Aspirin-Induced Decline in Prostacyclin Production in Patients With Coronary Artery Disease Is Due to Decreased Endoperoxide Shift

Analysis of the Effects of a Combination of Aspirin and n-3 Fatty Acids on the Eicosanoid Profile

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Background. It was the purpose of this study to determine the effects of the combination of aspirin (ASA) and fish oil, which is rich in n-3 polyunsaturated fatty acids, on the eicosanoid profile of patients with coronary artery disease. Specifically, we wanted to determine whether the ASA-induced reduction in prostacyclin production is due to inhibition of endothelial cell cyclooxygenase or to reduced endoperoxide shift from platelets and whether ASA negates the potentially beneficial effects of fish oil on the eicosanoid profile.

Methods and Results. Fourteen patients with clinically stable but advanced coronary artery disease received 12 g (n=8) or 16 g (n=6) of fish oil concentrate containing 6 or 8 g of n-3 fatty acids for 6 weeks. In addition to the fish oil, patients received increasing daily doses of ASA (50 mg, 100 mg, 325 mg, and 1,300 mg; the latter in four divided doses). Each dose was taken for 2 weeks. With fish oil supplementation, red blood cell phospholipid fatty acid content of arachidonic acid (AA) decreased and of eicosapentaenoic acid (EPA) increased so that EPA as a percent of AA increased from 2% to 26%. Serum thromboxane B2, which represents the production of TXA2 by maximally stimulated platelets, was suppressed by 38% on fish oil alone and by 97% or greater on all doses of ASA. Excretion of PG12-M, the main urinary metabolite of PG1 in (derived from AA), fell from 50±4 ng/g of creatinine to 42±2 ng/g on fish oil alone (p=0.02). On 50 mg of ASA per day, PG12-M excretion was 26±2 ng/g of creatinine (p<0.001 versus fish oil alone). On 100 mg and 325 mg of ASA per day, PG12-M was 24±3 ng/g and 27±3 ng/g, respectively (p=NS versus value on 50 mg per day). PG12-M, the main urinary metabolite of PG1 (derived from EPA), increased from 0.2±0.1 ng/g of creatinine to 4.9±0.7 ng/g on fish oil alone (p<0.001). In contrast with the marked ASA-induced decline in PG12-M, PG12-M excretion was not affected by the addition of ASA, even at the higher doses (4.6±0.7 ng/g and 4.9±0.5 ng/g on 325 mg per day and 325 mg four times daily, respectively).

Conclusions. Moderate-dose (325 mg per day or less) ASA taken once daily has no effect on PG1 production despite significantly reducing PG1 production. This suggests that endothelial cell cyclooxygenase is minimally inhibited by such doses of ASA and that a large percent of the PG1 produced in patients with advanced coronary artery disease derives from the transfer of prostaglandin endoperoxides from activated platelets to endothelial cells. The loss of these substrates accounts for the decrease in PG1 with moderate-dose ASA. Thus, the ASA-induced decrease in PG1 may in large part be an unavoidable consequence of ASA-induced platelet cyclooxygenase inhibition. ASA does not negate the potentially beneficial effects of n-3 fatty acids on the eicosanoid profile. (Circulation 1991;84:2286–2293)
Aspirin reduces cardiovascular events in a number of clinical settings. This presumably has been due to its inhibition of platelet thromboxane A2 (TXA2) production. In patients with atherosclerotic disease, however, ASA therapy also reduces the potentially beneficial production of prostacyclin (PGI2) by endothelial cells. This has led to a search for an optimal ASA dosage regimen that maximally suppresses TXA2 production but minimally reduces PGI2 production. The reason for the ASA-induced decline in PGI2 production is not clear, however. Although it may be due to direct inhibition of endothelial cell cyclooxygenase, fewer platelet–vessel wall interactions, and/or less endoperoxide shift from platelets after ASA may account for some part of the decline in PGI2. Endoperoxide shift, or transcellular metabolism of prostaglandin endoperoxides, which has been demonstrated in vitro, is the unidirectional transfer of prostaglandin endoperoxides, the precursors of TXA2 in the platelet and PGI2 in the endothelial cell, from the activated platelet to the endothelial cell, resulting in enhanced PGI2 production (Figure 1). Thus, some of the ASA-induced decline in PGI2 production by endothelial cells may be due to the irreversible inactivation of platelet cyclooxygenase, which reduces prostaglandin endoperoxide supply to the endothelial cell during platelet–vessel wall interactions. If so, the reduction in PGI2 production caused by ASA may be unavoidable and the focus of therapy should be suppression of platelet TXA2 production, particularly when treating patients with unstable angina in whom even low levels of residual TXA2 production might be deleterious. Alternatively, if the ASA-induced decline in PGI2 synthesis is due to inactivation of endothelial cell cyclooxygenase, then the theoretical importance of finding an optimal low-dose ASA regimen to reduce this potentially negative effect of ASA would increase.

