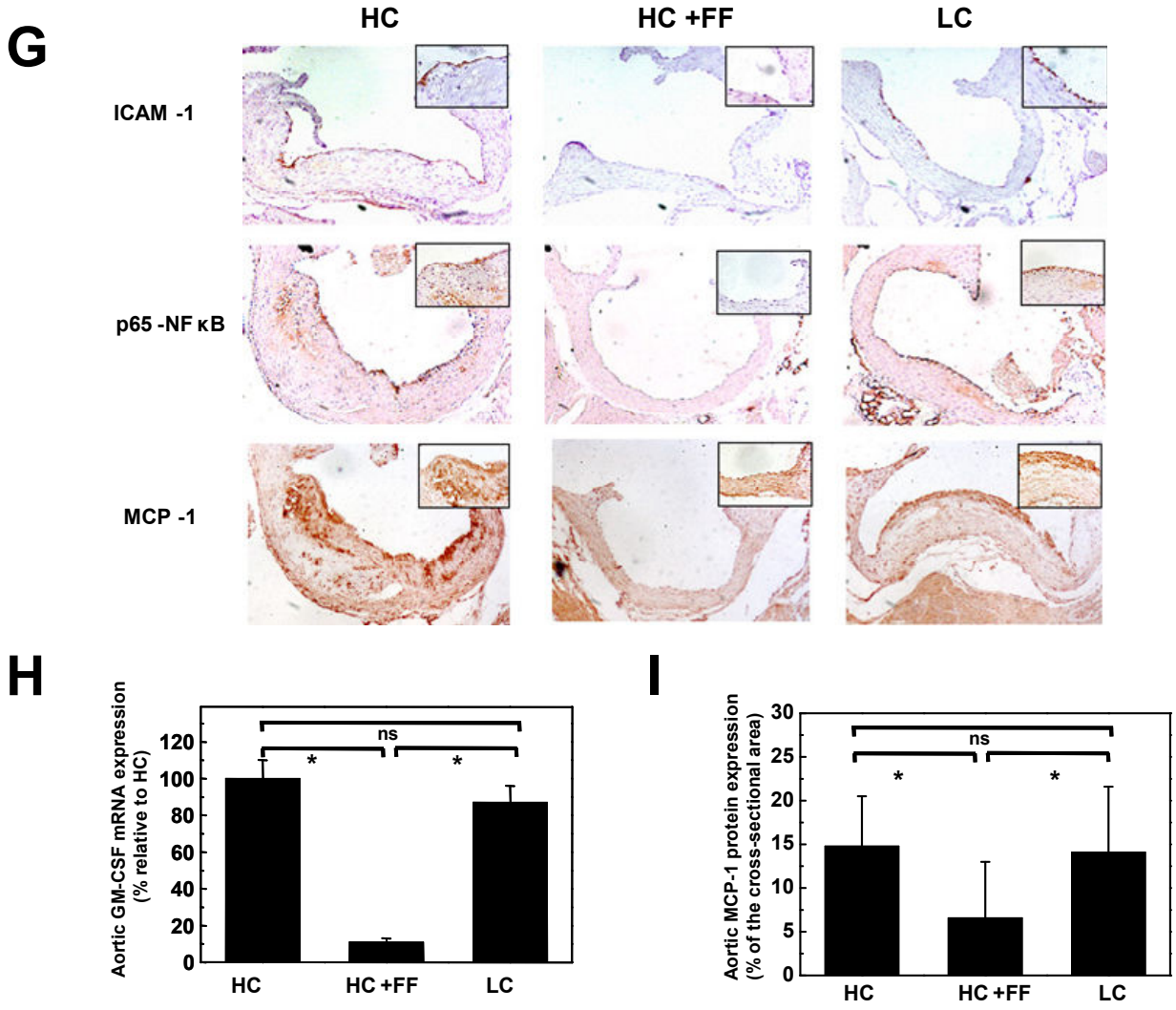

Figure 3. Effect of fenofibrate on the vascular inflammatory state. (continued) G, Expression of ICAM-1, p65-NFkB and MCP-1 protein in cross-sections of the aortic root (one representative photomicrograph out of n=7). **H,** Aortic GM-CSF mRNA expression (means ± SEM; n=5). **I**, Aortic MCP-1 protein expression. Means ± SD. *P<0.05, **P<0.01.

