Deuterium-reinforced polyunsaturated fatty acids protect against atherosclerosis by lowering lipid peroxidation and hypercholesterolemia

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Background and aims: Oxidative modification of lipoproteins is a crucial step in atherosclerosis development. Isotopic-reinforced polyunsaturated fatty acids (D-PUFAs) are more resistant to reactive oxygen species-initiated chain reaction of lipid peroxidation than regular hydrogenated (H-)PUFAs. We aimed at investigating the effect of D-PUFA treatment on lipid peroxidation, hypercholesterolemia and atherosclerosis development.

Methods: Transgenic APOE*3-Leiden.CETP mice, a well-established model for human-like lipoprotein metabolism, were pre-treated with D-PUFAs or control H-PUFAs-containing diet (1.2%, w/w) for 4 weeks. Thereafter, mice were fed a Western-type diet (containing 0.15% cholesterol, w/w) for another 12 weeks, while continuing the D-/H-PUFA treatment.

Results: D-PUFA treatment markedly decreased hepatic and plasma F2-isoprostanes (approx. 80%) and prostaglandin F2α (approx. 40%) as compared to H-PUFA treatment. Moreover, D-PUFAs reduced body weight gain during the study (54%) by decreasing body fat mass gain (87%) without altering lean mass. D-PUFAs consistently reduced plasma total cholesterol levels (approx. 25%), as reflected in reduced plasma non-HDL-cholesterol (28%). Additional analyses of hepatic cholesterol metabolism indicated that D-PUFAs reduced the hepatic cholesterol content (21%). Sterol markers of intestinal cholesterol absorption and cholesterol breakdown were decreased. Markers of cholesterol synthesis were increased. Finally, D-PUFAs reduced atherosclerotic lesion area formation throughout the aortic root of the heart (26%).

Conclusions: D-PUFAs reduce body weight gain, improve cholesterol handling and reduce atherosclerosis development by reducing lipid peroxidation and plasma cholesterol levels. D-PUFAs, therefore, represent a promising new strategy to broadly reduce rates of lipid peroxidation, and combat hypercholesterolemia and cardiovascular diseases.

1. Introduction

Atherosclerotic vascular disease, comprising heart attacks, stroke, aortic aneurysms, and peripheral vascular disease, is the...
most frequent cause of death in the Western world [1,2]. The impact of the atherosclerosis pandemic is predicted to increase worldwide over the next few decades, despite recent progress in lipid-lowering therapy [2,3].

Increased retention of low-density lipoprotein (LDL) in the vessel wall and subsequent oxidative modification is a crucial step in the pathogenesis of atherosclerosis [4]. Polyunsaturated fatty acids (PUFAs) can get oxidized through either enzymatic or non-enzymatic pathways. Non-enzymatic damage can be initiated by both 2-electron- and 1-electron-oxidants, through an addition to a double bond, or, more typically (mostly for 1-electron-oxidants) by H-atom abstraction off a bis-allylic methylene group. Once the radical is formed, the ensuing chain reaction of lipid peroxidation (LPO) multiplies the destruction. A smorgasbord of the downstream products of LPO include toxic carbonyls, which further exacerbate the atherosclerosis-related damage, through the modification of lipids [5] and apoB within LDL [6] (Fig. 1). Aldehyde-modified LDL-apoB is scavenged by macrophages in an uncontrolled manner, leading to foam cell formation and initiation of the atherosclerotic lesion [6]. Reactive carbonyls as well as oxysterols, cholesterol oxidation products, contribute to atherosclerosis mainly through their ability to induce inflammation, oxidative stress and apoptosis [7,8]. Isoprostanes are another LPO-derivative from PUFA oxidation (Fig. 1), although some (e.g. 8-iso-prostaglandin-F2) can also be produced enzymatically. These bioactive molecules can promote atherosclerosis development among others via inducing inflammation and enhancing endothelial/immune cell interaction [9].

