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EP2306 [2-(4-Biphenyl)-4-methyl-octahydro-1,4-benzoxazin-2-ol, hydrobromide], A Novel Squalene Synthase Inhibitor, Reduces Atherosclerosis in the Cholesterol-Fed Rabbit

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ABSTRACT

EP2306 [2-(4-biphenyl)-4-methyl-octahydro-1,4-benzoxazin-2-ol, hydrobromide] inhibits squalene synthase and lipid biosynthesis and possesses antioxidant properties. We hypothesized that EP2306 can effectively modify circulating lipids and reduce atherosclerosis in the cholesterol-fed rabbit. Animals were fed a high-cholesterol diet for 4 weeks followed by 4 (phase 1 and 2) or 12 weeks (phase 3) of drug treatment while on high-cholesterol diet. In phase 1, the dose-effect relationship of EP2306 on lipids and atherosclerosis was established, and its most effective dose was determined (2 mg/kg). This dose reduced significantly total cholesterol (512 $\pm$ 96 mg/dl before versus 320 $\pm$ 124 mg/dl after treatment, $p < 0.05$) and atherosclerotic lesions compared with control animals. In phase 2, the effects of 2 mg/kg EP2306, 2.5 mg/kg simvastatin, and their combination were assessed. Although no significant effect on lipid parameters was observed, there was a significant reduction (35 $\pm$ 5%, $p < 0.05$) of atherosclerotic lesions in animals treated with EP2306, a similar reduction with simvastatin, and a further reduction (48 $\pm$ 7%, $p < 0.05$) when the two agents were combined. In animals treated for 12 weeks with the drugs (phase 3), only EP2306 significantly reduced atherosclerotic lesions by more than 50%, whereas simvastatin alone or in combination with EP2306 had no effect. Treatment with EP2306 did not adversely affect liver transaminases or cause any histopathological changes on various organs of the animals. In conclusion, we have shown that EP2306 inhibits atherosclerosis in vivo, indicating potential as a novel therapeutic agent for coronary artery disease and other atherosclerosis-related disorders.

Atherosclerosis is a progressive disease characterized by the accumulation of lipids in the arterial wall and the involvement of cellular adhesion, migration, proliferation, and inflammation (Libby, 2002). It is unquestionably the main underlying pathology of cardiovascular disease, the leading cause of morbidity and mortality worldwide (Hennekens, 1998). There is evidence that elevated plasma levels of low-density lipoprotein (LDL)-cholesterol as well as oxidative modification of LDL are major risk factors for the development of atherosclerosis (Steinberg et al., 1989). Both primary and secondary prevention trials have shown that lipid lowering with 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors, hereafter referred to as statins, reduce morbidity and mortality from coronary heart disease (Group 4S, 1994; Shepherd et al., 1995). Apart from the lipid-lowering effect, statins have also direct antiatherosclerotic effects on the vessel wall (Bellosta et al., 1998). However, despite the results obtained with these drugs,

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ABBREVIATIONS: LDL, low-density lipoprotein; HMG-CoA, 3-hydroxy-3-methyl glutaryl coenzyme A; EP2306, 2-(4-biphenyl)-4-methyl-octahydro-1,4-benzoxazin-2-ol, hydrobromide; EP2302, 2-(4-biphenyl)-2-(3-nitrooxypropoxy)-4-methylmorpholine, hydrobromide; HC, high-cholesterol; HDL, high-density cholesterol; SGOT, (serum) glutamic oxaloacetic transaminase; SGPT, (serum) glutamate pyruvate transaminase; TAK-475, 2-(1-(2-((3*R*,5*S*)-1-(3-acetoxy-2,2-dimethylpropyl)-7-chloro-5-(2,3-dimethoxyphenyl)-2-oxo-1,2,3,5-tetrahydrobenzo[e][1,4]oxazepin-3-yl)acetyl)piperidin-4-yl)acetic acid; ER-27856, 5-{*N*-[2-butenyl-3-(2-methoxyphenyl)]-*N*-methylamino}-1,1-pentylidenebis(phosphonic acid)-tripivaloyloxymethyl ester; YM-53601, (*E*)-2-[2-fluoro-2-(quinuclidin-3-ylidene)ethoxy]-9*H*-carbazole monohydrochloride.

the majority of cardiovascular events are not prevented by treatment with statins. Numerous additional factors have been associated with the initiation and progression of atherosclerosis, including monocyte/macrophage accumulation at the subendothelial space, endothelial cell dysfunction, oxidized LDL uptake by macrophages and endothelial cells leading to foam cell formation, and oxidized LDL-induced inhibition of nitric oxide production. Therefore, novel agents targeting any of these steps in atherosclerotic lesion development might prove useful in clinical practice.

The main regulatory enzyme of the cholesterol synthesis pathway, HMG-CoA reductase, is inhibited by statins. However, statins also suppress the production of mevalonate, an intermediate in cholesterol biosynthesis. Mevalonate is also a precursor of nonsterol products that are vital for diverse cellular functions. Both major known side effects of statins (hepatotoxicity and myotoxicity) have been associated with inhibition of the synthesis of the nonsterol products (Kornbrust et al., 1989; Masters et al., 1995).

The enzyme squalene synthase catalyzes the conversion of farnesyl pyrophosphate to squalene, the first committed step in the de novo cholesterol biosynthesis. Squalene synthase inhibitors are candidate hypocholesterolemic agents that decrease circulating LDL-cholesterol by an increased expression of hepatic LDL receptors in a similar manner to statins (for review, see Charlton-Menys and Durrington, 2007). They are characterized by a more specific action that leaves other nonsterol products of mevalonate metabolism unaffected. Therefore, squalene synthase inhibitors have at least one theoretical advantage compared with statins as hypocholesterolemic and antiatherosclerotic drugs because of reduced side effects. Several squalene synthase inhibitors have been reported in the literature, including ER-27586 (Hiyoshi et al., 2000), YM-53601 (Ugawa et al., 2002a,b), and TAK-475 (Amano et al., 2003; Nishimoto et al., 2003). The latter, a 4,1-benzoxazepine-3-acetic acid derivative, is an effective lipid-lowering agent in a variety of animal models. TAK-475 has become the first squalene synthase inhibitor to enter phase III clinical trials to assess its ability to reduce LDL-cholesterol alone or in combination with statins or ezetimibe (Burnett, 2006; Davidson, 2007).

Several novel 2-biphenylmorpholine derivatives have been synthesized, and preliminary results for their hypolipidemic and antioxidant properties have been reported (Chrysselis et al., 2000, 2002). Two of these compounds, EP2306 and EP2302, have been shown to possess antioxidant properties both in vitro and in vivo (Tavridou and Manolopoulos, 2004) as well as to inhibit squalene synthase activity and lipid biosynthesis in vitro (Tavridou et al., 2006). The chemical structure of EP2306 is shown in Fig. 1. To the best of our knowledge, no reports exist on the antiatherosclerotic effects of squalene synthase inhibitors in animals or humans. In the present study, we hypothesized that EP2306 can effectively modify circulating lipids and reduce atherosclerosis in the cholesterol-fed rabbit. Our main finding is that both EP2306 and simvastatin reduce atherosclerotic plaque formation in this animal model. However, only the antiatherosclerotic effect of EP2306 persists after 3 months of treatment.

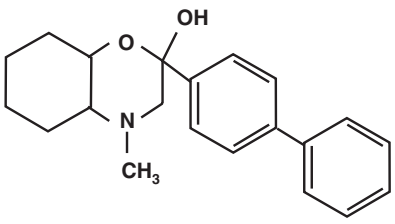

Fig. 1. Chemical structure of EP2306.