SMC content, a measure of plaque stability, in LC was comparable to that in HC (3.84±2.52% and 4.45±0.5% of the total lesion area, respectively), but markedly reduced in HC+FF (1.60±1.05%, P<0.05 vs. HC).

Fibrinogen and serum amyloid A (SAA) are two independent plasma inflammation markers which reflect the general systemic inflammatory state, and putatively participate in atherosclerotic lesion development. As shown in Figure 4, HC displayed higher plasma fibrinogen (3.3±0.5 mg/ml) and SAA (13.5±5.5 µg/ml) concentrations than chow-fed E3L

mice (1.8±0.3 mg/ml fibrinogen; 3.2±2.0 µg/ml SAA). LC diet did not (fibrinogen) or only moderately (SAA) reduce the inflammation markers, but fenofibrate markedly lowered plasma fibrinogen and SAA levels by 33% (P<0.01) and 61% (P<0.01), respectively, relative to LC.

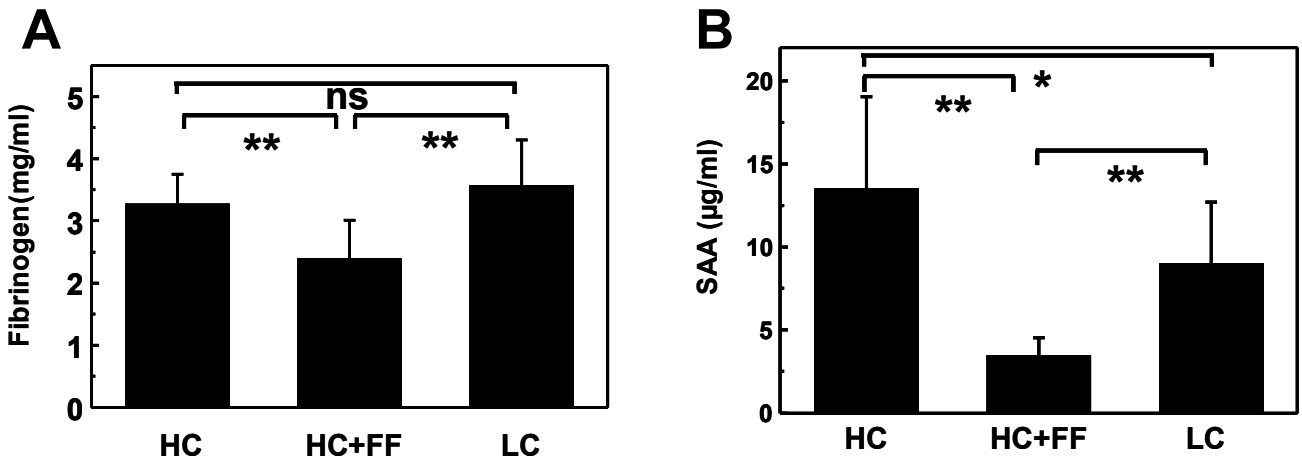

Figure 4. Effect of fenofibrate on circulating inflammation markers. Effect of fenofibrate on plasma concentrations of fibrinogen (**A**) and SAA (**B**) after 3 weeks of fenofibrate-treatment. Means ± SD (n≥10 mice per group). *P<0.01, **P<0.001.

Effect of fenofibrate on lipoprotein profiles and cholesterol efflux

Clinical data have shown a strong inverse relationship between plasma levels of HDL and its major apolipoprotein, apoA1, and the incidence of atherosclerotic vascular disease.

