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Exacerbation of atherosclerosis, hyperlipidemia and inflammation by MK886, an inhibitor of leukotriene biosynthesis, in obese and diabetic mice

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ABSTRACT

Leukotrienes are potent mediators of the inflammatory response and 5-lipoxygenase, the enzyme responsible for their synthesis, is dependent on its interaction with 5-lipoxygenase activating protein for optimum catalysis. Previous studies had demonstrated that macrophage infiltration into adipose tissue is associated with obesity and atherosclerosis in LDLR$^{-/-}$ mice fed a high fat-high carbohydrate. The present study was undertaken to determine whether inhibition of 5-lipoxygenase activating protein is efficacious in attenuating adipose tissue inflammation in LDLR$^{-/-}$ mice fed a high fat-high carbohydrate. 10-week old male LDLR$^{-/-}$ mice were fed a high fat-high carbohydrate diet for 22-weeks, with or without MK886 (40 mg/kg/day, *ad libitum*) a well-established 5-lipoxygenase activating protein inhibitor. All mice had an approximate 2-fold increase in total body weight, but a 6-week course of MK886 treatment had differential effects on adipose tissue size, without affecting macrophage accumulation. MK886 exacerbated the dyslipidemia, increased serum amyloid A content of high-density lipoproteins and caused a profound hepatomegaly. Dyslipidemia and increased serum amyloid A were concomitant with increases in atherosclerosis. In conclusion, MK886 paradoxically exacerbated hyperlipidemia and the pro-inflammatory phenotype in a mouse model of diet-induced atherosclerosis, possibly via a disruption of hepatic lipid metabolism and increased inflammation.

1. Introduction

It is well established that chronic and local inflammatory mechanisms contribute to the development of atherosclerosis. These mechanisms, associated with risk factors such as diabetes, smoking, excess body weight, fatty diet, sedentary lifestyle, elevated blood pressure and dyslipidemia, contribute to the production of a wide variety of inflammatory mediators that target cells of the immune system and the vascular wall and that regulate the development and resolution of inflammation (Libby et al., (2009) (Biros et al., 2022; Hopkins, 2013),. These mediators include growth factors, chemokines, cytokines and eicosanoids. Derived from the metabolism of arachidonic acid (C20:4; AA), eicosanoids are the products of three enzymatic pathways; the cyclooxygenases (COX-1/2), the 5- and 12/15-lipoxygenases (5LO, 12/15LO, respectively) and the cytochrome P450 (cyP450) monooxygenases (Fig. 1) and have been demonstrated to be involved with atherosclerotic lesion formation (Piper and Garelnabi, 2020). 5LO differs from other AA-metabolizing enzymes due to its requirement for interaction with FLAP in order to initiate the production of leukotrienes (LTs) (LTB_4, LTC_4, LTD_4 and LTE_4) (Dixon et al., 1990; Miller et al., 1990) and plays a prominent role in the development of inflammatory diseases, such as asthma (De Caterina and Zampoli, 2004; Funk, 2005; Lotzer et al., 2005). The cysteinyl LTs (CysLTs), namely LTD_4 and LTE_4, are implicated in a variety of inflammatory processes, including atherosclerosis (Singh et al., 2010). 5LO expression is largely restricted to leukocytes, which upon stimulation, synthesize and secrete LTs and trigger cell-specific responses (Gilbert et al., 2021) and while other steps in LT biosynthesis are subject to regulatory control, modulation of the expression and function of 5LO and FLAP are critical steps (Radmark and Samuelsson, 2010).

Diets rich in saturated fat and carbohydrates have been associated with weight-gain, insulin resistance (IR) and an increased risk of cardiovascular disease (Hruby and Hu, 2015; Kannel and Mcgee, 1979; Lutz and Woods, 2012) in a variety of mammalian species. Increased body

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weight is also a risk factor for the development of diabetes; hence, high-fat diets directly and indirectly contribute to risk of IR and diabetes. In addition, there is growing body of evidence that adipose tissues (AT) and the liver contribute to insulin sensitivity via the release of inflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukin-1-β (Il-1β) (Baker et al., 2011; Zatterale et al., 2019; Ziolkowska et al., 2021).

In obese mice, accumulation of macrophages in AT may be a key variable for systemic inflammation and IR (Subramanian et al., 2008; Weisberg et al., 2003; Xu et al., 2003), but the comprehensive mechanisms of how they are recruited into AT remains elusive. Chemoattractant molecules such as monocyte chemoattractant protein-1 (MCP-1) and other bioactive molecules have been proposed to mediate this process (Kanda et al., 2006; S. P. Weisberg et al., 2006). For example, studies have implicated an additional monocyte recruitment pathway, namely a complex containing a serum amyloid A-3 isoform (SAA3), in the accumulation of macrophages in in AT. In low-density lipoprotein receptor deficient ($LDLR^{-/-}$) mice fed a high fat-high carbohydrate (HFHC) diet, SAA was elevated, and like MCP-1, was chemotactic for monocytes and promoted atherogenesis (Chait et al., 2021).

FLAP expression is elevated in the AT of obese men (Kaaman et al., 2006), as well as in animals with experimental obesity and IR (Bäck et al., 2007) and CysLTs, metabolites of the 5LO pathway, stimulate TNF-α and MCP-1 synthesis in AT and contribute to low-grade inflammation and fat accumulation in C57BL6 mice fed a high-fat diet (Horrillo et al., 2010). A role for 5LO in hepatic steatogenesis was identified in leptin-deficient *ob/ob* mice, in which it was implicated in hepatic microsomal triglyceride (TG) transfer protein activity and the secretion of very low-density lipoprotein (VLDL) and apolipoprotein B (ApoB), with a significant protective observed with the pharmacological inhibition of 5LO (Lopez-Parra et al., 2008). However, the potential involvement of the products of the 5LO pathway in AT inflammation and diet-induced atherosclerosis in experimental obesity remains to be fully elucidated. In this study, we hypothesized that a well-established FLAP inhibitor and inhibitor of leukotriene biosynthesis, MK, would reduce AT inflammation and atherosclerotic lesion formation in $LDLR^{-/-}$ mice fed a diet high in saturated fats and carbohydrates (Graphical Abstract).

