Effect of dietary lipid on collagen- and adenosine diphosphate-induced platelet aggregation and thromboxane A_2 synthesis in the rat

Leonard A. Piché and Vhundi G. Mahadevappa

Cell Biology Research Group, Department of Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada

Dietary lipids containing different proportions of long-chain polyunsaturated fatty acids can affect platelet thromboxane A_2 formation and aggregation. In the present work, the effects of dietary lipid, from animal and vegetable sources, on collagen- and adenosine diphosphate (ADP)-induced thromboxane A_2 (measured as thromboxane B_2) production and aggregation in washed rat platelets were studied. In addition, plasma thromboxane B_2 levels in rats fed different dietary lipids were measured. Animals were fed 10% fat by weight as lard (LRD), corn oil, soy bean oil, canola oil (CAN), or cod liver oil (CLO) for a period of 7 weeks. Circulating thromboxane B_2 levels detected in platelet-poor plasma of the CLO-fed animals were significantly lower than those of rats fed all other dietary lipids. The platelets of CLO-fed animals synthesized significantly less thromboxane A_2 compared with those from other dietary groups following ex vivo stimulation of platelets with agonists such as collagen and ADP, with the exception of platelets from the LRD-fed animals. Ex vivo stimulation of platelets obtained from this group with collagen resulted in the synthesis of significantly greater levels of thromboxane A_2 compared with all other groups. However, aggregation responses to collagen and ADP were not significantly affected by dietary treatment, although relatively the lowest responses to these agonists were apparent in the CLO-fed and CAN-fed groups, respectively.

Keywords: Platelet aggregation; collagen; adenosine diphosphate; thromboxane A_2; dietary lipid.

Introduction

Numerous monounsaturated, polyunsaturated, and highly unsaturated fatty acids have been used in recent years in the preventive treatment of cardiovascular disease, but the mechanisms through which these fatty acids reduce the risk for cardiovascular disease remain unclear.[1,2] The search for the mechanisms of these fatty acids remains the object of intensive study. It is also unclear to what extent dietary lipids affect eicosanoid synthesizing capacity and functions of blood platelets, although it is known that dietary fatty acids are definitely a powerful means of controlling tissue fatty acid composition.[1-3] Many of the changes observed in platelet fatty acid composition and functions, particularly in animal models and humans given a fish oil diet containing high levels of long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been attributed to changes in the ratios of arachidonic acid and longer-chain highly unsaturated fatty acids of the n-3 series, such as EPA and DHA.[1,2]

There is still great interest in the possibility that dietary fatty acids of the n-3 series may play an important role in altering platelet arachidonic acid metabolism (e.g., thromboxane A_2 synthesis), thus affecting platelet functions (e.g., aggregation). In the present study, we investigated the effects of dietary lipids containing substantial quantities of fatty acids of one or both of these series as well as saturated fatty acids on collagen- and adenosine diphosphate (ADP)-

Address reprint requests to Dr. Mahadevappa, Cell Biology Research Group, Department of Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1.
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induced platelet thromboxane A_2 synthesis (measured as thromboxane B_2) and aggregation in the rat. Plasma thromboxane B_2 levels were also measured in these dietary groups.

Materials and methods

Animals and diet

Male weanling Wistar rats weighing 71 to 75 g (Charles River Canada Inc., St. Constant, Quebec, Canada) were individually housed in suspended stainless steel cages and maintained at 22 to 23°C on a 12-hour light-dark cycle. Animals were randomly assigned to one of five groups and fed one of the following diets ad libitum: lard (LRD), corn (CRN), soya (SOY), canola (CAN), or cod liver oil (CLO) (Santoquin 0.05% of oil) (*Table 1*). The fatty acid composition of the lipids used for diet formulation is presented in *Table 2*. The dry ingredients of the diet were mixed and frozen at −25°C. The lipid component of the diet was mixed with the dry ingredients thrice weekly, and the animals were provided with fresh diet daily. Food intake and body weight were recorded weekly for 7 weeks.

