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Acceleration of experimental diabetic retinopathy in the rat by omega-3 fatty acids

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Summary Omega-3 fatty acids exert several important biological effects on factors that may predispose to diabetic retinopathy. Potential pathogenetic mechanisms include platelet dysfunction, altered eicosanoid production, increased blood viscosity in association with impaired cell deformability and pathologic leucocyte/endothelium interaction. Therefore, we tested whether a 6-month administration of fish oil (750 mg Maxepa, 5 times per week), containing 14 % eicosapentaenoic acid (EPA) and 10 % docosahexaenic acid, could inhibit the development of experimental retinopathy of the streptozotocin-diabetic rat. The efficiency of fish oil supplementation was evaluated by measuring EPA concentrations in total, plasma and membrane fatty acids and by measuring the generation of lipid mediators (leukotrienes and thromboxanes). Retinal digest preparations were quantitatively analysed for pericyte loss, and the formation of acellular capillaries. Omega-3 fatty acid administration to diabetic rats resulted in a twofold increase of EPA 20:5 in total fatty acids, and a reduction of the thromboxane$_{2/3}$ ratio from 600 (untreated diabetic rats) to 50 (treated diabetic rats). Despite these biochemical changes, diabetes-associated pericyte loss remained unaffected and the formation of acellular, occluded capillaries was increased by 75 % in the fish oil treated diabetic group (115.1 ± 26.8; untreated diabetic 65.2 ± 15.0 acellular capillary segments/mm^2 of retinal area). We conclude from this study that dietary fish oil supplementation may be harmful for the diabetic microvasculature in the retina. [Diabetologia (1996) 39: 251–255]

Keywords Diabetic retinopathy, rat model, omega-3 fatty acids.

Early diabetic retinopathy both in humans and in experimental animal models is characterized by increased vascular permeability and capillary closure [1–4]. Although hyperglycaemia has been identified as the major underlying factor of retinopathy, the complex cascade of events leading to vascular occlusions remains undefined. Chronic hyperglycaemia affects cellular and matrix function by a variety of mechanisms such as increased polyol pathway activity, altered intracellular redox state, activation of protein kinase C and non-enzymatic glycation [5–8]. Hyperglycaemia also leads to intravascular alterations that can be linked to vascular occlusions. Activation of leucocytes and monocytes has been associated with retinal vascular occlusion in diabetic rats [9]. In diabetic patients, a reduction of erythrocyte deformability and an increase of whole-blood viscosity was found to be correlated with microangiopathy [10, 11]. Platelet aggregation is enhanced in diabetic patients and correlates with the development of diabetic angiopathy [12, 13]. The increased release of thromboxane A_2 upon platelet aggregation can induce further platelet aggregation and vasoconstriction resulting in decreased blood flow and vascular occlusion

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Abbreviations: EPA, Eicosapentaenoic acid; STZ, streptozotocin; AGE, advanced glycation end products; TxB, thromboxane; AU, arbitrary units; PMN, polymorphonuclear neutrophil granulocytes; RP-HPLC, reverse-phase-high performance liquid chromatography.

as observed in early retinopathy [14]. Lipid mediators derived from cyclooxygenase and lipoxygenase metabolism are regarded to play a crucial role in the above-described pathogenetic cascade.

Dietary supplementation with omega-3 fatty acids has been reported to exert a variety of beneficial effects by inhibiting arachidonic acid conversion to inflammatory lipid mediators and by providing an alternative substrate for lipid mediator synthesis, i.e. eicosapentaenoic acid (EPA). These observations led to the hypothesis that omega-3 fatty acids may be useful for prevention or treatment of diabetic retinopathy [15].

We therefore assessed the effect of dietary supplementation with omega-3 fatty acids on retinal morphometric parameters in an experimental model of diabetic retinopathy.

Materials and methods

Animals. Male 8-week-old Lewis rats weighing 200–260 g (ZVI, Hannover, Germany), were rendered diabetic by i.v. injection of 65 mg/kg body weight streptozotocin (STZ) (Boehringer Mannheim, Mannheim, Germany) after an overnight fast. Rats with blood glucose levels greater than 15 mmol/l one week after injection of STZ were randomized to receive either a diet of normal rat chow (Altromin, Lage, Germany), or the same diet supplemented with fish oil (750 mg/day, 5 days per week; Maxepa, Fresenius, Bad Homburg, Germany) containing 14 % EPA and 10.6 % docosahexaneic acids. Fish oil was administered by tube feeding for 6 months to ensure complete uptake and gave a calculated average dose of 500 mg omega-3 fatty acids $\cdot$ kg^{-1} $\cdot$ day^{-1}. This dosage was chosen to simulate current therapeutic regimens used in humans.