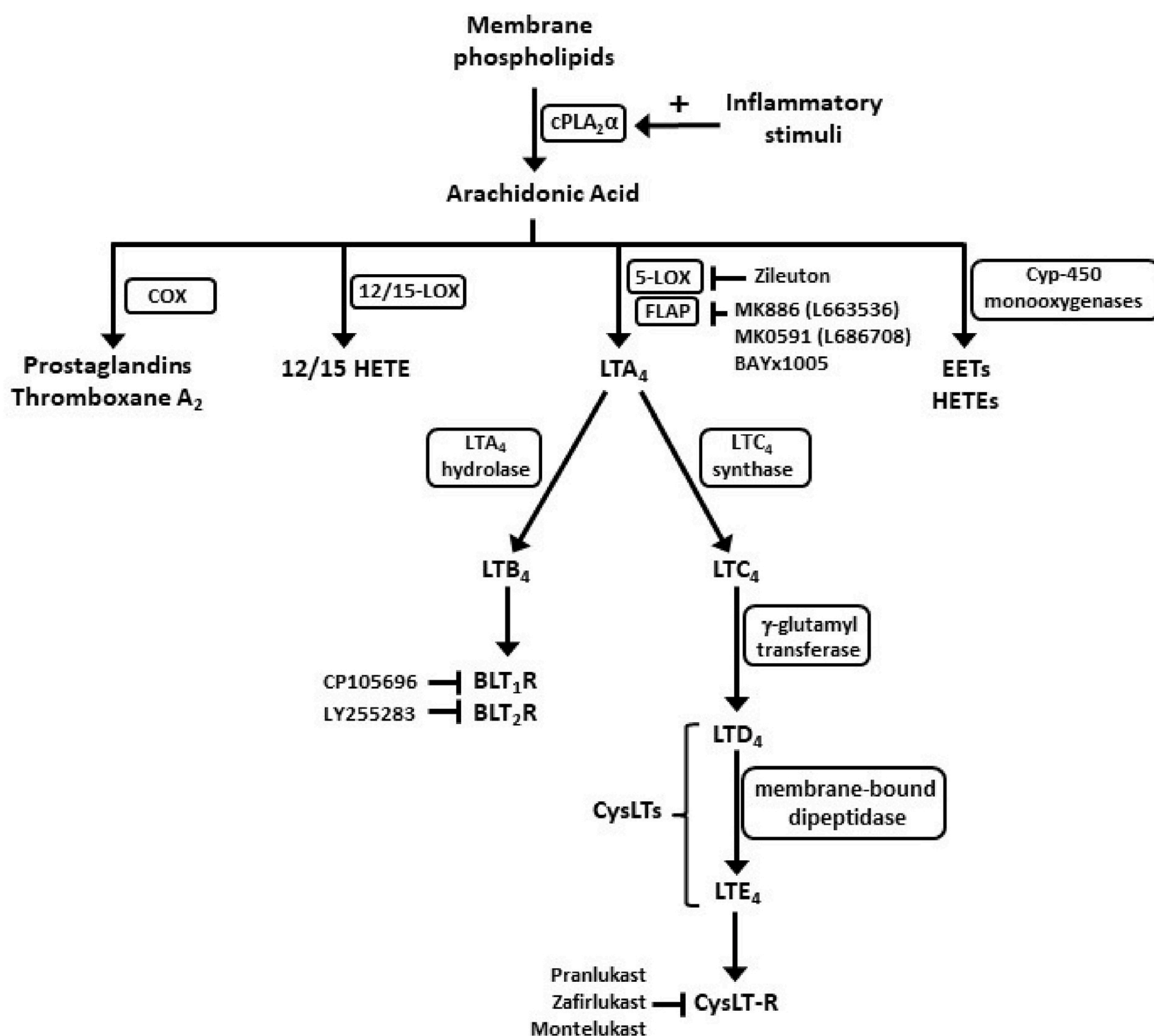


Fig. 1. Schematic illustration of the biosynthesis of eicosanoids from AA and sites of action of the pharmacological inhibitors of synthesis and effects of 5LO-derived mediators. Briefly, in an inflammatory setting, AA is liberated from membrane phospholipids by an inducible isoform of phospholipase A_2 and is consequently converted by cyclooxygenases (COX1/2), 12/15 HETE via 12/15 lipoxygenase, EET's/HETE's via cyp450 monooxygenase and leukotrienes via the 5-lipoxygenase pathways in a tissue and cell-dependent manner to a variety of bioactive eicosanoids. COX; Cyclooxygenase, TXA_2; Thromboxane A_2, HETEs; Hydroxyeicosatetraenoic acids, EETs; Epoxyeicosatrienoic acids, LOX; Lipoxygenase, LT; Leukotriene.

2. Materials and methods

This manuscript was organized to be consistent with the ARRIVE guidelines 2.0 and the ethics rules (https://doi.org/10.1371/journal.pbio.3000411) for experiments in animals.

2.1. Animals and study protocols

2.1.1. Study design, randomization & blinding

The Institutional Animal Care and Use Committee of the University of Washington in Seattle approved all experimental protocols and procedures. The study aims to compare hyperlipidemic mice fed a HFHC diet (control group) to hyperlipidemic mice fed with same diet but whose diet is supplemented with MK886 (MK), an inhibitor of FLAP (experimental group). In all experiments, animal designation (treated vs. non-treated) were randomized and were analyzed in a blinded fashion. Water and diet were available *ad libitum.* All mice were housed in temperature-controlled rooms with a 12-h light/dark cycle and examined daily for health and weighed throughout the study.

2.1.2. Sample size

Sample size was determined by the following: Each group was calculated to have an 80% chance (power) of detecting a difference between 2 means of untreated and MK-treated mice, if the true difference between the means is 1 standard deviation (2-tailed $\alpha = 0.05$ corresponding to a 95% confidence interval. The sample size was then adjusted (14 mice/group to 6 mice/group) in order to comply as a pilot study.

2.1.3. Experimental animals and inclusion & exclusion criteria

Mice were randomly assigned to 2 groups of littermates and housed in rooms on a 12 light/12 dark cycle with temperature set at 68–79 °F. All animals (3 mice/cage, 2 cages/group) were obtained from the same litter. 8-week old male LDLR$^{-/-}$ mice (C57BL6 background) were fed standard rodent chow for 10-weeks, placed on a HFHC diet (Supplemental Table 2) (No. F1850, Bio-Serv; Flemington, NJ) for an additional 16-weeks. One mouse was found dead in the cage at week 10 and excluded from all study endpoints. The chosen dose for MK was shown to have effective anti-atherosclerotic efficacy in other mouse models of atherosclerosis (Jawien et al., 2006). The mice were then treated with MK for 6-weeks and sacrificed at 32-weeks of age (Fig. 2A, Supplemental Fig. 2). MK (40 mg/kg/day; Merck, Whitehouse Station, NJ, Supplemental Fig. 1) was incorporated into the diet and administered *ad libitum.*

2.1.4. Outcome measures

Body weights (BW) were measured weekly. In selecting a dose for evaluating the actions of MK, Mice were fasted for 4 h prior to blood-draw on the day of sacrifice. Post-sacrifice, liver and inguinal, retroperitoneal, epididymal and mesenteric ATs were excised and weighed. Portions were either snap-frozen with liquid N_2 or fixed with 10% neutral-buffered formalin and embedded in paraffin wax. Frozen tissues were stored at −70 °C until use.