Materials and Methods

Materials. EP2306 was synthesized as described previously (Chrysselis et al., 2000). Simvastatin was obtained from ELPEN Pharmaceuticals (Pikermi, Greece). Cholesterol was purchased from Dolder AG (Basel, Switzerland).

Experimental Design. Male, New Zealand White rabbits (2.5–3.5 kg), fed a standard rabbit chow, were acclimatized for 1 week before experiments started. The rabbits were housed individually in cages and given water ad libitum. Subsequently, they were fed a high-cholesterol (HC) diet consisting of standard chow supplemented with 0.5% cholesterol for 4 weeks and then were randomly divided into several treatment groups. During treatment, all animals continued to receive an HC diet. Animals were treated daily by oral gavage with the compounds suspended in 34 mM NaCl/71 mM HCl (vehicle) as indicated. Control animals received an equal volume of vehicle only (placebo). The experimental design of the study is shown in Fig. 2. In phase 1, animals were allocated to six groups and received placebo (vehicle only), several doses of EP2306 (0.2–10 mg/kg), or simvastatin (2.5 mg/kg) daily for 4 weeks. In phase 2, animals were allocated to four groups and received placebo, 2 mg/kg EP2306, 2.5 mg/kg simvastatin, or their combination daily for 4 weeks. In phase 3, animals were allocated to four groups as in phase 2, but the duration of treatment was 12 weeks. The dose of simvastatin was chosen on the basis of previous experiments designed to rule out toxicity, and it was the maximum dose tolerated by the animals without any significant increase in deaths. The weight of the animals was recorded weekly to adjust the dose of drugs. Records of food intake did not show any significant difference among animals. General symptoms were assessed daily throughout the study. At the end of the experiments, animals were killed by exsanguination by cardiac puncture while under phenobarbital anesthesia.

The experiments were performed in accordance with 1) the guidelines of the Greek Government for the protection of animals as stated in article 4 of Law 1197/81, 2) the guidelines of Law 2015/92 for the protection of animals used for experimental or other scientific purposes, and 3) the Presidential Order 160/91 issued in compliance with the European Communities Council Directive of 24 November 1986 (86/609 EEC). The study was approved by the Department of Veterinary Services of the Prefecture of East Attica.

Biochemical Methods. Blood was collected after overnight fasting from the central ear artery. Serum was separated, stored at −80°C, and used within 1 week for biochemical measurements. Serum levels of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, glutamic oxaloacetic transaminase (SGOT), and glutamate pyruvate transaminase (SGPT) were determined enzymatically on an Abbott Alcyon 300 Analyzer using commercially available reagents (Abbott Laboratories, Abbott Park, IL).

Histopathology. After euthanasia, the lungs, the kidneys, the heart, the liver, the antrum of the stomach, the gallbladder, and the spleen were removed, washed, and their weight was measured. Macroscopic examination followed, and representative sections were taken from each one of the above organs. The samples were fixed overnight in 10% buffered formalin and, after routine processing, were embedded in paraffin. Routine histochemistry was performed on sections 3 μm in thickness, including staining with hematoxylin-eosin, trichrome, elastic van-Gieson, and oil-red (the latter done on fixed-frozen material).

All the above organs were examined under routine light microscopy to identify the degree of lipid deposition in the parenchyma and

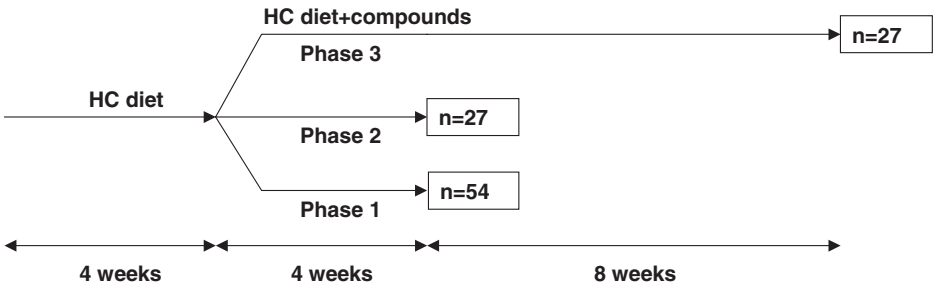

Fig. 2. Experimental design of the study.

the vessels, the degree of fibrosis, the extent and the type of the inflammation, and, if present, any other pathological changes. The pathologist who analyzed all these specimens was unaware of the compound administered to the animals.

Assessment of Atherosclerosis. Three samples (rings) from the ascending aorta were taken starting from the area above the ostia of the left main coronary artery, each one measuring approximately 1.5 to 2 mm in length. The same number of sections and all of them of similar length were taken from the descending aorta starting from the area immediately after the aortic arch. A random fragment from the carotid was also excised. All these samples were fixed in buffered 10% formalin and after routine processing, were embedded in paraffin. Tissue sections 3 μm thick were cut from paraffin-embedded blocks. The sections were dewaxed, dehydrated in graded alcohols, and stained with hematoxylin and eosin.

Serial sections were cut from all the six samples of both sides of the thoracic aorta and stained accordingly. Four parameters were analyzed, and two ratios were evaluated using an image analysis system (CAS; BD Biosciences, San Jose, CA), as follows: 1) the perimeter of the lumen (the perimeter of intima); 2) the perimeter or the length of the atherosclerotic lesions, the ratio b/a provides the percentage of the intima affected by atherosclerosis; 3) the area of the lumen; and 4) the sum of the atherosclerotic lesions occupying part of the area of the lumen, the ratio d/c provides the percentage of the luminal area affected by atherosclerosis. All the measurements from the samples of the ascending and the descending aorta were summarized, and the mean number was calculated. Apart from the usual atherosclerotic changes (e.g., fatty streaks composed of foamy macrophages filled with lipid), additional histopathological findings such as microcalcifications of the media, intramuscular degeneration, or hemorrhage were included in the histopathological analysis.

Statistics. Statistical analyses were performed using the Statistical Package for Social Sciences (version 10.0; SPSS Inc., Chicago, IL). Data are expressed as mean ± S.E.M. Values of p less than 0.05 were considered statistically significant. Wilcoxon's test was used to compare paired values before and after treatment with drugs within the same treatment. The effect of different treatments on lipid levels was assessed using one-way analysis of variance (Vickers and Altman, 2001) followed by post hoc Dunnett's multiple comparison analyses or Kruskal-Wallis test followed by Mann-Whitney U test as appropriate. The effect of treatment on atherosclerosis was examined using Kruskal-Wallis test followed by Mann-Whitney U test. Bivariate association between cholesterol lowering and antiatherosclerotic effect of EP2306 was examined using Spearman's correlation coefficient. For this purpose, total cholesterol values after treatment were subtracted from values before treatment; therefore, a negative correlation indicates a positive association between cholesterol lowering and antiatherosclerotic effect.

Results

Serum Lipid Concentrations and Atherosclerotic Changes before Treatment. Table 1 shows the changes of lipid parameters in rabbits fed an HC diet for 4 weeks without any additional compounds. Feeding with HC diet for 4 weeks resulted in significant increases in total and LDL-cholesterol (13.9- and 19.1-fold increase, respectively, over basal levels) but only modest increases in triglycerides and HDL-cholesterol (1.4- and 1.2-fold increase, respectively).

