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The relationships between dietary α-linolenic:linoleic acid and rat platelet eicosapentaenoic and arachidonic acids

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Increased dietary intake of α-linolenic acid (ALA) may be desirable to enrich tissue eicosapentaenoic acid (EPA; 20:5*n*-3) but competition between *n*-3 and *n*-6 fatty acids for enzymes involved in elongation and subsequent acylation will determine the relative proportions of phospholipid fatty acids. The aim of the present study was to examine the effects of altering the dietary ALA:linoleic acid (LA) ratio on rat platelet EPA and arachidonic acid (AA; 20:4*n*-6) concentrations. Sprague–Dawley rats were fed on diets containing 30% total energy as fat with approximately 10% each of saturated, monounsaturated and polyunsaturated fatty acids with one of the following ALA:LA values; 1:7, 1:4, 1:1 or 1·3:1 (nine rats per group). After 4 weeks, blood was withdrawn from the abdominal aorta and platelet fatty acids analysed. The proportion of EPA was greater at the 1:1 and 1·3:1 ratios compared with the 1:7 and 1:4 ($P < 0·05$), and a decrease in AA was observed ($P < 0·05$) at the higher ratios. It was established that the platelet EPA:AA value increased ($P < 0·05$) as the dietary ALA:LA value increased.

α-Linolenic acid:linoleic acid ratio: Eicosapentaenoic acid: Arachidonic acid: Rat platelets

It appears desirable to increase the eicosapentaenoic acid (EPA; 20:5*n*-3): arachidonic acid (AA; 20:4*n*-6) ratio in membrane phospholipids. Both these C20 fatty acids are substrates for production of eicosanoids, e.g. thromboxane and leukotrienes (Herold & Kinsella, 1986). EPA results in eicosanoids which are less aggregatory and inflammatory than those from AA and may lessen the tendency to thrombosis and atherosclerosis (Dyerberg *et al.* 1978; Terano *et al.* 1986). In Western diets the predominant polyunsaturated fatty acid is linoleic acid (LA; 18:2*n*-6) (Truswell *et al.* 1992) which is elongated to AA (de Gomez Dumm & Brenner 1975). The corresponding *n*-3 fatty acid, α-linolenic acid (ALA; 18:3*n*-3) is converted to EPA via the same enzymic pathways. Recent work confirms that the replacement of some LA with ALA may increase tissue EPA via its elongation (Renaud & Nordoy, 1983; Sanders & Roshani, 1983; Weaver *et al.* 1990; Mantzioris *et al.* 1995) but the efficiency of this conversion in man was previously questioned (Dyerberg *et al.* 1983). ALA is preferred to LA as substrate of Δ-6 desaturase (Emken *et al.* 1992) but AA is favoured over EPA as a substrate for acylation into phospholipids (Emken *et al.* 1992). Feeding EPA and by-passing the conversion step is obviously a more efficient way to increase tissue EPA but while habitual consumption of fatty fish will increase it and decrease AA, it may not be feasible to increase fish consumption for all people on a global basis. Therefore the use of ALA to increase tissue EPA warrants research.

The doses of ALA that have been used in previous studies have varied, as has the subsequent phospholipid enrichment. It appears that it is the *n*-3:*n*-6 fatty acid ratio, in particular ALA:LA, rather than the absolute amount of ALA which influences the incorporation. Increases in ALA:LA from 1:170 through to 1:4, 1:2 and 1:1 were associated with increasing percentages of EPA in rat plasma, lung and liver phospholipids

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(Hwang *et al.* 1988) while increases in the amount of ALA but with constant ALA:LA ratio produced no differences (Boudreau *et al.* 1991). Chan *et al.* (1993) reported that in men an ALA:LA value of 1:3 increased platelet EPA compared with 1:27 and 1:7, and that if the quantity of ALA was doubled while the ratio was kept constant no further increases occurred. Further confirmation that the ALA:LA ratio is important was provided by Emken *et al.* (1993) who found that the conversion of ALA to longer-chain metabolites, measured after infusions of deuterated ALA and LA, was reduced by 30% after feeding a diet with 29·8 g LA *v.* 15·1 g LA.

The present study was designed to extend previous studies. The rat studies in which the dietary ALA:LA ratio has been controlled have measured fatty acids in tissues other than platelets (Hwang *et al.* 1988; Lands *et al.* 1990). The dietary values of ALA:LA in the current study have been selected because they would be achievable in human mixed diets based on 30% total energy from fat with 10% energy from saturated fat and 10% energy from polyunsaturated fat. Previous studies have not been concerned with this. We would have preferred to study humans but with free-living conditions it is impossible to be certain that subjects have complied with the exact ratio. The rat provides a reasonable model because it has the same enzyme pathways as humans for elongation and desaturation of ALA to EPA (de Gomez Dumm & Brenner 1975; Crawford *et al.* 1976).

