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# Platelet thromboxane A<sub>2</sub> receptors in type I diabetes

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## SUMMARY

1. Human platelet thromboxane A<sub>2</sub> receptor expression on the membrane surface is possibly dynamically regulated by changes either in the ligand concentration or in membrane fluidity. An increased thromboxane A<sub>2</sub> production and a decreased membrane fluidity has been reported in diabetic patients.

2. In the present study the binding characteristics of platelet thromboxane A<sub>2</sub> receptors have been investigated in nine diabetic patients (type I) and in 15 healthy control subjects by a radioligand-binding method using 9,11-dimethylmethane-11,12-methane-16-[3-<sup>125</sup>I]-4-hydroxyphenyl]-13,14-dihydro-13-aza-15-tetranor-thromboxane A<sub>2</sub> as the radiolabelled ligand.

3. The maximum concentration of binding sites was 163 (sd 35) fmol/10<sup>8</sup> platelets ( $n=15$ ) with 987 (sd 209) receptors/platelet in controls, whereas in diabetic patients the maximum concentration of binding sites was 74.2 (sd 28) fmol/10<sup>8</sup> ( $n=9$ ) with 447 (sd 172) receptors/platelet ( $P<0.001$ ). The dissociation constants were 18 (sd 4) nmol/l and 21 (sd 6) nmol/l (not significant) in control subjects and in diabetic patients, respectively. Glycated haemoglobin, which is reported to reduce membrane fluidity, was found to be negatively correlated ( $r=0.60$ ,  $P<0.05$ ) with thromboxane A<sub>2</sub> receptor number in the diabetic patient group. On the contrary, a positive linear correlation between the equilibrium dissociation constant and glycated haemoglobin was found in diabetic patients ( $r=0.75$ ,  $P<0.01$ ).

**Key words:** diabetes, platelets, receptors, thromboxane A<sub>2</sub>.

**Abbreviations:**  $B_{max}$ , maximum concentration of binding sites; HbA<sub>1c</sub>, glycated haemoglobin;  $K_d$ , equilibrium dissociation constant; PTA-OH, 9,11-dimethylmethane-11,12-methane-16-[3-<sup>125</sup>I]-4-hydroxyphenyl]-13,14-dihydro-13-aza-15-tetranor-thromboxane A<sub>2</sub>; TxA<sub>2</sub>, thromboxane A<sub>2</sub>.

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## INTRODUCTION

An increased thromboxane A<sub>2</sub> (TxA<sub>2</sub>) production in response to aggregating stimuli has been reported in platelets from diabetic patients [1-3].

Specific TxA<sub>2</sub>-binding sites have been found on the platelet membrane surface [4, 5] and membrane receptors are often dynamically regulated by several factors. Among these a major role is attributed to changes in the ligand concentration [6-9] or to changes in the membrane fluidity [10-12]. An impaired membrane fluidity in blood cells and tissues has been also observed in diabetic patients and related to the increase in protein glycosylation [13, 14]. Therefore the increased TxA<sub>2</sub> synthesis and membrane viscosity could induce changes in platelet TxA<sub>2</sub>-binding sites in diabetic patients.

In the present paper the binding characteristics of platelet TxA<sub>2</sub> receptors in patients with type I diabetes mellitus have been investigated by using a <sup>125</sup>I-labelled derivative (<sup>125</sup>I-PTA-OH) of the 13-azapinane thromboxane antagonist, ONO11120 [5].

