Influence of Dietary Fats on Blood Coagulation and Prostaglandin Production in the Common Marmoset

Graeme H. Mcintosh, Edward J. McMurchie, Michael James, Carolyn A. Lawson, Felicia H. Bulman, and John S. Charnock

Common marmosets were fed a standard marmoset diet (REF) or diets supplemented with 12% (wt/wt) sunflower seed oil (SSO) or sheep fat (SF) for a period of 90 weeks. The values for coagulation indices, clotting time, and Russel viper venom time were consistent with decreased thrombotic tendency of platelets from animals on the SSO diet relative to the low fat, REF diet animals, while an increased tendency to thrombosis was observed with SF-fed marmosets. The SSO- and SF-supplemented marmosets showed a significantly reduced thromboxane (TXB\textsubscript{2}) generation from platelets aggregating to collagen (ASC) relative to the REF group, while at 50 \(\mu\text{g/ml}\) ASC this difference was maintained only by the SSO group. The SF diet-fed marmosets showed a reduced prostacyclin (measured as 6-keto-PGF\textsubscript{1\alpha}) generation from incubated aorta relative to the REF or SSO-fed groups, which were not different from each other. A reduced proportion of platelet phospholipid arachidonic acid (20:4, n-6) and increased \(\alpha\)-tocopherol concentration was consistent with the decreased aggregability and thromboxane generation of platelets from SSO-fed marmosets relative to the REF and SF groups. The SF diet-fed marmosets, on the other hand, showed minimal change in arachidonic acid, \(\alpha\)-tocopherol or platelet reactivity from the REF group. These differing responses to dietary fats are discussed in relation to the potential for the development of thrombosis and atherosclerosis.

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The influence that dietary fats have on thrombogenic factors and atherosclerosis has been reviewed.\textsuperscript{1-5} A significant component of thrombogenesis is contributed by the platelet and its reactivity, which has been shown to be subject to the influence of dietary fatty acids. Dietary fats can be identified as antithrombogenic, neutral, or thrombogenic depending on the way they influence platelet function and coagulation factors. Much of the work characterizing these influences has used laboratory rats, although they are a poor animal model for the study of atherosclerosis,\textsuperscript{6} because they metabolize fats differently from animal models functionally and phylogenetically closer to humans.\textsuperscript{7,8}

Previous studies\textsuperscript{9} have shown that the marmoset is a good model for studying atherosclerosis because blood coagulation and platelet aggregation responses are similar to those observed in humans\textsuperscript{10} and the fatty acid profile of cardiac membrane phospholipids undergo significant changes under the influence of sheep fat (SF) and sunflower seed oil (SSO) dietary fat supplements\textsuperscript{11,12} The aim of this study was to examine the influence of altered dietary fat saturation on platelet composition and function in the common marmoset (Callithrix jacchus) when it was fed dietary SSO and SF supplements for 90 weeks. The influence of dietary fat supplements on coagulation indices, platelet aggregation (using both collagen and ADP as aggregating agents), thromboxane generation from platelets, and prostacyclin from aorta provides a good basis for assessing thrombogenic risk associated with differing dietary fats.\textsuperscript{13} The prostanoid thromboxane has a proaggregatory influence on platelets, whereas prostacyclin from aorta provides a good basis for assessing thrombogenic risk associated with differing dietary fats.\textsuperscript{13} The proportion of platelets is a useful marker of thrombogenic potential.\textsuperscript{14,15}

Methods

Animals

Two groups of 18 male common marmosets about 12 months of age and weighing 305 ± 18 g (so) at the start of the experiment were used in this replicate study. Common marmosets at this age are 80% to 90% of their mature size and have a life expectancy of 12 to 15 years. Each group was split into three treatment groups which were matched for age and weight. The animals were kept in pairs in aluminum cages at a temperature of 26°C and a humidity of 50%. Fluorescent lighting was provided for 12 hours a day with 30 minutes of ultraviolet light daily. The marmosets were fed the experimental diets for 90 weeks during which period they gained an average of 43 g each; there was no difference in weight gain between treatment groups.
**Diet**