Weekly measurements of body weight and monthly blood glucose levels after an overnight fast were determined in triplicate (Beckman Glucose Analyser, Brea, CA, USA). One week before the end of the study, 50 µl of blood was obtained from the tail tip to measure glycated haemoglobin (Glyc Affin; Isolab, Akron, OH, USA). At the end of the study, blood was partially collected in plastic tubes containing 7.5 % EDTA or in heparinized plastic tubes by puncture of the abdominal aorta in the deeply anaesthesized rats for the analysis of plasma and non-esterified fatty acids, membrane fatty acids, and prostanoids.

Fatty acids and lipid mediators. Non-esterified plasma fatty acids were quantified by one-step rapid extractive methylation for gas chromatography as described. Briefly, plasma was spiked with heptadecanoic acid as internal standard, non-esterified fatty acids were converted to methyl esters by mixing with diazomethane ether, the ether layer was dried, redissolved in chloroform and transferred to gas chromatography on a Chrompack CP 9000, using a CP-88 fused silica capillary column (Chrompack AG, Bad Homburg, Germany). Fatty acid methyl esters were detected by a flame ionisation detector [16].

For quantitation of total plasma and cell membrane (erythrocytes, polymorphonuclear neutrophil granulocytes (PMN)) fatty acids, 30 µl plasma, 10^8 erythrocytes and 10^7 PMN were subjected to hydrolysis and methylation in 1 ml 2N anhydrous methanol/HCL. After evaporation, the residue was redissolved in methanol/water, extracted with hexane, evaporated, redissolved in chloroform and subjected to gas chromatography analysis.

Leukotriene and thromboxane production were measured in the supernatant of stimulated PMN or platelets. To induce maximum lipid mediator synthesis and secretion, cells were stimulated using 1 µmol/l of the calcium ionophore A23187 over 10 min. The measurements of EPA-derived 5-lipoxygenase products were performed to evaluate qualitatively the postulated competition of the alternate lipid mediator precursors EPA with arachidonic acid at the cyclooxygenase- and lipoxygenase pathway. Leukotrienes were quantified by reverse phase-high performance liquid chromatography (RP-HPLC) of non-methylated compounds and solid-phase-HPαC of methylated compounds as previously described in detail [17, 18]. Thromboxanes were measured by solid-phase extraction, RP-HPLC separation with high-capacity columns and post-HPLC ELISA as previously described [19].

Retinal preparations. At the end of the study, the eyes of the animals of each study group (non-diabetic, $n = 7$; untreated diabetic, $n = 6$; diabetic, treated with fish oil, $n = 6$) were removed under deep anaesthesia and immediately fixed in 4 % buffered formalin.

After retinal isolation, the samples were washed in distilled water for 75 min. A combined digestion – 5 % pepsin in 0.2 % hydrochloric acid for 1 h, then 3 % trypsin in 0.2 mol/l tris(hydroxymethyl)amino-methane (Tris) for 3.5 h – was used to isolate the retinal vessel system. The preparations were placed on glass slides, washed with distilled water, air-dried and stained with periodic acid/Schiff reagent (PAS)/haematoxylin [20, 21].One retina per animal was used for morphometric evaluation.

Quantitation of advanced glycated end (AGE) products was performed as described previously [21] with the exception that a DMRXE microscope (Leica, Wetzlar, Germany) was used instead of an Aristoplan microscope.

Acellular capillaries were quantitated by a modification of the method of Engerman and Kern [22]. Using a grid of 100 fields, 10 microscopic fields covering a total retinal area of 6.76 mm^2 were scored for the presence of acellular occluded vessel segments (integration ocular Olympus, 400 × magnification). Each field containing acellular capillary segments was recorded as positive, and values were normalized to mm^2 of retinal area.

The numbers of pericytes were counted in ten randomly selected fields of each retina using an image analysing system with a morphometric software program (CUE 2, Olympus Opticals Europe, Hamburg, Germany) and the total for each retina was expressed as µm^2 of the capillary area.

All morphometric evaluations were performed by two observers unaware of the identity of the samples being examined.

Statistical analysis

All parameters are given as mean ± SD. The significance of differences between groups was tested using one-way analysis of variance and the Student's-Newman-Keuls test [23].