METHODS

Animals and diets

Forty-five Sprague–Dawley specific-pathogen-free rats (8 weeks old) were fed on diets with varying ALA:LA ratios of 1:7, 1:4, 1:1 and 1·3:1 based on weight. Rats were housed in cages (three per cage) kept in a room at constant temperature (25°) with a 12 h light–dark cycle maintained. The animals were maintained on standard rat chow for 1 week (YS Feeds, Mouse Breeder, Young, NSW, Australia), ratio 1:12, until commencement of the modified diets which were fed for 4 weeks.

The diets were identical in macronutrients, i.e. 191 g protein/kg, 678 g carbohydrate/kg, 35 g fibre/kg and 131 g fat/kg feed, and had the same vitamin and mineral content, the only exception being the contribution of the oils to vitamin E. The amounts of saturated, monounsaturated and polyunsaturated fatty acids were similar. The fatty acid compositions of the diets determined using GC are shown in Table 1. Palm olein, palm stearin, sunflower-seed oil, rapeseed oil (EOI Foods Pty Ltd, Marrickville, NSW, Australia) and linseed oil (Melrose foods, Box Hill, Victoria, Australia) were mixed in various amounts to achieve the desired fat composition and the ALA:LA values. Animals had free access to feed. Fresh feed was put in each cage every weekday morning and enough for 2 d was placed on late Friday afternoon.

The study was approved by the Animal Care and Ethics Committee of the University of Sydney.

Blood collection

At the end of the 4 weeks feed was withheld overnight and the rats were anaesthetized with diethyl ether, used in a manner which prevented irritation of mucous membranes. After incision, blood was collected from the abdominal aorta of each rat, using a 23 G needle (Becton Dickinson, Singapore) and plastic syringes to which 1 ml of sodium citrate (38 g/l) had been added. Platelet-rich-plasma (PRP) was made by centrifuging the blood for 10 min at 119 ***g*** (25°). The PRP from three rats was pooled and centrifuged at 1000 ***g*** for 10 min to pellet the platelets. The resultant platelet pellet was washed three times in 2 ml Ca^{2+}-free Tyrode's solution (137 mM-NaCl, 12 mM-$NaHCO_3$, 2·7 mM-KCl, 0·4 mM-NaH_2PO_4 and

Table 1. *Fatty acid composition of the diets (g/100 g total fatty acids)*

Fatty acid	Rat chow	α-Linolenic acid:linoleic acid 1:7	1:4	1:1	1·3:1
14:0	2·7	0·9	0·9	1·0	0·9
16:0	19·0	26·2	24·9	28·1	24·2
18:0	9·2	4·9	4·7	4·4	4·8
20:0	0·3	0·3	0·3	0·4	0·3
22:0	tr	0·3	0·3	tr	0·2
Total SFA	31·2	32·6	31·1	33·9	30·4
14:1	1·6	tr	tr	tr	tr
16:1	1·2	0·3	0·4	0·3	0·1
18:1*n*-9	24·8	34·3	32·8	34·1	27·0
18:1*n*-7	ND	1·2	1·2	1·2	1·3
20:1	0·3	0·2	0·2	0·2	0·3
Total MUFA	27·9	36·0	34·6	35·8	28·7
18:2*n*-6	36·0	27·2	25·3	14·6	17·1
18:3*n*-3	3·0	3·7	6·7	14·3	22·8
20:4*n*-6	tr	tr	tr	tr	tr
20:5*n*-3	tr	0·4	0·4	0·4	0·4
22:6*n*-3	1·9	tr	0·9	1·0	0·3
Total PUFA	40·9	31·3	33·3	30·3	40·6
α-Linolenic:linoleic	1:12	1:7	1:4	1:1	1·3:1
n-3:*n*-6	1:7	1:7	1:3	1:1	1·4:1

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; tr, trace; ND, not detected.

5 mM-glucose). After the final wash the platelets were resuspended in 1 ml of fresh Tyrode's solution and stored at −80° pending methylation.