The following three diets were fed for a period of 90 weeks: 1) the standard or reference (REF) marmoset diet, which contained 25% protein, 5% fat, and 3% fiber; 2) the REF diet supplemented (12% wt/wt) with sheep fat (SF); and 3) the REF diet supplemented (12% wt/wt) with sunflower seed oil (SSO). The REF diet was ground to a fine powder, fat was added with water, and the diet was mixed mechanically before being repelleted and dried for 18 hours at 45°C. The REF diet was treated similarly but without the addition of fat supplements. The major fatty acids, cholesterol, and the α-tocopherol content of the diets are shown in Table 1. As may be seen, the REF diet contained a mixture of fatty acids of animal and plant origin, the SF diet was a rich source of saturated fatty acids, and the SSO diet was rich in linoleic acid (18:2, n-6) and α-tocopherol. The three diets were considered adequate for the supply of essential fatty acids, the REF and SF diets having, respectively, 1.5% and 2.0% energy as linoleic acid thus exceeding the minimum requirement (1% energy) for primates. The percentage of total energy supplied as fat in the REF diet was 10% and for the two fat-supplemented diets was 32%. The marmosets ingested these diets at a rate of approximately 14.5 g daily for the REF diet and 12 g daily for the SSO and SF diets.

**Hematology**

Blood drawn from fasted, conscious marmosets via the femoral artery was added to trisodium citrate (0.129 M) in the proportion 1 part of citrate to 9 parts of blood. Coagulation indices were determined on freshly drawn blood using a Schnittner and Gross twin channel coagulometer as previously described. Clotting time, prothrombin time, and Russell viper venom time were determined using platelet-poor plasma prepared by centrifuging freshly drawn blood at 1000 g for 15 minutes. Platelet aggregation was undertaken on blood drawn from the femoral artery of conscious marmosets at 66 and 85 weeks on the diets. Platelet-rich plasma (PRP) was prepared from citrated whole blood by centrifugation at 160 g for 8 minutes at room temperature (22°C), and platelet aggregation was undertaken at 37°C using a dual channel Payton aggregometer and recorder as described previously. Acid-soluble collagen (ASC-Type 1 Bovine Achilles Tendon — Sigma Chemical Company) and adenosine diphosphate (ADP — Sigma Chemical Company) were used as aggregating agents for PRP. The stock collagen was dissolved in 6% acetic acid and was stored at 0° to 4°C, being diluted to working standards with normal saline on the day of assay. The ADP stock standard was diluted with Tyrode’s buffer. The threshold concentrations of agonists at which platelets completely aggregated, and the percentage aggregation for set concentrations of ADP (8.0 μM) and ASC (12.6 μg/ml) were used as the response parameters. Platelet counts were determined in PRP using a Coulter Counter Model F (Coulter Electronics Incorporated, Bedfordshire, U.K.), and were adjusted to 250,000 platelets μl⁻¹ with autologous platelet-poor plasma prior to assay. All aggregations were completed within 3 hours after bleeding. Whole blood platelet counts were measured with a Coulter Counter ‘S' model.

**Prostaglandin Assay**

Platelet thromboxane generation was assayed in association with collagen-induced aggregation of PRP after 3 minutes at 37°C, the reaction being terminated by the addition of 5.5N acetic acid to the aggregation cuvette. The stable form of thromboxane (TXB2) was measured by radioimmunoassay (RIA) after neutralizing the acidified sample with 8% KOH. Rabbit antiserum was used at a 1:5000 dilution. There was minimal (<0.05%) cross reactivity of antiserum with the other common prostaglandin metabolites, PGF1α, PGF2α, and PGE2. Details of the methodology for TXB2 and 6-keto-PGF1α assay have been published previously.

At the conclusion of the experiment, the marmosets were killed by saffan anesthesia (intramuscular alphaxalone alphadalone acetate, Glaxo Laboratories, Middlesex U.K.) and exsanguination. The thoracic segment of the descending aorta was removed and 2 mm aortic discs weighing 1 mg each were incubated for 30 minutes at 37°C in 0.5 ml Tyrode’s buffer containing glucose (1 mg/ml) before the reaction was stopped by the addition of 1N HCl. By this means prostacyclin was converted to the stable end-product 6-keto-PGF1α. A trace of 3H 6-keto-PGF1α was added as a recovery marker and the prostacyclin was extracted by use of ethyl acetate. After evaporation of the ethyl acetate, the residue was dissolved in Na2CO3 solution (1mM), and the 6-keto-PGF1α content was determined by RIA by use of rabbit antiserum as previously described. Cross reactivity of the antiserum at 50% binding of 6-keto-PGF1α was: PGE2, 1.6%; PGF2α, 5%; and TXB2, <0.2%.

