Published in final edited form as:
Atherosclerosis. 2009 March ; 203(1): 59–66. doi:10.1016/j.atherosclerosis.2008.05.058.

Differential anti-atherosclerotic effects in the innominate artery and aortic sinus by the liver × receptor agonist T0901317

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Abstract

Activation of liver × receptors (LXRs) has been reported to reduce atherosclerosis in mouse models. However, this can be associated with enhanced liver de novo lipogenesis and elevation of plasma triglyceride-rich VLDL, which may limit its clinical use. In this study, we administered orally the LXR agonist T0901317 to male LDLR−/− mice fed a Western diet. This induced a persistent enhanced hypertriglyceridemia by largely increasing plasma triglyceride-rich VLDL. T0901317 treatment decreased atherosclerosis with a much more pronounced response and dose dependence in the innominate artery than in the aortic sinus. Lesions in the innominate artery were less complex containing mostly macrophage foam cells in T0901317-treated mice. However, in the aortic root, a significant reduction of atherosclerosis was seen only in the right coronary-related aortic sinus (RC) of T0901317-treated mice. Increasing the dose of T0901317 did not extend atheroprotection to the other sinuses of the aortic root. Lesions in the RC were less complex both in T0901317 and vehicle-treated mice with macrophage foam cells predominating. On the other hand, in T0901317-treated mice, the left coronary-related sinus (LC) lesions while not reduced in size, were more complex with a large fibrous cap and necrotic core, more collagen-positive areas, and variable macrophage foam cell content compared to vehicle-treated mice. These data suggest that activation of LXR by T0901317 had differential anti-atherosclerotic effects in two arterial regions in mice with hypertriglyceridemia.

Keywords

Hypertriglyceridemia; Atherosclerosis; LXRs

1. Introduction

LXRs, members of the nuclear receptor superfamily, are activated by oxysterols and regulate lipid and glucose metabolism and inflammation [1]. Activation of LXRs in macrophages promotes the efflux of free cholesterol (CH) and phospholipids from cells to nascent and mature HDL by up-regulating the LXR target genes ABCA1, ABCG1 and apoE [2–4], which reduces lipid accumulation in macrophages and foam cell formation. LXR agonists also downregulate expression of inflammatory genes in macrophages [5], and have been shown to reduce

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atherosclerosis in mice [6–8]. However, activation of LXRs in liver also triggers the expression of sterol regulatory element binding protein 1c (SREBP-1c), a sterol-responsive transcription factor, which in turn induces some of the key enzymes involved in fatty acid synthesis, such as fatty acid synthase (FAS) and acetyl-coenzyme A carboxylase (ACC). Expression of these enzymes initiates de novo lipogenesis and triglyceride (TG) accumulation that eventually causes fatty liver and increases the blood level of large TG-rich lipoproteins [9,10].

Since elevation of plasma TG is an independent risk factor for coronary heart disease [11], the utility of LXR agonists in prevention of atherosclerosis has been questioned [10]. However, the effect of hypertriglyceridemia on the response of atherosclerotic lesions to LXR activation has not been studied in detail. Transient elevation of plasma TG by an LXR agonist was observed in low density lipoprotein receptor knockout (LDLR−/−) male mice fed an atherogenic diet containing sodium cholate. Cholate is a natural agonist of farnesoid × receptor (FXR) that can lower plasma TG by down-regulation of short heterodimer partner (SHP) and LXR-associated SREBP-1c expression [7,12]. Transient hypertriglyceridemia, induced by LXR activation, did not prevent a robust dose-dependent reduction of atherosclerosis in the aorta sinus [7]. In another study, a persistent elevation of plasma TG was induced by the same LXR agonist in the same mouse model fed a Western diet without cholate. In this case, regression of preexisting atherosclerotic lesions in the aortic root was not observed [8]. The different outcomes of these two studies may be explained by two possibilities. LXR agonists may have less of an effect on pre-existing advanced plaques compared to early developing lesions. It is also possible that persistently elevated plasma TG induced by the activation of LXR in the liver may interfere selectively with LXR mediated effects in the aortic sinus.

To extend these studies, we fed LDLR−/− male mice a Western diet without cholate, a diet that has been found to induce persistent hypertriglyceridemia [13]. Corn oil was also not included in the diet studied here, since polyunsaturated fatty acids have several potential atheroprotective effects including an ability to inhibit the transcription of SREBP-1c by antagonizing ligand-dependent activation of LXR [14]. A potent LXR agonist T0901317, which induces liver lipogenesis, was administered at different doses leading to variations in plasma TG in LDLR−/− mice. Atherosclerotic lesions in two separate arterial regions as exemplified by the aorta sinus and innominate artery were analyzed in these mice.