Analysis of fatty acids

A modification of the method of Lepage & Roy (1986) was used to make fatty acid methyl esters. Each platelet preparation was methylated in triplicate. Solvents used in the extraction procedure were supplied by Rhone and Poulenc (Clayton South, Victoria, Australia) and acetylchloride and potassium carbonate by BDH (Poole, Dorset). The methyl esters formed in the benzene layer which was separated from the methanol layer by centrifugation and transferred to GC vials for chromatography. This method achieved $\geqslant 95\%$ recovery rate for all fatty acids from phospholipids. Methyl esters were assayed in duplicate using flame ionization capillary GC (model 5890A, Hewlett-Packard, North Ryde, Australia) with a fused carbon silica column (30 × 0·25 mm internal diameter) which was coated with cyanopropylphenyl (type DB 225, 25 mm thickness, 25% w/w coating) (J and W Scientific, Folsom, CA, USA). A two-step oven program, 170° for 2 min followed by a rise of 10°/min to 190° then 190° for 1 min followed by a rise of 5°/min to a final temperature of 220° for 34 min, allowed optimal separation of fatty acid methyl esters.

The fatty acid peaks in the samples were identified by comparing the retention times of the peaks in the sample with those of the fatty acids in GLC 68 fatty acid standard (Nu Check Prep, Inc., Elysian, MN, USA). This standard did not contain 20:5 or 22:5 so the individual methyl esters (Cayman Chemical Company, Ann Arbor, MI, USA) were analysed to determine their retention times. Any peaks that preceded that for 13:0 were not included because the program could not resolve these peaks well.

Statistical analysis

The percentages of each fatty acid at the different dietary ratios were compared with each other using ANOVA. *A posteriori* contrasts were made using the Sheffé *F* test with the Statview Student computer program (version 1.0, 1991, Abacus Concepts Inc., Berkeley, CA, USA). $P < 0.05$ was selected as the level of significance.

RESULTS

Table 2 shows the platelet fatty acid composition at each of the four dietary ratios. The overall proportion of saturated fatty acids was constant. The monounsaturated fatty acid content showed some variation but 18:1*n*-9 was unchanged. The relative amounts of total polyunsaturated fatty acids and highly unsaturated fatty acids (i.e. fatty acids with more than three double bonds) were maintained but the total *n*-3 fatty acids increased on the diets with the two highest ALA:LA values. The proportion of EPA doubled and the proportion of AA declined on the 1:1 and 1·3:1 diets. ALA doubled at the highest ratio and LA also increased. The platelet EPA:AA value increased on the 1:1 diet and further increased on the 1·3:1 diet.

DISCUSSION

The ratio platelet EPA:AA value increased when rats were fed on diets with ALA:LA ranging from 1:7 through to 1·3:1. The platelet phospholipid EPA incorporation was similar on both the 1:1 and 1·3:1 diets while platelet AA progressively declined at these ratios. This is in agreement with previous studies of the effect of the dietary ALA:LA value on liver, erythrocyte and lung phospholipids (Hwang *et al.* 1988; Lands *et al.* 1990).

Hwang *et al.* (1988) fed rats on diets with ALA:LA values of 1:4, 1:2 and 1:1 and found progressive increases in EPA and decreases in AA in plasma, lung and liver phospholipids. It appears that Lands *et al.* (1990) demonstrated increases in the percentage of EPA in liver and erythrocyte phospholipids up to a dietary ALA:LA of 1·94:1, although no statistical analysis is shown. At a dietary ratio of 2·74:1 the percentage of EPA declined. In the current study the C18 ALA and LA content of the platelet phospholipids increased on the 1·3:1 diet and, while not significant, a trend to decreasing highly unsaturated fatty acids (fatty acids with more than three double bonds) was observed. Hwang *et al.* (1988) observed increased percentages of ALA and LA in liver phospholipids at the 1:1 ratio. Whether the increase in the proportion of C18 fatty acids occurs because the pathways for synthesis of C20 fatty acids are saturated or because the process for acylation of C20 fatty acids into phospholipids is maximal cannot be discerned from the present study.

Lands *et al.* (1990) found that a dietary ALA:LA value of approximately 1·3:1 resulted in an erythrocyte EPA:AA value of 0·4 while in the current study the platelet value was 0·25. It may be that there are blood-cell-specific differences but while the membrane *n*-3:*n*-6 fatty acids ratio appears to be determined by the dietary ratio, the total proportions will be dependent on the overall supply of fatty acids and the period of consumption. Lands *et al.* (1990) fed their rat pups on special diets from conception through to birth, i.e. the mothers had been on modified diets. In the present study, rats were fed on ordinary chow until commencing the test diets. Chow contains predominantly LA which could be deposited in the adipose tissue thereby creating an additional supply of LA to the dietary source, which was not so in the other trial (Lands *et al.* 1990).

