Increased arachidonic acid incorporation into platelet phospholipids in Type 2 (non-insulin-dependent) diabetes

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Summary. Arachidonic acid uptake activity was measured in platelets obtained from 27 Type 2 diabetic patients and 18 age-matched control subjects. In both groups after 1 h incubation almost all the incorporated ¹⁴C-arachidonic acid was located in the phospholipids of the platelets. Arachidonic acid was predominantly incorporated into phosphatidylcholine. The radioactivity incorporated into platelets increased linearly with incubation time, up to 90 min. The linear increase was observed at arachidonic acid concentrations of 0.1–1.0 µg/ml in both groups. The rate of incorporation of radioactivity in diabetic platelets was about 1.4 times higher than that in control platelets at all arachidonic acid concentrations studied. The arachidonic acid uptake activity of diabetic platelets (577 ± 26 ng/60 min per 10^9 platelets) was significantly higher than that in control platelets (410 ± 26 ng/60 min per 10^9 platelets).

No significant correlations were found between the arachidonic acid uptake activity and fasting plasma glucose, total cholesterol or triglyceride levels. The arachidonic acid uptake activity of platelets was significantly higher in diabetic patients with proliferative retinopathy than in those with little or no background retinopathy. In addition, there were no significant differences between control and diabetic subjects in the uptake activity of platelets for linoleic acid and oleic acid. These data may explain the elevated arachidonic acid content in diabetic platelet phospholipids and enhancement of thromboxane synthesis in diabetes.

Key words: Arachidonic acid uptake, platelet phospholipids, Type 2 diabetes, retinopathy, platelet-rich plasma, non-esterified fatty acid.

Thromboxane A₂ (TXA₂), biosynthesized from arachidonic acid in platelet phospholipids, plays an important rôle in the process of platelet aggregation [1, 2]. It is postulated that the balance between platelet TXA₂ and vascular prostacyclin production might be a crucial step in the development of atherosclerosis and arterial thrombosis in diabetes mellitus. Recently, Kalofoutis and Lekakis [3] and we [4] have demonstrated that the arachidonic acid content of platelet phospholipids was increased in diabetes. It is generally accepted that the TXA₂ synthesis rate is regulated at the step of liberation of arachidonic acid from membrane phospholipids [5]. Therefore, increased esterified arachidonic acid content in phospholipids might modulate TXA₂ production in diabetes.

In this study, we examined the ¹⁴C-arachidonic acid uptake activity of platelets obtained from Type 2 (non-insulin-dependent) diabetic patients and age-matched control subjects, to clarify the mechanisms of increased arachidonic acid levels in platelet phospholipids in diabetes.

Subjects and methods

Subjects

The Type 2 diabetic subjects included in the study were 10 men and 17 women ranging in age between 48 and 86 years (mean 67 ± 2 years). Eleven patients were treated with insulin and 16 were treated with sulphonylureas. The control subjects (five men and 13 women, age range 46–79 years, mean 69 ± 2 years) had no history of diabetes or any other disease that would alter platelet aggregation. No subject had taken aspirin or any other drugs which might alter prostaglandin metabolism during the previous 2 weeks. Fasting plasma glucose levels were 4.94 ± 0.11 mmol/l in the control subjects and 9.89 ± 0.94 mmol/l in the diabetic patients ($p < 0.001$). Nineteen patients had diabetic retinopathy (11 background retinopathy and 8 proliferative retinopathy), and four with proliferative retinopathy also had persistent proteinuria. In the diabetic group, six patients had a history of cerebral infarction. No patient had a history of myocardial infarction.

Assay for non-esterified acid uptake activity of platelets

Venous blood was collected from an antecubital vein by a citrated plastic syringe. The final ratio between 3.8% citrate and blood was adjusted to 1:9. Platelet-rich plasma was prepared by centrifugation of blood at 110 g for 10 min at room temperature. The platelet-rich plas-

ma was recentrifuged at 990 g for 20 s to remove erythrocytes. The final platelet concentration in the platelet-rich plasma was $1.6\text{--}4.9 \times 10^8$ cells/ml. ^{14}C -labelled fatty acids (1 μg) (specific activity = 58.4 Ci/mol for ^{14}C -arachidonic acid, 56.0 Ci/mol for ^{14}C -linoleic acid and 59.9 Ci/mol for ^{14}C -oleic acid, respectively, Amersham International, Bucks, UK) suspended in 50 μl of 100 mmol/l Tris buffer (pH 9.0) with a small amount of propyleneglycol were added into 1 ml of platelet-rich plasma. Incubation was carried out at 30 °C for 60 min unless otherwise indicated and was terminated by centrifugation of the incubation mixture at 990 g for 10 min at 4 °C. The radioactivities of both the supernatant and the platelet pellet were counted by a liquid scintillation counter (Beckmann, California, USA).