Analysis of plasma apoA1 protein levels showed no significant differences between HC and LC, whereas a decrease of plasma apoA1 levels (58% reduction) and hepatic apoA1 mRNA expression levels (49% reduction) was found in HC+FF as determined by immunoblotting and RT-PCR, respectively (data not shown).

Figure 5A depicts the lipoprotein profiles for cholesterol after 12 weeks of experimental treatment. Grosso modo, the three experimental groups did not show significant differences in their HDL cholesterol levels, but in HC+FF and LC, the amount of VLDL cholesterol and IDL cholesterol was strongly reduced relative to HC. Strikingly, in HC+FF a peak emerged in the transition area between small IDL/LDL particles and large HDL particles (fractions 10-17). To further define the HDL-related fractions and to characterize the fenofibrate- induced peak at fractions 10-16, lipoprotein fractions were analyzed by SDS-PAGE and Western blotting (Figure 5B). In all groups, fractions ≥17 contained mainly the HDL-specific apoAI but only little amounts of apoE and no apoB. Fractions 12-16, corresponding to the fenofibrate-induced peak, were rich in apoB100 and apoE.

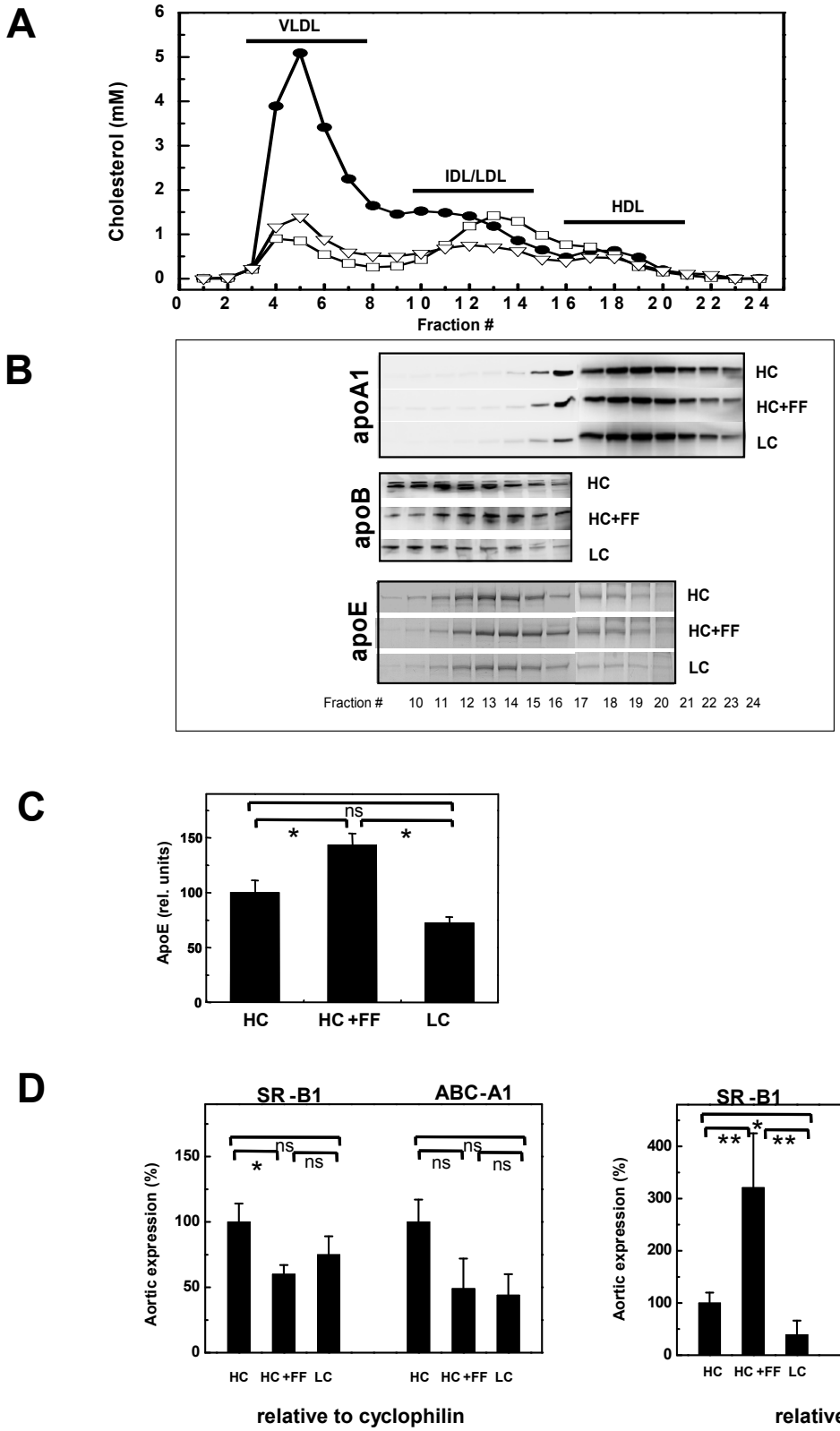

Figure 5. Effect of fenofibrate on lipoprotein profiles and aortic expression of reverse cholesterol transport parameters. A, Lipoprotein profiles after 12 weeks of treatment (plasma pools of n=17 mice). HC is indicated by solid circles, HC+FF by open squares and LC by open triangles. **B,** Apolipoprotein content in various fractions of the lipoprotein profile (derived from 5A). Equal volumes were loaded. Purified apolipoproteins served as positive controls (not shown). **C,** Effect of fenofibrate on apoE (total mouse apoE) protein level in plasma after 12 weeks of treatment (relative units). **D,** Effect of fenofibrate on aortic ABC-A1 and SR-B1 mRNA expression relative to cyclophilin (left) and relative to CD14 (right). Data represent means ± SEM (n=5 mice per group). *P<0.05; **P<0.01.

Analysis of unfractionated plasma showed in HC+FF, but not in LC, significantly increased plasma apoE levels (Figure 5C) indicating that fenofibrate treatment is associated with elevated plasma apoE levels.