Four rabbits were sacrificed after 4 weeks of receiving an HC diet. Atherosclerotic lesions of significant size had been developed in the whole length of the aorta, the coronary arteries, and the carotids in all four animals. In the ascending aorta, the mean perimeter ratio was 0.32, and the mean area ratio was approximately 0.074. In one of these animals, foci of calcifications were identified in the wall of the descending aorta.

Phase 1 Experiments. The aims of phase 1 experiments were to: 1) establish and characterize in detail the hypercholesterolemic rabbit model, 2) find the dose range of EP2306 that is effective in this model, and 3) assess macro- and microscopically as well as biochemically possible adverse or toxic effects in rabbits following treatment with EP2306. A dose-response curve was performed to examine the hypolipidemic effect of EP2306 (0.2–10 mg/kg). The levels of total, LDL-, and HDL-cholesterol as well as triglycerides before and after drug treatment are shown in Table 2. Total cholesterol was significantly reduced compared with pretreatment levels by 2 mg/kg EP2306, but no other dose of the compound caused any significant changes on the levels of lipid parameters in the blood. Simvastatin (2.5 mg/kg) failed to produce any significant changes in any of the lipids measured (Table 2).

Quantification of morphometric analyses of cross-sections of the ascending and descending aorta is shown in Fig. 3. The ratio of the perimeter as well as the area covered by atherosclerotic lesions was assessed for increasing doses of EP2306 (0.2–10 mg/kg) or 2.5 mg/kg simvastatin, and it was com-

TABLE 1
Levels of lipid parameters in rabbits fed a high-cholesterol diet for 4 weeks
Results are expressed as mean ± S.E.M.

Parameter	n	Baseline	4 Weeks of HC Diet	-Fold Increase	p
Total cholesterol (mg/dl)	92	56 ± 3	781 ± 50	13.9	<0.001
LDL-cholesterol (mg/dl)	92	24 ± 3	460 ± 32	19.1	<0.001
Triglycerides (mg/dl)	92	86 ± 4	123 ± 6	1.4	<0.001
HDL-cholesterol (mg/dl)	50	22 ± 1	27 ± 2	1.2	0.03

TABLE 2
Phase 1: Effect of EP2306 on serum levels of total cholesterol, LDL-cholesterol, triglycerides, and HDL-cholesterol (n = 5–12)
Results are expressed as mean ± S.E.M.

Parameter	Before Treatment	After Treatment
Total cholesterol (mg/dl)		
Placebo	526 ± 86	544 ± 120
EP2306		
10 mg/kg	474 ± 100	429 ± 134
2 mg/kg	512 ± 96	320 ± 124*
0.5 mg/kg	571 ± 123	563 ± 85
0.2 mg/kg	638 ± 168	577 ± 120
Simvastatin, 2.5 mg/kg	416 ± 158	338 ± 145
LDL-cholesterol (mg/dl)		
Placebo	311 ± 69	276 ± 56
EP2306		
10 mg/kg	251 ± 49	209 ± 94
2 mg/kg	254 ± 52	176 ± 69
0.5 mg/kg	272 ± 53	304 ± 92
0.2 mg/kg	261 ± 107	265 ± 105
Simvastatin, 2.5 mg/kg	235 ± 123	241 ± 116
Triglycerides (mg/dl)		
Placebo	132 ± 18	127 ± 26
EP2306		
10 mg/kg	163 ± 68	169 ± 70
2 mg/kg	105 ± 19	89 ± 34
0.5 mg/kg	106 ± 15	88 ± 6
0.2 mg/kg	175 ± 44	118 ± 3
Simvastatin, 2.5 mg/kg	94 ± 16	54 ± 3
HDL-cholesterol (mg/dl)		
Placebo	26 ± 3	30 ± 5
EP2306		
10 mg/kg	22 ± 4	25 ± 6
2 mg/kg	25 ± 5	23 ± 3
0.5 mg/kg	33 ± 8	25 ± 4
0.2 mg/kg	30 ± 7	31 ± 7
Simvastatin, 2.5 mg/kg	26 ± 7	27 ± 5

* $p < 0.05$ vs. pretreatment value.

pared with animals treated with placebo. A significant decrease in the ratio of the perimeter of the ascending and descending aorta covered by lesions was observed in animals treated with 2 mg/kg EP2306 (50–60% reduction) or simvastatin (~70% reduction) compared with animals treated with placebo (Fig. 3, A and B). The significant decrease in the ratio of the area covered by lesions after treatment with EP2306 was observed for several doses of EP2306 as well as for simvastatin (Fig. 3, C and D). Correlation analysis was performed to examine a possible association of the cholesterol-lowering effect of EP2306 with the decreased ratio of the perimeter or area of the ascending and descending aorta covered by lesions (Table 3). The different doses of EP2306 were examined separately or, to increase power, were combined. However, no such associations were identified as indicated by the lack of significant correlations between the reduction of total cholesterol and the ratio of the perimeter or area of the ascending or descending aorta covered by lesions.

Phase 2 Experiments. Phase 2 followed the same experimental design of phase 1. The specific aim of this set of experiments was to evaluate the effects of EP2306, at its most effective dose of 2 mg/kg (determined in phase 1), when given to the animals together with 2.5 mg/kg simvastatin. A group of animals receiving only simvastatin was also included. EP2306, simvastatin, or their combination did not significantly affect any of the lipid parameters measured, although there was a tendency of EP2306 to reduce both total (517 ± 106 before versus 413 ± 91 mg/dl after treatment) and LDL-cholesterol levels (263 ± 65 before versus 184 ± 58 mg/dl after treatment). Figure 4 shows the changes in atherosclerosis observed with the three treatments employed. There was a significant decrease of 35 ± 5% in the perimeter of the lumen covered by atherosclerotic lesions in animals treated with 2 mg/kg EP2306 compared with animals treated with placebo ($p < 0.05$). Simvastatin (2.5 mg/kg) caused a similar significant inhibition of atherosclerosis with EP2306. However, a further reduction of 48 ± 7% ($p < 0.05$) of atherosclerotic lesions was observed with the combination of EP2306 with simvastatin (Fig. 4, A and B). A less pronounced effect on atherosclerosis was observed according to the assessment of the area covered by atherosclerotic lesions but even in this case, the effect of the combination of EP2306 with simvastatin remained significant (Fig. 4, C and D). Correlation analysis was performed to examine a possible association of the total cholesterol-lowering effect of EP2306 with the decreased ratio of the perimeter or area of the ascending and descending aorta covered by lesions (Table 3), but, similarly with phase 1, no significant correlations were identified. However, when animals of phases 1 and 2 treated with 2 mg/kg EP2306 were combined, a significant ($p = 0.028$) association between lowering of total cholesterol and the perimeter of the ascending aorta covered by lesions was found (Table 3). It should be noted though that no such associations were seen between the other indexes of aortic atherosclerosis and total cholesterol lowering.

Figure 5 shows representative images of stained atherosclerotic lesions after treatment with EP2306, simvastatin, or their combination compared with animals treated with placebo. In the latter group, increased accumulation of foamy macrophages was observed in the subendothelial area, whereas in the former groups, there was less deposition of lipids with formation of a thin layer of foamy macrophages. The atherosclerotic lesions detected did not show any differences in their cellular composition. Foamy macrophages were the main cellular component along with occasional lymphocytes and proliferating smooth muscle cells. Occasional focal areas of intramural microcalcification were also identified.