2.1.4.1. Blood and plasma chemistry and hepatic lipid analysis. Plasma cholesterol and TGs were assayed using colorimetric assay kits (Amplex™ Red Cholesterol Assay Kit: Invitrogen; Waltham, MA; TG Assay Kit: Roche Diagnostics; Indianapolis, IN) and cholesterol content of lipoproteins was analyzed by fast-phase liquid chromatography (FPLC) fractions, as described previously (Lewis et al., 2004). Circulating SAA levels were measured by enzyme-linked immunosorbent assay in plasma and in lipoprotein fractions separated via FPLC from individual mice, as described previously (Subramanian et al., 2008). Blood glucose and non-esterified fatty acids (NEFA) were measured using a OneTouch® Ultra® glucometer (Lifescan Inc.; Milpitas, California) and the NEFA-HR (2) kit (FUJIFILM Wako Chemicals; Neuss, Germany), respectively. Neutral lipids were extracted from frozen livers of LDLR−/− mice using a modified method of Folch et al. (Folch, Lees and Sloan Stanley, 1957) and hepatic TG and cholesterol content were quantified as noted above and normalized to total sample tissue weight.

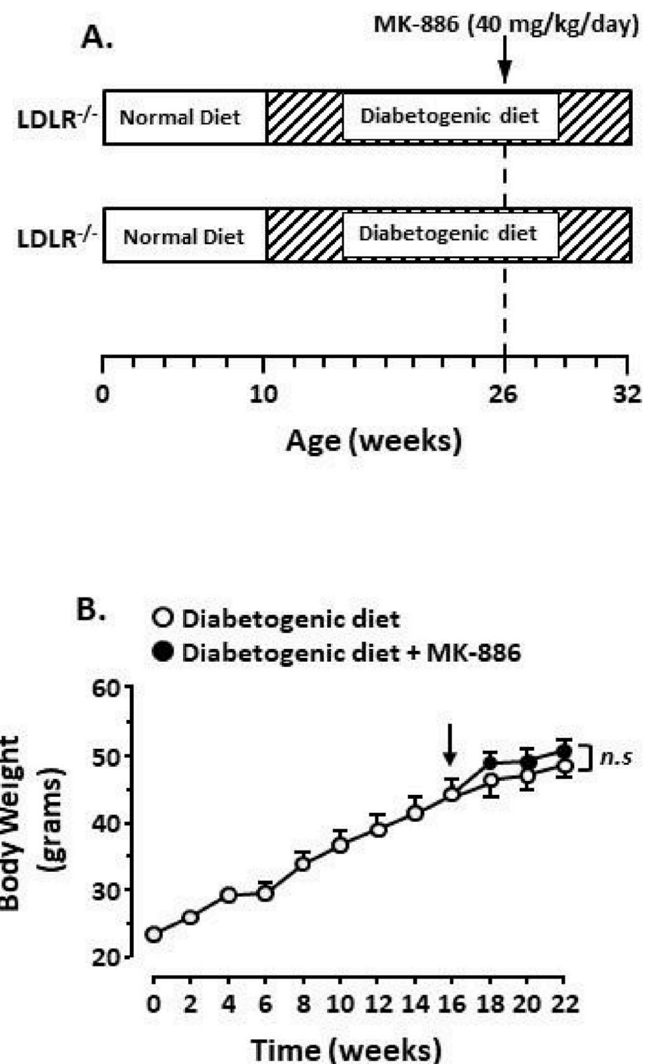


Fig. 2. MK-886 has no effect on body weight in LDLR$^{-/-}$ mice fed a diabetogenic diet. **(A)** Study design. Male LDLR$^{-/-}$ mice were fully weaned onto standard chow diet for 10-weeks. The diet was then changed into the HFHC at week 10. After 16-weeks, MK886 (40 mg/kg/day, arrow) was added for an additional 6-weeks. **(B)** Total body weights during and at the end of the study are shown (diabetogenic diet, open circles, n = 5; diabetogenic diet + MK, closed circles, n = 6). Results are shown as means ± SEM. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* analysis. *n.s.;* not statistically significant.

2.1.4.2. Atherosclerosis quantification and immunohistochemical analysis. Atherosclerotic lesions were analyzed in the aortic arch and aortic sinus. In the aortic arch, the aortae were collected, fixed in formalin-sucrose solution, cleaned from adventitia, split longitudinally, and pinned onto black-wax field using 0.1-mm-diameter stainless-steel pins (Fine Science Tools Inc.; Foster City, CA). To visualize atherosclerotic lesions, the aorta was washed with 70% ethanol, stained with 0.5% Sudan IV (Sigma-Aldrich, St Louis, MO) and washed again with 80% ethanol. Each picture was analyzed with Adobe Photoshop 6.0 (Adobe Systems Inc.; San Jose, CA), and the Sudan IV-positive lesion area was quantified using Image-Pro® (Media Cybernetics; Rockville, MD). All

analyses were made in a masked fashion and performed as follows. To visualize atherosclerotic lesions in the aortic sinus, area quantification for proteoglycans was performed on digital images of the aortic sinus stained with the Movat's pentachrome method (yellow: collagen, black: elastin, blue: proteoglycans, bright red: fibrin) (Lewis et al., 2004). Rat anti–Mac-2 antibody (1:5000, Cedarlane Laboratories; Ontario, CA) was used to detect macrophage content in epididymal AT, with Nova Red (Vector Laboratories; Burlingame, CA) as a peroxidase substrate to yield a red-brown reaction product. Nuclei were identified by counterstaining with hematoxylin. Area quantification for Mac-2 was performed on digital images of AT using Image-Pro® and counted, in a blinded manner, the number of Mac-2 positive cells per cross section.

2.1.5. Statistical methods

All data are expressed as mean ± standard error of mean (SEM) unless noted otherwise and analyzed using GraphPad Prism (GraphPad Software; La Jolla, CA) and presented following the recommendations made by Harvey J. Motulsky (Michel et al., 2020; Motulsky, 2015). Details of all statistical analysis are enumerate in the Figure Legends.Full comparative and between-group statistics of all measured end-points are listed in Supplemental Table 2.

3. Results

3.1. Body and tissue weights

To evaluate MK-induced effects on growth and BW, male LDLR$^{-/-}$ mice were fed a HFHC diet for 16 weeks and subsequently treated with MK for an additional 6 weeks (Fig. 2A). Prior to the termination of the study, one of the mice in the non-treated group died (Supplemental Figure 2). There was an approximate two-fold increase in total BW prior to initiation of MK treatment (26.9 ± 0.7 g vs 47.5 ± 1.7 g; 10-week vs 16-week mice, respectively, $P < 0.0001$, Fig. 2B), results similar to previously published reports (Subramanian et al., 2008). MK treatment caused a small increase in total BW (Fig. 2B–Table 1). Interestingly, MK also caused a marked hepatomegaly (Table 1), with the liver displaying a pronounced pale, whitish appearance (*unpublished observation*). Histopathologic analysis of MK-treated livers with Picrosirius Red did not reveal fibrotic damage in any of the tissues analyzed (*unpublished observations*). Hepatic TG content was elevated in MK-treated mice (10.05 ± 1.4 mg/mg tissue vs 17.97 ± 3.2 mg/mg tissue; untreated vs MK-treated, respectively, P = 0.0637, Supplemental Table 2). MK treatment had a differential effect on AT weights, increasing retroperitoneal and inguinal AT weights but without any effects on epididymal and mesenteric AT (Table 1, Supplemental table 1).

