The Antiatherogenic Effect of Fish Oil in Male Mice Is Associated with a Diminished Release of Endothelial ADAM17 and ADAM10 Substrates

Nancy Speck, Corinna Brandsch, Nadine Schmidt, Narges Yazdekhasti, Frank Hirche, Ralph Lucius, Gerald Rimbach, Gabriele I Stangl, and Karina Reiss

Abstract

Background: Growing evidence suggests that disintegrin and metalloprotease (ADAM) 17 (ADAM17) and ADAM10 contribute to the pathogenesis of vascular diseases. ADAM17 promotes inflammatory processes by liberating tumor necrosis factor α, and tumor necrosis factor receptor 1 (TNFR1). ADAM17 and ADAM10 modulate vascular permeability by cleaving endothelial adhesion molecules such as junctional adhesion molecule A (JAM-A) and vascular endothelial cadherin (VE-cadherin), respectively.

Objective: This study was designed to investigate whether a link might exist between the protective effects of fish oil (FO) supplementation against atherosclerosis and ADAM function.

Methods: Male LDL receptor knockout (LDLR−/−) mice and male wild-type (WT) mice were fed a Western diet (200 g/kg fat, 1.5 g/kg cholesterol) containing either 20% lard (LDLR−/−-lard and WT-lard groups) or 10% lard combined with 10% FO (LDLR−/−-FO and WT-FO groups) for 12 wk. Atherosclerotic lesion development and fatty acid composition of liver microsomes were evaluated. ADAM10 and ADAM17 expression was determined by quantitative real-time polymerase chain reaction and immunoblot analyses. Concentrations of soluble ADAM substrates in plasma and liver extracts were measured by ELISA.

Results: Dietary supplementation with FO markedly reduced development of early atherosclerotic lesions in LDLR−/− mice (LDLR−/−-lard group vs. LDLR−/−-FO group, mean ± SD: 29.6 ± 6.1% vs. 22.5 ± 4.2%, P < 0.05). This was not accompanied by changes in expression of ADAM17 or ADAM10 in the aorta or liver. No dietary effects on circulating TNFR1 (LDLR−/−-FO group mean vs. LDLR−/−-FO group mean: 1.22 ± 0.23 vs. 1.39 ± 0.28, P > 0.2) or IL-6R (1.06 ± 0.12 vs. 0.98 ± 0.09 fold of WT-lard group, P > 0.1), classical substrates of ADAM17 on macrophages, and neutrophil granulocytes were observed. However, a reduction in atherosclerotic lesions in the LDLR−/−-FO group was accompanied by a significant reduction in the circulating endothelial cell adhesion molecules JAM-A (LDLR−/−-lard group vs. LDLR−/−-FO group, mean ± SD: 1.42 ± 0.20 vs. 0.95 ± 0.56 fold of WT-lard group, P < 0.05), intercellular adhesion molecule 1 (1.15 ± 0.14 vs. 0.88 ± 0.17 fold of WT-lard group, P < 0.05), and VE-cadherin (0.88 ± 0.12 vs. 0.72 ± 0.15 fold of WT-lard group, P < 0.05), reflecting reduced ADAM activity in endothelial cells.

Conclusion: FO exerted an antatherogenic effect on male LDLR−/− mice that was accompanied by a reduced release of ADAM17 and ADAM10 substrates from endothelial cells. It is suggested that FO-decreased ADAM activity contributes to improved endothelial barrier function and thus counters intimal lipoprotein insudation and macrophage accumulation.

Keywords: ADAM, atherosclerosis, endothelial permeability, fish oil, LDLR knockout, shedding

Introduction

A disintegrin and metalloprotease 17 (ADAM17) and a disintegrin and metalloprotease 10 (ADAM10) play key roles in a spectrum of inflammatory processes and possibly contribute to events that underlie the development of atherosclerotic lesions. Both are present in atherosclerotic plaques and may contribute to plaque progression (1–3). ADAM17 liberates TNF-α from its membrane anchor, which becomes upregulated on macrophages when they enter into the inflammatory state. Canault et al. (4)
reported that expression of a noncleavable form of TNF-α blunted inflammatory responses in atherosclerotic lesions of apolipoprotein E knockout (apoE<sup>−/−</sup>) mice. Furthermore, lesion progression correlated with both increased immunostaining for ADAM17 in the vessels, and increased plasma concentrations of tumor necrosis factor receptor 1 (TNFR1), another substrate of ADAM17 on macrophages (2). These findings implicate ADAM17 upregulation on macrophages as a relevant event during development of the early atherosclerotic lesion.