Yet, oral antioxidants have not provided the obvious solution to this LPO problem. The most likely reason for this is that it has proven impractical to supplement an organism with sufficient antioxidants to block ongoing LPO chain reactions [10,11], specifically when endogenous protective antioxidant mechanisms are disrupted due to other underlying clinical conditions. In addition, some antioxidants may induce adverse effects as for instance, in the presence of an excess of LDL, vitamin E (z-tocopherol) may act as a pro-oxidant [12,13].

PUFAs are essential nutrients as they are not synthesized in mammalian tissues and have to be supplied through the diet. Following ingestion, PUFAs are quickly incorporated into lipid structures throughout the body. Deuterium is a stable hydrogen isotope that has natural abundance (150 ppm in ocean water) and is accordingly recognized by living systems as a normal, natural subtype of hydrogen. Deuterium incorporated into PUFAs at bis-allylic positions (D-PUFAs) gives rise to a well-known “kinetic isotope effect” [14], as a result of which, reactions involving cleavage of a C-H bond are slowed down in the C-D bond. As the abstraction step by reactive oxygen species (ROS) is repeated throughout the chain of LPO events, the protective effect of D-PUFAs is multiplied, thus resulting in a larger total beneficial effect when compared to exposure with normal (H-)PUFAs. As a result, we have been able to show that D-PUFAs, that are specifically deuterated at the bis-allylic positions, are resistant to LPO [15–18], and can mitigate several pathologies, including important aspects of cellular damage in Friedreich’s ataxia [19] and Parkinson’s disease [20,21].

As oxidative stress is a crucial step in atherosclerosis development and there is likely interplay between oxidative stress and lipid metabolism, we aimed to investigate the effect of D-PUFA treatment on LPO, hypercholesterolemia and atherosclerosis development in APOE*3-Leiden.CETP mice. This hyperlipidemic model is a well-established model for human-like lipoprotein metabolism. Unlike hyperlipidemic apoE- and LDLR-deficient mice, they have an intact, albeit attenuated, apoE-LDL clearance pathway for cholesterol-enriched lipoprotein remnants [22,23]. As
a result APOE*3-Leiden.CETP mice respond well to lipid-lowering and anti-atherogenic effects of e.g. statins [24], PCSK9 inhibition [25] and niacin [26]. Our results show that treatment with D-PUFAs markedly reduces the production of LPO products and plasma cholesterol levels thereby lowering the development of atherosclerosis.

2. Materials and methods

2.1. Mice, diets and general animal procedures

APOE*3-Leiden mice were crossbred with mice expressing human cholesteryl ester transfer protein (CETP) under control of its natural flanking regions to generate heterozygous APOE*3-Leiden.CETP mice [22]. Female APOE*3-Leiden.CETP mice of 10–12 weeks of age were housed under standard conditions (3–4 mice per cage) with a 12-h light/dark cycle and free access to food and water. At t = −4 weeks, mice were randomized based on 4-h fasted plasma lipids, age, body weight and body composition and divided into 2 groups.

Subsequently, mice were fed an AIN-93M-based ‘preloading’ diet (Research Diets, USA) containing either control PUFAs (H-PUFA group) or the iso-otope-reinforced PUFAs (D-PUFA group) for incorporation of the PUFAs in the body. No cholesterol was added yet to these preloading diets. The composition of all diets is presented in Supplemental Table 1. Specifically, these diets comprised 12% fat, which contained per fat fraction 65% saturated fatty acids (coconut oil 101, hydrogenated), 25% monounsaturated fatty acids (ethyl oleate), and 10% PUFAs (ethyl linoleate and ethyl linolenate in a 1:1 weight ratio; control H-PUFA group). In the D-PUFA group the H-PUFAs were replaced by iso-otope-reinforced D-PUFAs (ethyl 11,11-D2-linoleate and ethyl 11,11,14,14-D4-linolenate in a 1:1 weight ratio). After 4 weeks of feeding on this preloading diet, at t = 0, the diets of the mice were switched to the same diets as above, but now containing 0.15% cholesterol (‘Western-type diet’, WTD) for 12 weeks to induce development of atherosclerotic lesions. Food intake was monitored per cage twice per week. Body weight as well as total body fat and lean mass by EchoMRI-100 (EchoMRI, Houston, Texas), were determined every 4 weeks during the study. Animal experiments were approved by the Ethics Committee on Animal Care and Experimentation of the Leiden University Medical Center.

