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Effects of a *trans* isomer of arachidonic acid on rat platelet aggregation and eicosanoid production

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Abstract The addition of a *trans* isomer of arachidonic acid (20:4 Δ 14*trans*) to rat platelet suspensions inhibited the aggregation induced by 7.5 μ M of arachidonic acid. This inhibitory effect of 20:4 Δ 14*trans* was significant at concentrations of 7.5–22.5 μ M and the range of inhibition was 20% at an inhibitor/substrate ratio (I/S) 1 to 66% when I/S reached 3. However, the addition of its structural homolog (20:3n–9) or the natural isomer (20:4n–6) did not induce any modification of the platelet aggregation. In parallel, adding 20:4 Δ 14*trans* to the platelet significantly decreased thromboxane B₂ and 12-hydroxyheptadecatrienoic acid production. In contrast, the 12-lipoxygenase pathway was stimulated, as 12-hydroxy-eicosatetraenoic acid production increased up to 55% when the I/S reached 3. 20:3n–9, not being a substrate of the cyclooxygenase, did not induce any significant modification in the formation of thromboxane B₂ and 12-hydroxyheptadecatrienoic acid. 20:4 Δ 14t alone did not induce any platelet aggregation. However, this fatty acid was metabolized to a limited extent into two products that have still to be identified. One of them would be a product of the 12-lipoxygenase pathway.—Berdeaux, O., J. M. Chardigny, J. L. Sébédio, T. Mairot, D. Poullain, J. M. Vatele, and J. P. Noël. Effects of a *trans* isomer of arachidonic acid on rat platelet aggregation and eicosanoid production. *J. Lipid Res.* 1996. **37**: 2244–2250.

Supplementary key words platelet aggregation • *trans* polyunsaturated fatty acid • arachidonic acid • baicalein • cyclooxygenase • lipoxygenase • thromboxane • hydroxyeicosatetraenoic acid

Geometrical isomers of polyunsaturated fatty acids (PUFA) are formed during heat treatment of oils, including deodorization (1) and frying operations (2, 3). Consequently, isomers of linoleic and α -linolenic acids have been identified in oils taken from commercial operations (4). Among the different mono- and di-*trans* isomers of linolenic acid formed, 18:3 Δ 9c,12c,15t has been shown to be converted by rats to isomers of all *cis* 5,8,11,14,17 eicosapentaenoic acid (EPA) having a *trans*-17 bond (20:5 Δ 17*trans*) and to an isomer of all *cis* 4,7,10,13,16,19 docosahexaenoic acid (DHA) with a *trans*-19 bond (22:6 Δ 19*trans*) (5). Similarly, one isomer

of linoleic acid, the 18:2 Δ 9c,12t was also converted into an isomer of all *cis* 5,8,11,14 eicosatetraenoic acid (AA, arachidonic acid) with a *trans*-14 bond (20:4 Δ 14t) (6, 7).

All these long chain *trans* PUFA are incorporated into tissue lipids (7, 8) and recent studies carried out on the C20 and C22 n–3 fatty acids have shown that the *trans* PUFA have different effects compared to their respective *cis* homologues on platelet function and AA metabolism (9, 10). However, very little is known on the metabolic fate of the 20:4 Δ 14t, a *trans* isomer of AA. Arachidonic acid plays an important role in platelet aggregation as it is liberated from membrane phospholipids and subsequently oxygenated by cyclooxygenase into proaggregatory prostanoids (11). Moreover, the metabolism of AA and its effect on platelet function is modulated by other PUFA containing 20 and 22 carbons (12, 13) or less (14). Considering this important role of AA in platelet function, the effect of the geometry of the Δ 14 bond on inhibition of AA metabolism in isolated rat platelets has been examined.

MATERIAL AND METHODS

Chemicals

Both unlabeled and [1-¹⁴C]radiolabeled (specific activity: 2.11 GBq/mmoles) all *cis* 20:4n–6 were purchased from Sigma Chemicals (L'Isle d'Abeau, France) and

Abbreviations: PUFA, polyunsaturated fatty acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; TLC, thin-layer chromatography; PRP, platelet-rich plasma; HHT, 12-hydroxyheptadecatrienoic acid; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; TxB₂, thromboxane B₂; FFA, free fatty acid.