Blood collection procedure

Animals were anesthetized (Metofane; Pitman-Moore, Washington Crossing, NJ, USA) following the end of a 7-week period, and blood samples (8 to 10 ml) were obtained through direct cardiac puncture. Blood was drawn into 10-ml plastic syringes previously rinsed with heparin solution, then immediately transferred to polypropylene centrifuge tubes containing heparin in saline (final concentration, 50 U/ml blood) and gently mixed by inversion.

Preparation of washed platelets

Platelets were prepared essentially according to the procedure of Mustard et al.[4] for human platelets with minor modifications. Heparinized blood was centrifuged at 100 × *g* for 20 minutes; platelet-rich plasma (PRP) was removed and centrifuged again at 1,000 × *g* for 15 minutes at 37°C to sediment platelets. Platelet-poor plasma (PPP) was drawn off and stored frozen at −80°C until use for thromboxane B_2 measurement by radioimmunoassay. Platelets were washed twice and finally suspended in Tyrode's albumin buffer containing 2 mM Ca^{2+}, pH 7.35 (platelet suspension$_3$). Platelet number was determined using a Coulter Counter and was adjusted to 1.0×10^8/ml. Platelets (1×10^8 platelets/ml) were incubated in an aggrecorder for 1 minute (Payton Associates, Buffalo, NY, USA) with a stirring speed of 900 rpm and a temperature of 37°C; these platelets were allowed to remain for an additional 5 minutes. Following incubation of platelet suspensions for 5 minutes, indomethacin (final concentration, 25 μM) was added to the stirring platelets and suspensions were immediately transferred to polypropylene centrifuge tubes, stored on ice, and then centrifuged at 4°C to sediment the platelets from the medium. Aliquots of the resulting supernatant were stored in plastic cuvettes at −80°C until use for the measurement of thromboxane B_2.

Table 1 Composition of diets

Ingredients	Groups (wt%)				
	LRD	CRN	SOY	CAN	CLO
Vitamin-free casein	20	20	20	20	20
Choline chloride	0.2	0.2	0.2	0.2	0.2
Corn starch	10	10	10	10	10
Sucrose	50	50	50	50	50
Vitamin mix	1	1	1	1	1
Mineral mix	4.5	4.5	4.5	4.5	4.5
DL-Methionine	0.3	0.3	0.3	0.3	0.3
Fiber	4	4	4	4	4
Lard	10	0	0	0	0
Corn oil	0	10	0	0	0
Soya oil	0	0	10	0	0
Canola oil	0	0	0	10	0
Cod liver oil	0	0	0	0	10
Santoquin antioxidant	0.005	0.005	0.005	0.005	0.005

Table 2 Fatty acid composition of dietary lipids

Fatty acid	Fatty acids (wt%)				
	LRD	CRN	SOY	CAN	CLO
14:0/14:1	1.6	—	—	—	6.3
16:0/16:1	26.5	11.1	10.4	5.0	22.6
18:0	12.9	1.5	3.5	1.5	1.9
18:1	45.6	27.7	25.6	59.7	20.6
18:2n-6	10.2	56.7	50.2	21.3	3.1
18:3n-3	.5	1.4	7.8	8.5	1.0
18:4n-3	—	—	—	—	2.5
20:0	—	—	—	.5	—
20:1	1.2	.5	—	2.0	12.3
20:5n-3	—	—	—	—	8.4
22:1	—	—	—	—	7.4
22:5	—	—	—	—	1.1
22:6	—	—	—	—	7.7
Others	1.5	1.1	2.5	1.5	2.5

Only values for fatty acids representing >0.5% of total fatty acids are given.