Phase 3 Experiments (Long-Term Study). An additional experiment bearing an identical design to phase 2 was performed to investigate the long-term effects of EP2306, simvastatin, or their combination. For this purpose, the compounds were administered to the animals for 12 weeks on a background of HC diet. There was no reduction of serum total cholesterol or LDL-cholesterol after treatment with EP2306, simvastatin, or their combination (data not shown).

Figure 6 shows the changes in atherosclerosis observed after 3 months of treatment with the different treatments employed. There was a significant decrease by more than 50% of atherosclerotic lesions in the ascending (Fig. 6, A and C) and descending aorta (Fig. 6, B and D) in animals treated with EP2306 as assessed both by the perimeter and the area covered by atherosclerotic lesions ($p < 0.05$). However, simvastatin alone or in combination with EP2306 did not result in any significant reduction of atherosclerosis. Figure 7 shows representative images of stained atherosclerotic lesions after 3 months of treatment with EP2306, simvastatin, or their combination compared with animals treated with placebo. There was an extensive accumulation of foamy macrophages forming a thick layer protruding into the vascular

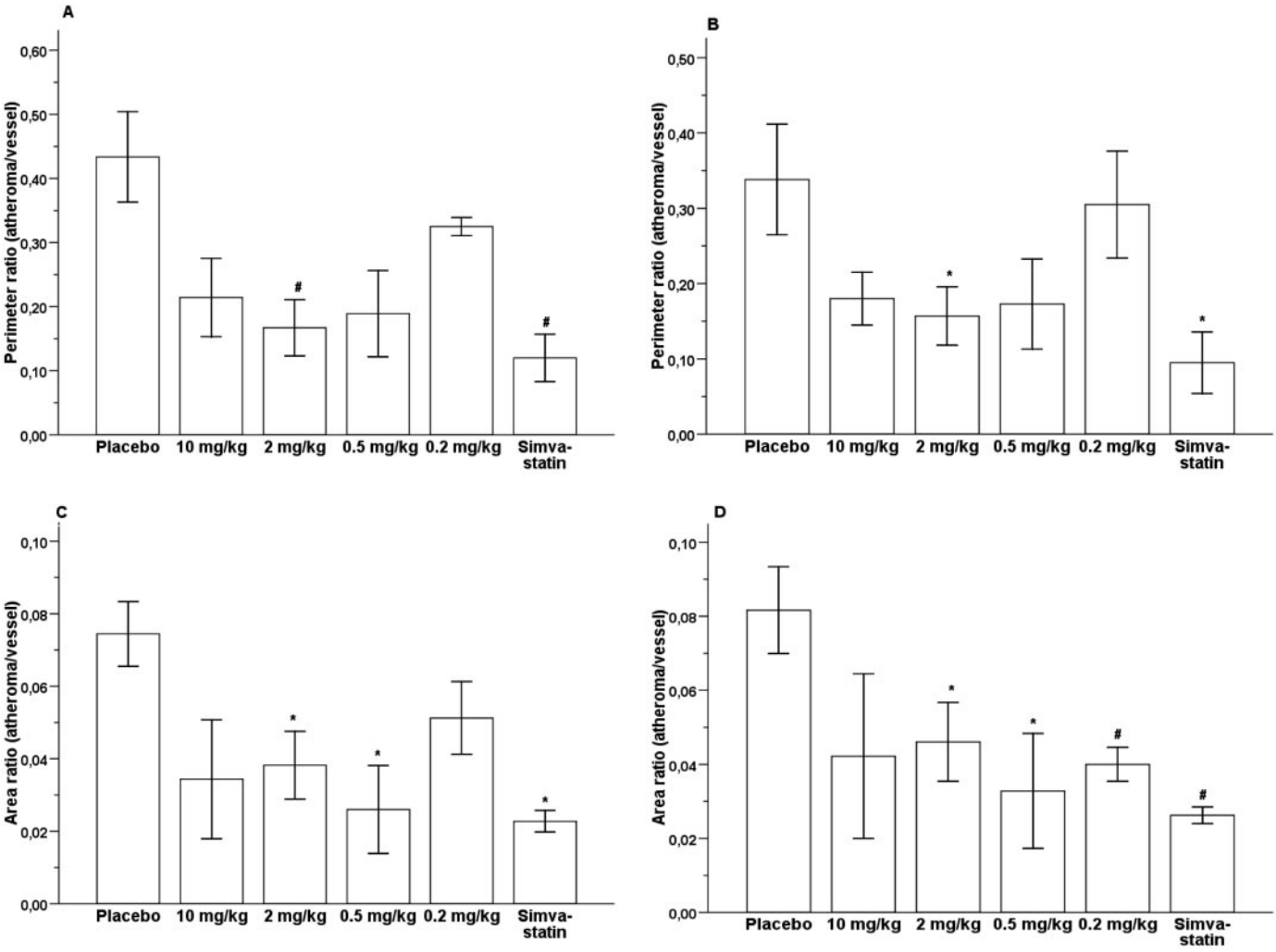

Fig. 3. Phase 1. Effect of EP2306 or simvastatin treatment on atherosclerosis in rabbits fed an HC diet for 8 weeks and concomitant oral administration of EP2306 or simvastatin for a 4 weeks treatment phase (4–8 weeks). Results are expressed as mean ± S.E.M. (n = 5–12). A and B, perimeter ratio covered with atherosclerotic lesions in the ascending and descending aorta, respectively. C and D, area ratio covered with atherosclerotic lesions in the ascending and descending aorta, respectively. *, $p < 0.05$; #, $p < 0.01$.

TABLE 3

Correlation analysis (r) between antiatherosclerotic action of EP2306 and total cholesterol lowering in phase 1 (n = 45) and phase 2 (n = 16) experiments

	Ascending Aorta		Descending Aorta	
	Perimeter	Area	Perimeter	Area
Phase 1				
Placebo	−0.16	−0.29	−0.43	−0.25
EP2306				
10 mg/kg	−0.41	0.45	0.11	0.32
2 mg/kg	−0.17	0.04	−0.26	0.06
0.5 mg/kg	0.40	0.40	0.60	0.40
0.2 mg/kg	0.40	0.20	−0.40	0.46
All doses	−0.31	0.26	−0.12	0.24
Phase 2				
Placebo	0.14	0.29	0.50	0.59
EP2306				
2 mg/kg	0.04	0.57	−0.21	0.31
Phases 1 and 2				
EP2306, 2 mg/kg	−0.49*	−0.07	−0.37	−0.12

* p = 0.028.

lumen in animals treated with placebo or the combination of EP2306 with simvastatin, whereas treatment with EP2306 or simvastatin resulted in thinner layers of foamy macrophages.

Effect of EP2306 or Simvastatin on Liver Transaminases. In phase 1, liver transaminases were measured in a subset of animals before and after 4 weeks treatment with different doses of EP2306 (0.2, 0.5, 2, and 10 mg/kg) or simvastatin (2.5 mg/kg) (Fig. 8). A small but significant decrease of SGPT levels was observed after treatment with EP2306 (46.2 ± 4.1 before versus 37.2 ± 2.9 U/l after treatment, p = 0.045). Simvastatin increased SGPT levels approximately 2-fold (40.6 ± 3.6 before versus 89.7 ± 39.2 U/l after treatment), but this difference did not reach significance. Treatment with EP2306 did not affect significantly SGOT levels, whereas there was a significant 3-fold increase of SGOT levels after treatment with simvastatin (30.0 ± 2.3 before versus 90.4 ± 18.9 U/l after treatment, p = 0.021).