Fractionation of platelet lipids

Platelet lipids were extracted from labelled platelet samples and separated by centrifugation of the mixture according to the method of Bligh and Dyer [6]. Separation into neutral lipid, non-esterified fatty acid (NEFA) and phospholipids was carried out by thin-layer chromatography with the following solvent system; n-hexane: diethyl ether: acetic acid (90:10:1, by volume). After development, the phospholipid zone was scraped off and then extracted with chloroform: methanol (1:2, by volume), and subfractionation of phospholipids was conducted by thin-layer chromatography with the solvent system of chloroform: methanol: acetic acid: water (75:45:12:3, by volume). Because phosphatidylinositol and phosphatidylserine showed the same R_F value in this solvent system, the phosphatidylinositol and phosphatidylserine zone was scraped and rechromatographed using the solvent system chloroform: methanol: acetic acid: formic acid: water (45:10:1:5:1, by volume) [7]. The radioactivities were detected by a Dünnschicht-scanner II (Laboratories Berthold, Wildbad, FRG) and the zones corresponding to the appropriate phospholipid standards were scraped off for the measurement of radioactivity by a liquid scintillation counter (Beckmann).

Biochemical assays

Fasting plasma glucose levels were estimated by a glucose-oxidase method using a Glucoroder-S automated glucose analyzer (Analytical Instruments, Tokyo, Japan). Plasma total cholesterol (Cholescolor-auto, Tokoboseki, Osaka, Japan [8] and triglyceride (Lipidos-auto N, Toyoboseki, Osaka, Japan) [9] were measured by an enzymatic colorimetric method. Plasma NEFA levels were assayed using an enzymatic spectrophotometric technique (Iatro-MA 701 NEFA, Iatron Laboratories, Tokyo, Japan) [10].

Statistical analysis

Results are presented as mean $\pm$ SEM. Statistical analysis was performed by Student's two-tailed t-test.

Results

When platelet-rich plasma was incubated with ^{14}C -arachidonic acid, approximately 15% of the radioactive arachidonic acid was taken up into platelets after 1 h. Radioactivity (99.4%) was incorporated into the phospholipid fraction. The effect of time on arachidonic acid incorporation into platelets is shown in Figure 1. The radioactivity incorporated into platelets increased linearly with time up to 90 min in both groups. The levels of uptake activity in diabetic patients were higher than in control subjects throughout the experiment. The radioactivity incorporated into platelets increased proportionally according to the increase in substrate con-

Fig. 1. Effect of incubation period on arachidonic acid uptake activity in platelets. Each point represents the mean of duplicate assays. Platelet-rich plasma was obtained from: ○—○ control subjects; ●—● diabetic patients. Each point represents the mean of three different subjects

Fig. 2. Effect of concentrations of ^{14}C -arachidonic acid on arachidonic acid uptake activity in platelets from: ○—○ control subjects; ●—● diabetic patients. Each point represents the mean of two different subjects. Duplicate assays were performed in each subject at each time-point indicated

centration (0.1–1.0 $\mu\text{g}/\text{ml}$ platelet-rich plasma) in both control and diabetic platelets (Fig. 2).

There was a linear relationship between arachidonic acid uptake activity and platelet numbers ranging from 1.6 to 4.9×10^8 cells/ml platelet-rich plasma. We could not find any significant difference in the platelet concentration in platelet-rich plasma between control and diabetic subjects ($2.49 \pm 0.17 \times 10^8$ and $2.35 \pm 0.14 \times 10^8$ cells/ml, respectively).

As summarized in Figure 3, the arachidonic acid uptake activity of diabetic platelets was significantly higher than that in control platelets (577 ± 26 versus 410 ± 26 ng/60 min per 10^9 platelets, $p < 0.001$). Among the diabetic group, platelets obtained from patients with proliferative retinopathy had significantly greater activity than those from patients with little or no background retinopathy (696 ± 45 versus 526 ± 25 ng/60 min per 10^9 platelets, $p < 0.01$).

Fig. 3. The incorporation of ^{14}C -arachidonic acid into platelet phospholipids in control and diabetic subjects. Number of subjects in each group is shown in parentheses, and line represents mean in each group

Table 1. Distribution of ^{14}C -arachidonic acid incorporated into platelet phospholipids in control and diabetic subjects

Phospholipids (ng/60 min per 10^9 platelets)	Control subjects (n=3)	Diabetic patients (n=4)	Difference ^a
Phosphatidylcholine	243 $\pm$ 19	350 $\pm$ 21	107
Phosphatidylinositol	58 $\pm$ 16	76 $\pm$ 9	18
Phosphatidylserine	27 $\pm$ 5	73 $\pm$ 8	46
Phosphatidylethanol- amine	61 $\pm$ 15	103 $\pm$ 16	42

^a Difference = (mean value of diabetic patients) – (mean value of control subjects). Results expressed as mean $\pm$ SEM

Table 2. The incorporation of ^{14}C -labelled fatty acid into platelet phospholipids in control and diabetic subjects

(ng/60 min per 10^9 platelets)	Control subjects (n=8)	Diabetic patients (n=9)
Arachidonic acid uptake	428 $\pm$ 50	613 $\pm$ 40 ^a
Linoleic acid uptake	75 $\pm$ 16	82 $\pm$ 13
Oleic acid uptake	77 $\pm$ 16	85 $\pm$ 13