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NEN (Les Ulis, France), respectively. Both unlabeled and [^{14}C]radiolabeled (specific activity: 1.94 GBq/mmoles) 20:4 Δ 14 $trans$ were obtained by total synthesis as previously reported (15). The radiolabeled fatty acids were diluted in ethanol with the respective unlabeled fatty acid in order to obtain to a specific activity of 0.37 GBq/mmol. 12 HPETE and baicalein were purchased from Biomol (Playmouth Meeting, PA). Other chemicals were from Merck (Darmstadt, Germany).

Platelet isolation

Ten-week-old male Wistar rats fed a standard chow (UAR, Villeneuve S/Orge, France), were lightly anesthetized with diethyl ether. Then, blood (10 mL) was withdrawn from the abdominal vein into plastic tubes containing 2 ml of anticoagulant buffer (acid citrate dextrose, ACD). Platelet-rich plasma (PRP) was obtained by low speed centrifugation (150 g for 18 min). PRP was centrifuged (1000 g for 18 min) to obtain a platelet pellet. Platelets were resuspended in Tyrode-HEPES, pH 7.35, buffer (16) and the platelet number was adjusted to $2 \times 10^8/\text{mL}$ before use.

Aggregation studies

Aggregation studies were performed according to the turbidimetric method of Born (17) and as already described for human platelets (9, 10, 18). Briefly, the platelet suspension (400 μL) was placed into a glass turbidity tube and warmed at 37°C in an aggregometer (Labintec, Montpellier, France). A small magnetic stirring bar was added 30 sec after. Calcium chloride ($0.6 \times 10^{-3} \text{ M}$) was added after 1.5 min. After 2 min, platelet activation was started by addition of solutions of the fatty acid in ethanol (1 μL of labeled AA, 3 nmol, 0.37 GBq, and 1 μL of unlabeled fatty acid (20:4 Δ 14 t or 20:3 n -9 or 20:4 n -6, 3 to 9 nmol in ethanol)) or 1 μL ethanol as a blank. In a second set of experiments, 1 μL of labeled 20:4 Δ 14 t (3, 40, or 80 nmol in ethanol, 0.37 GBq) and 1 μL of unlabeled AA (3 or 6 nmol in ethanol) or 1 μL unlabeled 12-HPETE (0.8 or 1.6 nmol in ethanol) or 1 μL ethanol as a blank was added. Thus, the final ethanol concentration was 0.5%. The aggregation was monitored for 4 min with a strip chart recorder. The 100% and 0% transmissions were adjusted by using buffer and platelet suspensions, respectively.

Inhibition of the 12-lipoxygenase pathway

The platelet suspension (400 μL) was placed into a glass turbidity tube and warmed at 37°C in the aggregometer and a small magnetic stirring bar was added 30 sec after. Baicalein ($30 \times 10^{-6} \text{ M}$) was added after 1 min and calcium chloride ($0.6 \times 10^{-3} \text{ M}$) was added after 3.5 min. The platelet activation was started by addition of 1

μL of labeled AA alone (3 nmol in ethanol, 0.37 GBq), or by addition of 1 μL 20:4 Δ 14 t (3 nmol in ethanol, 0.37 GBq) with 1 μL of 12-HPETE (1.6 nmol in ethanol).

Thin-layer chromatography of [^{14}C]AA or [^{14}C]20:4 Δ 14 $trans$ and of their metabolites

After 4.0 min of platelet incubation with fatty acids, the aggregation was stopped by addition of 500 μL of ethanol. The lipids were extracted with a mixture of chloroform-methanol 2:1 (v/v) and separated by TLC (19, 20). Briefly, a two-step development system was used. A first development (45 min) in a mixture of hexane-diethyl ether-acetic acid 60:40:1 (v/v/v) separated free acid ($R_f = 0.47$), 12-hydroxyheptadecatrienoic acid (HHT, $R_f = 0.24$), and 12-hydroxyeicosatetraenoic acid (HETE, $R_f = 0.30$) while polar lipid and TxB_2 ($R_f = 0.26$) were separated by using a second development (45 min) with a mixture of diethyl ether-methanol-acetic acid 90:2:2 (v/v/v). Thus 1.5 nmol [^{14}C]AA or 1.5 [^{14}C]20:4 Δ 14 t was used per 10^8 platelets. Radioactivity was detected by using an Automatic TLC-linear Analyser (model LB 2832, Berthold, Elancourt, France) and each metabolite was quantified by the determination of the radioactivity of each spot.