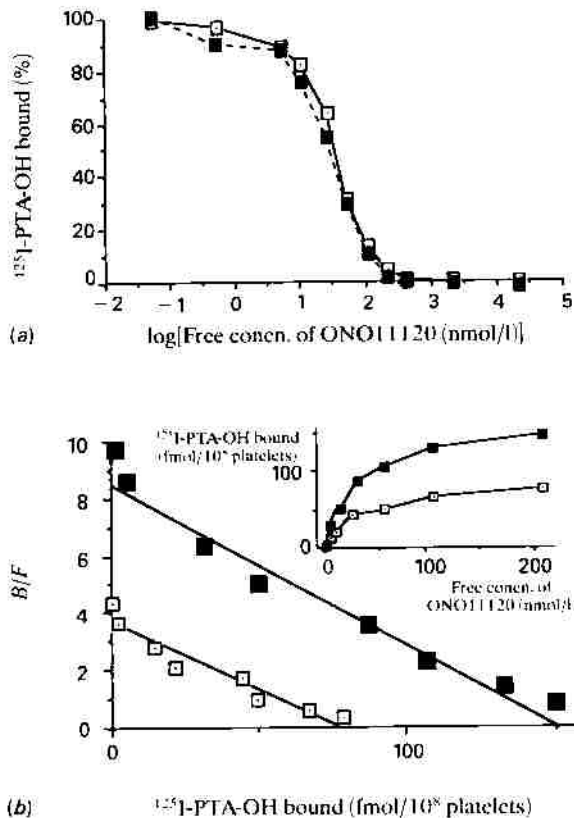
## METHODS

### Materials

ONO11120 (9,11-dimethylmethane-11,12-methane-16-phenyl-13,14-dihydro-13-aza-15-tetranor-TxA<sub>2</sub>) [5] was a kind gift of Professor S. Narumiya (Kyoto, Japan). <sup>125</sup>I-PTA-OH [2000 Ci/mmol; 74 000 GBq/mmol; 9,11-dimethylmethane-11,12-methane-16-[3-<sup>125</sup>I]-4-hydroxyphenyl]-13,14-dihydro-13-aza-15-tetranor-TxA<sub>2</sub>], the labelled hydroxylated form of ONO11120, was obtained from Amersham (Amersham, Bucks, U.K.). All the other reagents were obtained from Merck (Darmstadt, F.R.G.) and were of analytical grade.

### Subjects

Nine patients with type I diabetes mellitus were investigated. The clinical characteristics of the patients are reported in Table 1. Fluoroangiography was routinely performed and three patients had background retinopathy. All patients were normotensive and no clinical or



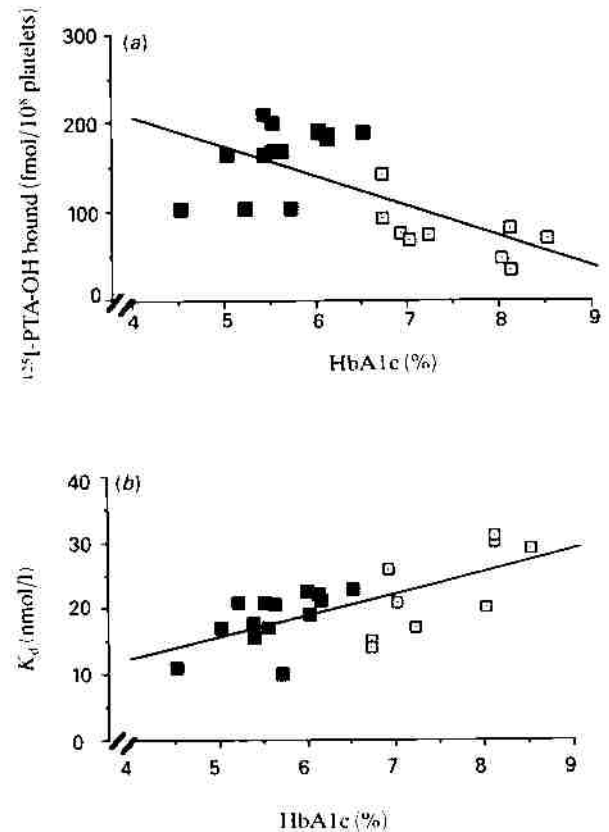
**Fig. 1.** (a) Curves of the displacement of specific  $^{125}\text{I}$ -PTA-OH binding to human platelets by ONO11120 in control subjects (■) and in diabetic patients (□). (b) Scatchard analysis of  $^{125}\text{I}$ -PTA-OH binding to platelets from control subjects (■) and diabetic patients (□). The saturation curve is depicted in the inset. B/F, Bound/free.

binding was 0.942 (SD 0.099;  $n=7$ ), suggesting the existence of a homogeneous individual class of binding sites without co-operativity.