Further to its proinflammatory effects, ADAM17 may act in concert with ADAM10 to directly influence endothelial barrier function. ADAM17 promotes leukocyte transmigration by cleaving the cell adhesion molecules vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and junctional adhesion molecule A (JAM-A) (5–7). Reduced cleavage of JAM-A by ADAM17 correlated with improved vascular barrier function in a mouse model of lipopolysaccharide-induced lung inflammation (8). ADAM10 cleaves vascular endothelial cadherin (VE-cadherin), a process that also directly enhances endothelial permeability (9).

Fish oil (FO) protects against development of cardiovascular diseases (10–13). It is a major source of n-3 PUFAs that exert a spectrum of beneficial effects on cardiovascular function (14–17). n-3 PUFAs such as DHA (22:6n-3) and EPA (20:5n-3) lower plasma TGs, regulate blood pressure, and enhance vascular integrity (18–20).

The present study was undertaken in order to investigate whether the antiatherogenic effects of FO might involve downregulation of ADAM expression or activity. Substrate processing is prone to regulation by many mechanisms, e.g., changes in cell membrane compartmentalization (21) or membrane fluidity (22), so this study focused on the quantification of shed substrates, which are a direct reflection of net enzymatic function. The LDL receptor knockout (LDLR<sup>−/−</sup>) model was chosen because, other than in the apoE<sup>−/−</sup> model, atherosclerosis can be influenced by n-3 and n-6 PUFA supplementation (23, 24). LDLR<sup>−/−</sup> mice were fed a Western diet including 20% lard (LDLR<sup>−/−</sup>-lard group) or 10% FO (LDLR<sup>−/−</sup>-FO group) for 12 wk before analysis. They were compared with similarly fed C57Bl6 wild-type (WT) mice (WT-lard and WT-FO groups) to discern whether the dietary effects on ADAM activity might also be observed without an atherosclerotic background.

This study represents a counterpart to investigations that have been conducted in hypercholesterolemic subjects. In those investigations, PUFA-rich diets were shown to have no effect on concentrations of circulating proinflammatory cytokines (25), but they caused a significant reduction in concentrations of the classical endothelial ADAM substrates ICAM-1 and VCAM-1 (26). The possibility lent itself to consideration that PUFAs might exert rather specific ADAM-repressing effects on the vascular endothelium. The objectives of the present study were to test this hypothesis and 1) compare the impact of dietary FO supplementation on the expression of ADAM10 and ADAM17 in WT and LDLR<sup>−/−</sup> mice; and 2) compare the impact of dietary FO supplementation on the generation of the soluble ADAM10 and ADAM17 substrates as indicators of ADAM function.

**Methods**

**Mice and diets.** A total of 24 4-wk-old male LDLR<sup>−/−</sup> mice (B6.129S7-Ldlrtm1Her/j) with an initial body weight of 16.3 ± 1.4 g (mean ± SD) were purchased from Jackson Laboratory, and 24 corresponding male WT mice (C57BL/6) with an initial body weight of 16.2 ± 0.8 g (mean ± SD) were purchased from Charles River Laboratories. Before starting the experiment, mice were fed a standard unpurified rodent diet (27) for 5 d. The WT mice and the LDLR<sup>−/−</sup> mice were then randomly assigned to 1 of 2 groups and were fed a Western diet (Supplemental Table 1) containing either 200 g/kg lard or 100 g/kg lard + 100 g/kg salmon fish oil (for detailed composition see Supplemental Table 2). By using the Western diet, it would be ensured that all LDLR<sup>−/−</sup> mice developed pronounced atherosclerotic lesions, which were assumed to be influenced by the dietary FO supplementation. The diets were supplemented with vitamins and minerals according to recommendations of the National Research Council (28) and are detailed in Supplemental Table 3. All diets were consumed ad libitum. Water was available from nipple drinkers throughout experiment. The mice were kept in Macrolon cages in a room maintained with controlled temperature (23 ± 1°C), humidity (50–60%), and lighting (0600–1800). All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt (approval number: 42502–5–34 MLU).