Platelet aggregation

Washed platelets (1 ml containing 1.0×10^8 platelets) were allowed to adjust to incubation conditions in an aggregometer for 1 minute (stirring speed, 900 rpm; 37°C), then treated as controls or stimulated with collagen (5 μg/ml, final concentration) or ADP (50 μM, final concentration; no fibrinogen was used) for 5 minutes. Platelet aggregation responses were recorded simultaneously (chart speed, 2 cm/min). After incubation for 6 minutes (1 minute adjustment + 5 minutes treatment), indomethacin (100 μl of a 250-μM solution) was immediately added to stirring platelets, and suspensions were immediately transferred to polypropylene centrifuge tubes, stored on ice, and then centrifuged to sediment platelets from the medium. Aliquots of the supernatant were stored in plastic

cuvettes at −80°C until required for the determination of thromboxane B_2 by radioimmunoassay.

Determination of thromboxane B_2 levels in platelet-poor plasma

Following sedimentation of platelets, PPP was drawn off, transferred to plastic storage cuvettes containing indomethacin (20 μM), and stored at −80°C. Platelet-poor plasma samples were analyzed for thromboxane B_2, a stable product of thromboxane A_2, using a radioimmunoassay kit from Amersham (Chicago, IL, USA). Briefly, [^{3}H]thromboxane B_2 was added to PPP samples along with thromboxane B_2 antiserum, charcoal was used to remove unbound thromboxane B_2, and samples were counted for radioactivity. Thromboxane B_2 values in PPP samples of rats from different dietary groups were determined from a thromboxane B_2 standard curve.

Determination of thromboxane B_2 in platelets

Thromboxane B_2 levels were measured in the supernatants obtained from stimulated and unstimulated platelets by radioimmunoassay as described in the previous section. Thromboxane B_2 values in these supernatants were obtained from a standard curve.

Statistical analysis

One-way analysis of variance and Tukey's test were used to test for significant differences between dietary groups. Results are expressed as means ± SEM unless stated otherwise.

Results

The level of circulating thromboxane B_2 detected in the PPP of CLO-fed animals was significantly lower than that of all other dietary groups (*Figure 1*). The circulating levels of thromboxane B_2 present in the PPP of LRD-, CRN-, SOY-, and CAN-fed animals were not significantly different from one another. Interestingly, circulating levels of thromboxane B_2 in the PPP of LRD-fed animals were relatively much higher than those from the CRN-, SOY-, and CAN-fed groups (*Figure 1*).

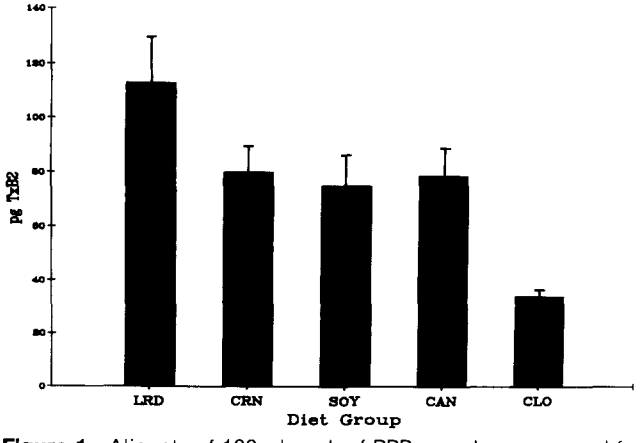

Figure 1 Aliquots of 100 μl each of PPP samples were used for the determination of thromboxane B_2 by radioimmunoassay. Values are expressed in pg/100 μl of plasma and represent the mean ± SE (n = 8)