In phase 2, SGPT levels were not significantly affected by EP2306 (51.7 ± 9.2 before versus 55.7 ± 10.4 U/l after treatment), simvastatin (45.0 ± 8.6 before versus 51.0 ± 11.3 U/l after treatment), or their combination (52.0 ± 6.1 before versus 68.0 ± 2.9 U/l after treatment). SGOT levels were not significantly modified after 1 month of treatment with EP2306 (34.7 ± 3.9 before versus 76.0 ± 36.5 U/l after treatment, p = 0.35), whereas they were significantly increased by

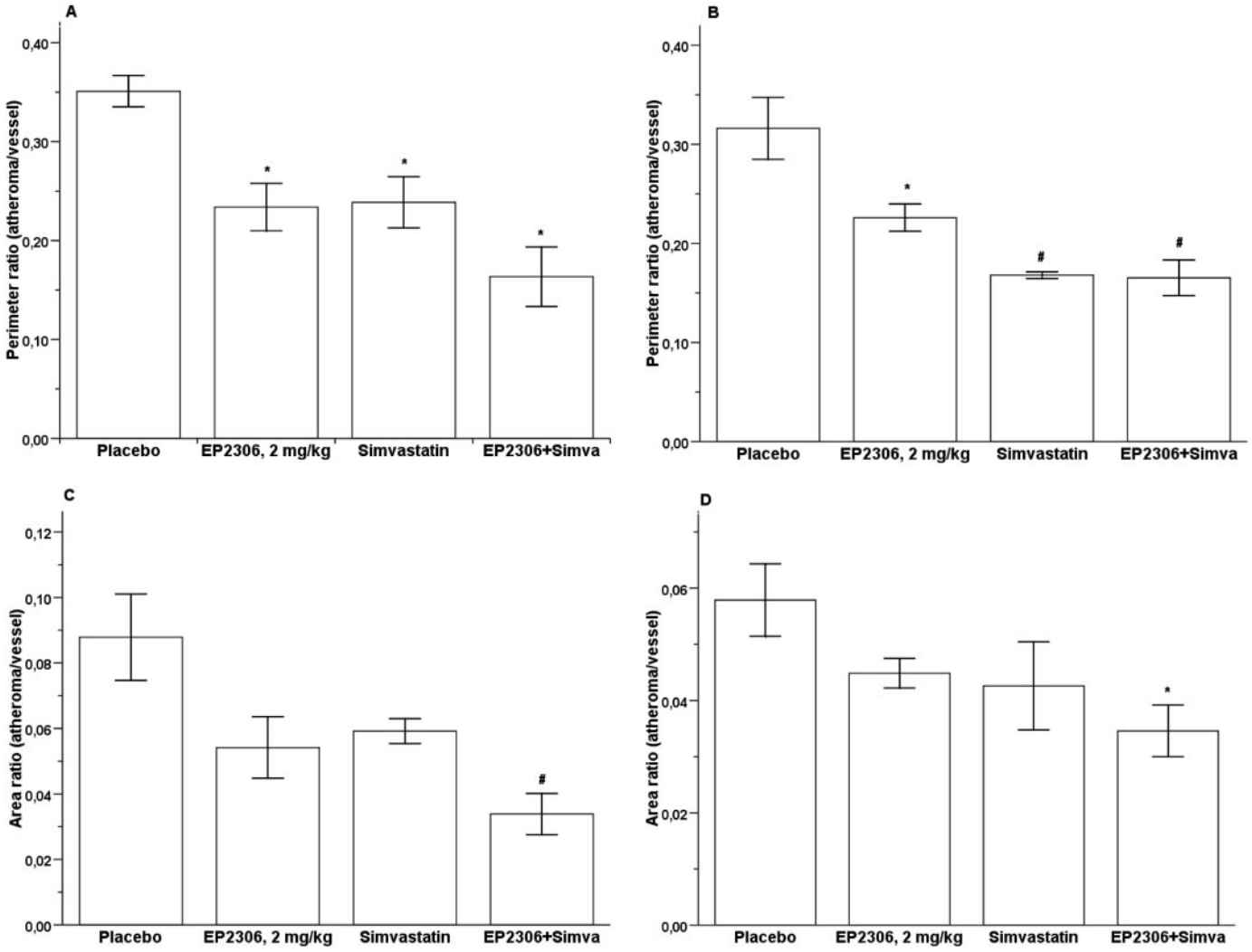

Fig. 4. Phase 2. Effect of treatment with EP2306, simvastatin, or their combination on atherosclerosis in rabbits fed an HC diet for 8 weeks and concomitant oral administration of EP2306 or simvastatin for a 4-week treatment phase (4–8 weeks). Results are expressed as mean ± S.E.M. (n = 4–8). A and B, perimeter ratio covered with atherosclerotic lesions in the ascending and descending aorta, respectively. C and D, area ratio covered with atherosclerotic lesions in the ascending and descending aorta, respectively. ∗, $p < 0.05$; #, $p < 0.01$.

simvastatin (33.7 ± 3.0 before versus 114.7 ± 20.7 U/l after treatment, $p = 0.05$) or their combination (27.0 ± 2.6 before versus 48.3 ± 2.9 U/l after treatment, $p = 0.045$).

Toxicity of EP2306 and Simvastatin on Various Organs. In all animals of the three phases of experiments, sections from various organs were also analyzed to detect any histopathological changes caused by EP2306, simvastatin, or their combination. The main findings per organ were the following.

Heart. Many atheromatic changes were observed mainly in intracardiac arteries with the form of subintimal accumulation of lipids. These features were present in the animals treated with placebo and to a lesser extent in the other groups. No evidence of myocarditis, necrosis, fibrosis, or thrombosis was seen.

Liver. Multifocal deposition of lipids in the form of micro- and macrovesicular fatty changes were observed. In addition, there was evidence of mild fibrosis in the periportal areas but no evidence of necrosis.

Lung. There was no evidence of edema, peribronchial inflammation, or vasculitis in any of the animals.

Kidney. No features of interstitial nephritis, glomerular changes, vascular lesions, necrosis, or thrombosis were observed.

Spleen. Numerous foamy macrophages were present in most of the animals fed with high-cholesterol diet.

Stomach. No evidence of interstitial inflammation, ulceration, necrosis, metaplasia, or atrophy was observed.

Gallbladder. No features of ulceration, inflammation, or necrosis were noted.

Discussion

The purpose of this study was to examine the effect of the novel 2-biphenylmorpholine derivative EP2306 on lipids and atherosclerosis in an animal model of atherosclerosis, the cholesterol-fed rabbit. EP2306 inhibits the rabbit and human liver squalene synthase with IC_{50} values of 33 and 63 μM, respectively (Tavridou et al., 2006). A significantly greater potency for inhibition of the enzyme has been reported for other squalene synthase inhibitors. For example, YM-53601 and TAK-475 inhibit human liver squalene synthase with