Saturation analysis showed saturation of the binding at a ligand concentration about 100 nmol/l (Fig. 1). Scatchard analysis yielded a straight line, indicating a single class of binding sites for  $^{125}\text{I}$ -PTA-OH (Fig. 1). The equilibrium dissociation constant ( $K_d$ ) and the maximum concentration of binding sites ( $B_{\text{max}}$ ) had values of 18.1 (SD 3.9) nmol/l ( $n=15$ ) and 163 (SD 35) fmol/ $10^8$  platelets respectively, with 987 (SD 209) binding sites per platelet ( $n=15$ ).

Scatchard analysis of data obtained in diabetic platelets also yielded a straight line (Fig. 2), with a slightly higher  $K_d$  of 21.1 (SD 6) nmol/l ( $n=9$ ). But the intercept with the  $x$ -axis ( $B_{\text{max}}$ ) was significantly lower ( $B_{\text{max}}=74.2$  (SD 28) fmol/ $10^8$  platelets;  $n=9$ ), with 447 (SD 172) binding sites per platelet ( $n=9$ ) ( $P<0.001$ ) (Table 2). The Hill coefficient for these data was similar to that obtained in control subjects.

Platelet counts and protein concentration in platelet suspensions were comparable in the two groups, as were



**Fig. 2.** (a) Negative linear correlation between  $^{125}\text{I}$ -PTA-OH bound and HbA1c in control subjects (■) and diabetic patients (□).  $y=341.12-33.52x$ ;  $r=0.66$ . (b) Positive linear correlation between  $K_d$  and HbA1c in control subjects (■) and diabetic patients (□).  $y=-0.69+3.28x$ ;  $r=0.66$ .

the percentage of megathrombocytes and mean platelet volume (Table 1). A significant negative linear correlation between platelet receptor number and HbA1c levels was observed both when all subjects were grouped together (Fig. 2) and in the diabetic patient group alone ( $r=0.60$ ,  $n=9$ ,  $P<0.05$ ). On the contrary, a positive linear correlation between  $K_d$  and HbA1c was observed both in all subjects (Fig. 2) and in diabetic patients alone ( $r=0.75$ ,  $n=9$ ,  $P<0.01$ ).

## DISCUSSION

The present findings indicate that in patients with type I diabetes mellitus, platelet TxA<sub>2</sub> receptors are reduced by about 50%. This result cannot be explained by a reduced volume of platelets in diabetic patients, since the mean platelet volume and the protein concentration in platelet suspension were similar in control subjects and in patients.

In this study diabetic patients were selected on the basis of the absence of cardiovascular complications, so that vascular damage is unlikely to be responsible for the

**Table 1. Clinical data for control subjects and diabetic patients**

Values are means  $\pm$  SD. Abbreviations: FBG, fasting blood glucose concentration; TAG, triacylglycerol concentration; TC, total cholesterol concentration; HDL-C, high-density lipoprotein cholesterol.

	Control subjects ( <i>n</i> = 15)	Diabetic patients ( <i>n</i> = 9)
Age (years)	55 $\pm$ 10	58 $\pm$ 7.5
Sex (M/F)	13/5	5/4
Weight (kg)	65 $\pm$ 10	60 $\pm$ 12
Duration of diabetes (years)	—	5.5 $\pm$ 5
HbA1c (%)	5.3 $\pm$ 0.6	7.5 $\pm$ 0.66
FBG (mg/dl)	103 $\pm$ 6	138 $\pm$ 31
TAG (mg/ml)	126 $\pm$ 19	141 $\pm$ 33
TC (mg/dl)	186 $\pm$ 32	191 $\pm$ 26
HDL-C (mg/dl)	49 $\pm$ 9	38 $\pm$ 11
Platelet no. ( $10^6$ ml <sup>-1</sup> )	242 $\pm$ 22	238 $\pm$ 35
Megathrombocytes (%)	6.8 $\pm$ 1.8	10.1 $\pm$ 2.2
Mean platelet volume (fl)	6.8 $\pm$ 0.2	7.1 $\pm$ 0.3
Protein content (mg/10 <sup>9</sup> platelets)	3.5 $\pm$ 0.7	3.6 $\pm$ 0.5

