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Inhibition of five lipoxygenase activating protein (FLAP) by MK-886 decreases atherosclerosis in apoE/LDLR-double knockout mice

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Abstract

Background Recent reports point to an important role of leukotrienes in atherogenesis. Leukotrienes are produced by 5-lipoxygenase co-operating with five lipoxygenase activating protein (FLAP). We hypothesized that MK-886, an inhibitor of FLAP, could attenuate the development of atherosclerosis in the atherogenic apolipoprotein E/low density lipoprotein receptor (apoE/LDLR) double knockout (DKO) mouse model.

Materials and methods Female apoE/LDLR-DKO mice at the age of 8 weeks were put on Western diet. The experimental group (n = 10) received the same diet as the control group (n = 10), but mixed with MK-886 (Merck, Rahway, NJ) at a dose of 4 μg per 100 mg of body-weight per day. At age 6 months the mice were sacrificed under anaesthesia.

Results Measured by the *en face* method, the percentage of area occupied by lesions in aortas in the control group was 25·15 ± 2·9%, whereas in the MK-886-treated group it was 11·16 ± 0·7% ($P < 0·05$). Lesion area measured by cross-section of aortic roots was 455 494 ± 29 564 μm^2 in the control group versus 263 042 ± 20 736 μm^2 in the MK-886-treated group ($P < 0·05$). The MK-886 did not change the plasma cholesterol lipoprotein profile as compared with the control mice. Finally, we show that MK-886 may increase plaque stability by decreasing the macrophage content as well as increasing the collagen and smooth-muscle cell content.

Conclusions Our results show for the first time that inhibition of FLAP by MK-886 reduces development of atherosclerosis in gene-targeted apoE/LDLR-DKO mice.

Keywords ApoE/LDLR-double knockout mice, atherosclerosis, FLAP, leukotrienes, MK-886.
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Introduction

Atherosclerosis is an inflammatory vascular disease of increasing incidence in the developed countries. During recent years it has become apparent that the 5-lipoxygenase pathway may play a important role in modifying the pathogenesis of atherosclerosis. Enzymes associated with the 5-lipoxygenase pathway are abundantly expressed in arterial walls of patients afflicted with various lesion stages of atherosclerosis of the aorta and coronary arteries. These data raise the possibility that antileukotriene drugs may be an effective treatment regimen in atherosclerosis [1].

Since 1992 mice have been an excellent model of atherosclerosis [2], namely the apolipoprotein E (apoE) knockout mouse model was developed [3–5]. More recently, apoE and the low density lipoprotein (LDL) receptor-double knockout (apoE/LDLR-DKO) mice provided numerous possibilities to study the pathogenesis and to test the effectiveness of treatments, in a model of atherosclerosis and hyperlipidaemia [2–4,6]. Indeed, apoE/LDLR-DKO mice fed by atherogenic Western diet greatly accelerated lesion formation [7]. The 5-lipoxygenase is abundantly expressed in atherosclerotic lesions of apoE and LDLR-deficient mice, appearing to co-localize with a subset of macrophages but not with all macrophage-staining regions [8].

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Interestingly, the effects of drugs interfering with lipoxygenase metabolism were not studied in respect to atherosclerosis to date. As the five lipoxygenase activating protein (FLAP) has been demonstrated to be a critical step in the control of 5-lipoxygenase activity, we hypothesized that its inhibition by MK-886 could suppress the development of atherosclerosis in this experimental model.

Materials and methods

Animals and treatment

Twenty female apoE/LDLR-DKO mice of the mixed C57BL/6 J × 129/SvJ background were obtained from Taconic (Bomholt, Denmark). The mice were maintained on 12-h dark/12-h light cycles in air-conditioned rooms (22·5 ± 0·5 °C, 50 ± 5% humidity) with access to diet and water *ad libitum*. At 8 weeks old all mice were put on a Western diet comprising 21% fat and 0·15% cholesterol by weight, respectively (Ssniff, Soest, Germany). The experimental group ($n = 10$) received the diet mixed with MK-886 (Merck, Rahway, NJ) at a dosage of 4 μg per 100 mg body-weight per day. The drug was mixed with the diet, without heating, by the manufacturer. The mice treated with MK-886 had the same food intake and the same body-weight after the experiment as the control mice. All animal procedures were approved by the Jagiellonian University Ethics Committee on Animal Experiments.