Table 1
Effects of MK on total body, liver and AT weights in LDLR$^{-/-}$ mice fed a high-fat high-carbohydrate diet.

	LDLR$^{-/-}$ Mice		
Tissue	HFHC	HFHC + MK	*P*-value
Total Body Weight (g)	48.6 ± 1.8	50.7 ± 1.6	0.4286
Liver (g/gBW)	0.046 ± 0.003	0.077 ± 0.004	***0.0003***
Epidydimal adipose tissue (g/gBW)	0.019 ± 0.0008	0.018 ± 0.001	0.4977
Mesenteric adipose tissue (g/gBW)	0.024 ± 0.002	0.025 ± 0.001	0.6644
Retroperitoneal adipose tissue (g/gBW)	0.015 ± 0.001	0.019 ± 0.0006	***0.0247***
Inguinal adipose tissue (g/gBW)	0.019 ± 0.003	0.029 ± 0.001	***0.0064***

Total body and individual tissues were weighed at end of week 32 of the study in MK-treated and untreated LDLR$^{-/-}$ mice. Tissue weights were normalized to total body weight and presented as grams of body weight (g/gBW). Data is presented as mean ± SEM. Data was analyzed using unpaired *t*-test. Full comparative statistics are displayed in Supplemental Table 1. HFHC; diabetogenic diet, MK; MK886.

3.2. Blood glucose, serum lipids and lipoprotein profiles

In order to determine whether MK treatment had any metabolic effects on HFHC-fed LDLR$^{-/-}$ mice, we measured fasting blood glucose, serum lipids and cholesterol content of lipoproteins. We evaluated systemic inflammation using SAA levels in the plasma and associated with lipoproteins as an indicator and feature of visceral obesity (Eklund et al., 2012; Subramanian et al., 2008). MK had no effect on fasting blood glucose levels but raised blood cholesterol levels (P = 0.0519, Table 2). Additionally, MK treatment had other hyperlipidemic effects, significantly increasing both plasma TG and NEFA levels (Table 2). To further elucidate the effects of MK on plasma lipoproteins, we analyzed the SAA and cholesterol content of very low density/intermediate density, low-density and high-density lipoproteins (VLDL/IDL, LDL and HDL, respectively). Lipoprotein profiles of MK-treated mice revealed dramatic increases in the cholesterol content of VLDL/IDL and LDL, but not HDL, fractions (Fig. 3A).

HFHC-fed LDLR$^{-/-}$ mice have previously demonstrated elevated levels of both circulating and lipoprotein-associated SAA (Subramanian et al., 2008), observations reproduced in the present study (Fig. 3B, open circles). Treatment with MK caused an additional elevation in circulating SAA levels, increases which did not achieve statistical significance (23.34 ± 3.23 μg/ml vs 48.43 ± 14.65 μg/ml; untreated vs MK-treated, respectively, P = 0.08, Table 2). However, treatment with MK caused a pronounced increase in SAA content of HDL in HFHC-fed mice, without any effects on VLDL/IDL and LDL fractions (Fig. 3B, closed circles).

3.3. Analysis of atherosclerotic lesion formation

To assess the impact of the changes in lipoproteins observed in MK-treated HFHC-fed LDLR$^{-/-}$ mice on atherosclerotic lesion development, we visualized the lesions in the aortic arch and in cross-sections of the aortic sinus (Fig. 4). Lesion area in the aortic arch were increased, as measured in both total and percentage of total aortic area in MK-treated mice (Fig. 4A–C). In the aortic sinus, lesion areas of MK-treated mice were notably increased compared to non-treated animals, with MK treatment causing a 3-4-fold increase in total lesion cross-sectional area (Fig. 4D and E).

3.4. Analysis of epididymal adipose tissue macrophage content

Previous studies had demonstrated that macrophage accumulation is limited to epididymal (intra-abdominal) AT depots of HFHC-fed LDLR$^{-/-}$ mice (Subramanian et al., 2008). In order to determine whether MK treatment had any effect on macrophages in epididymal AT, mac-2 positive cells were used as an indicator of macrophage content. The macrophage content of epididymal AT induced by HFHC diet was not affected by MK treatment (Fig. 5A). Additionally, analysis of gene expression (via real-time RT-qPCR) for MCP-1, 5LO and SAA in epididymal AT found no differences between treated and non-treated mice (*unpublished observations)*.

Table 2
Effect of MK-886 on blood glucose, lipids and SAA in LDLR$^{-/-}$ mice fed a high-fat high-carbohydrate diet.

	LDLR$^{-/-}$		
	HFHC	HFHC + MK	*P*-value
Blood Glucose (mg/dL)	266.8 ± 6.8	260.2 ± 11.0	0.0823
Cholesterol (mg/dL)	673.3 ± 75.1	912.2 ± 77.2	0.0519
TG (mg/dL)	192.9 ± 26.4	544.1 ± 51.8	***0.0043***
NEFA (mEq/L)	0.538 ± 0.058	0.718 ± 0.050	***0.0303***
SAA (μg/ml)	23.34 ± 3.23	48.43 ± 14.65	0.0823

TG; triglycerides, NEFA; non-esterified fatty acids, SAA; serum amyloid A. Data is presented as mean ± SEM. Data was analyzed using unpaired *t*-test. HFHC, high-fat high-carbohydrate diet; MK, MK886.