**Platelet Phospholipid Fatty Acids**

Platelets were prepared from PRP by centrifugation at 1200 g for 15 minutes, followed by resuspension of the
packed cells in 1% ammonium oxalate to remove erythrocytes. Platelets were then washed and centrifuged three times in EDTA-phosphate buffered saline, and stored as a pellet at -80°C prior to lipid extraction. Lipid extraction, phospholipid separation, fatty acid methylation, and gas chromatography procedures have been reported previously.

**Plasma and Platelet α-Tocopherol**

Blood was collected using EDTA as the anticoagulant and was centrifuged at 1200 g for 15 minutes. Plasma was assayed for α-tocopherol with high pressure liquid chromatography with fluorimetric detection. Washed platelets were prepared as above and assayed using a similar methodology. After washing, platelets were resuspended in saline and counted using a Coulter Counter Model F. The platelet membrane α-tocopherol concentration was expressed per 10^9 platelets. Because a strong correlation exists between tocopherol and triglyceride concentration, plasma triglyceride was assessed using an enzymatic method (Boehringer Mannheim GmH), and the tocopherol concentration was expressed per milligrams of triglyceride. Plasma cholesterol was assayed by gas chromatography.

**Statistical Analysis**

The significance of the differences between means and standard errors of means for the various experimental parameters were assessed using Student's t test for each comparison.

**Results**

The influence of dietary fats on coagulation indices in the marmoset was assessed after 50 and 90 weeks (at sacrifice) on the experimental diets. The effect of dietary fats on coagulation was identical at both times and the results obtained at 50 weeks are shown in Table 2. There was a significant (p<0.05) increase in both clotting time (CT) and Russel viper venom time (RVVT) as a result of the SSO supplement relative to the SF- and REF-fed groups. The SF group was only slightly reduced (NS) relative to the REF group. The other three indices assayed showed no significant change with fat supplements, although fibrinogen (F) was 27% higher (NS) on the SF diet than on the SSO diet. The influence of the two fat-supplemented diets on platelet counts in whole blood and on platelet aggregation after 66 and 85 weeks on the diets is shown in Table 3. These times were chosen to allow sufficient time on the diet to avoid the influence of other procedures on platelet function. At 66 weeks there was a significant difference (p<0.01) between the two fat diets in the threshold concentration of collagen (ASC) required to produce platelet aggregation, with the SSO group requiring a higher concentration (+77%) and the SF group, a lower concentration of ASC (-33%) relative to the REF group. Although

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**Table 2. Blood Coagulation Indices in Male Marmosets Fed the Reference and Fat-Supplemented Diets for 50 Weeks**

<table>
<thead>
<tr>
<th>Indices</th>
<th>REF (n = 5)</th>
<th>SSO (n = 5)</th>
<th>SF (n = 5)</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting time (mins)</td>
<td>1.28 ±0.05</td>
<td>1.92 ±0.22</td>
<td>1.22 ±0.08</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Prothrombin time (secs)</td>
<td>9.25 ±0.32</td>
<td>9.55 ±0.38</td>
<td>8.81 ±0.20</td>
<td>NS</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (secs)</td>
<td>31.29 ±1.57</td>
<td>32.34 ±1.62</td>
<td>27.18 ±2.23</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen (ng/dl)</td>
<td>168.5 ±15.8</td>
<td>140.2 ±22.6</td>
<td>178.3 ±19.7</td>
<td>NS</td>
</tr>
<tr>
<td>Russel viper venom time (secs)</td>
<td>20.2 ±1.5</td>
<td>28.4 ±2.8</td>
<td>17.2 ±1.1</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

*Significance by Student's t test. NS, not significant.

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**Table 3. Platelet Aggregation at 66 and 85 Weeks for Platelet-Rich Plasma (250,000/μl) from Marmosets Fed Fat-Supplemented and Reference Diets**