Statistical analysis

Results are expressed as means \pm SD. They were analyzed using the Statistical Analysis System (SAS Institute, Cary, NC). The PROC ANOVA procedure was used for the analysis of variance.

TABLE 1. Fatty acid profile of phospholipids of rat platelets (means \pm SEM of 6 determinations)

Fatty Acid	%
Saturated	
16:0	24.4 \pm 0.10
18:0	15.4 \pm 1.49
22:0	1.7 \pm 0.27
Monounsaturated	
16:1	0.7 \pm 0.10
18:1	6.5 \pm 0.21
20:1	0.4 \pm 0.04
22:1 n -9	0.2 \pm 0.05
24:1 n -9	1.2 \pm 0.10
Polyunsaturated	
18:2 n -6	9.5 \pm 0.47
18:3 n -3	0.1 \pm 0.06
20:2 n -6	0.8 \pm 0.08
20:3 n -6	0.6 \pm 0.07
20:3 n -9	tr
20:4 n -6	20.0 \pm 1.41
22:4 n -6	2.2 \pm 0.44
20:5 n -3	0.2 \pm 0.10
22:5 n -3	0.8 \pm 0.31
22:6 n -3	0.6 \pm 0.10
DMA	1.0 \pm 0.87

Results are means of 6 \pm SD independent determinations; tr, traces < 0.1%; DMA, dimethyl acetal.

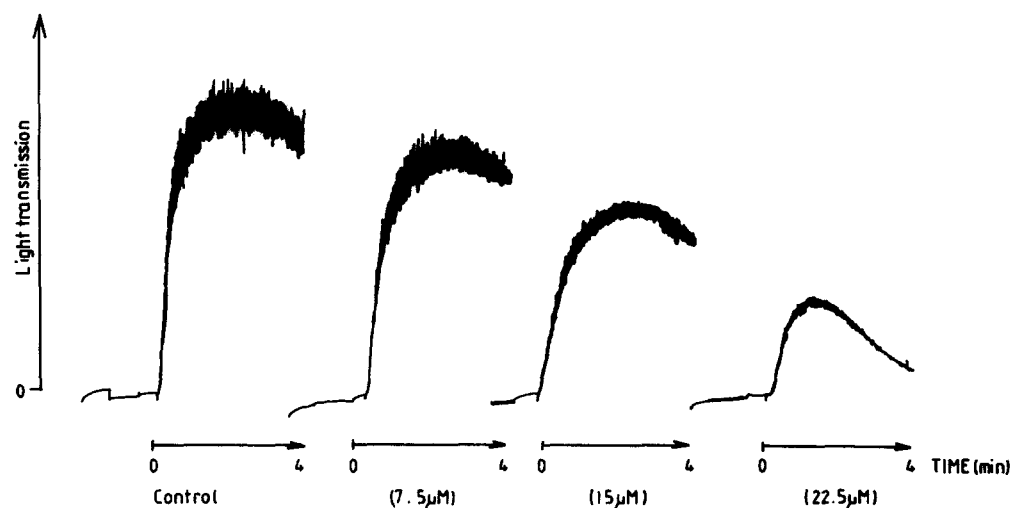


Fig. 1. Typical aggregation of rat platelets stimulated by 7.5 μM AA in the presence of increasing amounts of 20:4 Δ 14t.

RESULTS AND DISCUSSION

Earlier studies on the effects of long chain *trans* PUFA on platelet function and AA metabolism were effected using human platelets (9, 10). However, results showed a large variation within blood samples (9). Furthermore, analyses of the fatty acid profiles (21) showed that the platelets contained *trans* isomers of EPA and DHA. The 20:5 Δ 17*trans* represented up to 0.5% of the total fatty acids while a maximum value of 0.05% was observed for the 22:6 Δ 19*trans*. A partial chromatogram published by the same authors also showed the presence of a peak that eluted just ahead of the AA that could be the *trans* 20:4 Δ 14 isomer. For these reasons, we have carried out the present study using rat platelets as these showed a standard fatty acid composition (Table 1) with no detectable quantities of n-3 and/or n-6 *trans* PUFA.

