EFFECT OF EVENING PRIMROSE OIL ON PLATELET AGGREGATION IN RABBITS FED AN ATEROGENIC DIET

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Abstract Evening primrose oil (Oenothera biennis) is a rich source of ω-6 series fatty acids. We report here the effects of dietary supplementation with evening primrose oil (EPO) on platelet aggregation as the main factor in arterial thrombus formation in an experimental model of atherogenesis in rabbits. A total of 40 male white New Zealand rabbits were divided into four groups (n=10 animals/group): 1: normal diet, 2: atherogenic diet (ATD), 3: normal diet enriched with 15% EPO, 4: ATD + EPO. Each group was kept on the diet for 6 weeks. We determined serum lipid profile, platelet aggregation in whole blood, platelet thromboxane B2 production and platelet lipid peroxides. The atherogenic diet increased platelet aggregation (135% when ADP was used, and 185% when collagen was used as the inducer). Evening primrose oil reduced hyperaggregation to the values obtained in rabbits fed with the normal diet. Thromboxane synthesis was increased from 0.18 to 2.28 nmol/10⁹ platelets); EPO reduced this value to 1.38 nmol/10⁹ platelets. Lipid peroxides were increased by ATD from 0.27 to 0.81 nmol/10⁸ platelets; EPO prevented this increase (0.35 nmol/10⁸ platelets). In conclusion, EPO reduced platelet hyperaggregability in rabbits fed an atherogenic diet.

One of the most influential nutritional factors in arteriosclerosis is the consumption of a diet rich in saturated fatty acids (SFA) [1]. These compounds can increase plasma cholesterol [2] and alter platelet-vascular wall interactions [3]. Imbalances in the levels of SFA and unsaturated fatty acids (UFA), together with their pathogenetic consequences, can be corrected by adding direct precursors of UFA to the diet [4].

Evening primrose oil (Oenothera biennis) is a rich source of ω-6 series fatty acids [5], and has shown therapeutic effects in some skin diseases and in diabetic neuropathy [4,6-8]. This oil supplies ω-linoleic acid, a product that increases the production of series 1 prostaglandins; these in turn display antithrombotic activity [4].

Key words: Atherosclerosis, platelet aggregation, thromboxane, unsaturated fatty acid.
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We report here the effects of dietary supplementation with evening primrose oil (EPO) on platelet aggregation as the main factor in arterial thrombus formation, in an experimental model of atherogenesis in rabbits.

MATERIAL AND METHODS

Animals
A total of 40 male white New Zealand rabbits were used. The animals were 2 months old at the start of the experiment, and had a mean body weight of 2498 ± 36 g. The rabbits were randomly divided into four groups of 10 animals each. Group 1 animals (controls) were fed a normal diet (Lapin Entretien Ref/112, Laboratorios Panlab SL, Barcelona). Group 2 animals (atherogenic diet) received an atherogenic diet (fat-enriched diet supplemented with 1.33% cholesterol, Laboratorios Panlab SL). Group 3 animals (EPO diet) were given the normal diet enriched with 15% EPO (Oikos Pharmaceuticals, Fuengirola, Málaga, Spain). Group 4 animals (atherogenic diet + EPO) were fed with the atherogenic diet enriched with 15% EPO. Evening primrose oil was used because of its high content in linoleic acid. Table 1 shows the percentage of different fatty acids in each of the four diets tested, and of EPO alone. Each group was kept on the diet for 6 weeks. Food intake was recorded periodically to avoid differences between groups in the amount of feed consumed. The EPO-enriched diet was prepared fresh daily. The changes in body weight during the experiment are shown in Figure 1.

The study was carried out in a simple blind fashion, i.e., the persons responsible for each set of experiments were unaware of the which group the samples were from (type of diet, serum, blood, etc). The study protocol was approved by the University of Málaga Ethical Committee for animal care.

TABLE 1

Percentages of Fatty Acids Referred to the Total Amount of Food, in Different Diets and in Evening Primrose Oil.

<table>
<thead>
<tr>
<th></th>
<th>Normolipemic Diet (NLD)</th>
<th>Atherogenic Diet (ATD)</th>
<th>NLD+EPO</th>
<th>ATD+EPO</th>
<th>EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fat</td>
<td>2.10</td>
<td>12.17</td>
<td>20.30</td>
<td>19.80</td>
<td>100</td>
</tr>
<tr>
<td>MUFA</td>
<td>16.79</td>
<td>30.55</td>
<td>12.00</td>
<td>15.72</td>
<td>8.91</td>
</tr>
<tr>
<td>SFA</td>
<td>25.17</td>
<td>35.79</td>
<td>10.15</td>
<td>15.04</td>
<td>7.60</td>
</tr>
<tr>
<td>PUFA</td>
<td>51.45</td>
<td>27.67</td>
<td>73.93</td>
<td>55.41</td>
<td>81.41</td>
</tr>
<tr>
<td>SFA/PUFA</td>
<td>0.48</td>
<td>1.29</td>
<td>0.13</td>
<td>0.27</td>
<td>0.09</td>
</tr>
</tbody>
</table>

EPO: evening primrose oil; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids.
Sample collection
The animals were anesthetized with a subcutaneous injection of thalamonal (0.5 mL/kg body weight), ketamine (50 mg/kg body weight) and midazolam (2.5 mg/kg body weight). A medial laparotomy was made to obtain blood from the inferior vena cava. In some aliquots coagulation was prevented with 3.8% trisodium citrate (1:10), and 5 mL of blood was kept at 37°C for 45 min, then centrifuged at 4500 x g for 15 min at 4°C; serum was frozen at -80°C until the day of the experiment.