The full Materials and methods section is available in the Supplemental Materials and Methods.

3. Results

3.1. D-PUFAs efficiently incorporate into tissues

Female APOE*3-Leiden.CETP mice were pre-treated with preloading diets containing either iso-otope-reinforced D-PUFAs or control H-PUFAs (composition of the diets is presented in Supplemental Table 1). After 4 weeks at t = 0, cholesterol was added to these diets to induce hyperlipidemia and atherosclerosis development. At the end of the study, 12 weeks after Western-type diet (WTD)-feeding efficient D-PUFA incorporation was confirmed by measuring deuterium content on sections of the tails of mice. The difference between the D-PUFA (6982 ± 217‰) and the H-PUFA (−57 ± 5‰; p < 0.001) group corresponds to approx. 30% D-PUFA incorporation (i.e. D-PUFA fraction of total PUFA) based on previous studies [20,27]. This level of D-PUFA substitution is biologically relevant as approx. 10–20% is sufficient to terminate LPO [16].

3.2. D-PUFAs reduce lipid peroxidation products

As oxidative stress is a main contributor to the development of atherosclerosis and D-PUFAs have been previously shown to reduce LPO products in other models [15–20], we investigated whether D-PUFA treatment also reduces LPO in hyperlipidemic APOE*3-Leiden.CETP mice. Indeed, D-PUFA treatment markedly decreased F2-isoprostanes (F2-Isop; −72%) and prostaglandin F2α (PGF2α; −44%) levels in the liver (Fig. 2A). More importantly with respect to atherosclerosis development, D-PUFAs also markedly reduced plasma levels of F2-isoprostanes (−87%) and PGF2α (−40%) (Fig. 2B). D-PUFA treatment did not affect the liver toxicity markers ALAT and ASAT in plasma (not shown). These findings indicate that D-PUFAs potently reduce tissue and circulating LPO products in hyperlipidemic APOE*3-Leiden.CETP mice, without adverse effects on the liver.

3.3. D-PUFAs reduce body fat mass, while enhancing food intake

To study the effect of D-PUFAs on the metabolic phenotype of mice, body weight and body composition were monitored during the study. D-PUFAs reduced body weight (up to −9%; Fig. 3A) and body weight gain (up to −54%; Fig. 3B) during the study. D-PUFAs did not consistently affect lean mass gain (Fig. 3C), but effectively prevented body fat mass gain (up to −87%; Fig. 3D). Accordingly, the weight of gonadal white adipose tissue (gWAT) was reduced in the D-PUFA group as compared to the control H-PUFA group (−60%; Fig. 3E). These beneficial effects on body fat mass were not the result of decreased food intake as daily food intake even gradually increased in the D-PUFA group after 5.5 weeks of cholesterol-feeding (up to +33% after 12 weeks; Fig. 3F).

3.4. D-PUFAs lower plasma cholesterol levels and alter hepatic cholesterol homeostasis

Subsequently, we assessed the effect of D-PUFA treatment on lipid metabolism, in particular cholesterol metabolism, the main contributor to atherosclerosis development. D-PUFAs did not modulate fasting plasma triglyceride (TG) levels up to t = 4 weeks, but did increase plasma TG levels (up to +36%; Fig. 4A) in the last phase of the study when food intake was also increased. Under non-cholesterol-feeding conditions, D-PUFAs minimally increased fasting plasma total cholesterol (TC) levels (+14%; Fig. 4B). However, under cholesterol-feeding hypercholesterolemic conditions, D-PUFAs consistently reduced plasma TC levels (ranging from −20% to −37%). Combined, D-PUFA treatment resulted in clearly reduced plasma total cholesterol exposure during the complete study (−23%; Fig. 4C). The reduced plasma TC level (−4.6 mM at the endpoint) was confined to both reduced plasma non-HDL-C (−4.0 mM; −28%; calculated as TC minus HDL-C) and reduced plasma HDL-C (−0.7 mM; −48%) levels (Fig. 4D).