**Sample collection.** After food deprivation for 4 h, mice were killed by decapitation under light anesthesia. Blood was collected into EDTA tubes and plasma was separated by centrifugation at 3000 × g for 10 min at 4°C and stored at −20°C. Liver, lungs, aortic tissue, and kidney were harvested, immediately snap frozen in liquid N<sub>2</sub>, and stored at −80°C. For preparation of the aortic root sections, the vasculature was perfused with 0.9% NaCl. The ventricular edge and ~1 mm of the aortic root were immediately dissected under a stereomicroscope, cryo-mounted in mounting medium (Tissue Freezing Medium, Jung; Leica Instruments), snap-frozen in liquid N<sub>2</sub>, and stored at −80°C.

**Preparation of liver microsomes and FA analysis.** Subsequent to the preparation of liver microsomes (29), total lipids were extracted (30) and transmethylated (31). FA methyl esters were analyzed by a GC system (GC-2010, Shimadzu) fitted with a high polarity-FAs phase capillary column (30 m × 0.53 mm i.d.; Agilent Technologies) and a flame ionization detector. The individual FA methyl esters were identified by comparing their retention times with those of standards.

**Morphometric analysis of aortic roots.** To quantify atherosclerosis at the aortic root, frozen serial sections (7 μm thick) were prepared with the use of a cryostat (Leica CM 1850 UV, Leica Microsystems) beginning at the aortic valve (point 0). Four sections each were stained with hematoxylin and eosin for overview (sections 0 to 28 μm), von Kossa for vascular calcification (sections from 42 to 70 μm), Mallory-Cason’s trichrome for collagen structures (sections from 84 to 112 μm), and Oil Red O for vascular lipids (sections from 126 to 154 μm). Aortic lesions were histomorphologically characterized and quantified as described recently (27).

**Quantitative real-time PCR.** RNA was isolated with the SV Total RNA Isolation Kit (Promega) and reverse transcribed with the use of PrimeScript RT Master Mix (TaKaRa) according to the manufacturer’s protocols. Quantitative real-time PCR was performed with a StepOnePlus real-time PCR instrument (Applied Biosystems). The reaction mixture consisted of 10 ng cDNA, 0.25 μM of each primer (see below), and 5 μL SYBR Premix Ex Taq II Master Mix (TaKaRa) per 10 μL mixture. Values were calculated according to the ΔΔCt method, Gapdh was used as an internal housekeeping gene, and all values were normalized to the mean of the WT-lard group. The following sequences were
used as primer: Adam10 fwd CGCACAACCTCGGTGAAG rev CCGGAAGAGCTCTGATGCTG, Adam17 fwd CCTGATGGGCTAGCA- GAATG rev TGTGTTGCTGCTCCTGGAAG, Gapdh fwd TGGT- GAAGGTCGGTGTTGGAAC rev ATTCGCGCCTGTCACTGTC, 1cam1 fwd CCTGGTTCACGTTGTGATCC rev TCAGCCACTGAGTTC- CAAG.

ELISA. Tissue samples were homogenized in ice-cold 20 mM Tris-HCl, pH 6.8, supplemented with protease- and phosphatase-inhibitor cocktails (Roche), as well as 10 mM 1,10-phenanthroline, and then centrifuged at 100,000 × g for 45 min at 4°C to separate soluble from membrane-bound proteins. Membrane fraction was used for immunoblot analyses. A total of 50 µg of total protein per well was used for the tissue ELISA assay. Blood plasma was used either diluted or undiluted according to the range of the respective standard curves. DuoSet ELISA kit was from Cusabio. Values were normalized to the mean of the WT-FO group.

Immunoblot. The membrane fraction of tissue samples was resuspended in lysis buffer (1% Triton-X-100, 5 mM Tris, 1 mM EGTA, 250 mM saccharose, 10 mM 1,10-phenanthroline, pH 7.4) supplemented with a protease inhibitor cocktail (Roche), and samples were subsequently sonificated. Immunoblots for the analysis of ADAM17 (Figure 1C and D). In WT mice, vascular lesion areas and lower lipid deposits than those that received FO and lower aortic lesion areas and lower lipid deposits than those that received FO. (Figure 1A, B, and D). The vascular content of collagen was lower in the FO than in the respective lard group (Table 1).