Results

The efficacy of omega-3 fatty acid feeding was qualitatively evaluated by measuring EPA concentrations in total fatty acids, non-esterified fatty acids, mem-

Table 1. Plasma levels of total fatty acids, PMN and erythrocyte membrane fatty acid composition in treated and untreated diabetic rats and controls after 6 months. Lipid mediator release from in vitro stimulated thrombocytes and PMN, isolated and stimulated with the calcium ionophore A 23187 is also given

	Non-diabetic control	Untreated diabetic	Diabetic omega-3 fatty acid
A.			
Total fatty acids (μg/ml plasma)			
18:2	1247 ± 154	2017 ± 282	1510 ± 185
20:4	1280 ± 209	745 ± 157	606 ± 112
20:5	88 ± 13	66 ± 19	149 ± 32
PMN membrane fatty acids (μg/10^7 cells)			
18:2	2230 ± 65	2108 ± 112	2413 ± 99
20:4	4351 ± 207	4415 ± 231	4464 ± 264
20:5	ND	ND	203 ± 41
Ery. membrane fatty acids (ng/10^8 cells)			
18:2	1667 ± 109	2023 ± 262	1824 ± 199
20:4	5285 ± 148	4295 ± 231	4132 ± 351
20:5	ND	ND	185 ± 34
B. Lipid mediators			
Thromboxanes (ng/10^8 cells)			
TxB_2	71.1 ± 19.5	95.5 ± 17.2	41.2 ± 12.8
TxB_3	ND	0.16 ± 0.04	0.82 ± 0.10
Leukotrienes (pmol/10^7 cells)			
LTB_4	194.5 ± 16.6	173.5 ± 10.8	149.1 ± 18.5
LTB_5	ND	5.75 ± 0.61	13.32 ± 1.55

Plasma, PMN and erythrocytes of three animals each were pooled and subjected to fatty acid analysis as described in the method section. 18:2 linolenic acid (Ω 6); 20:4 arachidonic acid (Ω 6); 20:5 EPA (Ω 3).
Lipid mediator release was quantitated by analysis of the cell supernatant by a sequence of chromatographic procedures as described. Arachidonic acid-derived cyclooxygenase products (TxB_2) and lipoxygenase-derived products (LTB_4) are compared with the corresponding EPA-derived mediators (TxB_3/LTB_5). ND, Not detectable. Measurements were performed in duplicate and are given as mean ± SD

brane fatty acids of PMN, erythrocytes and thrombocytes. Additionally, the generation of lipid mediators (leukotrienes B_4 and B_5 and thromboxanes (TxB) B_2 and B_3) was used to estimate eicosanoid production in PMN and thrombocytes, respectively.

Omega-3 fatty acids were increased twofold in total fatty acids and only detectable in non-esterified acids of fish oil fed rats, while omega-6 fatty acids were decreased by approximately one fourth in total fatty acids of treated rats (Table 1). Non-diabetic rats had lower linoleic acid (18:2) and higher arachidonic acid (20:4) fatty acid levels, compared with untreated diabetic animals. Membrane content of EPA (20:5)was only detectable in PMN of treated diabetic rats (203 ng/10^6 cells), but not in either of the untreated groups. Similarly, EPA (20:5) was approximately 400 ng/10^8 platelets while undetectable in untreated diabetic rats (not shown). In erythrocyte membranes, fish oil feeding led to an EPA concentration of 185 μg/10^8 cells.

In vitro stimulation of isolated thrombocytes with the calcium ionophore A23187 resulted in a 50% reduction of TxB_2 and a fourfold increased release of TxB_3, reducing the $TxB_{2/3}$ ratio from 600 (untreated rats) to 50 (treated rats). Stimulation of isolated PMN with A23187 doubled the leukotriene LTB_5 production in the treated diabetic group (Table 1).

By these parameters, it was shown that dietary supplementation of omega-3 fatty acids led to a significant effect on plasma and membrane fatty acid composition. Predominant effects for cell membrane biophysical properties (membrane fluidity, peroxidation) and minor consequences on inflammatory and aggregatory cell behaviour may be the likely functional consequences.

After 6 months of diabetes, treated and untreated diabetic rats differed significantly from non-diabetic animals in terms of glycaemic control and body weight (Table 2). No differences in these parameters were observed between the treated and the untreated diabetic group.

The net effect of long-term fish oil feeding on diabetic retinopathy was quantitatively evaluated in retinal digest preparations (Table 2). Non-diabetic rats had a regular capillary appearance and no major areas of vascular occlusion. Pericyte counts were 2540 ± 170 cell nuclei/mm^2 of capillary area. The number of acellular occluded capillary segments was small (20.1 ± 6.0). No increased autofluorescence in precapillary arteriolar branching sites was detected

Table 2. Effect of omega-3 fatty acid supplementation on metabolic and retinal parameters

Groups/*n*	Non-diabetic control/7	Untreated diabetic/6	Diabetic omega-3 fatty acid/6
Starting body weight (g)	290 ± 15	287 ± 17	302 ± 16
Final body weight (g)	460 ± 13	224 ± 17[a]	226 ± 19[a]
Plasma glucose (mmol/l)	3.95 ± 0.18	26.17 ± 1.81[a]	26.27 ± 1.83[a]
Glycated haemoglobin (%)	4.3 ± 0.6	14.7 ± 2.9[a]	13.1 ± 1.9[a]
Pericyte numbers	2540 ± 170	2120 ± 200[a]	1980 ± 80[a]
Occluded capillary segments	20.1 ± 6	65.2 ± 15[a]	115.1 ± 26.8[b]
Retinal AGEs (AU)	164 ± 14	304 ± 56[a]	283 ± 33[a]

