Platelet Redox Balance in Diabetic Patients With Hypertension Improved by n-3 Fatty Acids

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OBJECTIVE—Patients with type 2 diabetes mellitus (T2DM) are at increased risk of developing cardiovascular disease, largely as a result of defective production of cardioprotective nitric oxide and a concomitant rise in oxidative stress. Dietary interventions that could reverse this trend would be extremely beneficial. Here we investigated whether dietary n-3 polyunsaturated fatty acid (n-3 PUFA) supplementation positively affected platelet nitro-ros-redox imbalance.

RESEARCH DESIGN AND METHODS—We randomized hypertensive T2DM patients (T2DM HT; n = 22) and age-and-sex matched hypertensive study participants without diabetes (HT alone; n = 23) in a double-blind, crossover fashion to receive 8 weeks of n-3 PUFAs (1.8 g eicosapentaenoic acid and 1.5 g docosahexaenoic acid) or identical olive oil capsules (placebo), with an intervening 8-week washout period. Platelet nitrite and superoxide were measured and compared before and after treatment. 8-isoprostane was determined by ELISA and subcellular compartmentalization of the NAD(P)H oxidase subunit p47-phox examined by Western blotting.

RESULTS—The n-3 PUFA supplementation reduced 8-isoprostane and superoxide levels in platelets from T2DM HT, but not HT alone, participants, without effect on nitrite production. This coincided with a significant decrease in p47-phox membrane localization and a similar reduction in superoxide to that achieved with apocynin. At baseline, a subcohort of T2DM HT and HT alone participants showed evidence of nitric oxide synthase (NOS)–derived superoxide production, indicating defective enzymatic activity. This was reversed significantly in T2DM HT participants after treatment, demonstrating improved NOS function.

CONCLUSIONS—Our finding that n-3 PUFAs diminish platelet superoxide production in T2DM HT patients in vivo suggests a therapeutic role for these agents in reducing the vascular–derived oxidative stress associated with diabetes.

Dietary interventions that could reduce the risk of development of cardiovascular disease (CVD) in patients with type 2 diabetes mellitus (T2DM) would clearly be advantageous instead of expensive pharmacological treatments. There is now considerable evidence demonstrating an association between enhanced oxidative stress and the incidence of CVD. In healthy vasculature, oxidative stress is kept in check by nitric oxide (NO) derived from endothelial NO synthase (NOS). Endothelial- and platelet-derived NO plays a pivotal role in maintaining normal vascular homeostasis and has atheroprotective effects, inhibiting smooth muscle cell proliferation, leucocyte adhesion, and aggregation of platelets (1–3). Diabetic vascular disease, in contrast, is characterized by altered NO bioavailability resulting from an increase in the amount of the NO scavenger superoxide (O₂•−), derived predominantly from NAD(P)H oxidase or from uncoupled endothelial NOS (1,4). Together, these alterations lead to endothelial dysfunction, enhanced platelet activity, and an increased risk of thrombosis (5–7). Despite a large body of evidence linking dysregulated NO production and enhanced oxidative stress to vascular disease, however, clinical studies with well-characterized antioxidant vitamins, such as vitamins C and E, have proved disappointing (8). The inability of antioxidants to be delivered and retained in the vascular bed may explain these results. Moreover, with increasing evidence of the importance of free radicals in normal physiologic function, a new paradigm is emerging that favors controlled free radical production. In this model, a therapeutically beneficial rebalancing of NO relative to O₂•− production is sought, rather than the complete suppression of free radical production, such as occurs after antioxidant administration (9,10). In this regard, we have recently shown that n-3 polyunsaturated fatty acids (n-3 PUFAs), the major constituents of oily fish, can modulate the nitroso-redox balance by increasing vasculo-protective NO and decreasing O₂•− production in endothelial cells (11). This finding may be extremely valuable for diabetic patients whose disease manifests as a high oxidative status. Furthermore, fish oil–derived n-3 PUFAs may also offer several other advantages. Importantly, they are easily absorbed and retained within cellular membranes, where they can act as a readily available supply of free radical–modifying agents. This bioavailability, coupled with their ability to specifically modulate the function of enzymes producing reactive oxygen species (ROS) and reactive nitrogen species, such as NO and NOS, suggests that, far from simply being free radical scavengers like other antioxidants, n-3 PUFAs may be therapeutically superior.