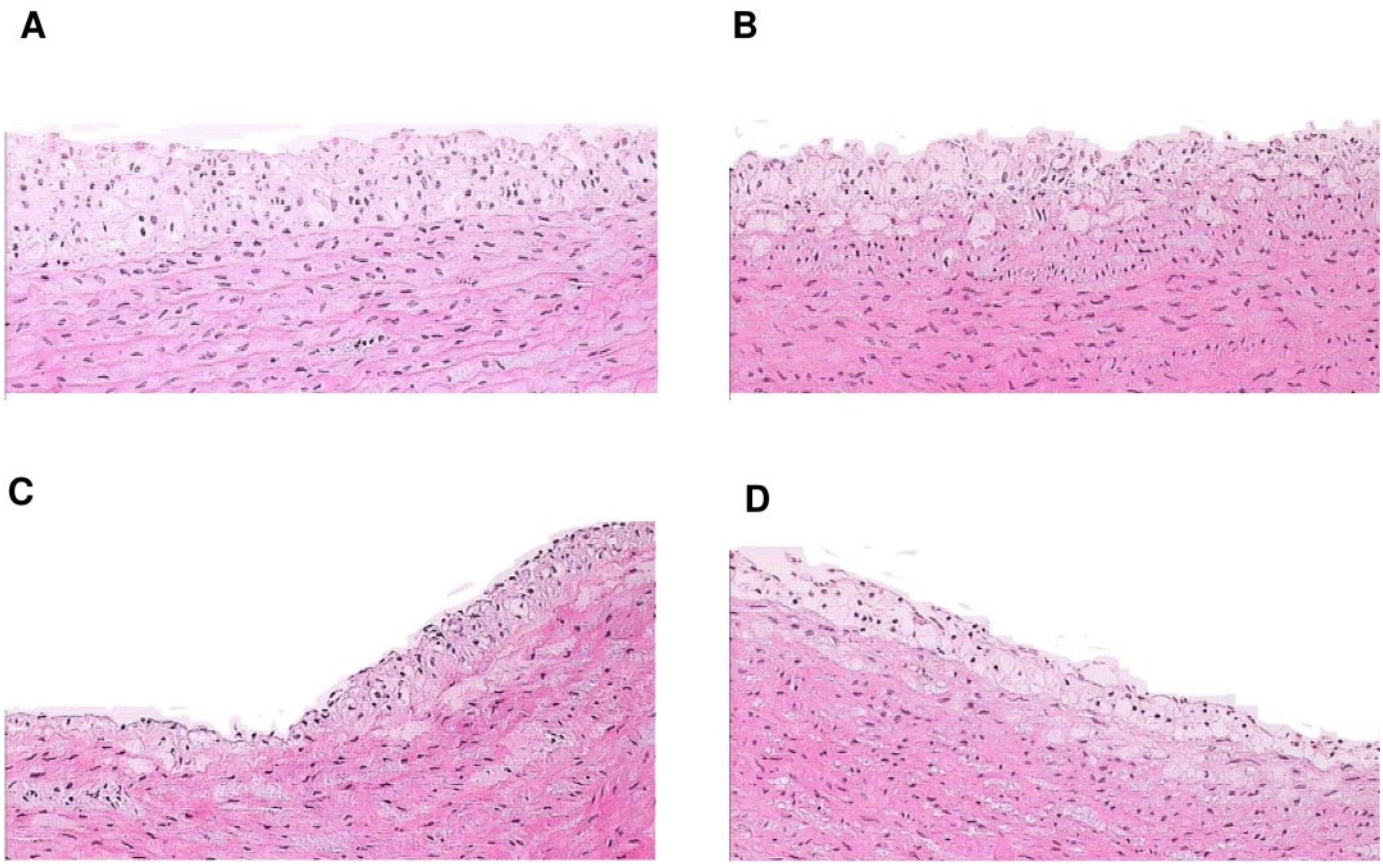

Fig. 5. Representative histopathological images of the aortas of rabbits fed an HC diet for 8 weeks and concomitant oral administration of EP2306 or simvastatin for a 4-week treatment phase (4–8 weeks). A, placebo; B, EP2306; C, simvastatin; D, coadministration of EP2306 with simvastatin.

IC_{50} values of 79 and 78 nM, respectively. However, it is not possible to directly compare the lipid-lowering properties of EP2306 with these compounds since different animal models were used for this purpose.

Despite the existence of several studies showing a lipid-lowering effect of squalene synthase inhibitors, so far, no data exist on the effect of any such compound on atherosclerosis in vivo. The experimental model of diet-induced atherosclerosis in rabbits has been extensively used to study the role of different pathways in atherogenesis as well as to evaluate the ability of pharmacological treatment to modulate atherosclerosis mainly because the atherosclerotic lesions in this model resemble those occurring in humans (Sugano et al., 1996; Yang et al., 1998). High cholesterol levels are easily achieved in rabbits fed an HC diet. However, the cholesterol-lowering effect of statins is a phenomenon restricted to higher animal species, including primates and man, because in lower animals, enzyme inhibition is followed by a dramatic rise of tissue enzyme levels blunting the plasma cholesterol-lowering response (Endo, 1992). Therefore, it is not unexpected that inconsistent results have been obtained regarding the cholesterol-lowering effect of statins in this animal model (Alfon et al., 1999; Hernandez-Presa et al., 2003). Moreover, the cholesterol-fed rabbit is not a good model for studying the mechanisms affecting triglycerides and HDL-cholesterol levels, as shown in previous reports where HC diet resulted only in modest changes in these lipids (Hernandez-Presa et al., 2003; Nachtigal et al., 2005). Our results confirm these reports because no significant changes were observed following 1 month of HC diet in the levels of HDL-cholesterol, whereas a significant but modest increase was observed in triglyceride levels.

In preclinical studies, several squalene synthase inhibitors have been shown to possess lipid-lowering properties (Hiyoshi et al., 2000; Ugawa et al., 2002; Amano et al., 2003; Nishimoto et al., 2003). So far, EP2306 has been shown to be an effective inhibitor of cholesterol and triglyceride biosynthesis in vitro (Tavridou et al., 2006). Moreover, it decreased total cholesterol, LDL-cholesterol, and triglycerides in plasma of Triton WR-1339-induced hyperlipidemic rats (Chrysselis et al., 2000). However, in the present study, we did not observe consistent reductions in total and LDL-cholesterol levels after treatment with EP2306 in the cholesterol-fed rabbit. One shortcoming of this model is that, to establish atherosclerotic lesions in these animals, serum cholesterol must reach much higher levels than those occurring in clinical situations. Further studies are required to evaluate the lipid-lowering effect of EP2306 in this animal model, performed in milder hypercholesterolemic conditions that would be more relevant to the clinical situation.

We found significant reductions of atherosclerotic lesions in animals treated with EP2306 compared with control animals. The weak and inconsistent effect of the compound on lipids and the lack of association between lipid-lowering and most atheromatic indexes assessed suggest that it is unlikely the antiatherosclerotic effects of EP2306 in HC rabbits to be solely mediated by the cholesterol-lowering properties of this drug. It appears that there are other mechanisms preventing atherosclerosis development in response to EP2306 in these