In this study, we used eicosapentaenoic acid (EPA) from fish oil as a “probe” to help clarify in vivo the role of endoperoxide shift in the ASA-induced decrease in PGI2 production in patients with atherosclerotic disease (Figure 1). The rationale for the use of EPA was based on its being a very poor substrate for platelet cyclooxygenase and thromboxane A2 formation but being more easily metabolized by endothelial cells to PGI2 and thromboxane B2, the metabolites of TXB2 (derived from EPA) in serum and urine, respectively, are only about 5% of the levels of TXB2 and 2,3-dinor-thromboxane B2 (metabolites of TXA2, derived from arachidonic acid), even after feeding of very large doses (up to 50 grams per day) of fish oil. Thus, any shift of three series (EPA-derived) endoperoxides from the platelet to the endothelial cell should be minimal. Therefore, if the ASA-induced decline in endothelial cell prostacyclin production is due largely to the loss of the supply of endoperoxides from platelets, then PGI2 production should decline far more than PGI1 production. If, on the other hand, the ASA-induced inhibition of PGI2 production is due primarily to endothelial cell cyclooxygenase inhibition, then PGI1 production should decline proportionally to PGI2. We report herein our findings on the effects of the combination of fish oil and increasing doses of ASA on the eicosanoid profile of 14 patients with coronary artery disease.

Methods

Patient Population and Study Design

Fourteen men and two women, nonsmoking patients (ages, 49–67 years) with coronary artery disease documented by cardiac catheterization, were entered into the study. All had suffered a myocardial infarction and four had undergone coronary artery bypass surgery. None had any cardiac events within the year before entry into the study.

All patients were withdrawn from ASA-containing products for a minimum of 3 weeks. Patients were then begun on 12 or 16 g of fish oil concentrate per day (Promega capsules, provided by Warner-Lambert Co., Morris Plains, N.J.), which supplies 6 or 8 g of n-3 polyunsaturated fatty acids, of which 4.2 or 5.6 g are EPA. After 6 weeks on the fish oil alone, patients received ASA in increasing doses in addition to the fish oil. Patients took 50 mg ASA in a once-daily dose for 2 weeks followed by 100 mg and 325 mg (each dose for two weeks) and finally 325 mg four times daily for 1 week.

The following fasting laboratory data were collected at baseline (before the fish oil), after 6 weeks on fish oil alone, after 2 weeks on the 50 mg, 100 mg, and 325 mg doses of ASA, and after 1 week on the 325 mg four-times-a-day dose: complete blood count including platelet count, serum TXB2, and 24-hour urine collections for urinary eicosanoids. Red blood cell and plasma phospholipid fatty acid analyses were performed on samples taken at baseline and at week 6, week 12, and week 13 (the end) of the study.