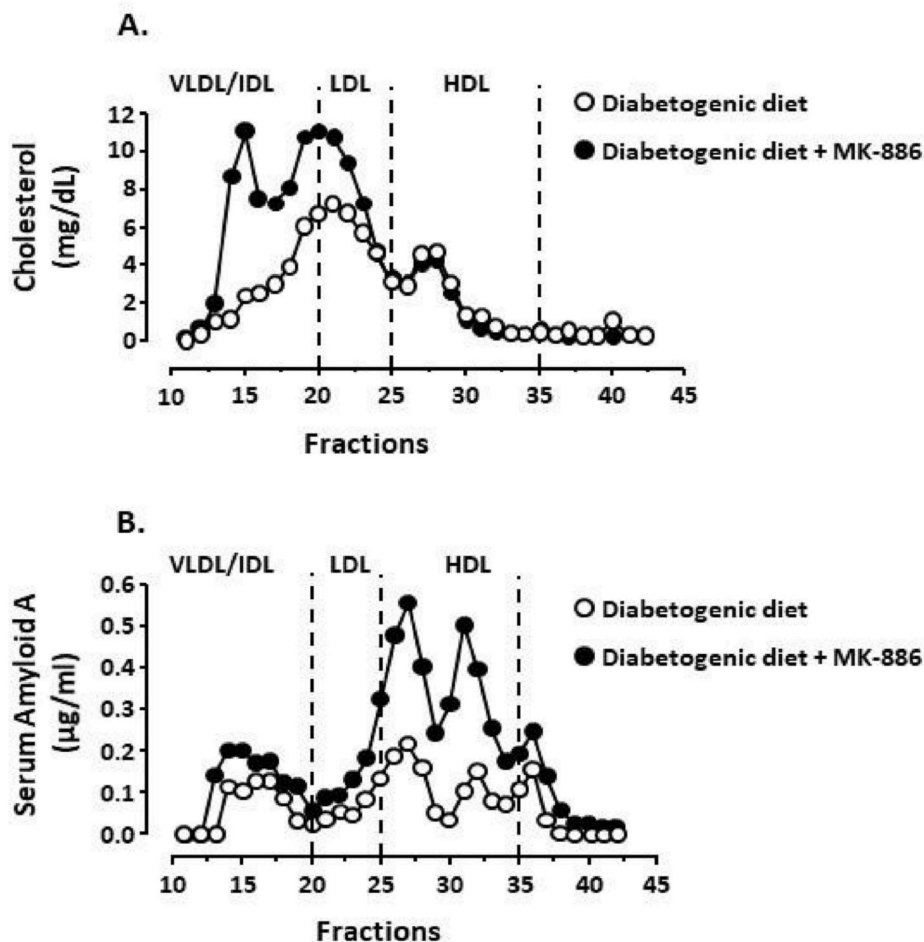


Fig. 3. MK-886 increases atherogenic and inflammatory lipoproteins in LDLR$^{-/-}$ mice fed a diabetogenic diet. 10-week-old LDLR$^{-/-}$ mice were placed on a HFHC with and without MK (40 mg/kg/day) for 6 weeks. Cholesterol **(A)** and SAA **(B)** distribution in lipoprotein fractions at the end of the study are shown (diabetogenic diet, open circles; HFHC + MK, closed circles).

4. Discussion

This study expanded upon previous reports evaluating the impact of dietary and pharmacological interventions on AT macrophage content and atherosclerosis in a dyslipidemic and diabetic murine model of atherosclerosis (Subramanian et al., 2008; Yoon et al., 2011). In this study, male LDLR$^{-/-}$ mice were fed a HFHC diet for 22 weeks, subsequently developing hyperglycemia, atherosclerosis, adipocyte hypertrophy and accumulation of macrophages in epididymal AT. This study recapitulated the obesogenic, hyperglycemic and hyperlipidemic effects of HFHC in male LDLR$^{-/-}$ mice, with similar elevations in blood glucose and cholesterol levels (Fig. 2B–Table 2). We also observed macrophage accumulation in epididymal AT and the presence of SAA in the VLDL/IDL, LDL and HDL fractions of plasma (Fig. 3B, open circles). To evaluate the involvement of 5LO in AT inflammation and atherosclerosis in our model, we used MK, an orally bioavailable FLAP inhibitor previously shown to be efficacious in reducing atherosclerotic lesions in apolipoprotein E-deficient mice lacking the dominant-negative transgene for CD4^{+} T-cells transforming growth factor β-type II receptor (ApoE$^{-/-}$ x CD4dnTβRII) and in ApoE$^{-/-}$-LDLR$^{-/-}$ double knockout mice (Bäck et al., 2007; Jawien et al., 2006).

The anomalous findings in our study became apparent as soon as we started analyzing the collected tissues, the most striking being the marked hepatomegaly observed in the MK-treated mice (>50% increase in liver weight), an effect that was concomitant with a distinct whitish appearance. Unfortunately, this observation was not recorded due to the lack of any on-site equipment. When analyzed, the TG and cholesterol content of the livers of MK-treated mice were elevated compared to non-treated mice (Supplemental Table 2). Similar paradoxical effects were observed when we analyzed other experimental end-points, as demonstrated in the Results section. These results left us perplexed, as nothing similar has been observed in our review of the published literature regarding the pharmacological inhibition of the 5LO pathway in animal models of inflammation and atherosclerosis.

To date, pharmacological disruption of the leukotriene pathway in animal models of atherosclerosis have resulted in somewhat ambiguous findings, ranging from a lack of any changes to attenuation of lesion size and morphology (Bäck, 2009; Riccioni et al., 2010). Studies utilizing MK to inhibit FLAP resulted in attenuated atherosclerotic lesion formation, without any concomitant changes in blood lipids and lipoproteins (Bäck et al., 2007; Jawien et al., 2006); results similar to those of a study which used a chemically distinct FLAP inhibitor, BAYx1005, to reduce lesion size in ApoE$^{-/-}$-LDLR$^{-/-}$ double knockout mice (Jawień et al., 2007). However, MK0591, a FLAP inhibitor in the same chemical family (Supplemental Fig. 1) as MK886, had no effect in abdominal aortic aneurysm formation in angiotensin II-infused ApoE$^{-/-}$ mice (Cao et al., 2007). In our study, administration of MK resulted in a paradoxical increase of pro-inflammatory plasma biomarkers, with elevations in blood and lipoprotein-associated SAA levels (Table 2 and Fig. 3B, respectively). The exacerbation of systemic inflammation was associated with increased lesion area in the aortic arch and the aortic sinus. Finally, MK had no effect on macrophage accumulation in epididymal AT (Fig. 5). A thorough review of the current and available literature reveals that the above-mentioned results are the only observations of pro-inflammatory