Procedures

At 6 months old the mice were sacrificed under anaesthesia: 10 mg of Thiopental (Sandoz, Vienna, Austria) i.p. by cervical translocation. Ten minutes before anaesthetizing, 1000 IU of Fraxiparine (Sanofi-Synthelabo, Santea, France) was injected into the peritoneum. Plasma was separated by centrifugation at 1000 g at 4 °C for 10 min and stored at –80 °C. The right atrium was incised and the heart was perfused by phosphate buffered saline (PBS) buffer through the apex of the left ventricle at a constant pressure of 100 mmHg. Next, the heart and the whole aorta were dissected.

Plasma lipoproteins

Total cholesterol and triglycerides were assayed using commercially available kits (Roche Molecular Biochemical, Alameda, CA). Size fractionation of lipoproteins was carried out by fast protein liquid chromatography (FPLC) using a micro-FPLC column, 30 × 0·32 cm, Superose6B (Amersham Pharmacia, Uppsala, Sweden) coupled to a system for on-line separation and subsequent detection of cholesterol. This was followed by 10 μL of plasma injected into the FPLC column of every animal, and the cholesterol content in lipoproteins was determined on-line using the commercially available MPR-2-1442-350 cholesterol assay kit (Roche, Meylan, France), which was continuously mixed with the separated lipoproteins at a flow-rate of 40 μL min^{-1}. Absorbance was measured at 500 nm, and data were collected every 20 s using EZCHROM software (Scientific Software, San Ramon, CA) [9].

Quantification of atherosclerosis

The heart and ascending aorta were embedded in Optimal Cutting Temperature (OCT) compound (Cell Path, Oxford, UK) and snap-frozen. Ten micrometer (μm)-thick cryosections were cut from the aortic root using a standardized protocol. Serial sections were cut from the proximal 1 mm from the aortic root. Eight sections were collected at 100-μm intervals starting at a 100-μm distance from the appearance of the aortic valves. After fixation in 4% paraformaldehyde (pH 7·0), sections were stained with Meyer's haematoxylin and oil red-O (ORO) (Sigma-Aldrich, St. Louis, MO). Oil red-O-stained sections were examined under an Olympus BX50 (Olympus, Tokyo, Japan) microscope and used for quantitative evaluation. Images of the aorta were recorded using an Olympus Camedia 5050 digital camera and stored as TIFF files of resolution 1024 × 768 pixels. Total area of the lesion was measured semi-automatically in each slide using ANALYSIS FIVE software (Soft Imaging System, Munster, Germany). For each animal a mean lesion area was calculated from eight sections, reflecting the cross-sectional area covered by atherosclerosis [10].

The aorta, from arch to bifurcation, was fixed in 4% formaldehyde, opened longitudinally, pinned onto black wax plates and stained with Sudan IV (Sigma-Aldrich, St. Louis, MO). Aortic lesion area and total aortic area were calculated using LSM IMAGE BROWSER software (Zeiss, Jena, Germany).

Sections were also stained with Masson's trichrome and orcein to visualize collagen and elastic fibres, respectively. Macrophage, collagen and smooth-muscle cell (SMC) contents in plaques were measured dividing the CD68, collagen and α-actin-stained cross-sectional area by the total lesional surface.