Laboratory Studies

Serum Thromboxane B2. Three milliliters of blood was drawn from a peripheral vein into a glass tube and incubated at 37°C for 60 minutes. The sample was then centrifuged at 2,000g for 15 minutes and stored at −20°C until acidic organic extraction and radioimmunoassay with the use of a sensitive and specific antibody (gift of Dr. L. Levine, Brandeis University, Waltham, Mass.) with a limit of detection of 0.8±0.13 pg per sample.

Urinary Thromboxane Metabolites. Urinary excretion of 11-dehydro-TXB2, the major urinary metabolite of TXB2, was analyzed with a stable isotope dilution assay by negative ion–chemical ionization gas chromatography/tandem mass spectrometry (GC/MS/MS). Urine samples from 24-hour collections were centrifuged and 2 ng of tetradeuterated 11-
dehydro-TXB₂ was added to a 2-ml aliquot of the supernatant. Samples were extracted on a phenyl boronate matrix, and the pentfluorobenzyl ester–trimethylsilyl ether derivative was then injected into a Finnigan TSQ 70 triple-stage GC/MS/MS operated in the negative chemical ionization mode. Fragments m/z 243/247 of the parent ion m/z 511/515 were monitored in the multiple ion detection mode for quantification.

**Urinary Prostacyclin Metabolites.** Urinary excretion of 2,3-dinor-6-ketoprostaglandin F₁₀ (PGI₂-M) and Δ17-2,3-dinor-6-ketoprostaglandin F₁₀ (PGI₁-M), the major urinary metabolites of PGI₂ and PGI₁, respectively, which are derived from arachidonic acid (AA) and EPA, respectively, was analyzed with a stable isotope dilution assay by negative ion–chemical ionization GC/MS.³⁸ Fifty milliliters of urine was extracted by the method of Falardeau et al.,³⁸ after addition of tetradeuterated PGI₂-M. Pentfluorobenzyl ester–methoxime-1 trimethylsilyl ether derivatives were prepared, and fragments m/z 586 and 584 of the endogenous PGI₂-M and PGI₁-M, respectively, were monitored as well as fragments m/z 590 of the tetradeuterated standard.

**Phospholipid Fatty Acid Analyses.** For determination of red blood cell and plasma phospholipid fatty acids,⁴⁴ lipids were extracted with chloroform–methanol (2:1), and the organic phase was evaporated to dryness under N₂. Samples were redissolved in chloroform and partitioned on a silicic acid column. The

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**FIGURE 1.** Schematic of the metabolism of eicosapentaenoic acid (EPA) and arachidonic acid (AA) via the cyclooxygenase (CO) pathway in the platelet and the endothelial cell of patients receiving fish oil supplementation, and possible mechanisms of aspirin (ASA)-induced reduction in PGI₂. With fish oil feeding, platelet and endothelial cell membrane phospholipid content of EPA increases and that of AA decreases modestly. Upon platelet activation, AA and EPA are released from membrane phospholipids and the platelet CO catalyzes their conversion to their respective 2- and 3-series prostaglandin endoperoxides (PGEPs). Thromboxane synthase converts PGEPs to thromboxane A₂ and A₃ (TxA₂ and TxA₃). These unstable eicosanoids are rapidly converted to the stable (and inactive) metabolites TxB₂ and TxB₃ and subsequently to a number of urinary metabolites including the 11-dehydro thromboxanes. In the endothelial cell, CO catalyzes the conversion of AA and EPA to the same 2- and 3-series PGEP intermediates as in the platelet, and prostacyclin synthase converts these to PGI₁ and PGI₃, respectively. Transcellular metabolism or shift of endoperoxides from activated platelets to the endothelial cell might occur at the level of the horizontal arrow. Thus, ASA-induced reduction in PGI₂-production might be secondary to a reduction in shift of 2-series PGEPs (labeled A) and/or reduced endothelial cell CO activity (labeled B). Relative proportions of the EPA- and AA-derived CO products are indicated by the sizes of the arrows. PGI₂-M and PGI₁-M are the main urinary metabolites of PGI₂ and PGI₁.
TABLE 1. Red Blood Cell Phospholipid Fatty Acid Content

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<th>Weeks of study</th>
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<tr>
<td>Arachidonic acid (%)</td>
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<tr>
<td>Eicosapentaenoic acid (%)</td>
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Values are percent of total phospholipid fatty acids.