2. Methods

2.1. Mice, diets and LXR agonist treatment

To examine the effects of LXR agonist on gene expression, fifteen week old male LDLR−/− mice in the C57BL/6 background obtained from Jackson Laboratory were fed a Western diet (Harlan TEKLAD, TD 88137) and treated by gavage with the LXR agonist T0901317 (Cayman Chemical Company) in a 20% microemulsion [15] at a dose of 0.5 mg/kg/day for 2 weeks. The mice in the control group (vehicle-treated) were treated with microemulsion without T0901317. Peritoneal macrophages flushed with ice-cold PBS from the peritoneal cavity were collected and pelleted by centrifugation. Cell pellets were resuspended in RNAlater (Ambion) and stored at −20°C. Intestine samples were also stored in RNAlater at −70°C.

To examine the effects of T0901317 on atherosclerosis, 8 week old male LDLR−/− mice in a C57BL/6 background were fed a Western diet for 12 weeks. Mice also were given LXR agonist T0901317 by gavage at doses of 0.2, 0.5, 1 and 2 mg/kg daily in a 20% microemulsion. Mice from the control group were given microemulsion without T0901317. This treatment was initiated at the time of introduction of the Western diet.

2.2. Analysis of atherosclerotic lesions

After 12 weeks of LXR agonist treatment, mice were fasted for 4 hours, anesthetized with ketamine and xylazine, and bled from the retro-orbital plexus. The mice were then perfusion fixed and the heart and aorta with its principal branches were isolated, embedded, and sectioned as described previously [16]. Lesions in the innominate artery were quantified from four digitally captured oil red O-stained 10-μm sections, each separated by 100 μm, and located between 150 and 450 μm distal to the branch point of the innominate artery from the aortic arch. Aortic sinus lesions were evaluated from three sections, each separated by 100 μm, beginning at the site of appearance of the coronary artery. Aortic roots were always oriented in the same way using a slice of liver as an orientation marker, so that individual sinuses could be separately evaluated. Hematoxylin and eosin (H & E), trichrome staining, sirius red staining, or immunostaining for macrophages using MoMa-2 was also performed on the sections. OpenLab software version 3.1.5 was used in the quantification.

2.3. RNA isolation and analysis of gene expression

Total RNA from mouse macrophages and intestine was isolated by using the RNeasy Mini System (QIAGEN). First strand cDNA was synthesized by utilizing the SuperScript III System (Invitrogen Life Technologies). Quantitative real time PCR was performed as described in the TaqMan Universal PCR Master Mix kit from Applied Biosystems. Specific mRNA levels were normalized to 36B4 mRNA levels and presented relative to the controls.

2.4. Analysis of lipids and lipoproteins

Plasma was prepared from blood and a 200 μl sample of plasma was fractionated on tandem Superose 6 FPLC columns [16]. CH and TG in the even-numbered fractions were measured by using commercial assay kits (Stanbio Laboratory). Liver tissue was homogenized and lipids extracted as described [17]. Extracted lipids were measured as described above.

2.5. Statistical analysis

Values are presented as means ± standard error of the mean (SEM). Differences between means were analyzed for statistical significance by using one-way ANOVA and Fisher's PLSD post hoc test or unpaired Student *t*-test. A statistically significant difference was set at $p<0.05$.

3. Results

3.1. Persistent induction of enhanced hypertriglyceridemia in T0901317-treated mice

For the study of the effect of LXR agonist on atherosclerosis, eight week old male LDLR−/− mice were fed a Western diet and gavaged daily with vehicle or different doses of T0901317 for 3 months. In comparison with vehicle-treated mice, a significant increase in VLDL-CH was observed in mice treated with 1 or 2 mg/kg/day of T0901317, while significant decreases in LDL-CH and HDL-CH were only found in mice treated with 2 mg/kg/day of T0901317 (Fig. 1A). Total plasma CH was significantly increased only in mice treated with 2mg/kg/day of T0901317 (Fig. 1A). On the other hand, total and VLDL-TG were clearly elevated in a dose-dependent manner in mice treated with T0901317 (Fig. 1B). Even the LDL-TG levels in mice treated with T0901317 at 1 and 2 mg/kg/day were also significantly elevated. Most of the increase in total TG in the treatment groups was due to increases in VLDL (Fig. 1B).

Treatment with 0.5, 1 and 2 mg/kg/day of T0901317 significantly raised the ratio of TG to CH in VLDL particles from 0.81 ± 0.11 in control group (n=10) to 1.24 ± 0.07 (n=11), 1.61 ± 0.13 (n=12) and 2.33 ± 0.13 (n=8), respectively in the T0901317 treatment groups ($p<0.05$, $p<0.001$ and $p<0.001$). Treatment with 1 and 2 mg/kg/day of T0901317 also substantially raised the ratio of TG to CH in LDL particles to 0.4 ± 0.04 (n=12) and 0.59 ± 0.07 (n=8) compared with

the control ratio of 0.24 ± 0.01 (n=10) ($p<0.05$ and $p<0.001$). Based on these data, activation of LXR by T0901317 induced a long-term enhanced hypertriglyceridemia in male LDLR−/− mice by mainly increasing TG-rich VLDL particles.