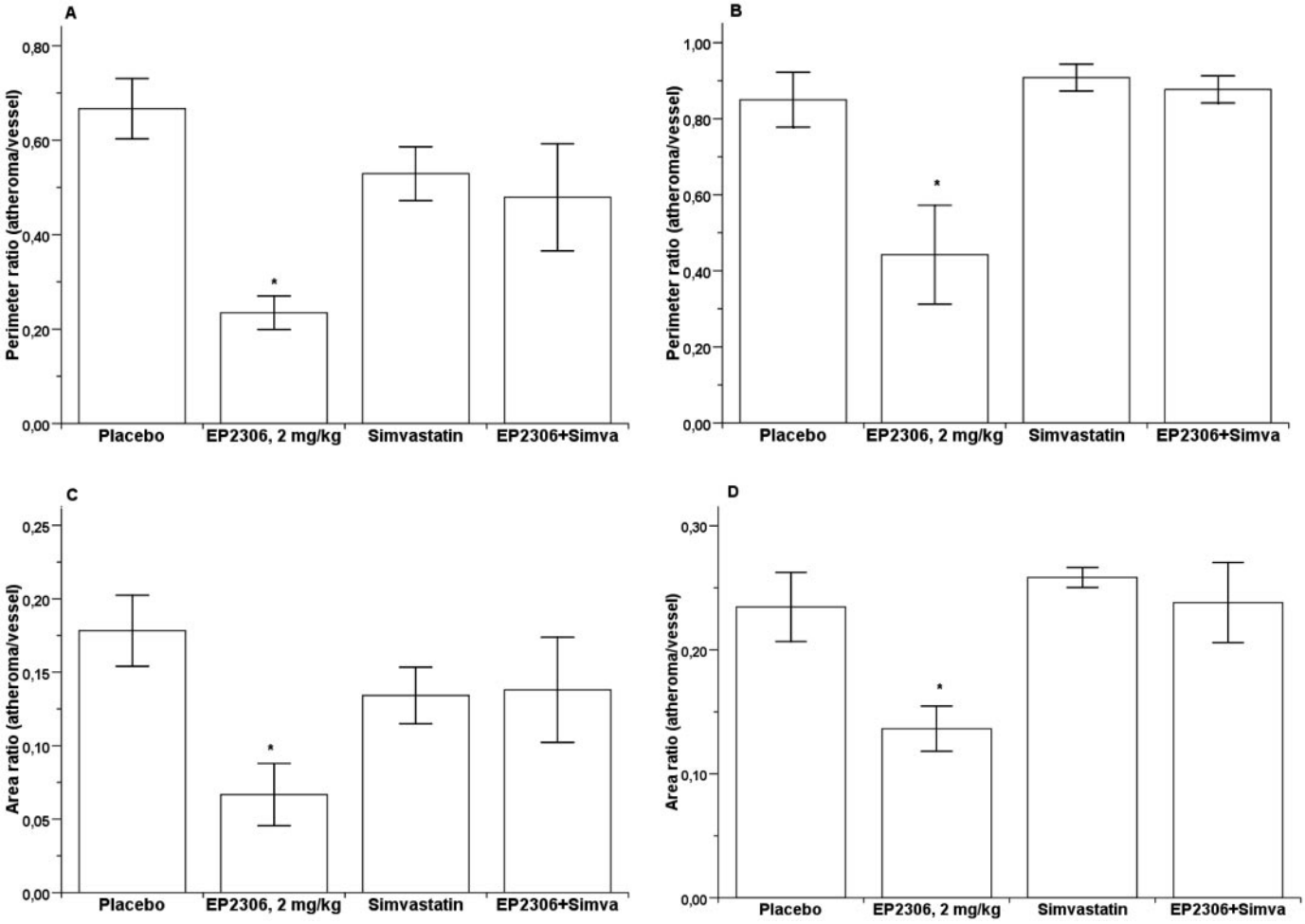

Fig. 6. Phase 3. Effect of treatment with EP2306, simvastatin, or their combination on atherosclerosis in rabbits fed a high-cholesterol diet for 16 weeks and concomitant oral administration of EP2306 or simvastatin for a 12-week treatment phase (4–16 weeks). Results are expressed as mean ± S.E.M. (n = 4–8). A and B, perimeter ratio covered with atherosclerotic lesions in the ascending and descending aorta, respectively. C and D, area ratio covered with atherosclerotic lesions in the ascending and descending aorta, respectively. *, $p < 0.05$.

animals. Interestingly, there is clinical evidence that inhibition of LDL-oxidation can inhibit atherosclerosis independently of lowering plasma cholesterol levels. Among statins, fluvastatin and simvastatin possess significant antioxidant properties (Franzoni et al., 2003). The antiatherosclerotic effects of fluvastatin have been extensively studied in the cholesterol-fed rabbit and have been mainly attributed to its antioxidant properties. Baetta et al. (2002) have shown that fluvastatin reduces tissue factor expression and macrophage accumulation in carotid lesions of cholesterol-fed rabbits in the absence of lipid lowering. Furthermore, fluvastatin, at a dose insufficient to reduce plasma cholesterol levels in cholesterol-fed rabbits, decreased atheromatous plaque formation by preserving endothelial function (Rikitake et al., 2001; Mitani et al., 2003). We have already shown that EP2306 inhibits dose dependently the in vitro oxidation of LDL induced by copper ions as well as 12-lipoxygenase activity (Tavridou and Manolopoulos, 2004). In addition, in hyperlipidemic rabbits treated with EP2306 for 4 weeks, there was a decrease in thiobarbituric acid-reactive substance levels and a significant increase in total peroxyl radical-trapping potential levels compared with control animals (Tavridou and Manolopoulos, 2004). Unfortunately, in the present study, no such markers of oxidative status were assessed to examine a possible association with the antiatherosclerotic effect of EP2306. However, these antioxidant properties of EP2306 could contribute to its antiatherosclerotic effect by several different manners. For example, decreased LDL-oxidation may suppress the expression of adhesion molecules by endothelial cells and the expression of tissue factor, colony-stimulating factor, and monocyte-chemoattractant protein-1 in macrophages, molecules that modulate the atherosclerotic process. All these findings suggest that inhibition of LDL-oxidation might contribute directly and/or indirectly to the antiatherosclerotic effects of EP2306 and the same appears to be true for simvastatin.

Although both EP2306 and simvastatin monotherapy as well as their combination significantly reduced atherosclerosis after 1 month of treatment, the results of the long-term study (3 months of treatment) showed a differential effect on atherosclerosis by these two compounds. EP2306 sustained its antiatherosclerotic effect for this longer period, whereas there was a reversal of the effect of simvastatin either administered as monotherapy or in combination with EP2306. A possible explanation of this finding could be differential toxicity exerted by the compounds studied in this long-term study, but because we did not find any differences in organ toxicity among treatment groups, this is not very likely.