Inhibition of the platelet response by 20:4 Δ 14t

Recent studies reported that *trans* double bonds might be recognized as a single bond by enzymatic systems. For example, 18:3 Δ 9c,12c,15t is incorporated in rat cardiolipins at the expense of linoleic acid (22) and 20:4 Δ 14t has been shown to be incorporated in phos-

pholipid classes that contain 20:3n-9 (23). This AA isomer presents a structure similar to 20:3n-9 with 3 *cis* ethylenic bonds in Δ 5, 8, and 11 positions. For this reason, we have compared the effects of 20:3n-9 and 20:4 Δ 14t on platelet aggregation stimulated by AA.

The typical response of rat platelets stimulated by 7.5 μM of arachidonic acid in the presence of increasing concentrations of 20:4 Δ 14t (from 0 to 22.5 μM) is presented in Fig. 1. In all cases, the addition of 20:4 Δ 14t to the platelet suspension decreased the platelet response. On the other hand, the addition of arachidonic acid or 20:3n-9 from 7.5 to 22.5 μM did not induce any modification of the platelet aggregation. Results of 12 determinations for each concentration are presented in Fig. 2. The inhibitory effect of 20:4 Δ 14t is significant whatever the concentration added to the platelet suspension and lies from 20% when the inhibitor/substrate ratio (I/S) was 1 to 66% when the I/S ratio reached 3.

The addition of 20:3 n-9 to the platelet suspension did not result in any modification of the platelet response. These data indicate that the observed effect is not due to the addition of a C20 fatty acid, but suggest that the *trans* isomer of arachidonic acid has specific effects on platelet function. O'Keefe et al. (9) and

TABLE 2. Effect of 20:4 Δ 14t on [$1\text{-}^{14}\text{C}$]AA (7.5 μM , 1.5 nmol per 10^8 platelets) metabolism by rat platelets

	Control	20:4 Δ 14t (7.5 μM)	20:4 Δ 14t (15 μM);	20:4 Δ 14t (22.5 μM)
Thromboxane B ₂	339 \pm 32.1 ^a	255 \pm 37.1 ^b	172 \pm 37.0 ^c	126 \pm 26.9 ^d
HHT	331 \pm 48.9 ^a	257 \pm 21.8 ^b	154 \pm 19.2 ^c	136 \pm 19.0 ^c
12-HETE	483 \pm 71.8 ^a	644 \pm 58.7 ^b	739 \pm 73.9 ^c	749 \pm 89.1 ^c
Phospholipids	163 \pm 20.2 ^a	117 \pm 18.3 ^b	104 \pm 11.9 ^c	98 \pm 13.2 ^c
Free AA	101 \pm 34.9 ^a	154 \pm 37.0 ^a	254 \pm 82.8 ^b	297 \pm 80.3 ^b

Results are expressed as pmol/ 10^8 platelets and are means of 12 \pm SD independent determinations. Values having a different superscript are significantly different ($P < 0.05$).

TABLE 3. Effect of 20:3n-9 on [^{14}C]AA (7.5 μM , 1.5 nmol per 10^8 platelets) metabolism by rat platelets

	Control	20:3n-9 (7.5 μM)	20:3n-9 (15 μM)	20:3n-9 (22.5 μM)
Thromboxane B ₂	339 \pm 32.1	360 \pm 31.0	344 \pm 50.9	343 \pm 44.2
HHT	331 \pm 48.9	396 \pm 48.9	348 \pm 58.3	334 \pm 53.7
12-HETE	483 \pm 71.8 ^a	395 \pm 45.1 ^b	405 \pm 40.1 ^b	401 \pm 44.6 ^b
Phospholipid	163 \pm 20.2 ^a	118 \pm 14.2 ^b	97 \pm 11.4 ^c	80 \pm 10.2 ^d
Free AA	101 \pm 34.9	154 \pm 36.8	254 \pm 83.5	297 \pm 79.8

Results are expressed as pmol/ 10^8 platelets and are means of $12 \pm \text{SD}$ independent determinations. Values having a different superscript are significantly different ($P < 0.05$).