3.2. T0901317 activates LXR target genes in intestine and macrophages

The level of LXR target gene expression in intestine and peritoneal macrophages from LDLR −/− mice treated for two weeks with T0901317 or vehicle was examined by using quantitative real-time PCR. In intestine, T0901317 treatment increased ABCA1 mRNA more than four fold, and induced ABCG5 and ABCG8 expression more than two fold (Fig. 2A). In peritoneal macrophages, significant increases in ABCA1 and ABCG1 mRNA but not apoE mRNA were observed in T0901317-treated mice (Fig. 2B). Increased expression of ABCA1, ABCG1, ABCG5 and ABCG8 after T0901317 treatment has the potential to protect against atherosclerosis in this animal model, especially as the first two genes are involved in reverse CH transport and the latter two genes are involved in the export of sterols from the liver and intestine.

3.3. Effect of LXR agonist on atherosclerotic plaque formation in two arterial regions

Atherosclerosis was examined at two sites within the vascular tree. In the innominate artery T0901317 significantly reduced atherosclerosis in a dose-dependent manner. Doses of 0.5, 1 and 2 mg/kg/day of T0901317 produced a 54%, 75% and 94% decline in atherosclerotic plaque area, respectively (Fig. 3A). T0901317 at 0.2mg/kg/day had no effect on atherosclerotic plaque area (Fig. 3A). Interestingly, no dose-dependent therapeutic effect was observed in the aortic sinus. T0901317 at 0.5 and 2 mg/kg/day significantly reduced aortic sinus plaque area by 31% and 29% (Fig. 3B). Treatment with 1 mg/kg/day of T0901317 did not significantly reduce atherosclerotic plaque area. Average lesion area was lowered by only 16% compared to lesions in control mice (Fig. 3B). Overall, the anti-atherosclerotic effect of T0901317 was much more pronounced in the innominate artery than in the aortic sinus.

To further address T0901317's efficacy on blocking development of atherosclerosis in the aortic sinus, the lesions in the three individual sinuses were examined. The areas on the left and right coronary sides are designated LC and RC respectively, and the third one is designated NC (Fig. 3C). Interestingly, all treatments with T0901317 remarkably reduced the plaque size in RC. The reduction was 37%, 71%, 58% and 73% for treatment with 0.2, 0.5, 1 and 2 mg/kg/day of T0901317, respectively (Fig. 3D). However, no significant change was observed in plaque area in the LC or NC in treatment groups (Fig. 3E and 3F).

The quality of the lesions in the various treatment groups was also evaluated. In general the lesions in the innominate artery after T0901317 treatment were not only smaller but also much less complex with a predominance of macrophage foam cells compared to the control group (Fig. 4A to 4C and 4F). In contrast, the lesions in the LC sinus of T0901317-treated mice were more complex with variable macrophage foam cell content, larger necrotic cores and thicker collagen-positive fibrous caps compared to vehicle-treated mice (Fig. 4D, 4E, 4G to 4I and Fig. 5), while the lesions in the RC and NC sinus were much less complex in both T0901317 and vehicle-treated mice (Fig. 4J to 4M). The average thickness of the fibrous cap in the LC sinus was significantly increased from 26.13 ± 4.21 μm (n=9) in vehicle-treated mice to 54.22 ± 5.95 μm (n=11) in mice treated with 1 mg/kg/day of T0901317 ($p<0.05$).

4. Discussion

The atheroprotective effects of T0901317 may operate either by influencing lipoprotein homeostasis or through mechanisms at the level of the vessel wall. In these experiments, treatment with T0901317 markedly increased plasma triglyceride levels with a more modest

increase in VLDL cholesterol (Fig. 1). Elevated plasma and VLDL triglyceride is thought to be a risk factor for the development of atherosclerosis [11]. Since significant decreases in atherosclerotic plaque area were observed under these proatherogenic conditions, it is most likely that T0901317 is affecting atherosclerosis development through effects on components of the vessel wall. Cells of the atherosclerotic lesion are known to express LXR, most notably macrophage foam cells [5,8,18] and endothelial cells [19]. T0901317 increases the level of genes involved in reverse cholesterol transport in the macrophage, which would by itself be expected to be atheroprotective. It is also known that LXR agonists inhibit expression of pro-inflammatory molecules e.g. iNOS, COX1, MCP-1, MCP-3 perhaps by limiting the activation of NF-κB [5]. The local anti-inflammatory action of LXR agonists may account for the thickened fibrous cap and more stable plaque observed in the LC region of the aortic root [5, 20]. However, this is not found in the innominate artery. In light of previous work demonstrating that a LXR agonist suppresses vascular smooth muscle cell proliferation and inhibits neointima formation in balloon-injured rat carotid arteries [21], the mechanism of LXR-mediated atheroprotective effects might be quite complicated.