Table: Phospholipid eluate was evaporated and redissolved in methanol with C17:0 and C20:3, n-6 added as standards. After methylation with methanolic hydrochloric acid, fatty acids were analyzed with a Hewlett-Packard 5890A gas chromatograph. Fatty acids were identified by comparison of retention times and quantified with the standards using a Hewlett-Packard 3393A integrator. Results are expressed as percent of total fatty acids.

**Statistical Analyses.** Results from the patients on 12 g of fish oil did not differ compared with those on 16 g; therefore, the data from the two groups are presented and analyzed together. Variables were compared by using the paired t test or Wilcoxon matched-pairs signed-ranks test. Differences were considered significant when probability was less than 0.01 after Bonferroni correction for multiple comparisons. Analysis of variance with repeated measures was used to compare the effect of the four different doses of aspirin on the various laboratory studies (BMDP 2v, Department of Biometrics, University of California at Los Angeles, 1990). Values are expressed as mean±SEM.

**Results**

After 6 weeks of fish oil supplementation, red blood cell phospholipid content of C20:4w6 (AA), the precursor of TXA2 from platelets and PGI2 from endothelial cells, decreased from 14.5±1.1% to 11.3±0.5% (p=0.07). AA content did not change throughout the remainder of the study (p=0.20, Table 1). Red blood cell phospholipid content of C20:5w3 (EPA, the precursor of TXA2 and PGI2) increased from 0.3±0.1% at baseline to 2.6±0.4% after 6 weeks of fish oil (p<0.001). EPA content did not change throughout the remainder of the study (p=0.70, Table 1). Red blood cell phospholipid content of EPA as a percent of content of AA increased from 2% to 26% during the study. Changes in plasma phospholipid fatty acids were similar (Table 2). These data confirmed that patients were compliant with the fish oil regimen. Furthermore, unstable levels of AA and EPA after the sixth week of the study could not have accounted for the changes in eicosanoid production described below. Serum TXB2 production in clotted whole blood (which reflects maximal ability of the platelets to produce TXA2) fell to 63±14% of control (p<0.01) after 6 weeks of fish oil supplementation alone (Figure 2). On 50 mg per day of ASA, serum TXB2 production was suppressed to 3.2±1.0% of control (p<0.008). On 325 mg per day of ASA, serum TXB2 production was less than 1% of control (p=0.03 versus value on 50 mg per day) and was undetectable in most patients (Figure 2).

In vivo production of TXA2, as estimated by urinary 11-dehydro-TXB2 excretion, fell insignificantly with the fish oil supplementation alone (598±77 ng/g versus 535±61 ng/g of creatinine) (Figure 3). On 50 mg per day of ASA, urinary 11-dehydro-TXB2 excretion fell to 168±23 ng/g of creatinine (p=0.001). Further increases in the ASA dosage to 100 mg per day and 325 mg per day led to further slight reductions in urinary 11-dehydro-TXB2 of borderline statistical significance (p=0.03) (Figure 3). However, on 325 mg four times daily, excretion of 11-dehydro-TXB2 did decline significantly further to 81±9 ng/g of creatinine (p<0.001 versus value at time E depicted in Figure 3). Because serum TXB2 production by platelets was suppressed to less than 1% of control values on 325 mg per day of ASA, this further decline on 325 mg four times daily probably represents suppression of thromboxane production by nonplatelet sources that may be more resistant to the effects of ASA.