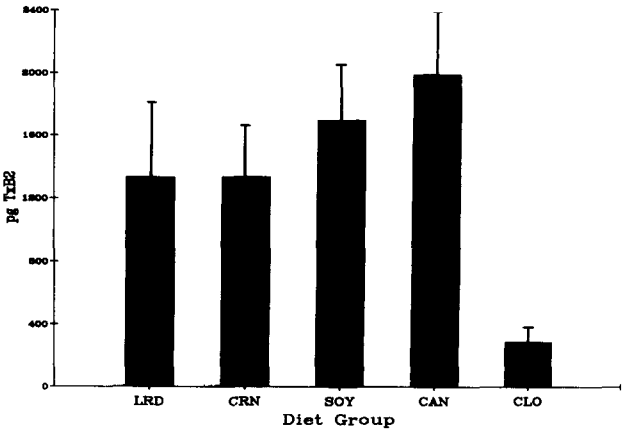

Figure 2 Platelets (1 × 10^8 platelets/ml) from different dietary groups were stimulated with ADP (50 μM) for 5 minutes at 37°C. Following incubation, indomethacin was immediately added (25 μM, final concentration). Suspensions were immediately transferred to polypropylene tubes and centrifuged. Appropriate aliquots of the supernatants were used for the determination of thromboxane B_2. Values are expressed as pg/1 × 10^8 platelets and represent the mean ± SE (n = 8)

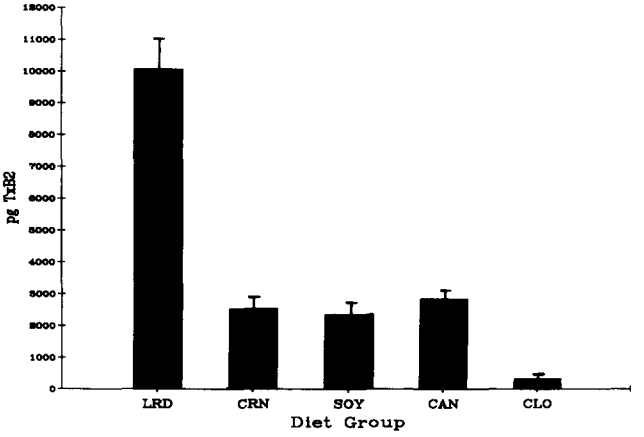

Figure 3 Platelets (1 × 10^8 platelets/ml) from different dietary groups were stimulated with collagen (5 μg/ml) for 5 minutes at 37°C. Following incubation, aliquots were prepared and assayed for thromboxane B_2 as described in *Figure 2*. Values are expressed as pg/1 × 10^8 platelets and represent the mean ± SE (n = 8)

Following ex vivo stimulation with ADP (50 μM), the platelets from CLO-fed animals produced significantly less thromboxane B_2 compared with all other dietary groups (*Figure 2*). The thromboxane B_2 levels obtained for these other groups (LRD-, CRN-, SOY-, and CAN-fed groups) did not vary significantly from one another (*Figure 2*). However, unstimulated platelets from these groups also had much higher basal levels of thromboxane B_2 compared with those from the CLO-fed group.

In platelets stimulated with collagen (5 μg/ml), the amount of thromboxane B_2 produced for CLO-fed ani-

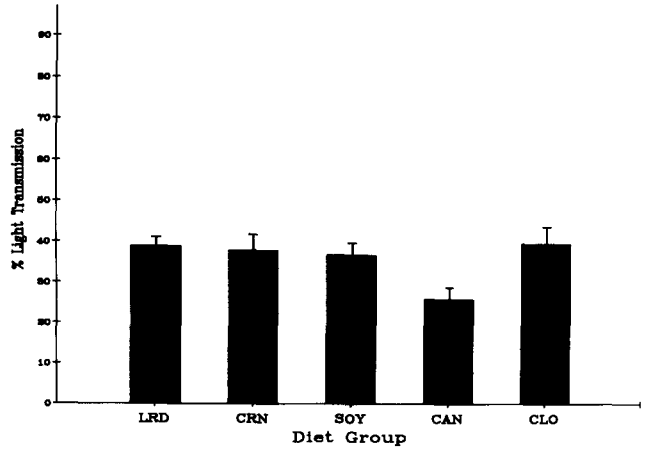

Figure 4 Platelets (1 × 10^8 platelets/ml) were stimulated with ADP (50 μM) for 5 minutes at 37°C in an aggrecorder. Aggregation responses to the effect of ADP were measured following the changes in percent light transmission relative to unstimulated platelet controls. Values are expressed as percent light transmission and represent the mean ± SE (n = 8)