A notable finding of the present study is that atherosclerosis in the innominate artery is particularly sensitive to treatment with the LXR agonist in a dose dependent fashion. Despite T0901317 induced hypertriglyceridemia, this LXR agonist still had strong atheroprotective effects in the innominate artery. While the effects on the innominate artery are clearcut, the response of the aortic root is less notable with modest though significant effects noted in only one of three sinuses. The persistent hypertriglyceridemia may play a role in this different response. Hypertriglyceridemia positively correlates with aortic sinus atherosclerosis but not innominate artery atherosclerosis (P Vanderlaan et al in preparation). Our studies and those of others have reported that the vascular response to various manipulations is frequently vascular site selective [22]. This selectivity is perhaps driven by the local hemodynamic profile or is a reflection of differences in rate of lesion growth and development at the various vascular sites. A recent report shows that laminar shear stress regulates LXR in vascular endothelial cells (EC) with an increase of LXR and LXR target gene expression in the thoracic aorta compared to that in the aortic arch [19]. Differences in LXR expression may also exist between the innominate artery and aortic sinus, and may affect T0901317-mediated atheroprotective effects in the two arterial regions. In a previous study using the same animal model and the same LXR agonist used in this study, a robust dose-dependent reduction of atherosclerosis was observed in the aortic sinus [7]. The difference between our findings and this earlier study indicates that some factors besides LXR expression may have a critical impact on T0901317-mediated atheroprotective effects in the aortic sinus. The major difference between the two studies is a transient hypertriglyceridemia in the previous study [7] compared to a persistent hypertriglyceridemia in this report.

The highest dose of T0901317 that we used in our study is 2 mg/kg/day. This is 5 fold lower than dosages used in similar studies [7,8]. Previous studies administered T0901317 in propylene glycol/Tween or PEG-400/Tween as vehicle, whereas we have used a microemulsion in this study to provide potentially greater bioavailability of T0901317. Since the response of plasma triglycerides to the highest dose in this work is similar in magnitude to the responses in previous studies [7,8], we believe that our microemulsion does indeed provide comparable bioavailability at the dosages used.

There are relatively few in vivo studies of LXR agonists influencing atherosclerosis in mouse models and no data from human studies. Our results suggest that more detailed studies of different arterial regions at various levels and duration of agonist treatment are required to understand the potential of LXR agonists as treatments for atherosclerosis. It is also important to examine the role of LXR expression in different cell types, for example in macrophages, in determining site-specificity of responses. Such studies are important to fully understand the

action of these potential therapeutic agents. It is clear from the above discussion that further work is required to unravel some of the complexities of the lipoprotein and atherosclerosis responses to LXR activation.

Acknowledgments

This research was partly supported by National Institutes of Health grants AT00850 and CA58073 and funding from Anagen Therapeutics Inc.

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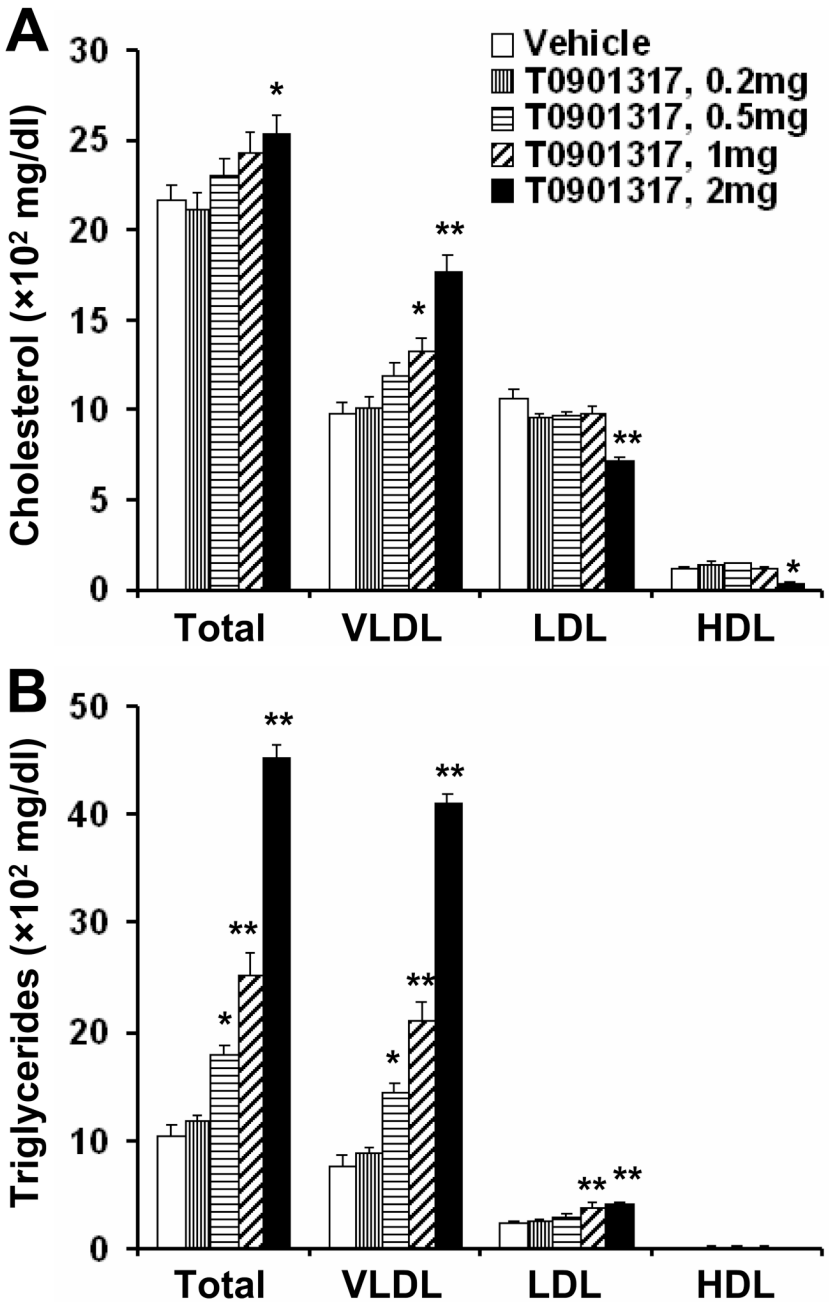