The health benefits of high oily fish consumption were first recognized in the Greenland Inuit, in whom n-3 PUFA consumption was correlated with a lower CVD mortality rate. Since that discovery, a substantial amount of evidence has accumulated to show that the high content of n-3 PUFAs, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are responsible for these health benefits. The n-3 PUFAs are believed to exert their therapeutic benefits through a
variety of mechanisms, including a reduction in antithrombotic and antiarrhythmic actions and improved endothelial function (12–14). Few studies, however, have investigated the potential benefits of n-3 PUFA treatment for diabetic patients (15–17).

Platelets contain all the components of a functioning NO-producing pathway, including the enzyme necessary for the production of the cofactor tetrahydrobiopterin (18,19), and are therefore capable of producing NO in response to platelet-specific agonists (20,21). In addition, they also contain all the subunits required for the assembly of the NAD(P)H oxidase system (7,22). Platelets thus represent an accessible cellular model for functional studies to explore free radical activity in relation to changes in NAD(P)H oxidase and NOS activity (7,20,22). Accordingly, in the current study we examined platelets to investigate the effects of n-3 PUFAs on NO and \( \text{O}_2^{-} \) bioactivities and on oxidative stress in a diabetic population.

**RESEARCH DESIGN AND METHODS**

**Materials and antibodies**

We obtained all reagents from Sigma (St Louis, MO) unless stated otherwise.

**Study design**

We recruited T2DM patients with hypertension (T2DM HT group; \( n = 22 \); duration of diabetes 6 ± 1 years) and age- and sex-matched control study participants with hypertension (HT alone group; \( n = 23 \)). A cohort of healthy individuals without treatment (control), collected as an adjunct to this study, was also used to provide baseline reference values and is included for illustrative purposes. We identified diabetic study participants eligible for the trial from clinical registers. Previous T2DM diagnosis was defined as a fasting plasma glucose level of >7.0 mmol/L or plasma venous sample level of >11.1 mmol/L 2 h after consuming a 75-g oral glucose load (23). Patients were eligible for the study if their records provided evidence of stable control of diabetes, defined as HbA1c levels of 6.5–10%. For the purposes of the study, we used fasting glucose levels throughout as a surrogate marker for diabetes status. We excluded participants if they had any history of overt CVD or established microcomplications and macrocomplications of T2DM or hypertension, such as retinopathy or nephropathy, as well as excluding those receiving insulin therapy. All study participants adhered to their regular diets and medications throughout and gave written, informed consent for all procedures. This study was approved by the local ethical committee of Queen’s University Belfast. Data from previous intervention studies revealed an SD of 0.39 pmol/10^8 platelets (after log transformation) for measurements of NO production. Thus to detect a 40% differential change in NO at a 5% significance level with a two-tailed hypothesis test, our study required 20 volunteers per group to achieve 90% power.

This was a randomized, double-blind, crossover study of 8 weeks of n-3 PUFA or placebo, with an intervening 8-week washout period. In brief, after randomization, study participants received daily for 8 weeks either four fish oil capsules (Omacor) containing 0.46 g EPA and 0.38 g DHA (Solvay Pharma) or four identical olive oil–containing capsules. We chose olive oil as the placebo to provide a calorie intake similar to that of the fish oil capsule, as described previously (17,24). After an 8-week washout period in which study participants did not receive any treatment, participants were then allocated the oil supplement that they had not previously received. We performed studies before treatment (baseline 1), after 8 weeks of treatment, after the 8-week washout (baseline 2), and after a further 8 weeks of treatment. This was in line with previous studies confirming that an 8-week treatment phase permits sufficient time for the incorporation of the fatty acids into cellular membranes (17).