Statistics. Statistical analyses were performed with the use of R software and GraphPad Prism. Values are expressed as means ± SDs. If not stated otherwise, 2-factor ANOVA was used to compare the effects of the diet (FO vs. lard), the genotype (LDLR−/− mice vs. WT mice), and their interaction. When 2-factor ANOVA revealed significant differences, a post hoc comparison was performed. In case of variance heterogeneity, by a Games-Howell test. Plasma IL-6 concentrations were compared between the 2 knockout groups with the use of Student's t test (Supplemental Figure 1A). Means were considered significantly different at P < 0.05.

Results

Body weight and FA composition of liver microsomes. One mouse in the WT-FO group failed to thrive and was killed after 9 wk. All other mice thrived and gained weight throughout the study. After 12 wk, body weights were similar in all 4 groups (body mass (g) mean ± SD: LDLR−/−/lard 29.1 ± 3.2; LDLR−/−/FO 29.1 ± 3.1; WT-lard 28.0 ± 2.4; and WT-FO 28.5 ± 1.9). Irrespective of the genotype, the liver microsomes of mice fed the FO diet had higher concentrations of EPA and DHA than mice fed lard. In parallel, concentrations of oleic acid (18:1n-9), linoleic acid (18:2n-6), and arachidonic acid (20:4n-6) were lower in the FO than in the respective lard group (Table 1).

Atherosclerotic lesion formation. LDLR−/− mice developed more vascular lesions than WT mice (Figure 1B). Among LDLR−/− mice, those that received FO had lower aortic lesion areas and lower lipid deposits than those that received lard (Figure 1A, B, and D). The vascular content of collagen was higher in LDLR−/− mice than in WT mice but was not influenced by the dietary fat (Figure 1C and D). In WT mice, vascular lesion sizes were small without showing differences in size and composition between lard- and FO-fed mice (Figure 1A–D). In all groups of mice, vascular calcification was negligible (data not shown).

Adam10 and Adam17 mRNA expression. ADAM17 is upregulated in many inflammatory diseases (34), and the mRNA expression of Adam10 and Adam17 was therefore analyzed in different tissues. We found that Adam10 expression

| TABLE 1 | FA composition in lipid extracts of isolated liver microsomes in male LDLR−/− and WT mice fed a diet containing either lard or FO for 12 wk1 |
|----------|-------------------------------------------------|-----------------|-----------------|-----------------|---------------|
|          | LDLR−/− mice                                    | WT mice         | P               |                |               |
|          | Lard    | FO    | Lard    | FO    | Diet | Genotype | Diet × genotype |
| FA        |         |        |         |        | <0.001 | 0.02 | 0.31 |
| 14:0      | 0.38 ± 0.08b        | 0.54 ± 0.16b     | 0.33 ± 0.04b     | 0.43 ± 0.11b     |         |        |
| 16:0      | 32.7 ± 1.79b        | 36.7 ± 1.90b      | 32.7 ± 2.59b     | 36.2 ± 2.58b     | <0.001 | 0.74 | 0.74 |
| 16:1      | 3.12 ± 0.42b        | 2.77 ± 0.32b      | 3.40 ± 0.53b     | 2.65 ± 0.55b     | <0.001 | 0.57 | 0.14 |
| 18:0      | 15.9 ± 1.72b        | 17.4 ± 1.80b      | 15.0 ± 0.66b     | 16.6 ± 0.33b     | 0.001  | 0.07 | 0.90 |
| 18:1-9    | 26.0 ± 2.04b        | 21.7 ± 2.18b      | 25.9 ± 2.09b     | 22.3 ± 2.84b     | <0.001 | 0.75 | 0.85 |
| 18:2-6    | 13.1 ± 0.94b        | 10.5 ± 0.73b      | 13.1 ± 0.85b     | 10.3 ± 0.53b     | <0.001 | 0.68 | 0.62 |
| 18:3-3    | 0.14 ± 0.04b        | 0.37 ± 0.07b      | 0.11 ± 0.03b     | 0.35 ± 0.12b     | <0.001 | 0.28 | 0.75 |
| 18:3-6    | 0.36 ± 0.08b        | 0.26 ± 0.06b      | 0.31 ± 0.04b     | 0.25 ± 0.08b     | <0.001 | 0.05 | 0.34 |
| 20:4-6    | 6.33 ± 0.91b        | 3.04 ± 0.89b      | 6.65 ± 2.18b     | 3.45 ± 1.05b     | <0.001 | 0.37 | 0.90 |
| 20:5-3    | 0.24 ± 0.05b        | 2.77 ± 1.23b      | 0.29 ± 0.11b     | 2.56 ± 1.06b     | <0.001 | 0.81 | 0.65 |
| 22:5-3    | n.d.                | 0.37 ± 0.21b      | 0.07 ± 0.12b     | 0.46 ± 0.20b     | <0.001 | 0.99 | 0.85 |
| 22:6-3    | 1.27 ± 0.36b        | 2.98 ± 2.29b      | 1.46 ± 1.15b     | 3.74 ± 2.44b     | <0.001 | 0.36 | 0.58 |
| 24:1      | n.d.                | 0.10 ± 0.15       | 0.06 ± 0.09      | 0.13 ± 0.14      | 0.01  | 0.15 | 0.74 |
| Σn-3      | 1.66 ± 0.37b        | 6.44 ± 3.63b      | 1.93 ± 1.18b     | 7.11 ± 3.59b     | <0.001 | 0.54 | 0.80 |
| Σn-6      | 19.8 ± 1.20b        | 13.8 ± 1.03b      | 20.1 ± 2.49b     | 14.0 ± 1.25b     | <0.001 | 0.62 | 0.93 |
| n-3:n-6   | 0.08 ± 0.02b        | 0.46 ± 0.23b      | 0.09 ± 0.04b     | 0.49 ± 0.20b     | <0.001 | 0.60 | 0.78 |