Chardigny et al. (10) reported that *trans* isomers of EPA and DHA had a weaker antiaggregant effect on human platelets than EPA and DHA themselves. However, the 17-*trans* isomer of EPA was not similar to AA and the 19 *trans* isomer of DHA was different from EPA, which are their respective structural analogs. These data are in keeping with a specific role of a *trans* double bond as compared with a saturated bond. To our knowledge, no study has yet reported such an interference between arachidonic acid and its main *trans* isomer.

The conversion of radiolabeled AA into platelet metabolites is presented in Table 2. Adding 20:4 Δ 14t to the platelet suspension significantly decreased thromboxane B₂ production, from 25% when the I/S ratio was 1 to 63% when the I/S ratio reached 3. These values are similar to the percentages of inhibition of aggregation (see above). The present data suggest an excellent correlation between the platelet response (aggregation) and the thromboxane synthesis, as has been suggested (24, 25). Moreover, the HHT production showed a similar decrease when the *trans* isomer of arachidonic

acid was added to the platelet suspension. Therefore, the cyclooxygenase pathway is greatly altered by the presence of 20:4 Δ 14t in the medium, suggesting a competition between arachidonic acid and its Δ 14*trans* isomer. Interestingly, the lipoxygenase pathway was stimulated, as 12-HETE production increased up to 55% when the I/S ratio reached 3. At the same time, the incorporation of arachidonic acid into platelet phospholipids was decreased slightly, with a concomitant increase of the remaining free fatty acid. Our data suggest that 20:4 Δ 14t may compete with AA at the cyclooxygenation step, leading to a decrease in TxA₂ production and of platelet aggregation. 20:3n-9, which is not a substrate of cyclooxygenase, did not induce any significant modification in the formation of TxB₂ and HHT from AA (Table 3). Nevertheless, the 12-HETE production decreased slightly, suggesting an inhibition of the lipoxygenase pathway. As this fatty acid is a substrate of 12-lipoxygenase, these results suggest a competition between 20:3n-9 and AA.

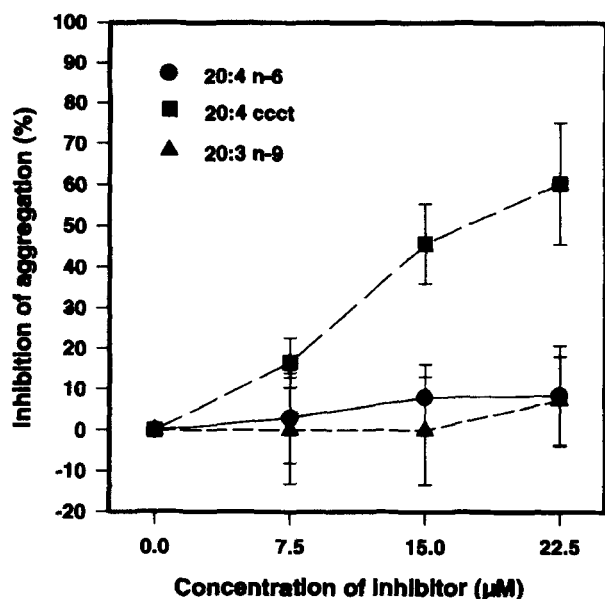


Fig. 2. Inhibition of rat platelet aggregation by increasing amounts of AA or 20:3n-9 or 20:4 Δ 14t. Results are means of 12 independent determinations. Values having a different superscript are statistically different ($P < 0.05$).