**Platelet isolation**

Study participants attended all trial appointments after having fasted overnight, and they refrained from taking their prescribed medications until after the assessment. After a 30-min rest period, we withdrew 120 mL of venous blood from the left antecubital fossa with a 19-gauge butterfly needle. Three aliquots were processed for routine biochemical assays. The remainder of the blood in 9.0-mL aliquots was run into each of 10 polypropylene tubes containing 1.0 mL 3.18% sodium citrate and gently mixed. We chose olive oil as the placebo to provide a calorie intake similar to that of the fish oil capsule, as described previously (17,24). After an 8-week washout period in which study participants did not receive any treatment, participants were then allocated the oil supplement that they had not previously received. We performed studies before treatment (baseline 1), after 8 weeks of treatment, after the 8-week washout (baseline 2), and after a further 8 weeks of treatment. This was in line with previous studies confirming that an 8-week treatment phase permits sufficient time for the incorporation of the fatty acids into cellular membranes (17).

**Measurement of \( \text{O}_2^{-} \) production**

Released \( \text{O}_2^{-} \) was measured in freshly isolated platelets by enhanced chemiluminescence detection with lucigenin as a luminescent substrate. Our group has previously validated this assay, described in detail elsewhere, to show that luminescence reflects \( \text{O}_2^{-} \) production as indicated by inhibition with superoxide dismutase and diphenyliodonium (21,25). Briefly, lucigenin was added to an aliquot of platelet suspension, and \( \text{O}_2^{-} \) production was measured before and after the addition of the stimulant phorbol 12-myristate 13-acetate (PMA; 10 \( \mu \)mol/L). We considered a stable baseline as indicative of the fact that that sample preparation had no effect on ROS production. Assays were carried out in the presence or absence of \( 1\text{-N}^\bullet \text{N}^{-}\text{nitro-1-arginine methyl ester (1-NNAME; 45 \( \mu \)mol/L) or apocynin (4-hydroxy-3-methoxyacetophenone 30 \( \mu \)mol/L; Fluka Chemie, Neu-Ulm, Germany).}

**Nitrite assay**

Freshly isolated platelets were stimulated with ADP (5 \( \mu \)mol/L) in the presence of \( \text{Ca}^{2+}/\text{Mg}^{2+} \) (2 Mmol/L), and fibrinogen were washed twice with Tyrode buffer. Two pellets were flash frozen in liquid nitrogen and stored at −80°C for subsequent platelet 8-isoprostane and membrane fatty acid analysis. A third pellet was separated into cytosol and membrane fractions for subsequent p47 content assessment and stored at −80°C. Finally, we resuspended the remaining two pellets in fresh Tyrode buffer in preparation for \( \text{O}_2^{-} \) or nitrite measurement. Estimation of platelet purity verified that the number of contaminating polymorphic nuclear cells was consistently below 0.05/10^6 platelets. Samples containing >1 erythrocyte/1000 platelets were discarded.
Table 1—Physical and clinical characteristics before and after fish oil or olive oil supplementation

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<td>135.6 ± 129.1</td>
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<td>LDL cholesterol</td>
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All data are given as mean ± SD. All measures are in mmol/L except as specified. Statistical analysis was performed on change data after fish oil supplementation and after olive oil placebo and was expressed as SD; bold entries indicate significant change from baseline; *, P < 0.05. Physical characteristic data was compared by t test; †, P < 0.05. Statistical analyses on pre-treatment baseline data were performed by ANOVA with Bonferroni post hoc test; ‡, P < 0.001.

RESULTS

Platelet phospholipid fatty acid profile

We confirmed compliance with fish oil intake by analysis of platelet fatty acid composition. Percentages of mem- brane EPA (HT alone, 0.17 ± 0.29; T2DM HT, 0.14 ± 0.01) increased. There was also a small decrease in arachidonic acid (AA) content after fish oil supplementation. Fatty acid content in other membrane lipids was not affected.

Determination of cyclooxygenase and prostanoid-associated PGH synthase activity

The study was designed as a two-period, two-treatment, crossover study with double blind stopping to the order of randomization. The washout period was 4 weeks. The washout period was 4 weeks. The washout period was 4 weeks. The washout period was 4 weeks.