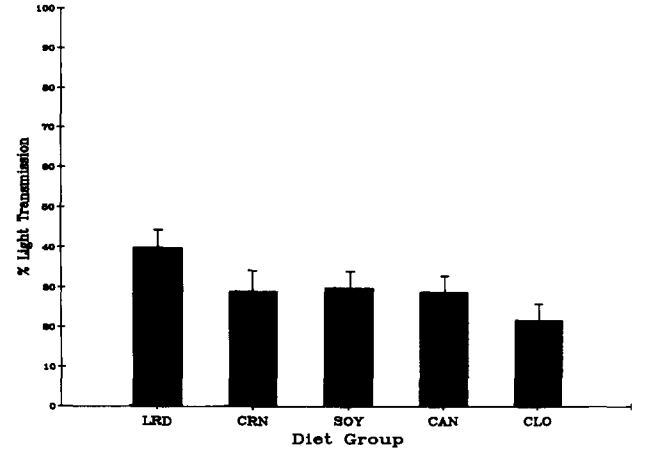

Figure 5 Platelets (1 × 10^8 platelets/ml) were stimulated with collagen (5 μg/ml) for 5 minutes at 37°C in an aggrecorder. The change in percent light transmission in the presence of collagen relative to unstimulated platelet controls represents a measure of aggregation. Values are expressed as percent light transmission and represent the mean ± SE (n = 8)

mals was again significantly less than that of all other dietary groups (*Figure 3*). On the other hand, the amount of thromboxane B_2 synthesized in collagen-stimulated platelets obtained from the LRD-fed animals was significantly greater than that of all other dietary groups, while the levels detected in stimulated platelets from all vegetable oil-fed animals did not vary significantly from one another. In general, the amounts of thromboxane B_2 formed in collagen-stimulated platelets were relatively much higher than those detected in ADP-stimulated platelets, with the exception of platelets from the CLO-fed group.

Ex vivo stimulation of platelets with ADP (50 μM) did not result in a significant difference in the aggregation response between dietary groups, including that from the CLO-fed group; however, it should be noted that the platelets of the CAN-fed animals produced the lowest aggregation response (*Figure 4*). Generally, aggregation responses of platelets from the CLO-fed groups to ex vivo stimulation with ADP were relatively higher than those from all other dietary groups, although no statistical difference was apparent between these groups.

In contrast, aggregation responses of platelets from the CLO-fed animals to ex vivo stimulation with collagen (5 μg/ml) were relatively lower than those from all other dietary groups, with the highest response being in the LRD-fed animals, but the differences in responses were not significantly different from those of the other (vegetable oil-fed) dietary groups (*Figure 5*). We also noted that rat platelets required more collagen to aggregate relative to human platelets.

Discussion

The circulating thromboxane B_2 level in the PPP of CLO-fed animals was less than 50% of that of all other dietary groups and this appears to reflect the decrease in the arachidonic acid found in the unstimulated platelets of these animals (unpublished observation). In a recent study in which lard and butter were fed to rats for 3 weeks at 10 to 50% energy (en%) of the diet, no significant difference in PPP-thromboxane B_2 was observed. Enrichment of the diet with butter resulted in significant decreases of arachidonic acid in plasma phospholipids. However, the changes in the arachidonic acid content of unstimulated platelets were not reported in this study.[5]

Using clotted whole blood and a comparable amount of dietary fat to that of the present study, similar tendencies in plasma thromboxane B_2 levels[6-12] or no change in serum thromboxane B_2[13] have been reported by others when n-3 fatty acids of vegetable or marine oils were fed to rats.