As D-PUFAs reduced plasma cholesterol levels we studied the effect of D-PUFA treatment on cholesterol metabolism in more detail by quantifying hepatic TC content as well as hepatic sterol synthesis markers (i.e. desmosterol, campesterol, sitosterol), cholesterol synthesis (i.e. desmosterol, lanosterol, dihydro-lanosterol) and cholesterol breakdown (i.e. 24OH-cholesterol, 7αOH-cholesterol, 27OH-cholesterol). D-PUFAs reduced the hepatic TC content (−21%; Fig. 4E). Interestingly, this was accompanied with reduced intestinal cholesterol absorption markers (i.e. cholesterol, campesterol) in the liver (Fig. 4F). Moreover, D-PUFAs enhanced hepatic markers of cholesterol synthesis (i.e. desmosterol, lanosterol, dihydro-lanosterol; Fig. 4G),
while also reducing most markers of cholesterol breakdown (i.e. 24OH-cholesterol and 7αOH-cholesterol were reduced, 27OH-cholesterol was increased; Fig. 4H). These data together indicate that D-PUFAs influence cholesterol metabolism, eventually resulting in a reduced hepatic cholesterol content and reduced plasma non-HDL-C levels.

3.5. D-PUFAs reduce atherosclerotic lesion development

Finally, we investigated whether the D-PUFA-mediated lowering of LPO and hypercholesterolemia resulted in reduced atherosclerosis development. Indeed, treatment with D-PUFAs reduced atherosclerotic lesion area throughout the aortic root of the heart (ranging from −23% to −30%; Fig. 5A–B), resulting in 26% lower mean atherosclerotic lesion area in the D-PUFA-treated mice as compared to H-PUFA-treated mice (79 ± 9 vs. 107 ± 12 × 103 μm²/cross section, respectively; Fig. 5C). Taken together, these findings demonstrate that D-PUFAs lower LPO as well as hypercholesterolemia, ultimately resulting in reduced atherosclerosis development in APOE*3-Leiden.CETP mice.
Fig. 4. D-PUFAs lower plasma cholesterol levels and alter hepatic cholesterol homeostasis.

 Plasma triglyceride (TG; A) and total cholesterol (TC; B) were determined at the indicated time points in APOE*3-Leiden.CETP mice fed a D-/H-PUFA-containing WTD (until t = 0 without cholesterol, thereafter containing 0.15% cholesterol, w/w). (C) The plasma total cholesterol exposure during the study was calculated. (D) At the end of the study, plasma non-HDL-cholesterol (non-HDL-C) and HDL-C were determined. In addition, hepatic cholesterol content (E) and hepatic markers of intestinal cholesterol absorption (F), cholesterol synthesis (G) and cholesterol breakdown (H) were measured. Values represent means ± SEM. (n = 18–20 per group). *p < 0.05, **p < 0.01, ***p < 0.001.
4. Discussion

Isotope-reinforced D-PUFAs are resistant to non-enzymatic LPO and effectively reduce oxidative stress in various experimental models [15–20], but their effect on lipid metabolism and atherosclerosis development had not been investigated before. In the present study, we show that dosing with ethyl esters of 11,11-D2-linoleic acid and 11,11,14,14-D4-linolenic acid markedly reduced LPO products under hypercholesterolemic conditions in APOE3- Leiden.CETP mice. D-PUFAs also reduced body weight gain by reducing body fat mass, under conditions of increased food intake. Moreover, D-PUFAs reduced plasma TC, concomitantly with reduced body fat mass gain. This was not the result of reduced food intake, in fact, food intake gradually increased over the course of the last 7 weeks. As a result, the D-PUFA cohort were exposed to more atherogenic dietetic cholesterol, making the reduced body fat mass is in line with previous studies showing that reducing oxidative stress reduces obesity [29,30], but studies on potential underlying mechanism(s) are scarce. Therefore, the main mechanism(s) underlying the D-PUFA-mediated reduced body mass gain remains to be identified, but may include reduced adipocyte differentiation as was observed in COX-2-deficient mice [31]. Overall, D-PUFAs thus beneficially influence the body composition despite increased food intake.