<table>
<thead>
<tr>
<th>Platelet aggregation</th>
<th>REF (n = 6)</th>
<th>SSO (n = 6)</th>
<th>SF (n = 6)</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>66 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole blood platelet count (× 10^3/μl)</td>
<td>416 ±41</td>
<td>505 ±34</td>
<td>565 ±42</td>
<td>NS</td>
</tr>
<tr>
<td>ADP threshold (μM)</td>
<td>4.33 ±0.80</td>
<td>5.33 ±0.84</td>
<td>3.33 ±0.42</td>
<td>NS</td>
</tr>
<tr>
<td>Aggregation (%) at 8.0 μM</td>
<td>26.7 ±2.3</td>
<td>26.8 ±4.0</td>
<td>41.7 ±3.2</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>ASC threshold (μg/ml)</td>
<td>9.45 ±1.41</td>
<td>16.73 ±2.61</td>
<td>6.3 ±0</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Aggregation (%) at 12.6 μg/ml</td>
<td>72.5 ±3.5</td>
<td>59.6 ±6.9</td>
<td>79.2 ±4.0</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>85 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASC threshold (μg/ml)</td>
<td>11.55 ±1.05</td>
<td>17.56 ±3.04</td>
<td>9.45 ±1.41</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Aggregation (%) at 12.6 μg/ml</td>
<td>77 ±4.3</td>
<td>56 ±14.5</td>
<td>63 ±8.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Significance as assessed by Student's t test. NS, not significant.
Figure 1. The production of thromboxane measured as TXB₂ from ASC-activated platelet-rich plasma of marmosets fed the reference and fat-supplemented diets for 85 weeks. The significance was assessed using Student's t test. *p<0.05, ***p<0.001 for comparisons of the sheep fat (SF) and sunflower seed oil (SSO) diet-fed marmosets relative to the standard (REF) diet group. TXB₂ = thromboxane.

with ADP aggregation, changes were observed, the threshold differences did not achieve statistical significance, whereas with 8.0 μM ADP, a significant difference (p<0.05) in the percent aggregation was observed between SF- and SSO-fed marmosets. The platelet counts in whole blood were significantly higher (p < 0.05) for the SF-fed animals relative to the REF diet group, but SF counts were not different from the SSO-supplemented marmosets. At 85 weeks also there was a significant difference (p<0.05) in the threshold for collagen aggregation between the fat-supplemented animals, the SSO-fed group requiring a higher concentration relative to the REF-fed and the SF-fed groups.

Thromboxane was generated from PRP stimulated at three different concentrations of collagen. With both SSO and SF dietary groups there was a reduction in platelet thromboxane production for all levels of collagen used, and this was statistically significant at 25 μg/ml collagen for the SSO-fed (p<0.001) and SF-fed (p<0.05) groups relative to the reference group (see Figure 1). At 50 μg/ml

Table 4. Prostacyclin Generation from Incubated Aorta of Marmosets Fed Fat-Supplemented Diets for 90 Weeks

<table>
<thead>
<tr>
<th></th>
<th>REF</th>
<th>SSO</th>
<th>SF</th>
<th>SSO vs. REF</th>
<th>SF vs. REF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TXB₂</td>
<td>5.86 ± 0.56</td>
<td>6.14 ± 1.03</td>
<td>4.85 ± 0.32</td>
<td>0.05&lt;p&lt;0.10</td>
<td>NS</td>
</tr>
</tbody>
</table>

Prostacyclin was measured as 6-keto PGF₁α by radioimmunoassay and expressed as ng/30 min/disc of aorta. REF, reference diet; SSO, sunflower seed oil diet; SF, sheep fat diet. Values are expressed as the mean ± SEM.

*Significance was assessed by Student’s t test. NS, not significant.

The influence of the fat supplements on platelet phospholipid fatty acid and α-tocopherol composition at 60 weeks is shown in Table 5. The data agreed with a repeat analysis of platelets at 80 weeks and only the 60-week data is shown. There were significant differences between the fatty acid content of platelets from SSO- and SF-supplemented marmosets, for all but 18:0. There was a marked increase in the proportion of 18:2 n-6 and a decrease in 20:4 n-6 in the platelets from marmosets fed the SSO diet, while the n-3 fatty acids fell relative to the REF group. For the SF-fed marmosets, the proportions of 18:1 n-9, 20:3 n-6, 20:4 n-6, 20:5 n-3, and 22:5 n-3 rose relative to the SSO-fed group. In α-tocopherol in platelets of SSO-fed marmosets relative to the SF-fed marmosets, which were slightly lower (ns) than the REF group.

Plasma cholesterol levels at 90 weeks showed a significant difference (p<0.05) between the SF and SSO groups; the SSO group (123 ± 7 mg/dl (mean ± SEM, n = 10) fell 10% relative to the REF group (137 ± 8 mg/dl, n = 10), while the SF group exhibited a 6% increase (145 ± 6 mg/dl, n = 11) relative to the REF group. Plasma triglyceride levels determined in half the number of animals had decreased by 13% with the SSO feeding (44.5 ± 4.4 mg/dl, n = 5) relative to the SF-fed group (51.2 ± 6.5, n = 5), although this was not significant. The REF group was 50.4 ± 6.3, n = 5. Plasma α-tocopherol concentration expressed per milligram triglyceride was 18.4 ± 1.5 μg/mg (n = 5) for the SF group, 25.3 ± 1.8 (n = 5) for the SSO group, and 17.4 ± 2.8 (n = 5) for the REF group. The SSO group was significantly elevated (p<0.02) relative to the SF group.