When diets containing less than one half the fat of the present study were fed to rats for 3 weeks, serum thromboxane B_2 levels were reported to be significantly less for Menhaden oil-fed animals (Menhaden oil contains high levels of EPA) but not for shark oil-fed animals (shark oil is a rich source of DHA) when compared with triolein/safflower oil controls.[14] When levels of dietary fat that were double those of the present study were fed to rats for 2 months, no significant difference was observed between the serum thromboxane B_2 levels of animals fed either various vegetable oils or beef tallow.[15]

Thus, from the present study and the majority of the studies cited above, it is apparent that the consumption of dietary n-3 fatty acids, particularly from marine oils, containing high levels of EPA and DHA by rats results in significantly lower circulating thromboxane B_2 levels in the plasma. Supplementation of CLO-containing long-chain polyunsaturated fatty acids such as EPA and DHA generally results in a marked decrease in the arachidonic acid content of other tissues in addition to platelets. Although it is likely that much of the PPP circulating thromboxane B_2 is derived from

platelets, it needs to be recognized that other tissues may also contribute to plasma thromboxane B_2. Thus, the decrease observed in the circulating plasma thromboxane B_2 levels in the CLO-fed rats may represent a decrease in the turnover of this platelet proaggregating substance following supplementation of cod liver oil. This decrease may occur through a reduction in the substrate level in tissues including platelets (a decrease in arachidonic acid levels in most tissues is generally observed following supplementation of CLO). However, the supplementation of CLO may also affect membrane-bound cyclooxygenase and thromboxane A_2-synthetase activities as well as the degradation of thromboxane B_2. A decrease in membrane-bound phospholipases that release arachidonic acid for the synthesis of thromboxane A_2 may also be affected by the supplementation of CLO.

In the present study, ex vivo stimulation of washed rat platelets of CLO-fed animals with ADP resulted in the synthesis of significantly less thromboxane B_2 than that of all other dietary groups. These results are in general agreement with the fact that the unstimulated platelets from animals in these other dietary groups contained significantly more of the thromboxane precursor arachidonic acid than those of the CLO-fed animals (unpublished observation). In a recent study in which 2 or 5 μM ADP (versus 50 μM of the present study) was used to stimulate PRP of rats fed either butter or lard at 10 to 50 en% of the diet, no increase in thromboxane B_2 production was observed compared with unstimulated platelets.[5] In comparison with unstimulated platelet thromboxane B_2 levels, we also found only a small increase in the net synthesis of thromboxane B_2 following ex vivo stimulation of platelets with ADP (50 μM). However, the differences in both the type of dietary fat and the level of ADP used are also likely to affect the net synthesis of thromboxane B_2 in ex vivo stimulated platelets in addition to the effect of decreased levels of thromboxane A_2 precursor, arachidonic acid.

Washed platelets from CAN-fed animals produced the lowest aggregation response to ADP stimulation (approximately 30% less than CRN-fed controls), but no significant differences between dietary groups were observed. In a recent study in which a comparable quantity of dietary fat (hydrogenated coconut oil or marine oil) to that of the present study was used, ADP stimulation of PRP did not result in a significant difference in aggregation.[10] More recently, similar results were obtained using ADP-stimulated PRP from rats fed 10 to 50 en% as either lard or butter for 3 weeks.[5] In another recent report, ADP stimulation of PRP from rats fed a 10% (by weight) fat diet that contained 2.5% (by weight) as marine oil was significantly lower when compared with all other groups studied.[16] However, no significant difference in aggregation was observed for groups consuming much greater quantities (5% to 10%, by weight) of their diet as marine oil when compared with alpha-linolenic acid (18:3 n-3)-rich controls. A similar lack of difference of washed rat platelets to ADP stimulation was reported recently for rats fed high-fat diets (40% versus 10%, by weight, of the present study).[17] In another study, in which rats were fed a high-fat diet (approximately 50 en% of diet) and ADP-induced aggregation of circulating rat blood was monitored using a filter loop technique, a longer obstruction time (a longer obstruction time indicates a lower thrombotic tendency) appeared to coincide with a low ADP response for the vegetable oils used.[18] They also report a low ADP response for whole oil-fed animals; this was associated with a short obstruction time. This association did not fit the pattern mentioned above. However, this oil was reported to contain 16% (by weight) as arachidonic acid, a fact which may help explain why whole oil did not fit the pattern observed with vegetable oils.