^a $p < 0.01$ versus control subjects. Results expressed as mean $\pm$ SEM

Table 3. Relationship between ^{14}C -labelled fatty acid uptake in platelets and plasma biochemical variables in diabetic subjects

	Fasting plasma glucose	NEFA	Total choles- terol	Trigly- ceride
Arachidonic acid uptake (n=27)	NS	NS	NS	NS
Linoleic acid uptake (n=9)	NS	$r = -0.807$ $p < 0.01$	NS	NS
Oleic acid uptake (n=9)	NS	$r = -0.826$ $p < 0.01$	NS	NS

NS = not significant

A more detailed examination of the distribution of incorporated ^{14}C -arachidonic acid in platelet phospholipids was performed. Arachidonic acid was predominantly incorporated into phosphatidylcholine in both control and diabetic subjects (Table 1). Although statistical analysis could not be evaluated due to the small number of experiments, the incorporation of ^{14}C -arachidonic acid into diabetic platelets tended to be higher than that into control platelets in all the phospholipid classes, especially in the phosphatidylcholine fraction.

To determine whether arachidonic acid uptake by diabetic platelets increased specifically, uptake activities for linoleic and oleic acid were examined simultaneously with those for arachidonic acid. The same trend of linearly increased linoleic and oleic acid incorporation into platelets was observed both in control and diabetic subjects up to 90 min incubation time. There were no significant differences in linoleic and oleic acid uptake between control and diabetic subjects (Table 2). The correlation between arachidonic acid uptake activity and plasma biochemical variable is shown in Table 3. The arachidonic acid uptake had no significant correlation with any of these variables in diabetes. On the other hand, the linoleic and oleic acid uptake activities were found to have significant negative correlations with plasma NEFA levels in diabetes (both $p < 0.01$).

Discussion

The accelerated platelet aggregation reported in diabetic patients suggests existence of abnormal arachidonic acid metabolism in diabetic platelets. TXA_2 , having an important rôle in platelet aggregation, is biosynthesized from arachidonic acid in platelet phospholipids. The arachidonic acid content in the phosphatidylcholine and phosphatidylinositol fraction is known to be an important modulating factor for TXA_2 synthesis [3, 11]. Therefore, increased arachidonic acid content in diabetic platelet phospholipids [3, 4] was considered as one possible cause of increased TXA_2 synthesis in diabetic platelets [12–14]. However, Jones et al. [15] recently reported a significant decrease in arachidonic acid levels in diabetic platelet phospholipids, a result quite different from ours. It has been demonstrated that the synthesis of prostaglandin precursors, polyunsaturated fatty acids, is influenced by insulin [16] and ageing [17]. Thus the discrepancy in results might be explained by a difference in type of diabetes: all the patients in our study had Type 2 diabetes, whereas the patients of Jones et al. probably had Type 1 diabetes as indicated by insulin-dependence and early onset of diabetes. Moreover, the difference in age of subjects (mean 45 years in their study and 67 years in ours) might be another possible reason. We demonstrated here that the accelerated arachidonic acid uptake activity of Type 2 diabetic platelets accounted for the increased arachidonic acid content in platelet phospholipids.

Although the uptake of arachidonic acid is affected by the number of platelets, control and diabetic subjects were similar in this respect. Phospholipid phosphorus levels in platelets might possibly alter arachidonic acid uptake activity, but were similar in control and diabetic subjects in our preliminary data (not shown). The fatty acid uptake activity of platelets might be influenced by NEFA levels in platelets and plasma. Bills et al. [18], however, demonstrated that free arachidonic acid levels in platelets are negligible. Moreover, the plasma free arachidonic acid levels in diabetic patients, determined by chemical ionization gas chromatography/mass spectrometry analysis, did not differ from those in control subjects (approximately 1.0 mg/l in both groups). Thus, the accelerated ^{14}C -arachidonic acid uptake activity of diabetic platelets may be involved in the quantitatively increased arachidonic acid content of platelets in diabetic patients.

The lack of significant correlation of arachidonic acid uptake activity of diabetic platelets with plasma NEFA levels, and the significant negative correlation of linoleic acid and oleic acid uptake with plasma NEFA levels (Table 3), are supported by our previous report [4]. We have now demonstrated a good correlation between the levels of fatty acids, excluding arachidonic acid, in platelet phospholipids and those in total plasma lipids. These results suggest that the fatty acid uptake activity of platelets is specifically accelerated for arachidonic acid in diabetic patients. The activity of an arachidonic-acid-specific arachidonoyl CoA synthetase in platelets discovered recently by Wilson et al. [19], might be activated in the diabetic state.

The arachidonic acid uptake activity of platelets was significantly higher in the diabetic patients with proliferative retinopathy than in those with little or no background retinopathy (Fig. 3). Production of TXA_2 by platelets after ADP, arachidonic acid or thrombin stimulation was higher in the diabetic patients with microangiopathy than in those without complications [12–14, 20]. On the other hand, prostacyclin synthesis was decreased in the diabetic patients with microangiopathy [21]. It is important to elucidate the mechanisms of abnormal arachidonic acid metabolism in diabetes in order to understand the pathogenesis of vascular complications in this disease.

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