Stimulation of platelets by 20:4 Δ 14t

In a second set of experiments, we tested the platelet response to stimulation with the *trans* isomer of arachidonic acid. Whatever the fatty acid concentration (7.5, 100, 200 μM), no significant modification of the light transmission was detected, indicating that 20:4 Δ 14t did not induce any aggregation of the platelets (6 independent measurements for each concentration). However, we tried to detect the formation of any metabolite from the radiolabeled 20:4 Δ 14t, using the same method as for radiolabeled arachidonic acid (see above). After 4 min of incubation, the radioactivity was recovered in the phospholipids (1-25% of total activity, depending of the substrate level) and in the remaining free fatty acid. One

TABLE 4. Platelet aggregation after stimulation of 2×10^8 platelets by [^{14}C]20:4 Δ 14t (7.5 μM , 1.5 nmol per 10^8 platelets) and increasing concentrations of AA (0-15 μM)

	Arachidonic Acid (μM)		
	0	7.5	15
Platelet aggregation (%)	0	42 \pm 4.4	29 \pm 6.4

Results are means \pm SD of 7 independent determinations.

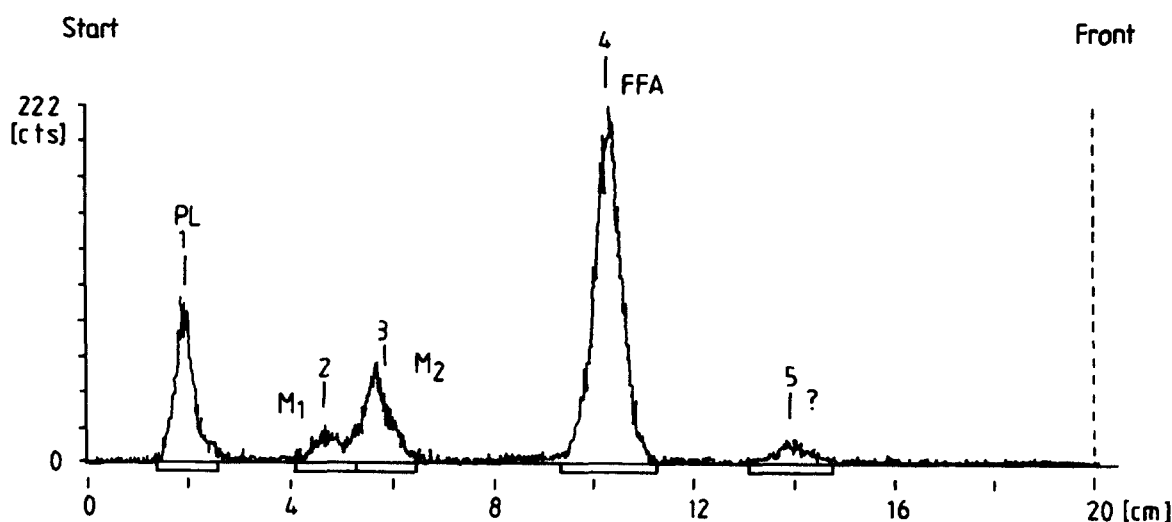


Fig. 3. Radiochromatogram obtained after elution of lipids extracted from rat platelets after 4 min of incubation in the presence of arachidonic acid (7.5 μ M) and radiolabeled 20:4 Δ 14t (7.5 μ M). The mobile phase was hexane–diethyl ether–acetic acid 60:40:1 (v/v/v).

small peak was detected at $R_f = 0.29$, suggesting the presence of traces of an unknown metabolite.

As Croset and Lagarde (13) have reported that arachidonic acid is needed to metabolize C20 polyunsaturated fatty acids by the lipoxygenase pathway, we have stimulated some platelet suspensions with both arachidonic acid (0, 7.5, 15 μ M) and radiolabeled 20:4 Δ 14t (7.5 μ M). The platelet response is presented in **Table 4**. The *trans* isomer of 20:4 alone did not induced any platelet aggregation as described above. When AA was also added, the aggregation of the platelets varied between 29 and 42% according to the concentration of agonist. After 4 min of incubation, the metabolites of radiolabeled 20:4 Δ 14t were analyzed by TLC (**Fig. 3**). The results are presented in **Table 5**. In these experimental conditions, the 20:4 Δ 14t fatty acid is incorporated into phospholipids (about 11% when arachidonic acid was also present). Moreover, two unknown peaks were detected at R_f 0.23 and R_f 0.29; they contained about 6 and 13% of the radioactivity, respectively. The more important peak ($R_f = 0.29$) was already detected when platelets were incubated in the presence of 20:4 Δ 14t without any arachidonic acid. These results indicate that the *trans* isomer of arachidonic acid is able to be metabolized by platelets. How-