Immunohistochemistry

For indirect immunohistochemistry acetone-fixed sections of ascending aorta were used. Sections were incubated overnight with primary antisera: Cy3-conjugated antiα-smooth-muscle actin (Sigma-Aldrich, St. Louis, MO) and rat antimouse CD68 (Serotec, Oxford, UK). Goat antirat IgGbiotinylated antibodies followed by DTAF-conjugated streptavidin (Jackson IR, West Grove, PA), respectively, were applied to visualize rat antibodies. Sections were examined using an epifluorescence Olympus BX50 microscope equipped with appropriate filter cubes to show Cy3 (red) and DTAF (green) fluorescence. Images were registered with a Camedia 5050 digital camera.

Statistical analysis

Results were expressed as mean ± SEM. The non-parametric Mann–Whitney *U*-test was used for analysis of the data and $P < 0{\cdot}05$ was considered as statistically significant.

Results

The MK-886 did not change the plasma cholesterol lipoprotein profile as compared with the non-treated mice (Fig. 1). Moreover, MK-886 did not change the level of cholesterol and triglycerides in blood, as compared with the control group (Table 1).

Measured by the *en face* method, the percentage area occupied by atherosclerotic lesions in aortas in the control group was $25{\cdot}15 \pm 2{\cdot}9\%$, whereas in the MK-886-treated group it was $11{\cdot}16 \pm 0{\cdot}7\%$ ($P < 0{\cdot}05$). Lesion area measured at the cross-section of aortic roots was $455\,494 \pm 29\,564\ \mu m^2$ in the control group versus $263\,042 \pm 20\,736\ \mu m^2$ in the MK-886-treated group ($P < 0{\cdot}05$) (Fig. 2).

We also studied macrophages as well as collagen and smooth-muscle cell content in plaque as indices of plaque stability. We found that the macrophage (CD68) content was decreased in plaques from mice receiving MK-886 (63% vs. 35%; $P = 0{\cdot}007$), whereas collagen (4% vs. 7%; $P = 0{\cdot}02$) and SMC (α-actin) content (8% vs. 15%; $P = 0{\cdot}03$) were increased in treated mice ($n = 10$ per group). Accordingly, the plaques from MK-886 treated mice seem to be more stable than those from the control mice (Fig. 3).

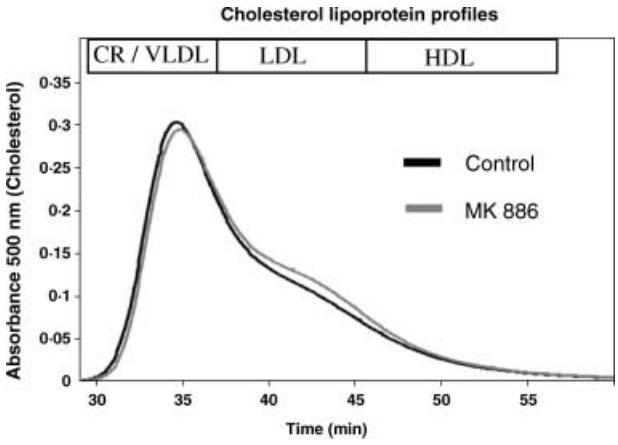

Figure 1 Fast protein liquid chromatography (FPLC) analysis of cholesterol lipoprotein profiles from sera of 24-week-old mice treated and non-treated with MK-886. Original tracings, representative for $n = 10$ (in each group) experiments.

Table 1 Cholesterol and triglycerides levels in control and MK-886 treated groups

Group	TCH (mmol L^{-1})	TG (mmol L^{-1})
Control ($n = 10$)	$26{\cdot}8 \pm 1{\cdot}3$	$2{\cdot}01 \pm 0{\cdot}1$
MK-886 treated ($n = 10$)	$25{\cdot}7 \pm 1{\cdot}1$ (NS)	$1{\cdot}9 \pm 0{\cdot}1$(NS)

NS, not significant difference between groups.