Fig. 1.

Plasma lipid levels in male LDLR−/− mice after 12 weeks of treatment with vehicle (control, n=10), or 0.2 (n=8), 0.5 (n=11), 1 (n=12), and 2 mg/kg/day (n=8) of T0901317. Significant differences between vehicle and T0901317-treated groups are indicated as * $P<0.05$, and ** $P<0.001$.

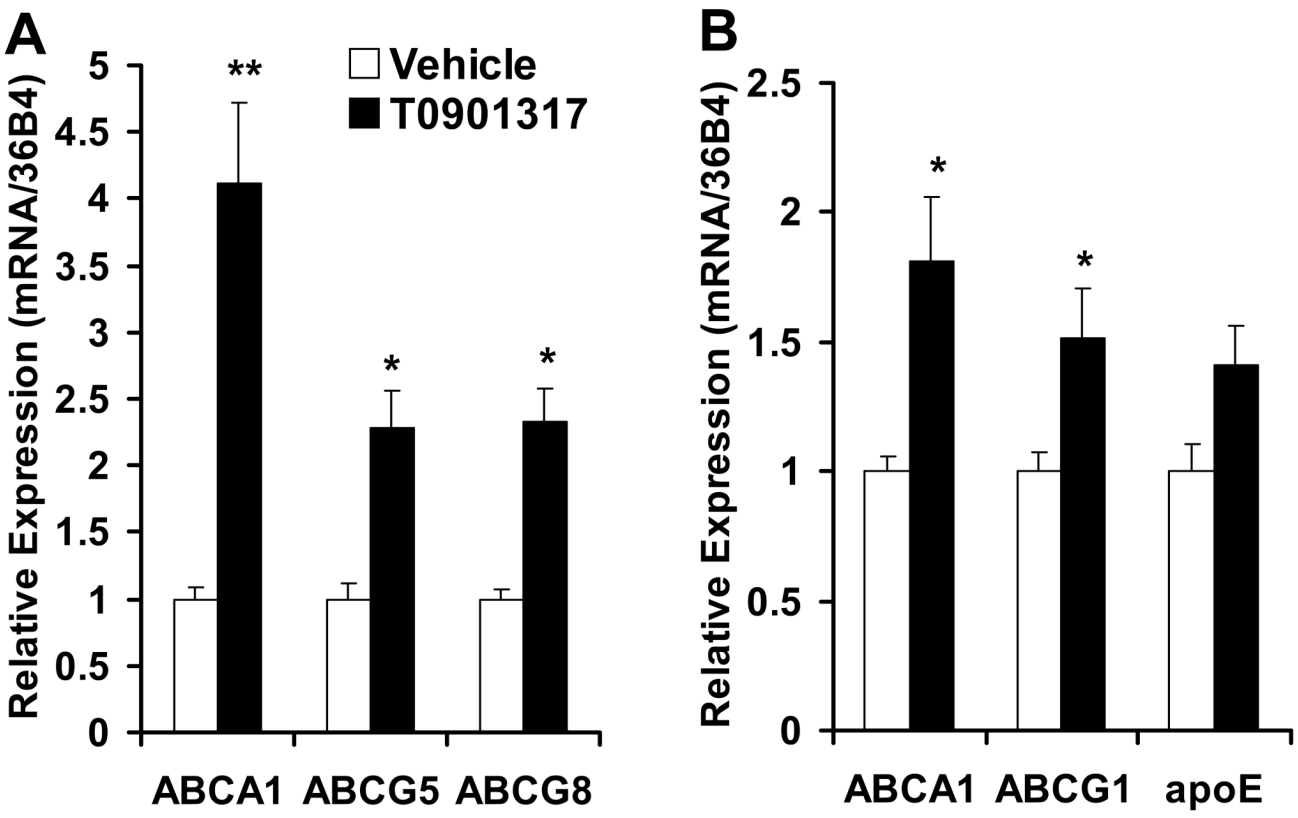

Fig. 2.