ever, the identification of these metabolites had not yet been possible, as the quantity produced is very low. The present data only allow us to demonstrate that two metabolites from 20:4 Δ 14t are formed after 4 min of incubation of rat platelets under our experimental conditions. The metabolite M1 (R_f 0.23) presented a R_f close to the one of HHT whereas M2 (R_f 0.29) had a R_f close to that of 12-HETE. The formation of 12-HETE requests 2 double bonds at the Δ 8 and Δ 11 positions (26). This structural requirement is also present in the 20:4 Δ 14t, 20:3n–9, as another eicosaenoic acid, is a poor substrate of the 12-lipoxygenase as it needs a certain peroxide tone to be oxygenated (13). Thus, 20:3n–9 is better converted by the 12-lipoxygenase in the presence of arachidonic acid or its lipoxygenase product, 12-HPETE (13). This is also the case for 20:4 Δ 14t as shown in **Table 5** where the formation of M1 and M2 is favored in the presence of 12-HPETE (2–4 μ M). According to these data, one could speculate that 20:4 Δ 14t is substrate of lipoxygenase like its structural homolog, 20:3n–9. In order to confirm this hypothesis, we have used baicalein as a selective 12-lipoxygenase inhibitor (27).

Different assays showed that baicalein at a concentration of 30 μ M inhibited by 50% the 12-lipoxygenase

TABLE 5. Labeled metabolites (pmol/ 10^8 platelets) formed from [$1\text{-}^{14}\text{C}$]20:4 Δ 14t (7.5 μ M, 1.5 nmol per 10^8 platelets) after 4 min incubation in the presence of arachidonic acid (AA) or 12-HPETE

	Control	AA, 7.5 μ M	AA, 15 μ M	12-HPETE, 2 μ M	12-HPETE, 4 μ M
M1 ($R_f = 0.23$)	nd	73.7 \pm 22.09 ^a	74.2 \pm 21.01 ^a	58.5 \pm 7.06 ^b	73.3 \pm 17.73 ^a
M2 ($R_f = 0.29$)	tr	136.7 \pm 25.41 ^a	155.1 \pm 33.91 ^a	110.2 \pm 13.27 ^b	159.7 \pm 27.87 ^a
Free 20:4 Δ 14t	892.2 \pm 62.72 ^a	840.2 \pm 51.98 ^a	856.6 \pm 26.97 ^a	1079.25 \pm 30.70 ^b	929 \pm 24.99 ^a
Phospholipids	293.7 \pm 60.59 ^a	136.5 \pm 25.1 ^b	140.7 \pm 34.14 ^b	154.2 \pm 16.74 ^b	169.6 \pm 37.85 ^b

Results are expressed as pmol/ 10^8 platelets and are means \pm SD of 6 independent determinations. Values having a different superscript are significantly different ($P < 0.05$); nd, not detected; tr, trace.

pathway from AA. Further aggregation studies were conducted using [$1\text{-}^{14}\text{C}$]20:4 Δ 14t in the presence of 12-HPETE (4 μM) and baicalein (30 μM). The results indicated that addition of baicalein in platelet suspension significantly decreased the formation of M2 by 60% (159.7 ± 27.87 versus 63.0 ± 8.83 for 6 independent determinations) without modification of the incorporation into platelet phospholipids. These data suggest that 20:4 Δ 14t is metabolized into M2 by the 12-lipoxygenase pathway in presence of 12-HPETE, as is its structural homolog, 20:3n-9. In this case, M2 would be a geometrical isomer of 12-HETE. The presence of baicalein also decreased slightly the formation of metabolite M1, but its origin is still unclear. Further studies will be carried out in order to identify these two unknown metabolites.

In summary, the present data allow the following conclusions: *i*) the Δ 14trans isomer of arachidonic acid is not an agonist for rat platelet aggregation; *ii*) it is able to antagonize the proaggregant effects of arachidonic acid; and *iii*) it is metabolized to a limited extent into at least two products that have to be identified. ■

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