PGI2 production, as determined by PGI2-M excretion, fell slightly on the fish oil alone (50±4 to 42±2 ng/g creatinine, p=0.02) (Figure 4, left panel). This decline was partially offset by the EPA-induced rise in PGI2 production (0.2±0.1 to 4.9±0.7 ng/g of creatinine, p<0.001) (Figure 4, right panel).

With institution of 50 mg per day of ASA, PGI2-M excretion fell significantly from 42±2 to 26±2 ng/g creatinine, 62% of the value on fish oil alone (p<0.001) (Figure 4, left panel). PGI2-M excretion did not change further as the dose of ASA was increased.

TABLE 2. Plasma Phospholipid Fatty Acid Content

<table>
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<td>Arachidonic acid (%)</td>
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<tr>
<td>Eicosapentaenoic acid (%)</td>
<td>1.1±0.4</td>
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Values are percent of total phospholipid fatty acids.
Discussion

The purpose of this study was to determine whether endoperoxide shift contributes significantly to the increased production of PGI2 in patients with coronary artery disease and whether the ASA-induced reduction in PGI2 production in these patients is due to a decrease in the supply of prostaglandin endoperoxides from activated platelets to the endothelial cells (reduced endoperoxide shift) or to a direct inhibition of vessel wall cyclooxygenase. We used EPA as a “probe” to further clarify the reasons for the reduction in PGI2-M with ASA. Our data suggest that the majority of the low-to-moderate-dose, ASA-induced fall in PGI2-M excretion in patients with atherosclerosis is due not to inhibition of endothelial cell cyclooxygenase but to a reduction in endoperoxide shift from platelets to endothelium. When we gave 50 mg, 100 mg, or 325 mg of ASA per day to patients who had received 6 weeks of EPA supplementation, PGI2-M excretion was reduced by nearly 40%. Despite this marked decrease in PGI2-M excretion, PGI2-M excretion did not change. If this suppression of PGI2-M excretion had been due primarily to inhibition of endothelial cell cyclooxygenase, there should have been an equivalent reduction in mean PGI2-M excretion of 2 ng/g of creatinine, a change that we should have detected easily with our assay.

The belief that a significant percentage of the ASA-induced reduction of PGI2 production is due to direct inhibition of endothelial cell cyclooxygenase has led to several studies of the effects of low-dose ASA or long dosing intervals on eicosanoid profiles. Specifically, these studies were designed to find an optimal dose of ASA that would maximally inhibit TXA2 production while minimizing the reduction in PGI2.18–24 Our data suggest that because the moderate-dose, ASA-induced reduction in PGI2 production in patients with coronary artery disease is largely due to decreased endoperoxide shift from platelets, it is
unavoidable if the goal of treatment is to suppress platelet cyclooxygenase activity. Endoperoxide shift occurs in vitro between platelets and endothelial cells,\textsuperscript{29,29} subendothelial smooth muscle cells,\textsuperscript{31} and lymphocytes.\textsuperscript{30} The endoperoxide shift hypothesis suggests that more frequent platelet–vessel wall interactions in vivo will supply greater amounts of endoperoxides (PGH\(_2\)), the precursor of TXA\(_2\) and PGI\(_2\)), to the endothelial cell, and consequently, PGI\(_2\) production will increase. This hypothesis is supported by the fact that PGI\(_2\) production is elevated in patients with active atherosclerosis.\textsuperscript{20} When the platelet is no longer able to produce endoperoxides after cyclooxygenase acetylation with ASA, PGI\(_2\) production falls markedly in these patients. One would expect, and Knapp et al\textsuperscript{22} have confirmed, that PGI\(_2\) production would be lower and fall much less dramatically in response to ASA in young, healthy patients (with fewer platelet–vessel wall interactions and therefore less opportunity for endoperoxide shift) than in patients with atherosclerosis.