A major mechanism by which apoA1 and HDL reduce atherosclerosis is by promoting cholesterol efflux from peripheral macrophages and returning it to the liver for excretion into the bile (RCT). Cholesterol efflux involves several gene products, including ATP binding cassette (ABC)-A1 cholesterol transporter and the scavenger receptor class B type I (SR-B1), each of which could be a target for stimulating this process. Aortic immunostaining revealed a predominantly macrophage-associated expression of both cholesterol transporters as demonstrated by a strong overlap with a staining specific for macrophages (Supplementary information I; available online at http://atvb.ahajournals.org). RT-PCR analysis showed that aortic ABC-A1 and SR-B1 mRNA expression levels in HC+FF and LC were comparable, but reduced when compared with HC (Figure 5D, left). Taking into consideration that ABC-A1 and SR-B1 are mainly expressed in macrophages and the macrophage content is diminished in HC+FF (compared to HC and LC; see also Fig.3A/B), ABC-A1 and SR-B1 mRNA levels were also determined relative to the mRNA concentration of a macrophage marker, CD14. Relative to CD14, the mRNA expression levels of ABC-A1 and SR-B1 were comparable in HC and LC, but a significant increase was observed in HC+FF (Figure 5D, right). These data point to an elevated expression of ABC-A1 and SR-B1 per macrophage with fenofibrate and are in agreement with published data.[20,37]

Also, in a separate experiment, peritoneal macrophages isolated from E3L mice treated with fenofibrate for 3 w displayed significantly increased SR-B1 mRNA expression levels when compared to placebo-treated E3L mice (not shown). Enhanced cholesterol efflux from the vasculature may thus contribute to fenofibrate's pleiotropic, anti-atherosclerotic effect.

For comparison, SR-B1 and ABC-A1 expression was also analyzed in livers. In LC, decreased mRNA expression levels of ABC-A1 and SR-B1 were found (33±4% and 62±13% of values in HC, respectively). In HC+FF, hepatic ABC-A1 mRNA expression remained high (99±7% of HC), but hepatic SR-B1 mRNA expression levels were strongly reduced (13±5% of HC), also in comparison with LC (Supplementary information I).