Discussion

Lipoxygenases are enzymes that catalyze a stereo-specific dehydrogenation and subsequent dioxygenation of polyunsaturated fatty acids with a 1,4-*cis*-pentadiene structure [11]. Of special interest for atherosclerosis is the arachidonate 5-lipoxygenase, which was originally identified in polymorphonuclear leukocytes [12], but was recently demonstrated to be over-expressed in macrophages [13] and in other cell types [14]. This enzyme generates an unstable epoxide intermediate, leukotriene A_4 (LTA_4), which is an important precursor of LTB_4, LTC_4 and other cysteinyl leukotrienes. Initial observations as well as the use of drugs affecting 5-lipooxygenase metabolism were mainly conducted in asthma and other inflammatory diseases [15]. However, increasing understanding of the role of inflammation in atherosclerosis brought attention to the potential role of leukotrienes and their metabolism. Indeed, 5-lipoxygenase was recently identified to importantly contribute to atherosclerosis in both mouse models and humans [8,16,17]. De Caterina *et al.* have shown that leukotriene B_4 is produced in human atherosclerotic plaques [18]. Later Aiello *et al.* showed that LTB_4 receptor antagonism reduced monocytic foam cells in mice [19]. Lotzer *et al.* pointed that macrophage-derived leukotrienes differentially activate $cysLT_2$-Rs via paracrine stimulation and $cysLT_1$-Rs via autocrine and paracrine stimulation during inflammation and atherogenesis [20].

Therefore, a hypothesis has been put forward that leukotriene-inhibiting drugs developed to treat asthma might protect the cardiovascular system against atherosclerosis [15]. There are numerous potential targets that could be useful in the intervention of leukotriene metabolism in atherosclerosis. Interestingly, an 18-kDa microsomal protein termed FLAP was found to be critical for the regulation of 5-lipoxygenase activity and biosynthesis of leukotrienes within certain compartments of plasma membrane. The role of FLAP in atherosclerosis was additionally confirmed in humans by Helgadottir *et al.* [21] who showed that genetic polymorphisms of FLAP are associated with myocardial infarction and stroke by increasing leukotriene production and inflammation in the arterial wall. In the present report, we extend these observations by showing that inhibition of FLAP by MK-886 causes attenuation of atherogenesis in the experimental model of atherosclerosis.

MK-886 not only decreased the extent of atherosclerotic changes, but also made the plaques more stable. MK-886 increased collagen and elastic fibres as well as α-smooth-muscle actin staining in lesions. In contrast, the proportion of the lesional area stained by macrophage marker CD68

