ORIGINAL ARTICLE

Effects of diltiazem on platelet activation and cytosolic calcium during percutaneous transluminal coronary angioplasty

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Aims: To evaluate effects of diltiazem on platelet hyper-reactivity in situations associated with endothelial injury and their possible relationship to cytosolic calcium concentration.

Methods: Blood samples were collected at seven time points from 35 patients undergoing percutaneous transluminal coronary angioplasty (PTCA) who received combined diltiazem and aspirin/ticlopidine therapy or aspirin/ticlopidine therapy alone. Platelet expression of glycoprotein IIb/IIIa and P-selectin, production of thromboxane B₂, and cytosolic calcium concentration were measured, respectively, by whole blood flow cytometry, radioimmunoassay, and fluorospectrophotometry. The effects of diltiazem of different concentrations on expression of glycoprotein IIb/IIIa and P-selectin were also studied in vitro in blood samples from patients with chronic stable angina.

Results: Of the two treatments, aspirin/ticlopidine therapy did not prevent an acute increase of expression of glycoprotein IIb/IIIa and P-selectin and plasma thromboxane B₂ five minutes and 10 minutes after first inflation and 10 minutes after PTCA, whereas combined diltiazem and aspirin/ticlopidine therapy had a significant inhibitory effect. In the group receiving aspirin/ticlopidine therapy, there was a short term increase of platelet [Ca²⁺]_i immediately after PTCA which was significantly reduced by diltiazem treatment. Expression of glycoprotein IIb/IIIa and P-selectin was significantly inhibited in vitro by diltiazem in the concentration of 200 ng/ml or higher, but not 50 ng/ml.

Conclusions: Combined diltiazem and aspirin/ticlopidine therapy significantly inhibited platelet activation that continued in the presence of conventional aspirin/ticlopidine treatment. Antiplatelet effects of diltiazem were probably a consequence of reduction of platelet [Ca²⁺]_i and may only be achieved in higher than therapeutic concentrations.

Pathological coronary arterial thrombosis is an important complication of percutaneous transluminal coronary angioplasty and intracoronary stent implantation, which contributes to acute ischaemic events and chronic vessel restenosis.^{1,2} Platelet activation in response to multiple factors by several mechanisms primarily mediates the occurrence of arterial thrombosis.^{3,4} Changes in cytosolic calcium concentration play a central part in platelet activation as an upstream event which further interacts with intracellular targets to evoke functional and structural changes of platelets.⁵ Agents that block calcium signalling may exert their beneficial action by inhibition of platelet activation. Antiplatelet effects of calcium antagonists have been documented in vitro and in clinical studies.⁶ The available data indicate that diltiazem may be more potent than verapamil and the dihydropyridines in inhibiting ADP induced and collagen induced aggregation in vitro.⁷ Mehta *et al* found that therapeutic concentrations of diltiazem attenuated thromboxane B₂ generation stimulated by various agonists in platelet-rich plasma.⁸ Zucker *et al* reported that diltiazem (120–360 mg daily for a week) significantly inhibited platelet aggregation and ATP release in vitro by agonists in 25 healthy subjects.⁹ However, few controlled clinical in vivo studies have been performed in patients with coronary artery disease, particularly those undergoing coronary angioplasty.

Direct assessment of surface expression of integrins such as glycoprotein (GP) IIb/IIIa and P-selectin on individual platelets can be measured by whole blood flow cytometry. We investigated the expression of these antigens and changes of cytosolic calcium concentration in patients treated with diltiazem during percutaneous transluminal coronary angio-

plasty (PTCA), in comparison with a control group. The purpose of the study was to evaluate effects of diltiazem on platelet hyper-reactivity in situations associated with endothelial injury and their possible relationship to cytosolic calcium concentration.