Statistical analysis

All data are given as mean ± SD. All measures are in mmol/L except as specified. Statistical analysis was performed on change data after fish oil supplementation and after olive oil placebo and was expressed as SD; bold entries indicate significant change from baseline; *, P < 0.05. Physical characteristic data was compared by t test; †, P < 0.05. Statistical analyses on pre-treatment baseline data were performed by ANOVA with Bonferroni post hoc test; ‡, P < 0.001.

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compositions of EPA and DHA were unchanged after placebo supplementation.

Clinical characteristics and lipid profile
We found baseline differences in diabetes-related indices such as fasting glucose, BMI, and elevated heart rate (Table 1). The blood pressure measurements in the T2DM HT group were lower than those in the HT alone group, reflecting the stricter clinical control of hypertension in the former group. LDL levels were also significantly increased in the HT alone group and were unchanged by the trial interventions. There were no other significant differences in the physical characteristics, including sex, between the two groups. Also, with the exception of antidiabetic medication, statistical comparison showed no significant difference in treatments between participant groups, including statin and aspirin intake. Importantly, the crossover study design, whereby every individual acted as his or her own control, largely controlled for these baseline differences and minimized the confounding influences of medications and interindividual variability observed in larger studies.

Unsurprisingly, we observed a significant fish oil–mediated lowering of triglycerides in both subject groups (Table 1). All other parameters were unchanged, apart from a decrease in diastolic blood pressure after fish oil supplementation in the HT alone group. No period effects or treatment-by-period interactions were apparent for any measurement variable, confirming the adequacy of the 8-week washout period.

Total 8-isoprostane in platelets before and after supplementation
Supplementation with n-3 PUFAs significantly reduced 8-isoprostane levels in platelets isolated from T2DM HT study participants (Fig. 1A).

Reduced PMA-stimulated $O_2^-$ production in platelets
PMA-stimulated $O_2^-$ was increased markedly in the T2DM HT group compared with controls and HT alone participants (Fig. 1B) and was significantly reduced after fish oil supplementation. In contrast, olive oil produced no significant change in PMA-stimulated $O_2^-$ in T2DM HT study participants, verifying the specificity of the n-3 PUFA–mediated effect.

Figure 1—Determination of total 8-isoprostane, $O_2^-$, and nitrite in platelets before and after dietary fish oil or olive oil supplementation. A: Supplementation with n-3 PUFAs significantly reduced 8-isoprostane levels in platelets isolated from T2DM HT study participants. HT alone, n = 14; T2DM HT, n = 19. B: PMA-stimulated $O_2^-$ production was measured by lucigenin chemiluminescence. Fish oil treatment significantly reduced PMA-stimulated $O_2^-$ production in the T2DM HT group. We observed no significant difference for HT alone participants receiving either fish oil or olive oil supplementation. HT alone, n = 20; T2DM HT, n = 21. C: Fish oil treatment did not significantly alter ADP-stimulated nitrite production. HT, n = 19; T2DM HT, n = 21. Data represented are mean ± SEM. *P < 0.05; **P < 0.01.
Effect on nitrite production
At baseline, in comparison with controls, both T2DM HT and HT alone groups exhibited an overall trend toward increased nitrite production in response to the calcium-dependent agonist ADP (Fig. 1C). ADP stimulated nitrite production was unchanged after treatment.

Reduced NAD(P)H-dependent $O_2^-$ production
Elevated levels of $O_2^-$ in the baseline T2DM HT state were significantly inhibited by the addition of apocynin, an inhibitor that, depending on the cellular environment, which can act as an inhibitor of the NAD(P)H oxidase or function as a $O_2^-$ scavenger (Fig. 2A). In contrast, after fish oil treatment, we found no significant inhibition of $O_2^-$ production by apocynin in T2DM HT participants, suggesting that $O_2^-$ production was already maximally decreased. p47-phox is a subunit of the NAD(P)H oxidase that must be associated with the plasma membrane for the enzyme to be activated, so its subcellular compartmentalization is a useful surrogate marker of NAD(P)H oxidase enzymatic activity. Western blotting indicated that in controls the presence of p47-phox predominated significantly in cytosolic fractions versus membrane fractions. In contrast, at baseline in T2DM HT and HT alone study participants, p47-phox localization was shifted from the cytosolic to the membrane fraction (Fig. 2B). After fish oil supplementation in the T2DM HT group, there was a significant reduction in membrane-associated p47-phox, such that the membrane/cytosolic localization ratio became normalized to that found in controls. This change in p47-phox subcellular localization was not observed in fish oil–treated HT alone participants.