1 Values are means ± SDs, n = 12, except for WT-FO, n = 11. Labeled means in a row without a common letter differ significantly, P < 0.05. FO, fish oil; LDLR−/−, LDL receptor knockout; n.d., not detectable (detection limit <0.04 g/100 g FAs); WT, wild-type; WT-FO, wild-type mice fed a diet containing 10% lard and 10% fish oil.

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The cytokine became detectable in the plasma of LDLR-/- mice and IL-6 was also not measurable in plasma of WT mice. However, there was no difference in the ratio of the inactive prodomain, which is required to generate the active enzyme. MCP-1 plasma was not observed in other organs, such as the kidney or lung (data not shown). Neither Adam17 nor Adam10 mRNA expression was influenced by dietary FO supplementation.

To investigate whether the high level of Adam17 expression would be reflected by enhanced protein expression, liver extracts were subjected to ADAM17 immunoblot analysis. No significant differences could be observed (Figure 2D and E). One mechanism modulating ADAM activity is the release of the prodomain from the enzyme, which is required to generate the active enzyme. However, there was no difference in the ratio of the inactive proform of ADAM17 and the mature proform as evidenced by densitometric analysis (Figure 2F). Similarly, we found no genotype-specific or nutrition-dependent differences in ADAM10 or ADAM17 localization by immunohistochemical analyses of aortic root sections in the different groups (data not shown).

Concentrations of circulating ADAM substrates. To discern whether early atherogenesis in LDLR-/- mice might be reflected by increased concentrations of leukocyte-derived inflammation markers, we first determined the plasma and liver concentrations of soluble TNF-α, IL-6, and MCP-1 (Supplemental Figure 1). TNF-α was not detectable in the plasma of any group (data not shown) and IL-6 was also not measurable in plasma of WT mice. The cytokine became detectable in the plasma of LDLR-/- mice, but no impact from the FO diet was discerned. MCP-1 plasma concentrations were significantly higher in LDLR-/- mice than in WT mice, but again, no significant changes were incurred by the FO diet. Measurements of hepatic IL-6 and MCP-1 generated essentially the same negative results, and FO supplementation did not reduce cytokine concentrations in any case.

The ADAM17 substrates IL-6R, L-selectin, and TNFR1 were then analyzed. Soluble TNFR1, IL-6R, and L-selectin were not elevated in the LDLR-/--lard group compared with the WT-lard group (Figure 3A–C). FO supplementation led to no significant reduction in soluble IL-6R and soluble TNFR1 in the LDLR-/- mice, even though L-selectin release was significantly diminished in the LDLR-/--FO group compared with the LDLR-/--lard group.