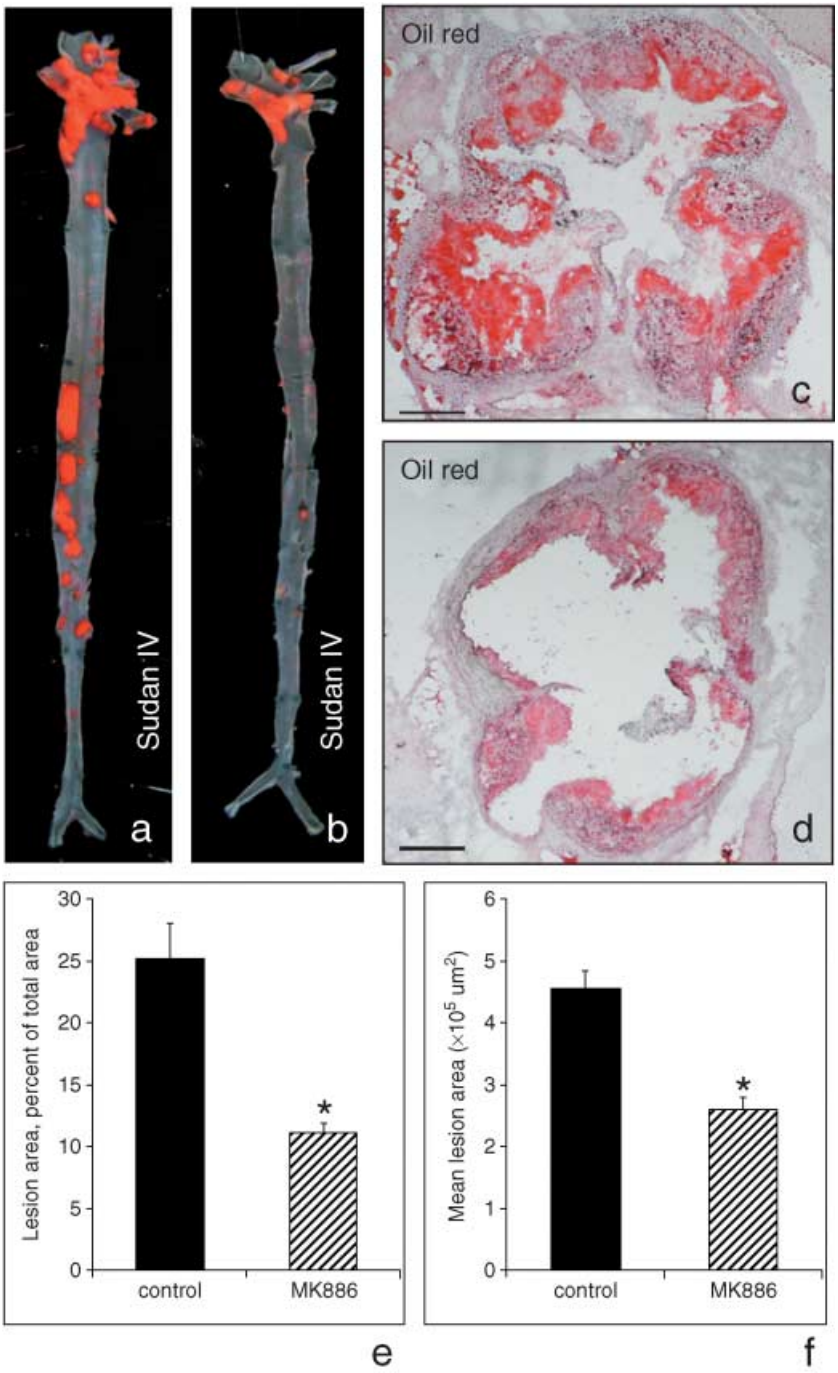

Figure 2 Treatment with MK-886 decreases atherosclerosis in age-matched mice. Sudan IV-stained *en face* preparations of aortas from control (a) and MK-886-treated (b) apoE/LDLR-double knockout mice. (e) Lipid lesion area in the entire aorta (percentage of total aortic surface area) in control and MK-886-treated apoE/LDLR-double knockout mice (n = 10 per group). Results are presented as mean ± SEM, $^{*}P < 0{\cdot}05$. Representative micrographs showing oil red-O-stained lesions in (c) control and (d) MK-886-treated apoE/LDLR-double knockout mice. Bars = 200 μm .(f) Lesion size in the aortic root (μm^2) stained by ORO in 6-month-old control and MK-886-treated apoE/LDLR-double knockout mice (n = 10 per group). Results are presented as mean ± SEM, $^{*}P < 0{\cdot}05$.

was lower in MK-886-treated animals. Reduced collagen as well as infiltration of macrophages in lesions have been linked to a propensity for plaque rupture [22].

Pharmacological antagonism of the LTB_4 receptor was shown to be protective in three atherosclerosis-susceptible mouse strains [19]. Interestingly, our preliminary results suggest that montelukast, an antagonist of the cysteinyl leukotriene receptor, also decreases atherosclerosis in apoE/LDLR-DKO mice (unpublished data, Jawien *et al.*, 2006), but to a lesser extent than MK-886.

To our knowledge, this is the first report that shows the effect of FLAP inhibition on atherogenesis.