Decreased NOS uncoupling
We have previously noted that patients can be subcategorized according to their response to L-NAME (21), and this was used to assign T2DM HT and HT alone study participants at baseline into coupled and uncoupled NOS groups (Fig. 3). In all, 41% of the HT alone group and 33% of the T2DM HT group exhibited an increase in $O_2^-$ in response to L-NAME, demonstrating an inhibition in NO production (Fig. 3A). In the remaining participants (59% and 67% of the HT alone and T2DM HT groups, respectively), incorporation of L-NAME reduced $O_2^-$ detection in accordance with L-NAME inhibition of NOS-mediated $O_2^-$ production, suggesting the enzymatic uncoupling of NOS (Fig. 3B).

After both fish oil and olive oil treatments, the coupled T2DM HT and HT alone study participants exhibited an increase in $O_2^-$ in response to L-NAME similar to that observed at baseline (Fig. 3A), indicating that NOS status remained unchanged. Significantly, in the uncoupled HT alone and T2DM HT cohorts, after both fish oil and olive oil suppletions inhibited $O_2^-$ production by L-NAME was reduced compared with baseline, although only in the latter participant group did this effect reach statistical significance. In the T2DM HT study participants, which is in agreement with observations in experimental models (30). Fish oil supplementation markedly reduced this membrane association, suggesting lower NAD(P)H oxidase activity. Measurement of NO levels in vascular disease settings is often complicated by a paradoxical increase in NOS expression. The additional protein, however, is dysfunctional and acts as a source of oxidative free radicals along with NO, resulting in elevated nitrite and concomitant $O_2^-$ accumulation (31). Thus an accurate assessment of altered NO bioavailability requires an assessment of the functional status of NOS. Although the current study did not demonstrate a measurable effect on nitrite production, evidence for a change in the functional status of NOS, in terms of its ability to produce NO or $O_2^-$, was found. In the T2DM HT study participants classified as the uncoupled NOS group, fish oil and olive oil supplements both decreased NOS-derived $O_2^-$ production significantly. These results indicate that both oils reduce the proportion of uncoupled NOS present. Together, these findings suggest that the ability to restore normal NOS function is common to both n-3 PUFA and olive oil, whereas the effects of n-3 PUFA treatment on decreased ROS production from NAD(P)H oxidase are unique to the fish oils. In summary, these findings indicate an overall n-3 PUFA–mediated improvement in global platelet redox status. Indeed, this is in agreement with previous research in our group showing that supplementation...
with n-3 PUFAs improved forearm blood flow in diabetic patients (17).

Mechanistically, there are several ways in which fish oils may mediate the effects observed in this study. First, the presence of the unsaturated double bonds increases lipid bilayer fluidity, thereby preventing the sequestering of signaling molecules to cholesterol-rich plasma membrane microdomains, such as caveolae and lipid rafts where endothelial NOS and NAD(P)H reside. For endothelial NOS, such a shift leads to a physical separation from its inhibitory partner caveolin-1, leading to an increase in basal NO production (32,33). This effect on membrane fluidity is a feature shared, albeit to a lower extent, by monounsaturated fatty acids like oleic acid, the major n-9 PUFA constituent in olive oil. This may explain the olive oil–mediated improvement in NOS function that we saw.

Second, the incorporation of PUFA displaces AA from the plasma membrane to accommodate the influx of EPA and DHA, effectively reducing AA concentrations. Any reduction in AA, an activator of NAD(P)H oxidase, inhibits NAD(P)H activity (34).