A different and clearer pattern emerged when concentrations of soluble endothelial cell adhesion molecules were compared. ADAM17 releases endothelial JAM-A (6), ICAM-1 (35), and VCAM-1 (5, 36), whereas ADAM10 is a major sheddase of VCAM-1, ICAM-1, and VE-cadherin (5, 36). The concentrations of soluble JAM-A were significantly increased in the LDLR-/--lard group compared with the WT-lard group (Figure 3D). This increase was totally abrogated by FO supplementation. Whereas concentrations of ICAM-1, VCAM-1, and VE-cadherin were not significantly elevated in the LDLR-/--lard group compared with the WT-lard group, FO supplementation significantly reduced plasma concentrations of ICAM-1 and VE-cadherin in the knockout mice (Figure 3E and G). Because an increased expression of ICAM-1 has been described in atherosclerotic lesions, we also compared the aortic mRNA expression of this molecule between the different groups, but did not find any significant difference (Supplemental Figure 2).

Soluble ADAM substrates in the liver. The above findings raised the possibility that the effects of FO supplementation might be exerted rather selectively at sites on which dyslipidemia primarily had an impact, i.e., the aorta and the liver. Therefore, we analyzed the soluble fraction of liver extracts for ADAM17 and ADAM10 substrates (Figure 4). The LDLR-/--lard group showed significantly elevated concentrations of soluble JAM-A compared with the WT-lard group, whereas all other marker molecules analyzed did not show significant differences between these 2 groups. Interestingly, FO supplementation reduced...
shedding of ADAM substrates in the liver. The following 4 markers were significantly lower in the LDLR\(^{-/-}\)-FO group compared with the LDLR\(^{-/-}\)-lard group: soluble TNFR1, soluble IL-6R, soluble JAM-A, and soluble ICAM-1. Moreover, the FO diet significantly reduced concentrations of soluble VCAM-1 and soluble VE-cadherin in WT mice. The diet-induced reduction of ADAM substrate release was not observed in other organs, such as kidney or lung (data not shown).

**Discussion**

Atherosclerosis is widely believed to be a chronic inflammatory disease with possible involvement of macrophage-derived cytokines (37, 38). Elevated plasma concentrations of the ADAM17 substrate TNFR1, soluble IL-6R, soluble JAM-A, and soluble ICAM-1. Moreover, the FO diet significantly reduced concentrations of soluble VCAM-1 and soluble VE-cadherin in WT mice. The diet-induced reduction of ADAM substrate release was not observed in other organs, such as kidney or lung (data not shown).

However, the concept that early atherogenesis is generally driven by macrophage-derived cytokines does not receive support from our findings. Despite clear lesion development, the classical macrophage and leukocyte-derived inflammatory markers TNF-\(\alpha\), MCP-1, IL-6, TNFR1, IL-6R, and L-selectin were not elevated in the plasma of the LDLR\(^{-/-}\)-lard group compared with WT-lard controls. The negative finding with regard to TNFR1 contrasts with previous data obtained in apoE\(^{-/-}\) mice and may reflect differences that are inherent in animal models of atherosclerosis. In apoE\(^{-/-}\) mice, lesions already develop in animals on a normal unpurified diet and atherogenesis is much more accelerated by lard feeding than in LDLR\(^{-/-}\) mice. In their study, Canault et al. (2) found that the TNFR1 concentrations in apoE\(^{-/-}\) mice were already more than 2 times as high as in the WT group even before high-fat feeding started. Wang et al. (48) found increased amounts of TNF-\(\alpha\) in the plasma of LDLR\(^{-/-}\) mice fed an atherogenic diet high in butterfat (20% fat, w:w) and cholesterol compared with those consuming a control diet low in butterfat (4% fat, w:w) and cholesterol for 32 wk. Of note, there was no effect on the TNF-\(\alpha\) release from elicited peritoneal macrophages, indicating that the increased plasma concentrations originated from other cells.
As expected, lesion development in lard-fed LDLR
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mice was markedly reduced by dietary FO supplementation. However, this had no impact on circulating concentrations of the macrophage-derived inflammatory markers. The effects of FO on the release of TNFR1, IL-6R, and L-selectin in WT mice were mixed and presently not amenable to interpretation.

The present findings are in accordance with published data on human subjects. Skulas-Ray et al. (25) showed that circulating concentrations of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in obese hypertriglyceridemic subjects are not reduced by PUFA-rich diets. Those and our findings would be in line with the concept that systemic inflammation may be more characteristic of late rather than early atherogenesis (42, 43) such as we are dealing with in the present mouse model and that is underway in overweight and hypercholesterolemic humans before development of clinical symptoms.