Discussion

Isocaloric sunflower seed oil (SSO) and sheep fat (SF) supplements influenced blood coagulation and platelet ag-
The aggregating response to collagen with SSO feeding showed a large standard error relative to the other two dietary groups, and this may have influenced the reactivity of platelets in this group. This suppression of prostacyclin generating potential is similar to the influence of an atherogenic diet on rabbit aorta as reported by Gryglewski et al., but is at variance with the effect of butter on prostacyclin production observed in rabbits by Galli et al. The latter authors suggested, however, that the long-term effects of the diet might be different from the 3-week effect observed.

Platelet phospholipid fatty acids showed a reduction in arachidonate associated with a significant elevation of linoleate from SSO feeding. This effect has been observed in the phospholipids of the hearts of marmosets fed similar diets and in the pig (McMurchie and McIntosh, unpublished data), but it has not been observed in the rat. In this respect, the rat is markedly different from the marmoset and pig in fatty acid lipid metabolism. The inhibitory influence of dietary linoleate on thromboxane production and platelet aggregation observed by this study and in the rabbit by others may be explained in the reduced availability of arachidonate as a substrate for cyclooxygenase and lipoxygenase. However, other factors besides the concentration and availability of substrate have been proposed to have a significant influence on prostanooid production. The small change produced in fatty acids with the SF-fed group is probably indicative of both the similarity in dietary fatty acid composition relative to the REF diet, and the absence of essential fatty acid (EFA) deficiency. There was no increase in 20:3 n-9 in the plasma of the SF-fed marmosets relative to the SSO diet group. This fatty acid can significantly enhance coagulation and platelet aggregation. Also the dietary levels of linoleic acid (18:2 n-6) of all diets exceeded the 1% energy required to avoid the variable dietary level of vitamin E. Platelets are a useful measure of tocopherol status because they are less subject to day-by-day dietary variation than is plasma tocopherol concentration. This study showed clear evidence of increased platelet concentrations of α-tocopherol with
SSO feeding (26% over REF, and 54% over SF-fed animals) associated with a doubling of the dietary concentration of vitamin E with the addition of SSO. This may have contributed towards the inhibition of aggregation observed with this group because vitamin E both inhibits platelet aggregation at high concentrations and modulates prostacyclin/thromboxane production by its influence on prostanoid production. This study does not allow separation of these influences.

The influences of dietary fats on circulating plasma cholesterol and triglyceride were similar to those observed in the rat, in that an SSO-supplemented diet produced reductions in triglyceride and cholesterol, while an SF supplement produced a slight elevation in plasma cholesterol. The cholesterol content of platelet membranes might be expected to reflect these plasma changes and thereby influence platelet reactivity in the directions observed, but it was not measured in this study.

In summary, in marmosets there was a direct influence of the polyunsaturated SSO supplement in reducing the thrombogenic potential of the blood. Platelets were influenced in their aggregating and thromboxane generating potential in a similar direction. This may be due to the decreased availability of the precursor arachidonate in membrane phospholipids, to increased membrane α-tocopherol, or to both. Clotting factors were affected similarly. Feeding SF had an opposite effect which was less substantial in the changes produced relative to the low-fat reference group. Therefore, although the SF changed the various parameters in a direction expected to favor thrombogenesis, this effect was not as great as that of SSO in the opposite direction. A significant influence of the SF supplement on platelet function may have been the reduced prostacyclin production, although more work is required to confirm this observation and examine the likely cause. It is clear from this study in the marmoset that linoleic acid has an inhibitory influence on thrombogenesis and thus on the risk of coronary heart disease, as has been reported in studies using other species. Insofar as this study used a primate species fed fats commonly available in the Australian diet over a significant period of time, it represents a useful assessment of some of the potential risks to humans associated with these dietary fats.

Acknowledgments

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Index Terms: marmosets • dietary fats • coagulation indices • platelet aggregation • prostacyclin • thromboxane • α-tocopherol • fatty acids

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