Third, n-3 PUFAs also have a high affinity for eicosanoid-synthesizing enzymes such as cyclooxygenase and lipooxygenase and can be metabolized to specific EPA- or DHA-derived eicosanoids that are less inflammatory and thrombogenic than their n-6 PUFA–derived counterparts (6). The production of eicosanoids is dependent on an n-6 unsaturated bond, which is absent from oleic acid. Thus it is possible that the fish oil–specific effects we observed are due in part to a decrease in n-6 PUFA–derived metabolites in conjunction with a concomitant increase in their n-3 PUFA–derived counterparts (35).

Raised triglyceride levels are a major risk factor for CVD in diabetic patients. Thus the finding that Omacor, currently prescribed for hyperlipidemia, had the ability to decrease triglyceride levels in our study population is clearly advantageous. Interestingly, the decrease in triglyceride levels was more marked in the T2DM HT participants and correlated with decreased ROS production. Mechanistically, this may be the result of a decrease in levels of oxidized LDL, which is known to inhibit NOS function by increasing its association with caveolin-1 (36). Furthermore, diabetes is a chronic inflammatory disease in which enhanced levels of circulating inflammatory cytokines act to exacerbate endothelial dysfunction. In this regard, n-3 PUFAs are known to modulate proinflammatory cytokine expression and also reduce cytokine signaling events by displacement of cytokine receptors from their downstream signaling partners, suggesting a further means by which fish oil may indirectly alter redox mechanisms (37,38).

Much of the advice for diabetic patients regarding fish consumption is

Figure 2—Inhibition of platelet derived ROS production by apocynin and p47-phox subcellular localization within T2DM HT platelets. A PMA-activated platelet production of $O_2^{-}$ measured by lucigenin-enhanced chemiluminescence was inhibited in the presence of 30 μmol/L apocynin. Fish oil supplementation abolished this inhibition in T2DM HT study participants. B: T2DM HT and HT alone platelets were isolated before and after treatment. Platelet extracts (40 μg), membrane (m), and cytosol (c) prepared from control, T2DM HT, or HT alone blood were separated on 12% SDS-PAGE, electrophoretically transferred to PVDF membrane, and analyzed with specific antiserum raised against p47-phox protein. Shown is a representative blot. The p47-phox expression was analyzed by Western blotting and quantified by measuring integrated optical density. Platelets from T2DM HT study participants showed a reduction in p47-phox membrane localization after exposure to fish oil. Data present are mean ± SEM; n = 5–9. *P < 0.05; **P < 0.01; ***P < 0.001.
based on the higher incidence of CVD in T2DM and is extrapolated from studies on the non-T2DM population or derived from prospective studies (39). T2DM patients have an enhanced oxidative burden compared with non-T2DM patients; therefore, studies confirming efficacy of fish oil supplementation for the former are extremely important. One of the few large scale studies to focus on individuals with T2DM recently reported no significant benefit of fish oils with respect to reducing cardiovascular mortality (40). In common with our study, all study participants received a similar ratio of EPA to DHA. In contrast, however, they received a lower dose (1 g/day) than we used here (3 g/day). Perhaps more importantly, the underlying disease pattern was markedly different, with patients only enrolled if they had already had a cardiovascular event. In contrast, our study focused on study participants without advanced disease and accordingly investigated markers known to be elevated in the early stages of endothelial dysfunction. Future large scale studies, such as ASCEND (A Study of Cardiovascular Events in Diabetes) should clarify whether fish oil supplementation in T2DM patients with no previous CVD would be beneficial in preventing disease progression.

In summary, by using well-characterized markers of platelet and vascular dysfunction, our findings support a model whereby supplementation with n-3 PUFAs decreases ROS production and oxidative stress and improves NOS function in T2DM study participants. These favorable effects on redox state in platelets support a cardioprotective role for n-3 PUFAs in patients with T2DM.

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D.M.M. codirected research, analyzed data, and wrote the manuscript. F.O., recruited study participants and analyzed data. M.M. and A.B.D. performed research and analyzed data. G.E.M. directed research and reviewed the manuscript. G.E.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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