The blank drawn on the possible role of macrophage-derived ADAM substrates in early atherogenesis contrasted with the results obtained upon examination of substrates released by the proteases in cells primarily affected by lipid metabolism. Quantitative mRNA analyses were first performed to exclude that any observed effects might have been due to altered expression of the proteases. Interestingly, Adam17 expression was significantly increased in the aorta and liver, but not in other cell types.

**FIGURE 3** Plasma concentrations of ADAM10 and ADAM17 substrates in male WT and LDLR
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mice fed a diet containing either lard or lard and FO for 12 wk. Concentrations of the metalloprotease substrates IL-6R (A), TNFR1 (B), L-selectin (C), JAM-A (D), ICAM-1 (E), VCAM-1 (F), and VE-cadherin (G). Values are normalized to the WT-lard group and are means ± SDs, n = 12 for all groups except WT-FO, n = 11. Two-factor ANOVA with P values of the factors D, G, or GxD. Means without a common letter differ significantly, P < 0.05. ADAM10, disintegrin and metalloprotease 10; ADAM17, disintegrin and metalloprotease 17; D, diet; FO, fish oil; G, genotype; ICAM-1, intercellular adhesion molecule 1; IL-6R, interleukin 6 receptor; JAM-A, junctional adhesion molecule A; LDLR
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, LDL receptor knockout; s, soluble; TNFR1, tumor necrosis factor receptor 1; VCAM-1, vascular cell adhesion molecule 1; VE-cadherin, vascular endothelial cadherin; WT, wild-type; WT-FO, wild-type mice fed a diet including 10% lard and 10% fish oil; WT-lard, wild-type mice fed a diet including 20% lard.

**FIGURE 4** Concentrations of ADAM10 and ADAM17 substrates in the liver of male WT and LDLR
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mice fed a diet containing either lard or lard and FO for 12 wk. Hepatic concentrations of the metalloprotease substrates TNFR1 (A), IL-6R (B), JAM-A (C), ICAM-1 (D), VCAM-1 (E), and VE-cadherin (F). Values are normalized to the WT-lard group and are means ± SDs, n = 6 for all groups except WT-FO, n = 5. Two-factor ANOVA with P values of the factors D, G, or GxD. Means without a common letter differ significantly, P < 0.05. ADAM10, disintegrin and metalloprotease 10; ADAM17, disintegrin and metalloprotease 17; D, diet; FO, fish oil; G, genotype; ICAM-1, intercellular adhesion molecule 1; IL-6R, interleukin 6 receptor; JAM-A, junctional adhesion molecule A; LDLR
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, LDL receptor knockout; s, soluble; TNFR1, tumor necrosis factor receptor 1; VCAM-1, vascular cell adhesion molecule 1; VE-cadherin, vascular endothelial cadherin; WT, wild-type; WT-FO, wild-type mice fed a diet including 10% lard and 10% fish oil; WT-lard, wild-type mice fed a diet including 20% lard.

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organs, such as lung or kidney, of LDLR−/− mice compared with the WT controls. It is noteworthy that augmented Adam17 RNA expression was not reflected by increased protein expression. This finding was in accordance with Santiago-Josefat et al. (49), who showed that protein concentrations of ADAM17 are controlled posttranscriptionally. Unsurprisingly, the concentrations of shed ADAM substrates were entirely comparable between LDLR−/− mice and their WT counterparts.

Atherosclerotic lesion development is driven by insudation of atherogenic lipoproteins into the intima and monocyte emigration to these sites. Both events are influenced by the action of endothelial cell–based ADAM10 and ADAM17. Cleavage of cell adhesion molecules augments vascular permeability and promotes leukocyte emigration. JAM-A was conspicuously the only adhesion molecule that showed significantly elevated concentrations in the plasma of the LDLR−/−-lard group compared with the WT-lard group. JAM-A is located in the tight junctions of endothelial cells and plays an important role in controlling barrier function via homophilic interaction. Upon endothelial activation, JAM-A translocates to the luminal surface of the cell, where it plays a key role in directing monocyte traffic to the underlying lipoprotein deposits, and is then shed in quantity into the bloodstream. Because other ADAM adhesion-molecule substrates that do not display this trafficking behavior were not elevated, we speculate that the JAM-A increase rather reflects changes in localization than in ADAM activity. Of interest is the finding that the antiatherogenic effect of FO supplementation was reflected in the significant reduction in the plasma concentrations of soluble JAM-A and ICAM-1, classical substrates of ADAM17, as well as VE-cadherin, the preferential substrate of ADAM10. A slight reduction was also noted for the third ADAM17 substrate, VCAM-1.