Discussion

In this report we evaluated the anti-atherosclerotic effect of the hypolipidemic drug fenofibrate, a compound that not merely lowers cholesterol but reportedly also exhibits

potential vasculoprotective effects besides cholesterol-lowering, including anti-inflammatory effects. Comparison of fenofibrate-treated mice with a cholesterol-matched low-cholesterol (LC) group allowed us for the first time to quantify and to comprehensively characterize pleiotropic anti-atherosclerotic effects of a fibrate *in vivo*.

Most importantly, we demonstrate that the anti-atherosclerotic effect of fenofibrate by far exceeds the effect that can be ascribed to its total plasma cholesterol-lowering activity *per se*. The effect of cholesterol-lowering alone on atherosclerosis has been assessed in the LC group which displayed a markedly reduced aortic plaque load. This was accompanied by a moderate reduction of lesion number and severity and a relatively small decrease in plasma SAA concentrations. As compared to LC, the fenofibrate-treated HC+FF group displayed an *additionally* reduced aortic plaque load, lesion number, cross-sectional lesion area and plaque severity. These additional effects in HC+FF were paralleled by a strong reduction of aortic inflammation (aortic macrophage content and density; monocyte adhesion; ICAM-1, MCP-1, GM-CSF, p65-NFκB expression) and systemic inflammation (SAA, fibrinogen), and increased levels of factors important for cholesterol efflux from vascular macrophages (apoE; ABC-A1, SR-B1).

Data from recent clinical studies support the notion that activities of fibrates independent of normalizing plasma lipid levels may account for much of their cardioprotective effect.[11] For example, the reduction of coronary heart disease-related mortality achieved with gemfibrozil in the VA-HIT prevention trial can be explained for only about 25% by changes in major lipid and lipoproteins, leaving most of the benefit of fibrate therapy as yet unexplained.[16] The mechanisms responsible for this outcome are presently a matter of speculation, and the few animal studies that demonstrate fibrate effects unrelated to lipid-lowering are only suggestive in the context of cardiovascular disease.[15,18] Our data unequivocally demonstrate and quantify for the first time that fibrates can reduce atherosclerosis beyond the effect attributable to their total plasma cholesterol-lowering effect *per se* and provide a molecular explanation for these additional beneficial effects.

Chronic subacute inflammation is a driving force of atherosclerotic lesion development[5] and is reflected by elevated levels of systemic plasma inflammation markers among which CRP, SAA and fibrinogen. These liver-derived markers have been demonstrated to independently predict future cardiovascular risk[8,28], and CRP reduction after cholesterol-lowering therapy is associated with improved clinical outcomes.[29] Patients treated with fenofibrate at a dose comparable to the dose used in this study display reduced plasma levels of CRP, SAA and fibrinogen after only 4 weeks of therapy.[15] Here we demonstrate

that fenofibrate reduces plasma SAA and fibrinogen levels in the HC+FF group beyond lowering plasma cholesterol *per se* (as assessed in cholesterol-matched LC group). This additional effect is in accordance with the downregulation of plasma CRP and fibrinogen levels by fenofibrate in absence of an effect on plasma cholesterol in human CRP transgenic mice by a mechanism involving quenching of the pro-inflammatory transcription factor NFκB.[18]

In E3L mice, fenofibrate reduced plasma SAA and fibrinogen levels after already 3 weeks of treatment, i.e. before atherosclerotic lesions have developed under the conditions applied. [22,23]

Since SAA and fibrinogen may participate in pro-atherogenic processes of early lesion evolution and promote lesion development[28,30], downregulation of their effective plasma concentrations by fenofibrate may possibly contribute to the observed pleiotropic anti-atherosclerotic effects of the drug.