During cholesterol-feeding, D-PUFAs consistently reduced fasted plasma TC levels. This reduction was mainly attributed to reduced non-HDL-C levels and was also accompanied by a reduced hepatic cholesterol content at the end of the study. In-depth analyses of hepatic cholesterol metabolism showed that D-PUFAs reduce markers of cholesterol absorption, indicative of reduced intestinal cholesterol absorption. This reduced intestinal cholesterol absorption is likely key to the D-PUFA-mediated beneficial effects on cholesterol metabolism. First, the cholesterol-lowering effects of D-PUFAs are dependent on dietary cholesterol as plasma TC levels are decreased only during cholesterol-feeding and not under non-cholesterol-feeding conditions. Second, it is well-known that inhibition of cholesterol absorption via genetic (i.e. Niemann-Pick C1 like 1-deficiency) or pharmacological (i.e. ezetimibe) modulation reduces the hepatic cholesterol content and plasma non-HDL-C levels in hyperlipidemic mice [32,33]. Our results indicate that this is a cholesterol-specific effect and not the result of a general reduction in chylomicon production and secretion, as plasma TG levels are unaffected or even increased. Reports on modulation of oxidative stress or inflammation in relation to intestinal cholesterol absorption are scarce. Interestingly, Stöger et al. [34] recently reported an anti-inflammatory mouse model with reduced intestinal cholesterol absorption and plasma TC levels, but the underlying mechanism(s) remain obscure. As treatment with D-PUFAs affect many mediators and possibly thus many processes, the exact mechanism(s) underlying the reduced intestinal cholesterol absorption upon D-PUFA treatment may be complex. Potential mechanism(s) may include modulation of the gut microbiome and/or bile acid metabolism in the gut [35,36], and modulation of (local) immune cells [37,38]. For example, dendritic cells, which are present in the inner lining of the intestines, may play a key role. Antioxidative strategies protect dendritic cells against degeneration [39], and increasing the lifespan and immunogenicity of dendritic cells reduces plasma cholesterol levels (via an unexplored mechanism) [37]. Together, one could speculate that D-PUFAs improve the lifespan and
immunogenicity of the dendritic cells in the intestines, thereby reducing dietary cholesterol absorption and reducing plasma cholesterol levels. However, more detailed studies are required.

We also observed that D-PUFAs increase hepatic cholesterol synthesis and reduce hepatic cholesterol breakdown. While D-PUFAs reduced the cholesterol breakdown markers 24OH-cholesterol and 7αOH-cholesterol, 27OH-cholesterol levels were increased. The latter is possibly the result of CYP7A1 down-regulation, and may thus also be indicative of reduced cholesterol breakdown. These effects on hepatic cholesterol synthesis and cholesterol breakdown are likely secondary to the reduced intestinal cholesterol absorption in an attempt to maintain homeostasis. Hepatic cholesterol synthesis is well-known to increase upon reduced intestinal cholesterol absorption [32,33]. Presumably, D-PUFA treatment also decreases plasma non-HDL-C mainly as a consequence of the reduced intestinal cholesterol absorption and subsequent reduced hepatic cholesterol content. It is known that reducing the hepatic cholesterol content, for example by ezetimibe or statins, enhances the hepatic LDL receptor expression, consequently lowering plasma non-HDL-C levels [33]. However, also potential local antioxidative effects of D-PUFAs may have influenced these hepatic pathways of cholesterol synthesis and cholesterol breakdown directly. As outlined above for the intestinal cholesterol absorption, D-PUFAs affect many mediators and possibly thus many processes, and the exact underlying mechanism(s) may be complex. Taken together, our findings indicate that D-PUFAs lower the hepatic cholesterol content by reducing the intestinal cholesterol absorption and possibly by additional (local) antioxidative effects, thereby ultimately reducing plasma non-HDL-C levels.