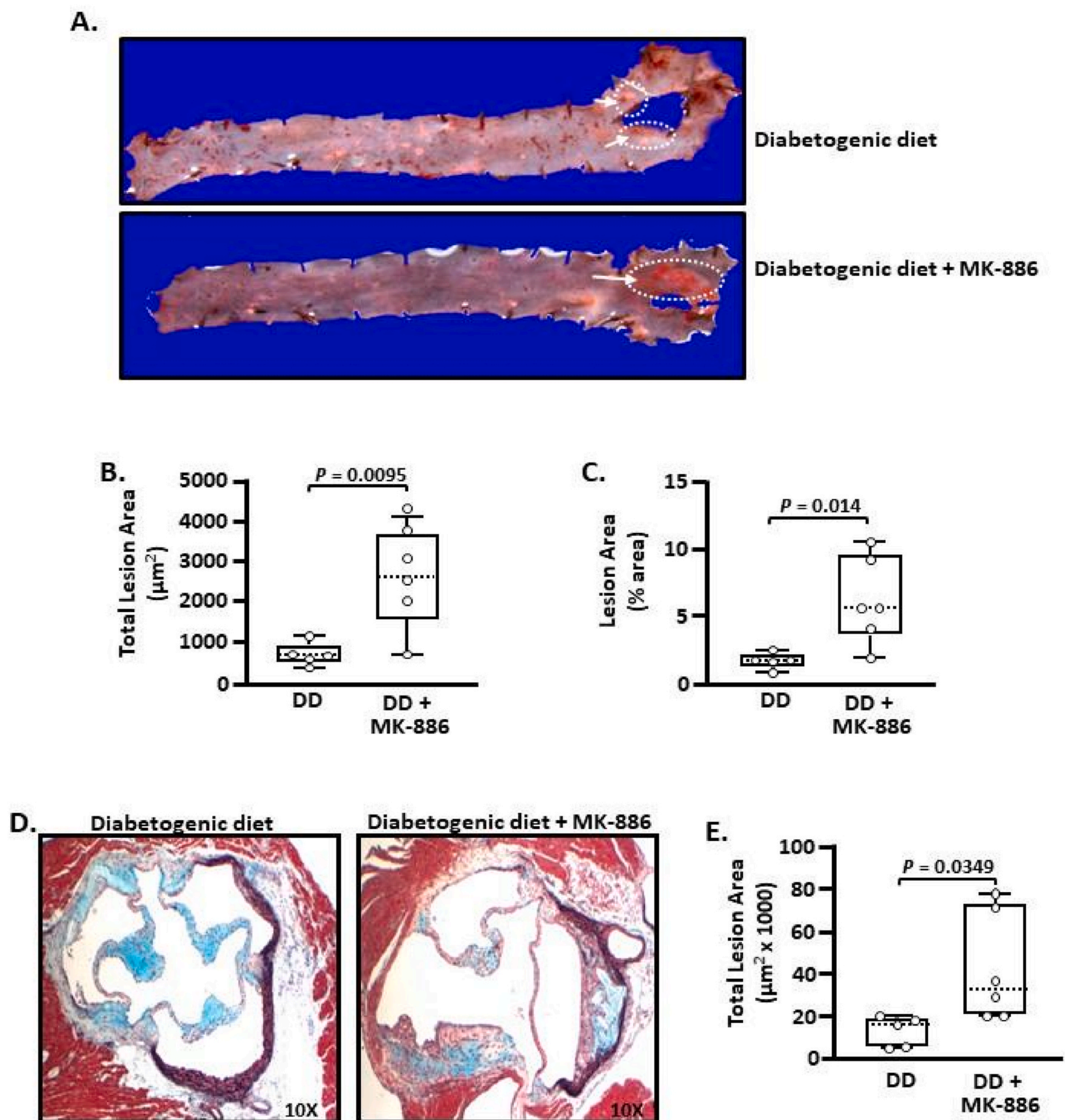


Fig. 4. MK-886 increased atherosclerotic plaque formation in the aortic arch and aortic sinus in LDLR$^{-/-}$ mice fed a diabetogenic diet. (A) Aortas were prepared for *en face* analysis and stained with Oil Red O. LDLR$^{-/-}$ mice were fed a HFHC and treated with MK developed larger atherosclerotic lesions compared with untreated LDLR$^{-/-}$. **(B, C)** Quantitative analysis of whole aortas revealed more extensive lesion area of *en-face*-prepared aortas of MK-treated LDLR$^{-/-}$ compared with LDLR$^{-/-}$ mice. **(D)** Representative photomicrographs and of aortic sinus lesion areas of untreated and MK-treated LDLR$^{-/-}$ mice fed a diabetogenic diet, stained using Movat's pentachrome stain. **(E)** Quantitative analysis of lesion areas in the aortic sinus. Data was analyzed using unpaired *t*-test and presented using box-and-whisker plots showing the range, quartiles and the median (dashed line) of the data. Full comparative statistics are displayed in Supplemental Table 1.

and hyperlipidemic effects of MK in animal models of atherosclerosis and AT inflammation. What are the possible variables that can explain these results?

4.1. Difference in mouse models of atherosclerosis

The majority of investigations into the dietary and genetic influences on atherogenesis are performed in the atherosclerosis-susceptible C57BL6 mouse with either an ApoE or the LDLR deficiency, with ApoE$^{-/-}$ mice being used more frequently. While there are similarities in the process of atherogenesis in both models, there are distinct differences (Getz and Reardon, 2016). The initial study observing the 5LO pathway as contributing to atherosclerosis occurred in LDLR$^{-/-}$ mice (Mehrabian et al., 2002). However, the majority of the studies using pharmacological interventions solely to disrupt the 5LO pathway have occurred in ApoE$^{-/-}$ mice (Bäck, 2008), with the western diet-fed LDLR$^{-/-}$ mouse being used to test the efficacy of the dual COX/5LO inhibitors HMB-TZD and BHB-TZD serving as quasi-exceptions (Choi et al., 2010, 2011). Analysis of gene expression of the components of the 5LO pathway in ApoE$^{-/-}$ mice demonstrated upregulation of FLAP in the vascular wall when compared to the C57BL6 mouse (Bäck, 2008), perhaps making this model more susceptible to FLAP inhibition. We have not found any such comparison of 5LO and its accessory proteins performed in LDLR$^{-/-}$ mice compared to wild-type mice, but arguably, a less robust difference in 5LO expression may make this model of atherosclerosis less susceptible to FLAP inhibition. Indeed, while a full comparison of the components of the 5LO pathway in atherosclerosis-susceptible ApoE$^{-/-}$ and LDLR$^{-/-}$ fed a HFHC would go a long way in elucidating this proposed reason, such endeavor is outside of the scope of this pilot proposal. Initiating another age- and