The findings in rats cannot be applied directly to humans. In the current study the dietary ratios were selected because they were within the range that could be achieved in human diets using diet containing 30% energy as fat with recommended percentages of energy from saturated and polyunsaturated fat (American Heart Association, 1988). In Western

Table 2. *Fatty acid composition (g/100 g total fatty acids) of platelets from rats fed on diets with different α-linolenic: linoleic acid (ALA: LA) values*

(Mean values with their standard errors for three samples, each representing a pool of three rats)

Time (weeks)...	0		4							
ALA:LA...	Rat chow*		1:7		1:4		1:1		1·3:1	
Fatty acid	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Total SFA	57·1	1·5	54·5	0·4	53·8	0·6	54·4	0·6	55·0	0·9
18:1*n*-9	5·8	0·5	6·9	0·4	9·5	1·2	7·8	0·5	7·5	0·2
Total MUFA	12·7	0·6	$13·1^{ab}$	0·4	$15·0^{b}$	1·0	$12·7^{ab}$	0·3	$12·6^{a}$	0·2
18:2*n*-6	7·9	0·2	$9·5^{a}$	0·3	$8·8^{a}$	0·4	$10·1^{ab}$	0·2	$11·0^{b}$	0·5
18:3*n*-3	0·4	0·0	$0·4^{a}$	0·0	$0·4^{a}$	0·0	$0·6^{a}$	0·3	$1·1^{b}$	0·1
20:4*n*-6	13·1	0·4	$14·9^{b}$	0·6	$15·0^{b}$	0·6	$13·3^{ab}$	0·3	$11·4^{a}$	0·6
20:5*n*-3	1·9	0·1	$1·0^{a}$	0·0	$1·2^{a}$	0·1	$2·5^{b}$	0·2	$2·6^{b}$	0·1
22:5*n*-6	0·9	0·1	$1·2^{c}$	0·1	$1·0^{bc}$	0·1	$0·7^{ab}$	0·1	$0·6^{a}$	0·1
22:5*n*-3	1·2	0·1	0·8	0·1	0·7	0·1	1·3	0·2	1·1	0·2
22:6*n*-3	0·5	0·1	0·7	0·1	0·8	0·1	0·8	0·1	0·7	0·1
Total PUFA	30·2	1·0	32·4	0·9	31·3	1·2	32·6	0·7	32·4	0·7
Total HUFA	21·9	0·7	22·4	0·8	22·1	0·9	21·9	0·7	20·3	1·0
Total *n*-6	21·7	0·9	25·6	0·9	24·8	1·0	24·1	0·5	22·8	1·8
Total *n*-3	4·0	0·2	$2·9^{a}$	0·2	$3·1^{a}$	0·1	$5·2^{b}$	0·4	$5·0^{b}$	0·4
20:5/20:4	0·15	0·01	$0·07^{a}$	0·00	$0·08^{a}$	0·00	$0·18^{b}$	0·01	$0·25^{c}$	0·02

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids (> 3 double bonds).

[a, b, c] Mean values within a row not sharing a common superscript letter were significantly different ($P < 0·05$, Scheffé F test).

* Rat chow was 3% total energy as fat with ALA:LA of 1:12. The zero time on rat chow was not included in the analysis of variance.

societies all the ratios tested may be considered high as in Australia the dietary ALA:LA may be as low as 1:30 (Truswell *et al.* 1992). Using rapeseed oil an ALA:LA value of 1:3 can be achieved and this can rise to 1·4:1 if linseed oil is used. The threshold value of ALA:LA that will result in significant EPA enrichment and an increase in EPA:AA value in human platelets appears to be less than that required by the rats in the current study and lies between 1:7 and 1:3 (Chan *et al.* 1993). In the current study the platelet EPA:AA value increased from the 1:4 to 1:1 to 1·3:1 diet and the maximum platelet incorporation which could be achieved remained undetermined. It was the decline in AA that was responsible for the further increase in EPA:AA value at the 1·3:1 dietary ratio because the EPA concentration was unchanged. The rat demonstrated declines in platelet AA at the higher ratios, but in humans, AA incorporation is unaffected by ALA intake (Chan *et al.* 1993; Mantzioris *et al.* 1995). Thus whether feeding the same ALA:LA values to humans would result in similar persistent increases in platelet EPA:AA is questionable.

In conclusion, the present study establishes that rat platelet phospholipids demonstrate changes in EPA:AA as the dietary ALA:LA value increases. The value at which EPA incorporation becomes maximal in humans requires further investigation as does the value for which EPA:AA in platelets is greatest and has maximal biological advantage.

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