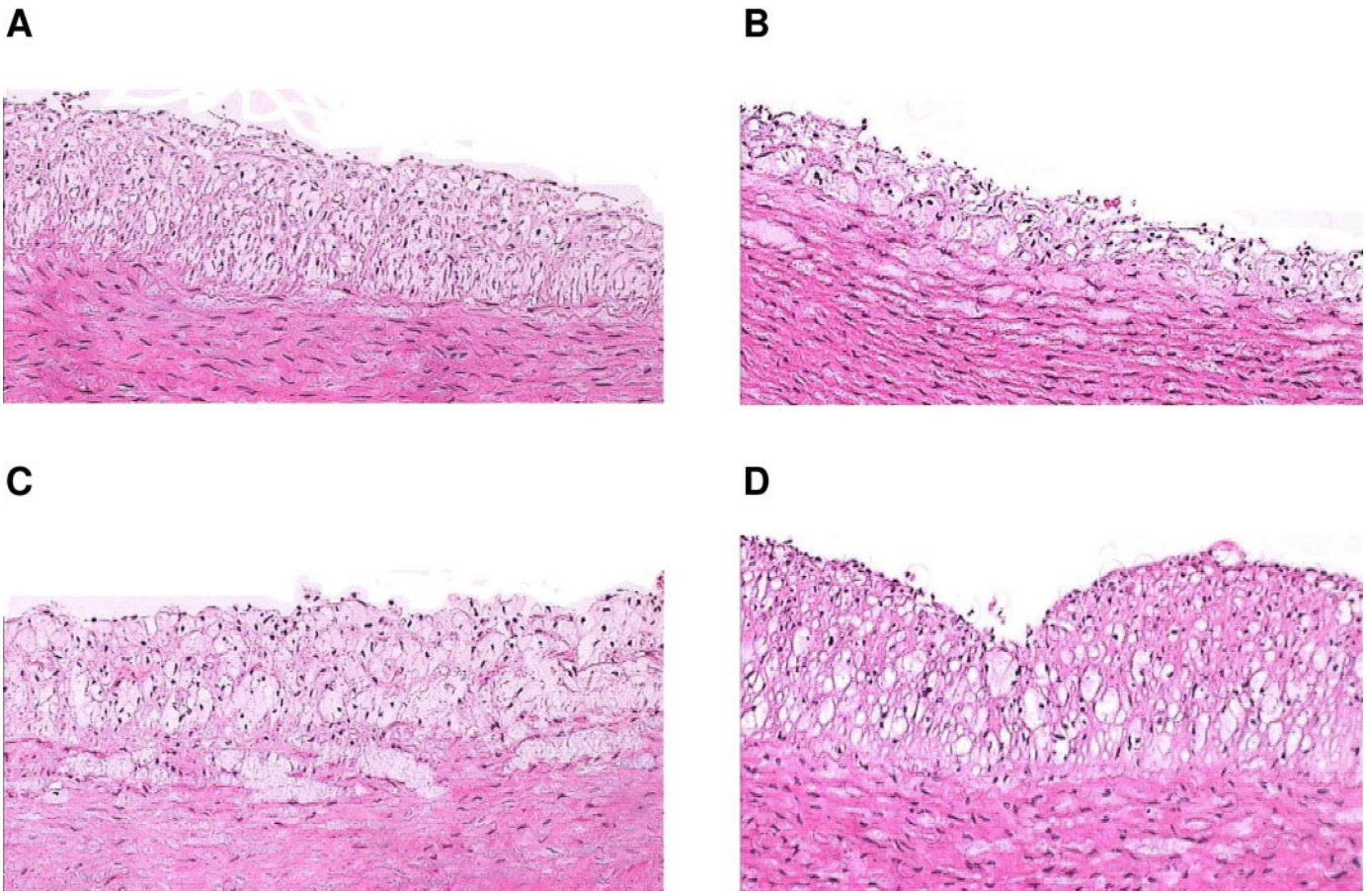

Fig. 7. Representative histopathological images of the aortas of rabbits fed an HC diet for 16 weeks and concomitant oral administration of EP2306 or simvastatin for a 12-week treatment phase (4–16 weeks). A, placebo; B, EP2306; C, simvastatin; and D, coadministration of EP2306 with simvastatin.

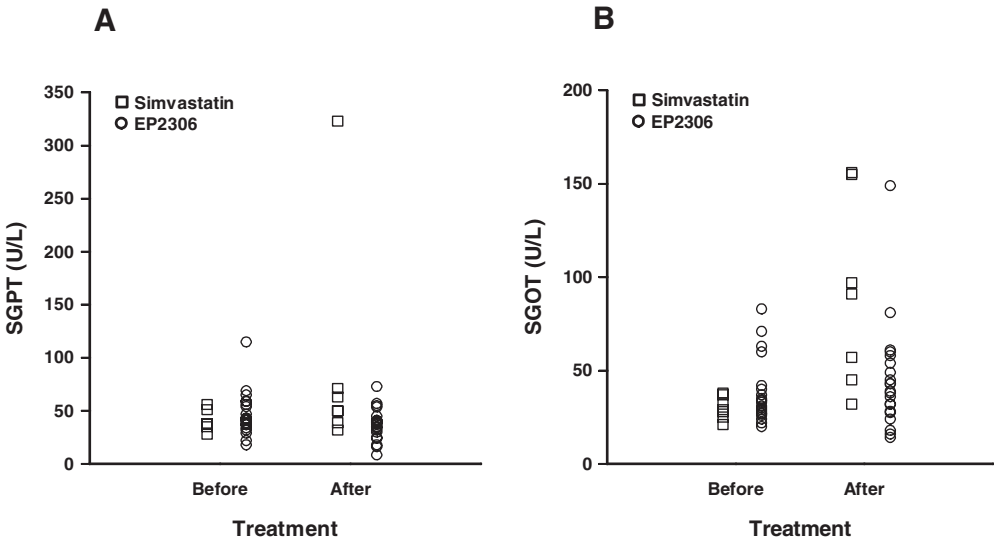

Fig. 8. Effect of EP2306 (0.2, 0.5, 2, and 10 mg/kg, n = 21–23) or simvastatin (2.5 mg/kg, n = 7) treatment for 4 weeks on serum liver transaminases SGPT (A) and SGOT (B).

Interestingly, our finding with simvastatin is in agreement with earlier reports questioning the sustained efficacy of statins. Long-term administration of statins was shown to be associated with the escape phenomenon, in which plasma cholesterol levels of human subjects are initially reduced but return to pretreatment levels (Pazzucconi et al., 1995). These investigators classified patients treated with statins (including simvastatin) in three groups: 1) responders, 2) nonresponders, and 3) response losers. In routine clinical practice, this is often masked either by increasing drug dosage or by adding drugs with different modes of action. The mechanism by which HMG-CoA reductase inhibitors induce the escape phenomenon has been a subject of considerable research and is believed to involve their causing inhibition of not only

cholesterol but also mevalonate derivatives such as farnesol. For example, Ugawa et al. (2002b) described an animal model of the escape phenomenon observed in humans and showed that the squalene synthase inhibitor YM-53601 consistently decreased plasma non-HDL-cholesterol in this model, whereas pravastatin lost its hypolipidemic effect between 17 and 27 days of treatment.

Hepatotoxicity remains one of the concerns of statin use, and frequent follow-up of liver markers is recommended. To exclude such an adverse effect with EP2306 treatment, we examined the effect of EP2306 on serum liver transaminases. In comparison with simvastatin, EP2306 showed a more favorable effect both on SGOT and SGPT enzymes, indicating a lack of hepatotoxicity. Moreover, there was no evidence of toxicity of EP2306 on any other vital organs including the heart, liver, lungs, kidney, spleen, stomach, and gallbladder. Overall, the safety profile of EP2306 on this animal model was good, and this is encouraging given the safety concerns raised for many of the therapeutic agents aiming at the cardiovascular system.

Hitherto, strategies for prevention of cardiovascular disease mainly focus on improving dyslipidemia with the use of statins. Frequently, monotherapy with statins or any other agent is insufficient. Meta-analyses have shown that even with the most aggressive treatment with statins, the reduction of the risk for major coronary events is only 30% (LaRosa et al., 1999). Clearly, there is a need for combining drugs with different or complementary mechanisms of action to achieve the proposed lower LDL-cholesterol therapeutic targets. Recently, the effects of ezetimibe, an inhibitor of dietary cholesterol absorption, have been described (Sudhop et al., 2002; Garcia-Calvo et al., 2005), and the agent is currently widely used as an add-on hypolipidemic agent to statins (Gagne et al., 2002; Davidson et al., 2004). Squalene synthase inhibitors might prove to be an additional useful tool in the context of combination therapy of hyperlipidemia.

In conclusion, we have shown that EP2306 reduces the development of atherosclerotic lesions in an animal model of diet-induced atherosclerosis. This is the first report showing an inhibitory effect of a squalene synthase inhibitor on atherosclerosis in vivo. Although more research is needed to further elucidate its mechanisms of action and its pharmacological profile in humans, such a molecule that confers vascular protection indicates potential as an additional therapeutic approach for coronary artery disease and other atherosclerosis-related disorders.

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