LXR target gene expression in mouse intestine (A) and peritoneal macrophages (B). Male LDLR−/− mice were treated for two weeks with 0.5 mg/kg/day of T0901317 (n=7) or vehicle (n=8). Specific mRNA levels were measured by quantitative RT-PCR and presented relative to controls. Significant differences between control and T0901317-treated groups are indicated as * $P<0.05$, and ** $P<0.001$.

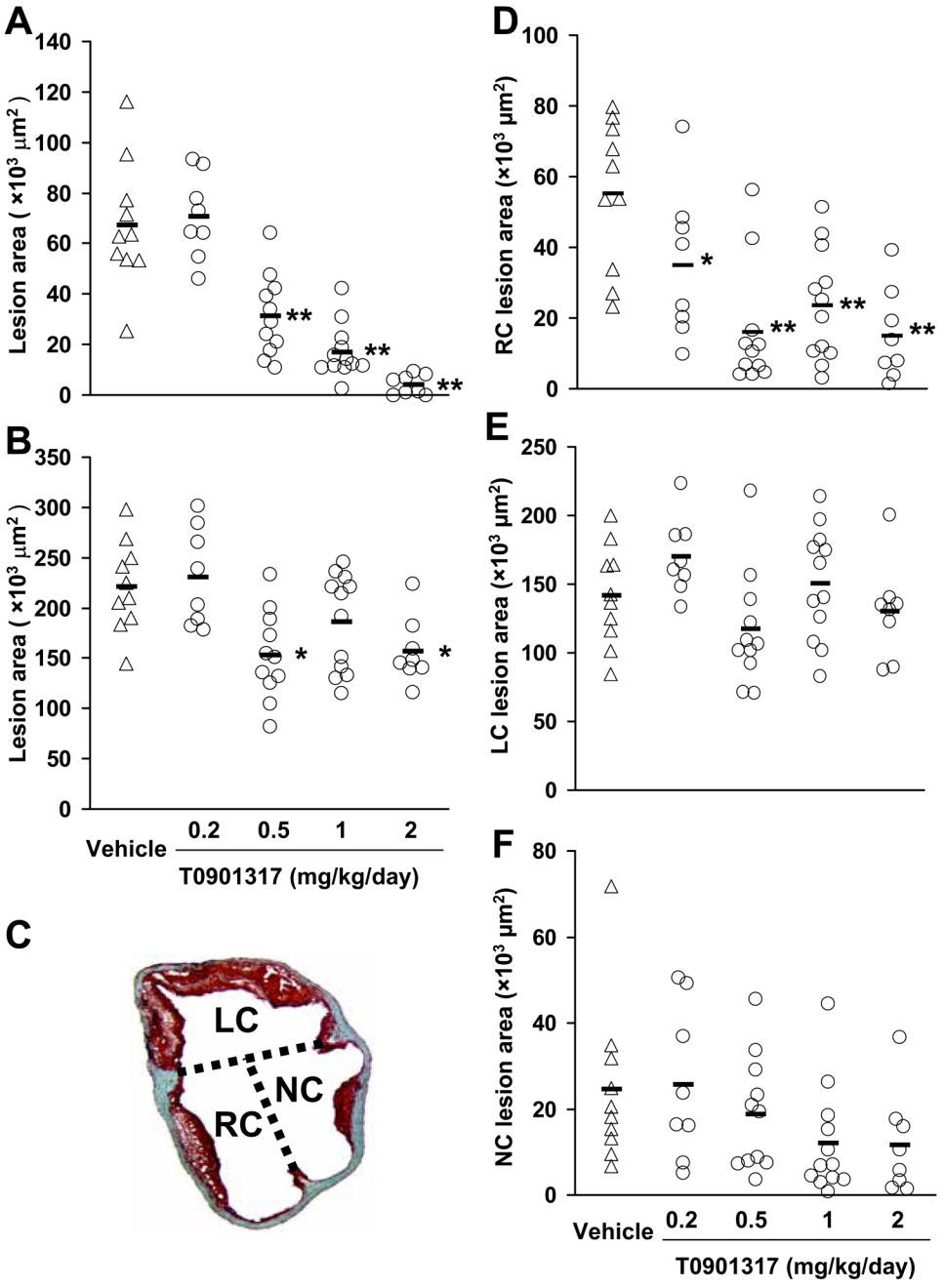

Fig. 3.