Our data provide evidence that fenofibrate exerts direct anti-inflammatory effects in aorta: the macrophage-density in atherosclerotic lesions was 86% smaller in HC+FF when compared to LC. This strong effect of fenofibrate on the aortic macrophage content may be explained by the observed suppression of endothelial monocyte adhesion in combination with the reduced aortic expression of ICAM-1 (which mediates the interaction between circulating monocytes and endothelial cells), MCP-1 (which stimulates the transmigration of monocytes through the endothelial cell layer) and GM-CSF (which augments the proliferation of vascular macrophages). In line with our data, a decrease of aortic MCP-1 mRNA expression was also observed in the descending aortas of fenofibrate-treated apoE-deficient mice.[20] Downregulation of GM-CSF by a PPARα-activator has to our knowledge not been reported before. The molecular mechanism underlying these anti-inflammatory vascular effects may well be the capacity of fenofibrate to suppress NF-κB expression and activity (Figure 3 this paper and [13,14,18,31]), a transcriptional master regulator which controls the expression of adhesion molecules, MCP-1 and GM-CSF.

Low HDL-cholesterol levels constitute a risk factor for CVD and are, independently of LDL-cholesterol levels, predictive for the risk of a cardiovascular event.[32] Several clinical studies demonstrated that fibrate treatment results in a significant increase of HDL levels.[11] This effect in humans can be explained by a PPARα-dependent upregulation of apolipoprotein apoA1 (reviewed in [33]). In rodent liver, apoA1 is negatively regulated by fibrates, including fenofibrate[33,34], a finding that was confirmed in this study.

Elevated plasma triglyceride levels represent an independent risk factor for cardiovascular disease. Fenofibrate treatment reduced plasma triglyceride concentrations by >80%. This result together with the changes observed in lipoprotein profile points to changes in the lipid content of lipoproteins and their size; this may modulate the atherogenicity of the lipoproteins. Triglyceride-rich lipoproteins even if smaller in the IDL fraction may be more atherogenic than triglyceride-poor IDL particles. Furthermore, these changes in physicochemical characteristics of lipoproteins may also affect their catabolism by tissues and cells, thus contributing differentially to atherosclerosis.

The cholesterol transporters ABC-A1 and SR-B1 are important for the efflux of macrophage-laden cholesterol from peripheral tissues and thereby for RCT.[10,35,36] Our finding that the macrophage expression of ABC-A1 and SR-B1 in aorta is elevated by fenofibrate may be of relevance for the anti-atherosclerotic activities of this compound, and is in line with several other studies showing that PPARα-activators stimulate the ABC-A1 pathway to induce cholesterol removal from human macrophage foam cells.[20,37] Our *in vivo* effects of fenofibrate on ABC-A1 and SR-B1 as well as apoE expression may all contribute to enhanced aortic cholesterol efflux and thereby to reduced progression of atherosclerosis.

Taken together, we describe and quantify for the first time in a comprehensive way that the PPARα-agonist fenofibrate reduces atherosclerosis more than can be achieved by lowering total plasma cholesterol alone. We provide a mechanistic explanation for these additional beneficial effects by demonstrating diminished recruitment of monocytes/macrophages to atherosclerosis-prone sites of the aorta, reduced vascular inflammation, decreased expression of pro-atherogenic liver-derived acute phase reactants, and stimulation of factors involved in vascular cholesterol efflux. Our data underline the potential use of PPARα-agonists in the treatment of atherosclerosis and support the view that pharmaceutical intervention beyond standard LDL-lowering strategies may further reduce atherogenesis.

Acknowledgements: We thank A. Jie for excellent technical assistance. The Netherlands Organization for Scientific Research NWO and the Netherlands Heart Foundation NHS supported this work (NWO grant VENI 016.036.061 to R.K. and NHS grant 2002B102 to L.V. and K.T.). We thank Aventis Pharma, Frankfurt, Germany (support to H.P.) and the 'New Initiative Systems Biology' research program of TNO (support to T.K. and R.K.) for supporting parts of this study.

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