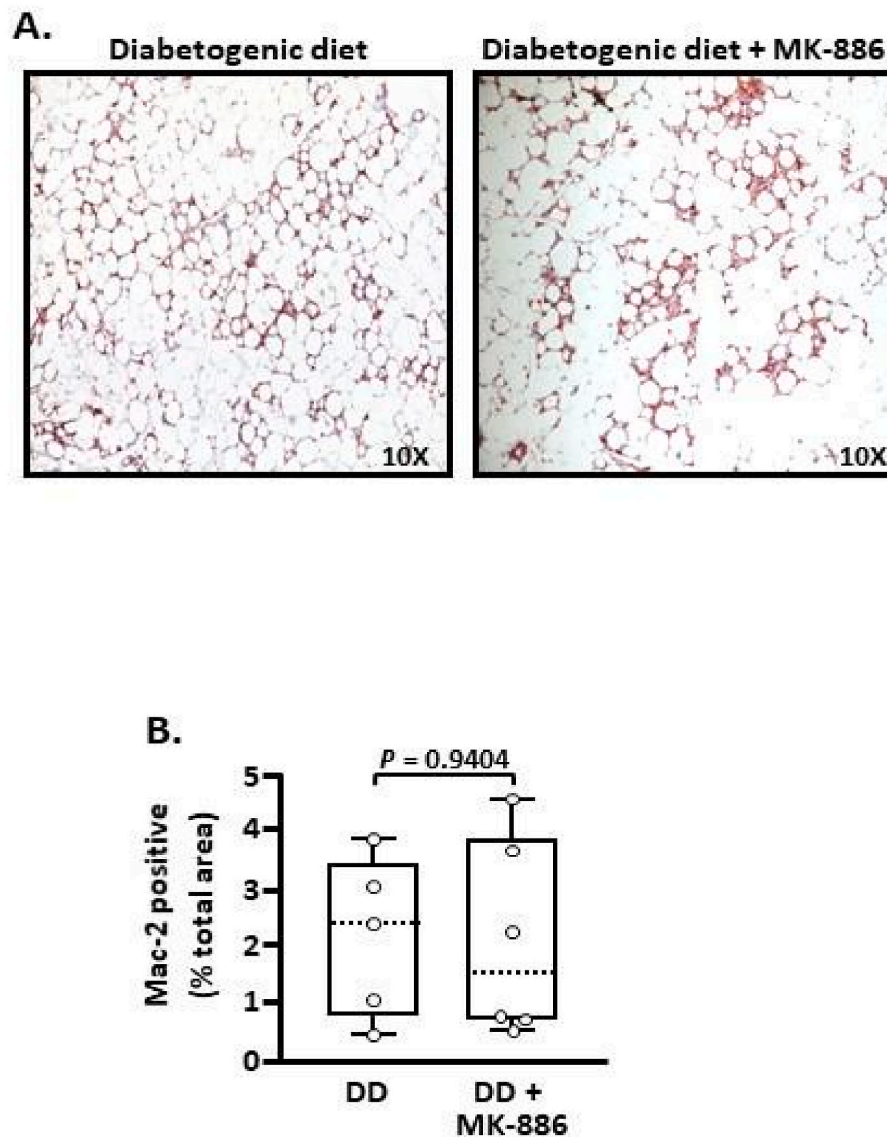


Fig. 5. MK-886 has no effect on macrophage accumulation in epididymal adipose tissue of LDLR$^{-/-}$ mice fed a diabetogenic diet. (A) Representative photomicrographs of epididymal AT from MK-treated and untreated LDLR$^{-/-}$ mice fed a diabetogenic diet. **(B)** MK treatment has no effect on macrophage accumulation in epididymal AT of LDLR$^{-/-}$ mice fed a diabetogenic diet. Data was analyzed using unpaired *t*-test and presented using box-and-whisker plots showing the range, quartiles and the median (dashed line) of the data. Full comparative statistics are displayed in Supplemental Table 1.

gender-matched study would add variables such as differences in the lot number of the diets and changes in the microbiome of mice bred and maintained in a different facility and hence, will be methodologically inappropriate (Motulsky, 2015)

4.2. Off-target effects of MK-886 on PPARα

Development of MK was initiated on its ability to inhibit activated polymorphonuclear leukocytes, with the intent of attenuating hypersensitivity reactions (Friedman et al., 1993; Gillard et al., 1989). However, off-target effects of MK occur in cells other than leukocytes, ranging from an induction of mitochondrial depolarization in prostate cancer cells (Gugliucci et al., 2002) to the phosphorylation of glutamate receptors in embryonic neuronal cells (Imbesi et al., 2007). Critical to our observations, MK is also demonstrated to be a noncompetitive inhibitor of the peroxisome proliferator-activated receptor (PPAR)-α, evaluated using a reporter assay system (Kehrer et al., 2001). Expression of PPARα is pronounced in tissues with high rates of lipid metabolism (such as the liver) and it regulates the expression of genes that promote fatty acid synthesis and oxidation (Kane et al., 2009).

A brief review of the literature revealed that while the majority of studies using MK to inhibit the activity of PPARα have occurred in *in vitro* and *ex vivo* settings, approximately 1/3 have occurred in *in vivo* models, indicating that the off-target effect on PPARα is not an artifact of cell culture.[2] We have previously demonstrated that selective activation of PPARα results in the induction of hepatic fatty acid oxidizing genes in a murine model of diabetes and obesity (Askari et al., 2014). Other investigators have also demonstrated that PPARα$^{-/-}$ mice develop hepatic inflammation when fed a high-fat diet and display signs of steatohepatitis with significant tissue infiltration of lymphocytes (Cha et al., 2007; Guerre-Millo et al., 2001; Stienstra et al., 2007). The accumulated observations in this study lead us to hypothesize that this off-target effect of MK, namely inhibition of hepatic PPARα and consequent inhibition of

[2] We searched MEDLINE/Pubmed databases for non-FLAP activities of MK886 in both *in vivo* and *in vitro* settings in. We focused on using “MK886” and “PPARα inhibition” as search terms, resulting in 113 citations.

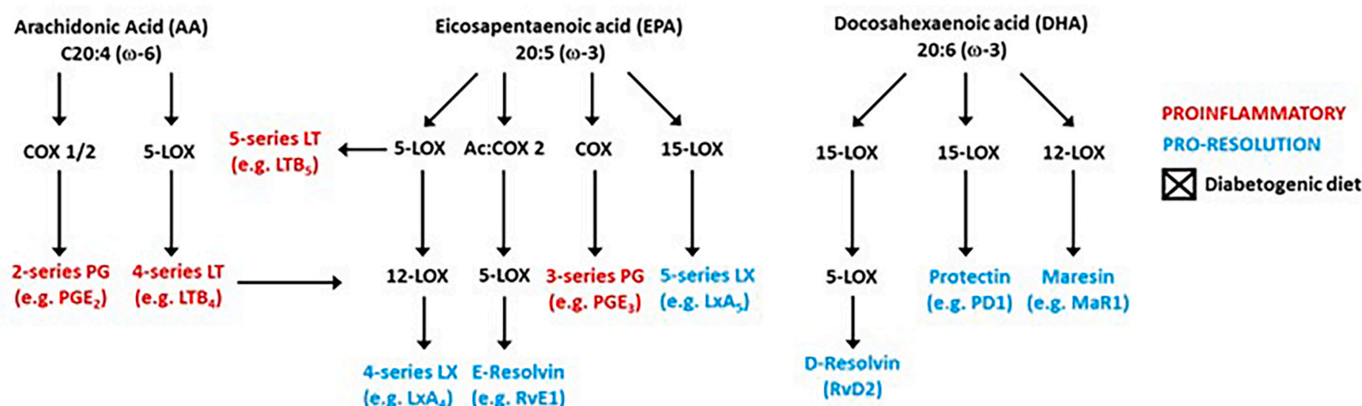
fatty acid oxidation, has occurred in our model and resulted in hepatic lipid accumulation (in the form of TG-rich lipoproteins) and increasing hepatic and systemic inflammation in LDLR$^{-/-}$ mice fed a HFHC.

4.3. Pro-inflammatory effects of MK-886

Until recently, it was widely thought that only sites for regulation of inflammation occurred in its initiation and progression. AA-derived products of the 5LO pathway (Fig. 1) are an important driving force in the initiation and progression of a variety of inflammatory conditions, including atherosclerosis (De Caterina and Zampoli, 2004; Funk, 2005; Hopkins, 2013; Lotzer et al., 2005; Piper and Garelnabi, 2020). However, AA is not the only polyunsaturated fatty acid (PUFA) whose metabolites are engaged in the inflammatory process.