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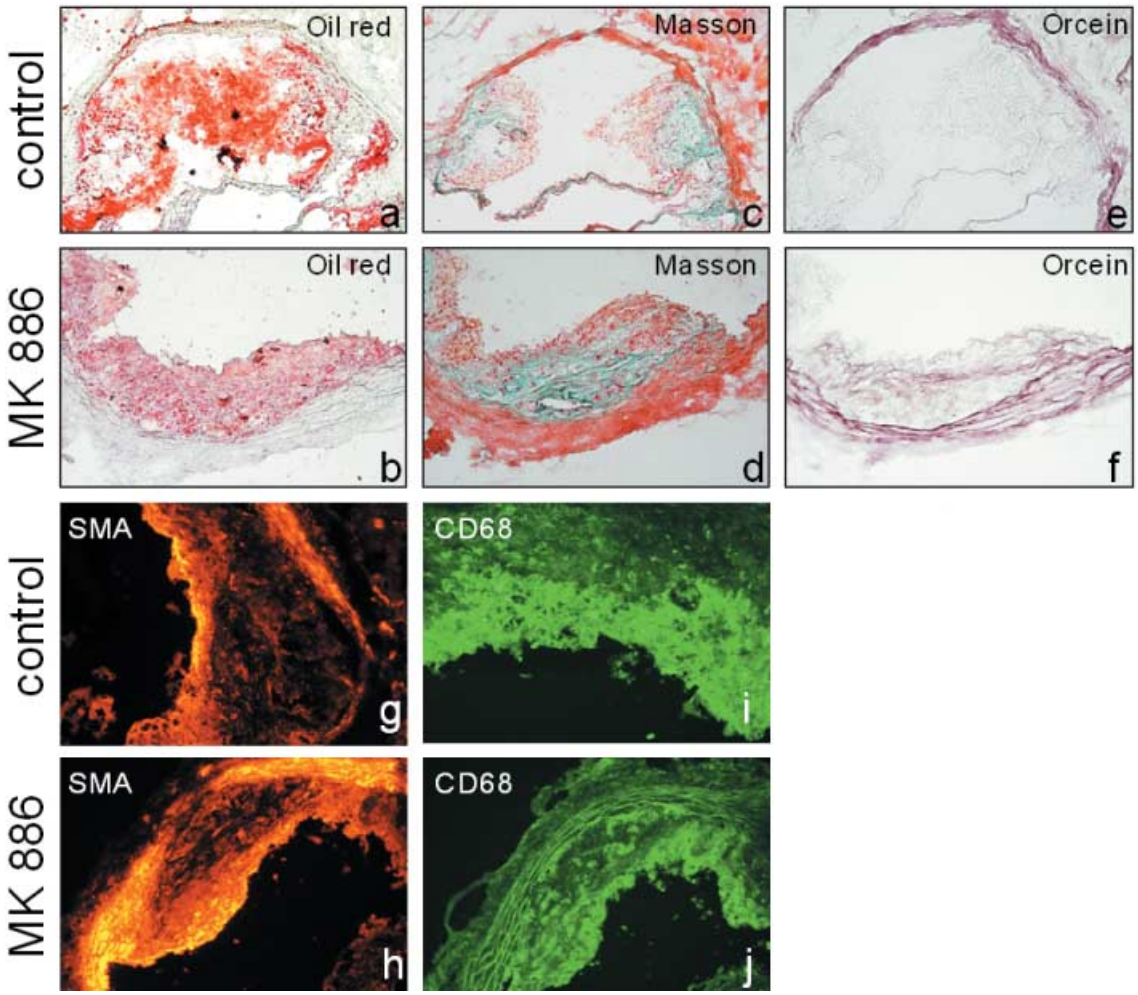

Figure 3 Lesion composition in 24-week-old mice treated with MK-886 as compared with control group. Representative micrographs showing lesions in control and MK-886-treated apoE/LDLR-double knockout mice stained with oil red-O (a,b). Masson's trichrome (collagen in green) (c,d) and orcein (e,f). Immunohistochemical staining for α-smooth-muscle actin (α-actin) (g,h) and CD68 (i,j) (all × 400).

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