Analytical techniques
To determine the basic serum lipid profile we used standard spectrophotometric techniques to measure the concentration of total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides.

Platelet aggregometry. We determined platelet aggregation in whole blood with the electrical impedance method [9] in a Chrono-Log model 540S aggregometer (Chrono-Log Corp., Haverton, PA, USA). Aggregation was induced with different concentrations of adenosine diphosphate (ADP) or collagen (Menarini Diagnostica, Barcelona, Spain). In each experiment the change in electrical impedance was recorded 10 min after the aggregant was added. Concentration-effect curves were drawn from the percentage aggregation obtained at each concentration of inducer, and the concentration of inducer that produced 50% of the maximal aggregation (EC50) was calculated.

![Graph of body weight changes](image)

Changes in body weight during the experiment in normal rabbits (open symbols) or rabbits fed an atherogenic diet (solid symbols), without (circles) or with (triangles) 15% supplementation with evening primrose oil.
Platelet production of thromboxane B₂. Thromboxane B₂ (stable metabolite of TxA₂) was measured by radioimmunoassay (Amersham International plc, Little Chalfont, Buckinghamshire, England, with [³H]thromboxane B₂). The sample of whole blood without anticoagulant was placed in a bath at 37°C for 45 min and then centrifuged at 4500 x g at 4°C for 15 min. The serum was removed and kept frozen at -80°C until analysis. To assess the possible influence of platelet number on platelet TxB₂ production, we used the formula described by Carter & Hanley [10]:

\[ \text{TxB₂ (nmol/10⁹)} = \text{TxB₂ (nmol/L)} \times \frac{1-(\text{hematocrit/100})}{\text{platelet number (cells x 10⁹/L)}} \times 10⁹. \]

Enzymatic lipid peroxidation. Platelet-rich plasma was used to measure enzymatic lipid peroxidation [6] by incubating samples at 37°C with 200 μmol/L arachidonic acid, and then adding 100 μmol/L indomethacin 5 min after the arachidonic acid. Then 500 μL of 0.5% thiobarbituric acid in 20% trichloroacetic acid was added, and the sample was centrifuged at 10 000 x g for 3 min. The supernatant was separated and incubated at 100°C for 15 min, and absorbance was measured spectrophotometrically at 532 nm. Lipid peroxides (main component malondialdehyde) were calculated by comparing the results with a standard curve prepared with malondialdehyde-bis-diethyl-acetal.

Fatty acids were quantified by gas chromatography. Samples were previously dried, methylated and extracted with hexane, then injected into a Hewlett-Packard Model 5890 (Avondale, PA, USA) gas chromatograph.

Statistical analysis

All results were expressed as the mean ± standard error of the mean (SEM). The groups were compared with analysis of variance, and significance of the differences was determined with Bonferroni correction. All analyses were done with the Statistical Package for Social Sciences (SPSSx, version 3.0, Microsoft, Seattle, WA, USA).

RESULTS

Effect of the atherogenic diet

Feeding with a diet rich in SFA led to mixed hyperlipemia: hypercholesterolemia and hypertriglyceridemia (Table 2). The levels of HDL-cholesterol decreased.

Platelet aggregation in whole blood was increased in hyperlipemic rabbits regardless of whether ADP or collagen was used as the inducer (Fig. 2). The EC₅₀ values were significantly lower in hyperlipemic animals (Table 3).

Platelet production of thromboxane B₂ and malondialdehyde (Table 4) were nearly 30-fold greater in hyperlipemic animals than in controls.
TABLE 2.
Basic Lipid Profile in the Different Groups of Animals.

<table>
<thead>
<tr>
<th></th>
<th>Normolipemic diet (NLD)</th>
<th>NLD+EPO</th>
<th>Atherogenic diet (ATD)</th>
<th>ATD+EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>83 ± 13</td>
<td>82 ± 14</td>
<td>1918 ± 195^a</td>
<td>1365 ± 149^b</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>110 ± 20</td>
<td>105 ± 12</td>
<td>560 ± 75^a</td>
<td>276 ± 34^b</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dL)</td>
<td>29 ± 5</td>
<td>30 ± 5</td>
<td>19 ± 2^a</td>
<td>31 ± 4^b</td>
</tr>
</tbody>
</table>

EPO: evening primrose oil. ^p=0.05 vs NLD; ^b=0.05 vs ATD.

Effect of evening primrose oil
The administration of EPO to hyperlipemic rabbits reduced cholesterol values by 25%, reduced triglyceride levels by 51%, and increased HDL-cholesterol by 64% (Table 2).