In other studies in which only low levels of purified fatty acid or 10 or less en% of the diet was lipid (versus approximately 20 en% of diet in the present study), no significant differences in aggregation response to ADP stimulation of PRP were observed.[14,19-21] In one of these studies, however, perfusion of PRP through isolated aortae prior to ADP stimulation did result in a significantly lower threshold dose response to ADP in animals that consumed marine oil.[20]

Thus, based on the present results and the foregoing discussion, it appears that neither the type nor the level of dietary fat significantly alters ADP-induced platelet aggregation in the rat. Neither the decrease in the level of arachidonic acid, a precursor of thromboxane A_2, and possibly of other fatty acids, nor the increase in n-3 fatty acids (EPA/DHA) in platelet membranes seem to affect ADP-induced platelet aggregation. These findings are in general agreement with the fact that ADP-induced platelet aggregation is not dependent on thromboxane A_2 and other endoperoxides. However, the reasons for the observed respective decrease and increase in platelet aggregation in CAN- and CLO-fed rats compared with CRN-fed animals remain unclear.

In the present study, the thromboxane B_2 levels detected in platelets stimulated ex vivo with collagen were significantly lower in CLO-fed groups and significantly greater in LRD-fed groups than those from all other dietary groups, while those of the animals receiving vegetable oils did not vary significantly from one another. These results appear to be in parallel with our findings that the unstimulated platelets of the CLO-fed animals contain the least and those of the LRD-fed animals contain the most arachidonic acid (unpublished observation), although the difference between these groups in terms of thromboxane B_2 and arachidonic acid levels were not of the same magnitude. In studies with collagen-stimulated PRP of rats fed a comparable amount of lipid to that of the present study, to which purified or concentrates of n-3 fatty acids contributed graded levels up to approximately one half the energy of the diet, thromboxane B_2 levels were significantly reduced.[22] Similar results were reported recently for collagen-stimulated thromboxane A_2 synthesis by the whole blood of rats fed 50 en% palm oil versus 5 en% sunflower seed oil.[23] When sub-

stantially higher levels of fat, linseed, or marine oils (approximately double those of the present study) were fed to rats, either no significant difference[17] or approximately one half the level of thromboxane B_2 has been reported for collagen-stimulated washed rat platelets.[24-26] Thus, the results on fatty acids of platelets and those on thromboxane B_2 levels reported in this study again suggest a poor correlation between the levels of arachidonic acid and of thromboxane A_2 synthesized in ex vivo-stimulated platelets in different dietary groups.

In the present study, the type of dietary lipid did not significantly affect collagen-induced aggregation in washed rat platelets.

Collagen-induced aggregation responses of PRP from rats fed levels of lipid similar to those of this study and which contained substantial quantities of purified alpha-linolenic acid (18:3 n-3) or a fish oil concentrate were similarly unaffected by the type of dietary lipid.[10,22] On the other hand, the aggregation response of washed platelets from rats fed double the amount of fat of the present study was either significantly lower in animals fed linseed versus safflower oil or cocoa butter[27] and sardine oil versus corn oil[26] or remained unchanged when a variety of vegetable and marine oils or their products were fed.[17] Similarly, in other rats fed 50 en% as fat, no change in obstruction time was observed (a larger obstruction time indicating a lower thrombotic tendency) in animals fed palm versus sunflower seed oil[23] or sunflower seed oil versus cod liver oil.[25] Aggregation response of collagen-stimulated PRP from rats fed approximately one half the level of fat of the present study which contained small amounts of enriched marine oils or purified EPA were unchanged[14] or significantly decreased.[19,28]

Thus, while moderate (as used in the present study) and, in some cases, high levels of different dietary lipid do not affect collagen-induced aggregation response of rat platelets (whole blood, PRP, and washed), some high-fat diets have been shown to affect this response in washed platelet suspensions. The reasons for the different results with high dietary fat are not clear and require further study.

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