The termination of inflammation was long assumed to be a passive process, requiring only the cessation of production of soluble mediators and their eventual removal by processes such as dilution and diffusion. However, in recent years it has been discovered that the process of resolution and termination is an active one, requiring, in part, the synthesis of mediators derived from other species of dietary PUFAs, collectively called “Specialized Pro-resolving Mediators” (SPMs) (Nathan and Ding, 2010). These SPMs are further categorized into the lipoxins, resolvins, protectins and maresins class of mediators and are derived from AA, eicosapentaenoic acid (20:5; EPA) and docosahexaenoic acid (22:6; DHA). The synthesis of SPMs is dependent on the sequential catalysis of the PUFAs by COX2, 5LO, 12/15LO and on the dietary availability of the PUFAs (Fig. 6A) and are best explained in other publications (Dalli and Serhan, 2017; Nathan and Ding, 2010; Serhan and Chiang, 2004). Resolvin and lipoxin biosynthesis has been shown to be MK-sensitive in cell culture (Lehmann et al., 2015) while resolvins and protectins regulate inflammation and macrophage function in AT in obese people (Maciejewska-Markiewicz et al., 2021) and high fat-fed mice (Claria et al., 2012; Titos et al., 2011). For example, lipoxin A_4, a product of the sequential metabolism of AA by COX and 5LO, is protective against AT inflammation, IR and obesity in a high fat diet-fed mouse (Elias et al., 2016). Could the extensive (16-week) exposure to the HFHC and MK treatment resulted in the loss of ability to resolve inflammation and help explain our paradoxical results (Fig. 6B)? While we do not have any direct evidence demonstrating the loss of SPMs as the cause of the increased inflammation and pro-atherosclerotic effect of MK in our study, there is an indication that this may have been the case. In a concurrently-run experiment with the same diet and duration of MK treatment (6-weeks) but shorter duration of HFHC (8-weeks vs 16-weeks), MK had no observable effect on lesion formation

A.



B.

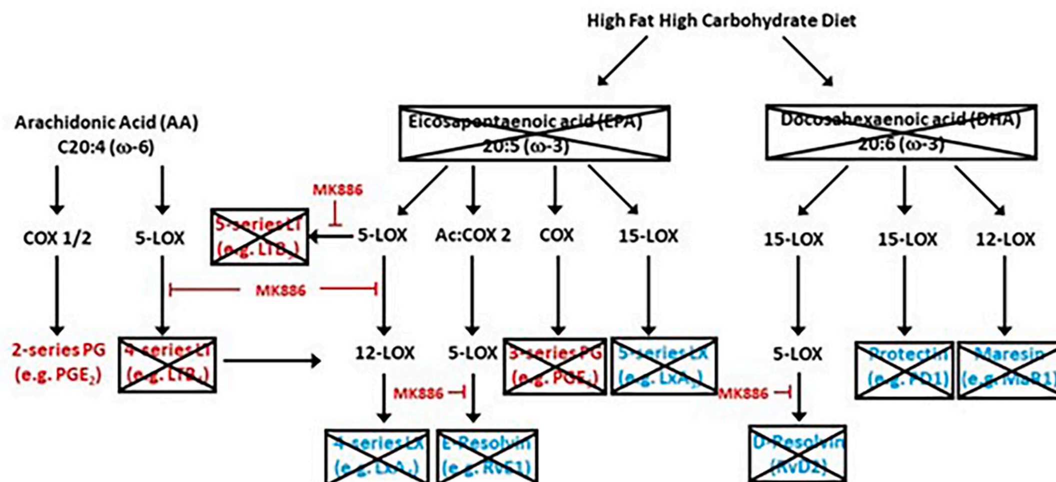


Fig. 6. **Hypothetical effects of FLAP inhibition by MK-886 and by HFHC on the formation of inflammatory and pro-resolving products of the enzymatic catalysis of arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid.**

(*unpublished observation*), leading us to conclude that the extensive exposure to the HFHC may lead to a decrease to the PUFA substrates required for the production of SPMs. Unfortunately, we don't have any direct evidence of the role of SPMs in the exacerbation of atherosclerosis, systemic inflammation and the lack of any anti-inflammatory effects in AT in our mice and further analysis of target tissues for SPM is currently beyond the scope and capability of the authors. It's important to note that dietary fatty acids are integral to immune homeostasis in other pathologies (Del Corno et al., 2021). Concerning models of hyperlipidemia and atherosclerosis, a western diet was pro-inflammatory and led to changes in phospholipid species of the plasma membranes of T-lymphocytes, leading to their enhanced activation (Pollock et al., 2016). Our HFHC has a larger percentage of saturated fats and a smaller share of PUFAs when compared to a western diet (Subramanian et al., 2008; Surwit et al., 1988); therefore, it is possible that the extended duration of our experiment could cause wholesale changes in the acyl groups of membrane phospholipids, especially in the cells of the immune cells, leading to an attenuation of SPM synthesis. In addition, other investigators have found that an imbalance between SPMs and LTs in the atherosclerotic plaques of the aortic sinus in LDLR$^{-/-}$ mice fed a western diet (Fredman et al., 2016). A 17-week duration of this experiment resulted in a dramatic (>80-fold) decrease in 5LO-derived SPMs, similar to effects seen in ApoE$^{-/-}$ mice fed a high-fat diet for a similar duration (16 weeks), with decreases in resolvin D2 and maresin 1 (Viola et al., 2016). A comparison of the duration of these experiments (17- and 16-weeks) to our study (22 weeks) lends credence to this explanation.

While the limited sample size of this study could be construed as a limitation, the only measurements that an increase in sample size could influence are that of plasma SAA, plasma cholesterol and hepatic TGs. Since the changes observed are exactly the opposite of what was expected and without any precedent in published literature, any additions to sample size in order to tailor the means and standard deviations is not appropriate (Michel et al., 2020; Motulsky, 2015). This study was designed as a preliminary one and the statistical analysis and sample size were pre-determined before any interventions.

4.4. Conclusion

We speculate that the combination of events stated above resulted in a "multiple-hit" model of atherosclerosis exacerbation (Fig. 6). Specifically, we propose that the genetic background (LDLR$^{-/-}$ model, hit1) may limit the efficacy of FLAP inhibitors, the long duration (16-weeks, hit 2) of a diet high in saturated fats and carbohydrates could result in a loss of ability to resolve inflammation and an off-target effect of MK (inhibition of hepatic PPARα activity and subsequent increase in hepatic adiposity, hit 3), resulted in a scenario that led to the excess hepatic and systemic inflammation, increases in pro-atherogenic lipoproteins and exacerbation of atherosclerosis in the obese and diabetic LDLR$^{-/-}$ mouse.

CRediT authorship contribution statement

Katherine Keever: Data curation, Validation, Writing – review & editing. **Bardia Askari:** Conceptualization, Methodology, Software, Investigation, Writing – original draft.

Conflict of interest

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors did **NOT** use any AI or AI-associated Technologies in the writing process. The authors take full responsibility for the content the publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphar.2024.100203.

Data availability

Data will be made available on request.

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