Finally, D-PUFA treatment reduced atherosclerotic lesion formation, which is in line with the reduced systemic oxidative and inflammatory status and reduced plasma non-HDL-C levels. Previous experimental studies also show that antioxidative strategies such as with vitamin E [40,41] and probucol [42] in most cases reduce atherosclerosis development in rodent models. However, numerous large clinical trials with antioxidants (typically vitamin E or beta carotene) in atherosclerosis have been consistently negative [43]. This does not necessarily challenge the importance of oxidative modification of LDL in the pathogenesis of atherosclerosis as well as the clinical potential of D-PUFA treatment. Rather, the choice or dosage of antioxidants could have been suboptimal, particular in view of a still unresolved issue of the relative importance of enzymatic versus non-enzymatic, as well as two-electron versus one-electron processes in LDL oxidation [13,44]. Indeed tocopherol, an accepted lipophilic chain-terminating antioxidant, can actually serve as a pro-oxidant in an LDL-rich environment, thus exacerbating the generation of LPO [12,13]. We propose that specific inhibition of non-enzymatic LPO, e.g. by D-PUFAs, may prove to be beneficial when targeting atherosclerosis and this process is likely superior to general antioxidiant-based inhibition or employing nonsteroidal anti-inflammatory drugs (NSAIDs). It is important to realize that D-PUFAs are not antioxidants: they are not quenching the ROS and do not disturb the redox status or antioxidant-ROS ratio, and so are unlikely to disturb normal ROS-mediated signalling pathways. Our results thus show that D-PUFAs reduce experimental atherosclerosis development by reducing both LPO and hypercholesterolemia. D-PUFAs may hence represent a promising alternative approach to reduce oxidative stress, including reducing the risk factors for, hypercholesterolemia and atherosclerosis in humans.

The limitations of the current study include a need to better understand how D-PUFAs reduce intestinal cholesterol absorption. In addition, the effects of D-PUFAs on body fat mass gain, while increasing food intake, is not well understood. Further studies are warranted to resolve the potential use of D-PUFAs for obesity-related therapeutic indications. A strength of our study is that we made use of isotopic-reinforced PUFAs instead of conventional antioxidant therapies which so far have failed therapeutically. Furthermore, we made use of hyperlipidemic APOE*3-Leiden.CETP mice which have a lipoprotein metabolism that closely resembles that of humans.

In conclusion, our data demonstrate that D-PUFAs limit body weight gain, improve cholesterol handling and reduce the development of atherosclerotic lesions by reducing systemic, and likely local, LPO and by reducing plasma non-HDL-C levels. The reduced plasma non-HDL-C levels probably mainly result from reduced intestinal cholesterol absorption, though additional mechanisms cannot be excluded. Future studies should elucidate whether D-PUFA treatment also reduces LPO, hypercholesterolemia and cardiovascular disease risk in humans.

Conflict of interest

Financial support and D-PUFA reagents were provided by Retrotupe, Inc. Lex H.T. van der Ploeg and Mikhail S. Shchepinov receive compensation from and hold stock in Retrotupe, Inc. The other authors have no conflicts to declare.

Author contributions

J.F.P.B., P.C.N.R and M.S.S. designed the experiments with the help from I.M.M, and L.H.T.v.d.P. Experiments were performed and data analysed by J.F.P.B., I.M.M., G.L.M., E.P., G.H. and D.L. Data were interpreted by J.F.P.B., D.L. and P.C.N.R. M.S.S., C.M. and L.H.T.v.d.P. provided intellectual contributions throughout the project. J.F.P.B. and M.S.S. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2017.06.916.

References