Atherosclerosis in the innominate artery (A), aortic sinus (B) and three parts of the aortic sinus (C) named RC (D), LC (E), and NC (F). Male LDLR−/− mice on a Western diet were gavaged daily for 12 weeks with vehicle (control, n=10), 0.2 (n=8), 0.5 (n=11), 1 (n=12), or 2 mg/kg/day (n=8) of T0901317. Atherosclerotic lesions were measured as described in Methods. Significant differences between vehicle and compound treated groups are indicated as * P<0.05, and ** P<0.001.

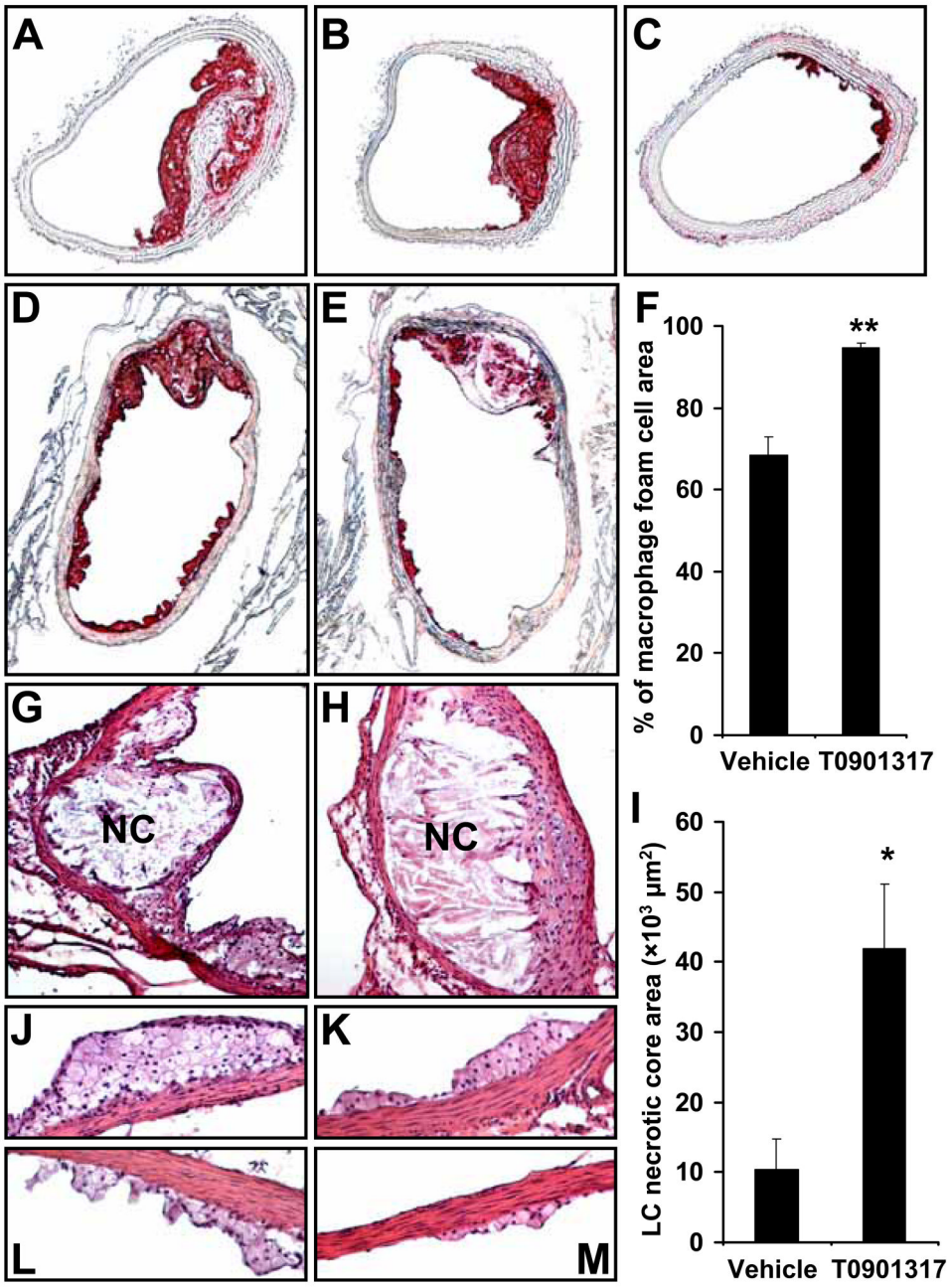

Fig. 4.