instrumental evidence of peripheral artery disease or ischaemic heart disease was found (Doppler examination, electrocardiogram at rest and on exercise). The control group was composed of 15 clinically healthy subjects of equivalent age. No subject or patient had taken any drug for at least the 15 days preceding the study. All were non-smokers. Informed consent was obtained from each subject investigated.

#### Blood sampling and platelet isolation

After the subjects had fasted overnight blood was withdrawn by clean venepuncture between 08.00 and 09.00 hours and 15% (v/v) acid-citrate-*b*-glucose (National Institutes of Health, Formula A) was immediately added. Platelets were washed by centrifugation as described previously [15]. The platelet-rich plasma was adjusted to pH 6.5 with acid-citrate-*b*-glucose, and centrifuged at 1800 *g* for 30 min at room temperature (20–22°C). Platelets were then resuspended in 10 ml of phosphate buffer (8 mmol/l Na<sub>2</sub>HPO<sub>4</sub>, 2 mmol/l NaH<sub>2</sub>PO<sub>4</sub>, 10 mmol/l KCl, 135 mmol/l NaCl, pH 7.2) and again recentrifuged at 1800 *g* for 30 min at room temperature. The supernatant was discarded and the platelets were resuspended in assay buffer containing 138 mmol/l NaCl, 5 mmol/l MgCl<sub>2</sub>, 1 mmol/l ethylene glycol bis(aminoethyl ether)tetra-acetate and 25 mmol/l Tris/HCl, pH 7.5. When necessary assay buffer was added to obtain a platelet concentration of 10<sup>9</sup> platelets/ml.

Platelet and megathrombocyte counts were performed by using an electronic counter (Platelet Analyzer 810; Baker Instruments, Allentown, PA, U.S.A.). Megathrombocytes were evaluated as a percentage of platelets counted in the 20–27  $\mu^3$  region. Protein concentration in platelet suspensions was assessed by the method of Lowry *et al.* [16]. Glycated haemoglobin (HbA<sub>1c</sub>) was evaluated with a column chromatographic method [17].

#### Binding studies

A portion of the platelet suspension (0.1 ml) was incubated for 10 min at room temperature in a final volume of 0.2 ml with <sup>125</sup>I-PTA-OH at a final concentration of 0.05 nmol/l (2000 Ci/mmol); plus ONO11120 at increasingly selected concentrations (0–4000 nmol/l). Non-specific binding was considered as the residual radioactivity measured after the addition of ONO11120 at a final concentration of 20000 nmol/l. Non-specific binding of 0.05 nmol/l <sup>125</sup>I-PTA-OH amounted to 25–35% of total bound radioactivity.

After 10 min incubation, 4  $\times$  4 ml of ice-cold buffer was added to each tube to stop the reaction, and the content was rapidly filtered under reduced pressure through Whatman GF/C glass microfibre filters. Previous time course experiments showed that under these conditions binding equilibrium was reached [14]. The entire washing procedure was completed within about 15 s. Filters were dried under an air flow and were counted in a Beckman  $\gamma$ -counter with an overall efficiency of 50%. The total binding at each concentration of the displacement curves at equilibrium was determined by dividing the decay per minute (d.p.m.) of each platelet pellet by the calculated specific activity in d.p.m./mol [5, 15]. The analysis of these binding isotherms was performed as described by Scatchard [18].

#### Statistical analysis

If not otherwise indicated, all data given in the text are means (SD). Each experiment was performed in triplicate and a displacement curve was plotted for each subject. The results were analysed by using Student's *t*-test for unpaired data and by linear regression analysis.

#### RESULTS

The curve of the displacement of <sup>125</sup>I-PTA-OH binding by ONO11120 is shown in Fig. 1. The Hill coefficient for the

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