The concentration dependence of platelets in hyperlipemic animals treated with EPO was significantly reduced (Fig. 1). The EC50 values in these animals were nearly double those in rabbits that were not given EPO (Table 3).

TABLE 3.
Concentration of Inducers that Produced 50% of the Maximal Platelet Aggregation in Whole Blood (EC50).

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Normolipemic diet (NLD)</th>
<th>NLD+EPO</th>
<th>Atherogenic diet (ATD)</th>
<th>ATD+EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (μmol/L)</td>
<td>2.54 ± 0.23</td>
<td>2.39 ± 0.84</td>
<td>1.08 ± 0.18^a</td>
<td>2.50 ± 0.49b</td>
</tr>
<tr>
<td>Collagen (µg/mL)</td>
<td>0.57 ± 0.07</td>
<td>0.50 ± 0.20</td>
<td>0.20 ± 0.04a</td>
<td>0.39 ± 0.05b</td>
</tr>
</tbody>
</table>

EPO: evening primrose oil. ^p=0.05 vs NLD; ^b=0.05 vs ATD.
Platelet Production of Thromboxane B₂ (TxB₂) and Malondialdehyde (MDA).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normolipemic diet (NLD)</th>
<th>NLD+EPO</th>
<th>Atherogenic diet (ATD)</th>
<th>ATD+EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>TxB₂ (nmol/10⁸ platelets)</td>
<td>0.18 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>2.28 ± 0.17ᵃ</td>
<td>1.38 ± 0.15ᵇ</td>
</tr>
<tr>
<td>MDA (nmol/10⁸ platelets)</td>
<td>0.27 ± 0.01</td>
<td>0.21 ± 0.02</td>
<td>0.81 ± 0.06ᵇ</td>
<td>0.35 ± 0.04ᵇ</td>
</tr>
</tbody>
</table>

EPO: evening primrose oil. ᵃp<0.05 vs NLD; ᵇp<0.05 vs ATD.

Platelet production of thromboxane B₂ was significantly reduced, although it was not as low as in normolipemic animals. Lipid peroxide production was inhibited, with values approaching those in normolipemic rabbits (Table 4).

FIG. 2.

Concentration-dependent curves of platelet aggregation induced by ADP or collagen after 6 weeks on a normolipemic (open symbols) or hyperlipemic (solid symbols) diet, without (circles) or with (squares) 15% supplementation with evening primrose oil.
DISCUSSION

Our findings show that the platelet hyperactivity caused by feeding with a diet rich in SFA was reduced when 15% EPO was added to the diet. Platelet hyperaggregability in hyperlipemic states has been documented in several studies [11-14]. Increased thromboxane synthesis [13] was reported in patients with hypercholesterolemia, and some authors have ascribed an important role in platelet hyperaggregability to oxidized low-density lipoproteins [15,16].

The reduction in platelet aggregability in hyperlipemic rabbits did not affect normal platelet reactivity. Horrobin et al. [17] suggested that EPO might show this behavior, and postulated that ω-6 polyunsaturated acids might complement supplementary metabolic pathways when these are altered in certain disorders, while leaving these pathways unaffected under normal circumstances.

Evening primrose oil may inhibit platelet functioning by reducing hyperlipemia, or by increasing prostaglandin synthesis [17,18]. The prostaglandin PGE\textsubscript{1} stimulates adenyl cyclase, and thus increases cAMP. However, we cannot rule out an effect, however small in quantitative terms, on thromboxane synthesis (Table 4). Several authors have demonstrated that dietary supplementation with ω-3 polyunsaturated fatty acids [19-21] or certain components of monounsaturated fatty acids [22] reduces platelet aggregation, mainly through metabolic competition between the different prostanoid pathways, which reduces thromboxane A\textsubscript{2} synthesis and increases other prostaglandin series (thromboxane A\textsubscript{3} and prostaglandin I\textsubscript{3}) with a weaker ability to favor aggregation. Dietary supplementation with EPO may act through PGE\textsubscript{1}, a common prostaglandin in platelets, whereas series 3 prostaglandins are infrequent under physiological conditions.

The ability of EPO to reduce lipid peroxide production (Table 4) in compatible with earlier reports from our laboratory that the administration of EPO to hyperlipemic rabbits reduced the tissue lipid peroxide production induced by free radicals, and enhanced the antioxidant effect of glutathione [23]. In view of the ability of lipid peroxides to modulate prostaglandin synthesis [24], this effect may also play a role in the ability of EPO to curtail platelet function in hyperlipemic animals. Because 400 mg/day of vitamin E was administered to each rabbit in both the normal and atherogenic diets supplemented with EPO, the presence of vitamin E in the EPO preparation might have played an important role in this antiperoxidizing effect.

In conclusion, EPO reduces platelet hyperaggregability in rabbits fed an atherogenic diet. This effect may constitute the basis for further research on ways in which EPO, alone or in association with other platelet antiaggregants, might prevent thrombotic complications arising from arteriosclerosis.

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REFERENCES