The possibility consequently emerges that FO supplementation might differentially influence ADAM function, with endothelial cells representing one type of responders. Our findings again agree very nicely with a previous clinical study, in which it was found that PUFA-rich diets led to significant decreases in circulating concentrations of ICAM-1, E-selectin, and VCAM-1 (26). The decreases observed in humans were in the same range as discerned here. The simplest explanation for such significant changes in plasma concentrations of endothelial markers is that PUFAs reduce ADAM activity in these cells.

The liver is the central organ involved in lipid uptake and metabolism. A lard diet induces hepatic steatosis in LDLR−/− mice that is suppressed by FO supplementation (50). Interestingly, it was reported recently that ADAM inhibition with the metalloprotease inhibitor Marimastat reverses hepatic steatosis and improves insulin resistance in mouse models of diet-induced obesity and leptin deficiency (51). On the contrary, it was shown that increased ADAM17 activity in mice lacking the endogenous ADAM17 inhibitor tissue inhibitor of metalloproteinase 3 contributed to hepatic steatosis and insulin resistance (52). Our present findings are of interest against this background. The FO diet significantly reduced the amounts of soluble TNFR1 and IL-6R in the liver extracts of LDLR−/− mice, and a similar tendency was also observed in the WT mice. In this organ, hepatocytes are the major source of TNFR1 and IL-6R, so the speculation might appear justified that the beneficial effects of FO on hepatic steatosis actually involve functional downregulation of ADAM17. The overall FO-dependent reduction in shedding of endothelial substrates in the liver is reminiscent of and in accord with the pattern observed systemically.

FO supplementation of a Western diet was observed to decrease the release of soluble ADAM substrates in the liver and in plasma. The effect was visible in LDLR−/− mice as well as in WT mice, which agreed with other studies demonstrating vascular dysfunction also in WT mice on a high-saturated–fat diet and the beneficial effects of FO in these mice (24). FOs have hypolipidemic effects and reduce the mRNA expression of genes coding for lipogenic enzymes such as fatty acid synthase in hepatic cells. Moreover, FOs protect vascular endothelial cells by decreasing oxidative stress and by activation of endothelial NO synthase (20, 53–55).

Oxidative stress activates both ADAM10 and ADAM17, whereas NO inhibits the shedding of ADAM17 substrates (56). These factors may contribute to the reduced functionality of ADAMS in the FO-fed mice observed here. Such considerations may form the starting point for future analyses, but it is apparent that elucidation of molecular mechanisms underlying the interplay between PUFAs and ADAMS will require a broad spectrum of in vitro and in vivo studies. Cell culture systems are mandatory, but the results obtained may not always match in vivo findings. For example, although without measurable effects in vivo, dietary PUFS do lead to reduced inflammatory cytokine production by peripheral blood mononuclear cells (57), and they also exert direct anti-inflammatory effects in a human acute monocytic leukemia cell line (58).

In sum, evidence is provided here that dietary FAs can directly modulate ADAM10 and ADAM17 activity in vivo. A decreased release of endothelial adhesion molecules that was observed with FO intake would contribute to endothelial barrier improvement and thus counteract atherogenesis. Endothelial dysfunction has been linked to many cardiovascular disease risk factors, including diabetes, hypertension, and hypercholesterolemia. Type 2 diabetes is also characterized by elevated circulating concentrations of ADAM17 substrates (59), and a high-fat diet supplemented with FO improves metabolic features associated with type 2 diabetes, such as hepatic steatosis, in mice (60). In humans, serum long-chain n-3 PUFA concentration is associated with a reduced risk of type 2 diabetes (61). Decreased ADAM10 and ADAM17 activity might be one factor that contributes to the beneficial effects of FO in the context of vascular dysfunction and fatty liver diseases.

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References

19. Schwellenbach LJ, Olson KL, McConnell KJ, Stolcpart RS, Nash JD,
15. Calder PC. The role of marine omega-3 (n-3) fatty acids in inflamma-
13. Kris-Etherton PM, Harris WS, Appel LJ. Fish consumption, fish oil,
7. Pruessmeyer J, Ludwig A. The good, the bad and the ugly substrates for
5. Singh RJ, Mason JC, Lidington EA, Edwards DR, Nuttall RK, Khoka

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