Our data that suggest minimal ASA-induced suppression of endothelial cell cyclooxygenase activity must be reconciled with those in vitro studies of vascular endothelial cells in culture and ex vivo studies of vascular segments that have shown profound and prolonged (lasting up to 48 hours) inhibition of cyclooxygenase activity and suppression of PGI\(_2\) synthesis.\textsuperscript{14,18,19} Such profound suppression has not been a universal finding, however. \textsuperscript{18} Czervionke et al,\textsuperscript{18} studying primary cultures of human umbilical vein endothelium, demonstrated significant recovery of thrombin-stimulated synthesis of PGI\(_2\) within 2 hours of removing ASA from the culture medium. Recovery of PGI\(_2\) synthesis was inhibited by cycloheximide. Thus, significant resynthesis of the proteins necessary for PGI\(_2\) production begins quite rapidly after ASA. Because the ASA elimination half-life in humans may be as little as 20 minutes,\textsuperscript{45} new protein synthesis could theoretically lead to a relatively rapid recovery of PGI\(_2\) production. The reasons for these disparate findings are not clear. Different methodologies may be partly responsible. In those studies showing prolonged suppression of PGI\(_2\) synthesis, PGI\(_2\) production was stimulated by exogenous addition of AA as opposed to agonist-stimulated synthesis of PGI\(_2\) from AA of endogenous phospholipids. More importantly, whole-vessel estimates of the rate of return of PGI\(_2\) production may overestimate the duration of the suppression of PGI\(_2\) synthesis by endothelial cells because much of the PGI\(_2\) synthesized by these vessel fragments comes from nonendothelial tissues, which appear to resynthesize cyclooxygenase/PGI\(_2\) synthase more slowly.\textsuperscript{21} Weksler et al\textsuperscript{21} used a method that excludes the contribution of vascular smooth muscle cells in the assay of production of PGI\(_2\) by saphenous veins to demonstrate rapid return of PGI\(_2\) production after removal of ASA and concluded that cyclooxygenase turnover in endothelium is quite rapid. Another study, in subjects given a single 600-mg dose of ASA, demonstrated full recovery of maximally stimulated (by bradykinin) PGI\(_2\) synthesis within 6 hours and 50% recovery at 3 hours.\textsuperscript{46} Some recovery was evident as early as 90 minutes after the ASA dose. Because we found no decline in PGI\(_1\) synthesis, our data support the conclusion that moderate-dose, ASA-induced suppression of endothelial cell cyclooxygenase activity is short-lived.
Our results have implications for the use of ASA in patients with vascular disease. Because much of the fall in PGI₂ production appears to be secondary to inhibition of platelet cyclooxygenase and reduced supply of endoperoxides, the use of a low, “PGI₂-sparing” dose of ASA may be less important than maximal suppression of platelet thromboxane production, especially because there is some evidence that more complete suppression of TXA₂ production (greater than 99%) leads to less platelet aggregability than does less complete (approximately 95%) suppression. Thus, a mid-range dose of ASA (100–325 mg per day) might be more protective for patients with unstable coronary syndromes in whom thromboxane production is markedly elevated. Maseri has pointed out that the capacity of platelets to generate thromboxane is so great that if this capacity is not nearly completely abolished, there might be sufficient residual cyclooxygenase activity to generate significant TXA₂ concentration locally at a site of marked platelet activation.

Conclusions
Concurrent ASA therapy does not negate the potentially beneficial effects of n-3 fatty acid supplementation. That is, EPA metabolism through endothelial cell cyclooxygenase is not sufficiently inhibited by even moderate-dose ASA to reduce PGI₂ production in patients with chronic coronary artery disease. A large percentage of the PGI₂ produced by patients with atherosclerosis derives from endoperoxides supplied to the endothelial cell by activated platelets, and the loss of these substrates accounts for the majority of the reduction in PGI₂-M excretion with moderate doses of ASA.

References


KEY WORDS • arachidonic acid • eicosapentaenoic acid • cyclooxygenase • thromboxane
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