Analysis of atherosclerotic lesion composition. Lesions of the innominate artery (A–C) and aortic sinus (D, E) from mice treated with vehicle (A, D), or 0.5 (B), 1 (C, E) mg/kg/day of T0901317 were immunostained with MoMa-2 antibody to detect macrophages. (F) The percentage of macrophage foam cell area in the innominate artery lesions from vehicle (n=8) and 1 mg/kg/day of T0901317 (n=10) treated mice. H & E staining was also performed on LC (G, H), RC (J, K) and NC (L, M) lesions from vehicle (G, J, L) or 1 mg/kg/day of T0901317 (H, K, M)-treated mice. (I) Quantitative analysis of necrotic core area in LC lesions (n=8 in vehicle and n=10 in 1 mg/kg/day of T0901317 treated mice). Original magnification: 100 ×

(A–C), 40 × (D, E) and 200 × (G, H, J–M). Significant difference between vehicle and compound treated group is indicated as * $P<0.05$, and ** $P<0.001$.

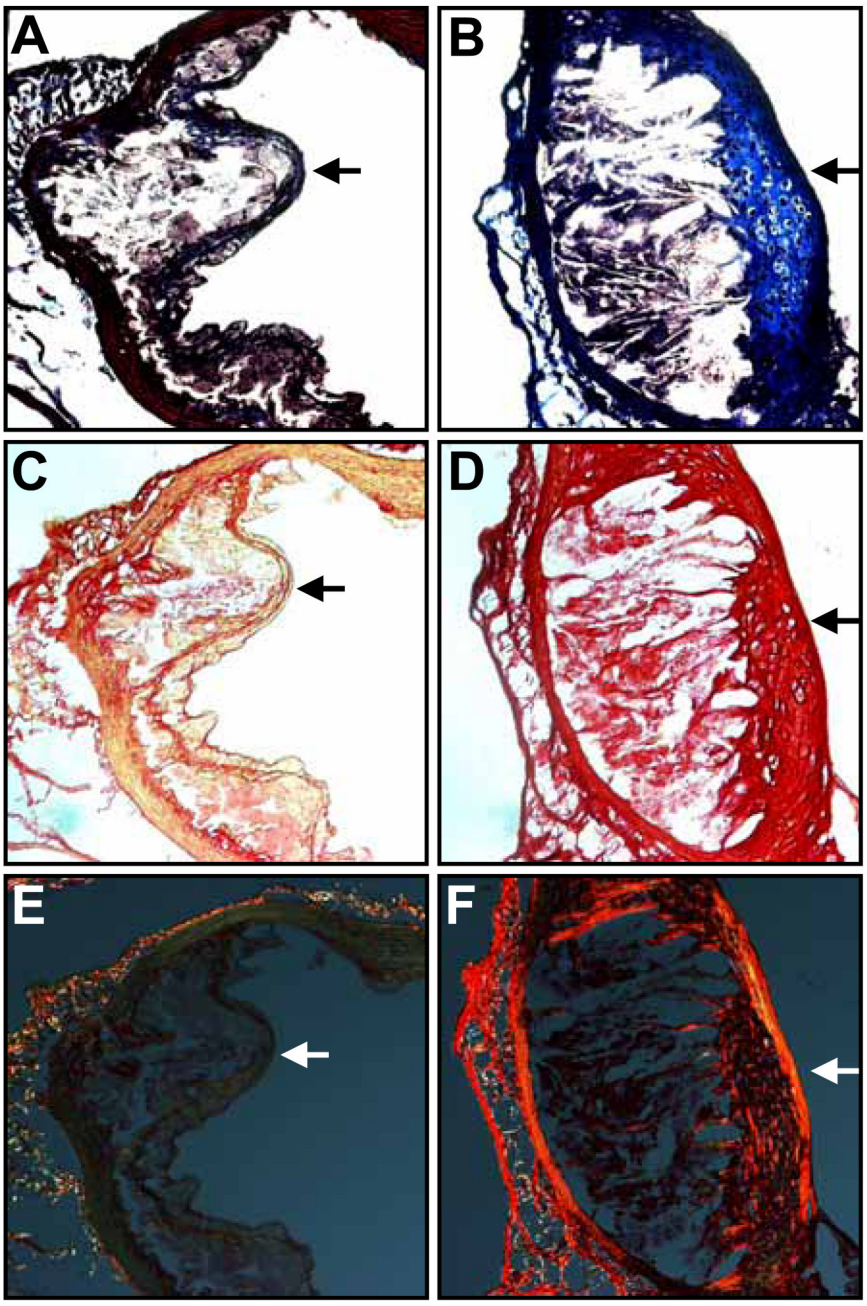

Fig. 5.
Collagen staining of LC lesions from vehicle (A, C, E) or 1 mg/kg/day of T0901317 (B, D, F)-treated mice. (A, B) Representative photomicrographs from trichrome staining (Masson). (C, D) Representative photomicrographs from sirius red staining. (E, F) Representative polarized photomicrographs from sirius red staining. Arrows indicate the fibrous cap. Original magnification: 200 ×.