Data (mean ± SD) were analysed by analysis of variance and differences between groups were tested as described in Materials and Methods. [a] $p < 0.001$ vs control; [b] $p < 0.001$ vs untreated diabetic. Pericyte numbers are given as numbers of cell nuclei/mm^2 of capillary area. Numbers of occluded acellular capillary segments are given per mm^2 of retinal area

(164 ± 14 arbitrary units (AU)). In contrast, retinae from rats diabetic for 6 months had a high proportion of scattered fields showing capillary irregularities. A significant loss of pericytes was observed (2120 ± 200; $p < 0.01$ vs non-diabetic rats) together with a significant increase of acellular occluded capillary segments (65.2 ± 15; $p < 0.001$ vs non-diabetic rats). Autofluorescence quantitation of corresponding fields yielded a significant increase in this group (304 ± 56 AU; $p < 0.001$ vs non-diabetic rats).

Dietary supplementation of fish oil at a dose that was found to effectively alter fatty acid composition of the plasma and cellular membranes did not lead to any detectable vascular benefit in this model. Pericyte loss was unchanged (1980 ± 80; P N.S. vs untreated diabetic rats) and the number of acellular capillaries increased to $115.1 \pm 26.8/mm^2$ of retinal area ($p < 0.01$ vs untreated diabetic rats). Retinal autofluorescence was not changed during this treatment (283 ± 33 AU; P N.S. vs untreated diabetic rats).

Discussion

Data obtained in this study indicate that omega-3 fatty acid supplementation in a dosage corresponding to currently applied treatment protocols used in humans not only fails to ameliorate, but even accelerates retinopathy in the STZ-diabetic rat model.

This result contrasts with the hypothesis that treatment with omega-3 fatty acids is beneficial for diabetic retinopathy [15]. This hypothesis resulted from experimental and clinical data showing that omega-3 fatty acids could restore hyperglycaemia-induced alterations, such as platelet aggregation [24–26], vasoconstriction [27, 28] endothelial permeability [29], chemotaxis [30], erythrocyte deformability, blood viscosity and granulocyte /endothelium interactions [31–33].

However, the mechanism(s) that led to retinopathy deterioration in our study remain(s) unknown. One hazard of fish oil treatment in diabetes is the deterioration of glucose control, as measured by plasma glucose and glycated haemoglobin [34]. In our study, neither of these parameters differed in either the treated or the untreated diabetic groups, so that hyperglycaemia as a factor of worsened retinopathy in the fish oil fed group can be excluded.

Supplementation of polyunsaturated fatty acids may have caused accelerated microvascular damage, because polyunsaturated fatty acids are highly susceptible to oxidative modification [35]. Moreover, the diabetic state is characterized by increased oxidative stress due to increased production of reactive oxygen species and a decrease in antioxidative defence mechanisms [36]. Lipid hydroperoxides which can be generated through increased oxidative modification of polyunsaturated fatty acids correlate with structural changes of the inner and outer retina [37]. Furthermore, the increased formation of AGE products increases cellular oxidative stress by the AGE-receptor (RAGE) [38]. Although not measured directly, a possible mechanism by which omega-3 fatty acids could increase retinal capillary damage is the potentiation of oxidative stress to capillary cells by their pro-oxidant properties in the diabetic milieu.

A further potentiation of the oxidative damage might also result from the relative depletion of antioxidant substances such as vitamins E and C. Vitamin C is necessary for the regeneration of vitamin E. Since ascorbate is known to be deficient in diabetes and no additional vitamin C was given to the fish oil treated rats, a cascade of pro-oxidant events from peroxidation of omega-3 fatty acids to pro-oxidant effects of oxidized vitamins E or C could be implicated in the accelerated retinopathy [39–43].

It is unknown whether dietary fish oil supplementation would lead to retinal capillary damage in the absence of diabetes. This important question arises from the unexpected results of this study and needs to be addressed separately. However, it is unlikely that fish oil would damage retinal capillaries under non-diabetic conditions due to the undisturbed balance between pro- and antioxidant mechanisms and the propensity of endothelial cells to increase antioxidant protection in compensation [44].

In conclusion, our study shows that dietary supplementation of fish oil, rich in omega-3 fatty acids does not inhibit the development of experimental diabetic retinopathy but, rather causes its acceleration. Extrapolation of these data to diabetic patients is difficult for many reasons. However, it appears that fish oil supplementation as an adjuvant therapy for diabetic retinopathy is potentially harmful unless sufficient antioxidant protection is assured. Clinical studies should provide definitive data.

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