SUBJECTS AND METHODS

Reagents

Diltiazem hydrochloride (Herbesser, Tanabe Seiyaku, Japan) is a benzothiazepin derivative calcium channel blocker that can be administered orally or intravenously. The following murine monoclonal antibodies were used in flow cytometry: fluorescein isothiocyanate (FITC) labelled PAC-1 (Becton Dickinson, San Jose, CA, USA), specific for activation dependent epitope of platelet GP IIb/IIIa complex; FITC labelled anti-CD62P (Fluka, Buchs, Switzerland) specific to the internal α -granule membrane protein P-selectin expressed on the surface of activated platelets; matched controls for isotype, fluorochrome, and protein concentration were run in parallel to all monoclonal antibodies: FITC labelled IgG1 (Sigma Chemical, St Louis, MO, USA) and FITC labelled IgM (Becton Dickinson, San Jose, CA, USA). The cell permeant fluorescent probe fura-2/AM (fura-2 acetoxymethyl ester) for detection of cytosolic calcium concentration was from Sigma Chemical, St Louis,

Abbreviations: FITC, fluorescein isothiocyanate; GP, glycoprotein; PTCA, percutaneous transluminal coronary angioplasty

MO, USA. Plasma thromboxane B_2 was measured by radioimmunoassay using a commercial kit (RIA Institute, Beijing, China).

Study groups

Thirty five patients undergoing PTCA were studied. They were divided sequentially into a control group of 20 patients receiving aspirin/ticlopidine therapy, and a diltiazem treated group of 15 patients receiving combined diltiazem and aspirin/ticlopidine therapy. Standard diltiazem therapy includes oral administration of 90–180 mg daily for 3–5 days before PTCA, a 10 mg bolus injection immediately before PTCA, which is followed by a 1.5 hour, 20 mg/hour infusion, and oral administration of 90–180 mg daily for seven days after PTCA. Both groups received standard aspirin/ticlopidine therapy which begins with ticlopidine, a 250 mg initial dose, plus aspirin, a 300 mg initial dose 12 to 24 hours before PTCA, which is followed by ticlopidine, 250 mg twice daily, plus aspirin, 300 mg daily for 30 days.

The study was approved by the ethical committee of the First Affiliated Hospital of Zhejiang University. All patients gave written consent to participate in the study.

Blood sampling

A heparin coated 5Fr Amplatz catheter was used for cannulation of the coronary sinus from the femoral vein. The two syringe method was used to obtain blood samples from the coronary sinus. Blood samples were collected at seven time points. Collection points from before PTCA included a baseline sample <1 hour before PTCA. At this point samples were collected from both the coronary sinus and peripheral vein. During PTCA blood samples were collected from the coronary sinus five minutes and 10 minutes after first inflation. Three other samples were obtained by venipuncture at 10 minutes, 24 hours, and 48 hours after PTCA. A preliminary study demonstrated that aspirating blood through the long catheter did not cause artificial platelet activation.

Analysis of GP IIb/IIIa and P-selectin expression

Blood samples were prepared for flow cytometric analysis using the whole blood method described previously.¹⁰ Five microlitres of citrated blood were added within 15 minutes after sampling to 50 μ l of HEPES buffered saline containing 5 μ l of appropriate concentrations of monoclonal antibodies. After gentle mixing, the samples were incubated for 20 minutes at room temperature, then fixed with 1 ml of 1% paraformaldehyde to inhibit further activation.

Analysis was performed using a Coulter Epics XL flow cytometer (Coulter Electronic Ltd, Luton, UK). The platelet population was identified from its light scattering property and its identity confirmed using an isotype control.

Results are expressed as a binding index calculated from the percentage of platelets positive for the marker and the mean fluorescence intensity for each sample from the following equation: binding index = (percent positive $\times$ mean fluorescence intensity) $\times 100^{-1}$.

Measurement of plasma thromboxane B_2

Thromboxane B_2 was measured as the stable metabolic product of thromboxane A_2 by radioimmunoassay. The technique has been described previously.¹¹ After centrifugation at 1500 g for 10 minutes, supernatants were stored at -20°C until analysed. A standard curve was obtained with thromboxane B_2 standards. Thromboxane B_2 levels in the test samples were derived from this standard curve. With this technique, the specificity is 100% for thromboxane B_2 . Intra-assay and interassay variation coefficients were respectively 8.7% and 10.4%. All measurements were made within one month after collection of samples.

Measurement of cytosolic calcium concentration in platelets

Fresh blood was immediately mixed with 1/9 by volume of citrate-citric acid-dextrose anticoagulant. Platelet-rich plasma was obtained by centrifuging whole blood at room temperature for 20 minutes at 100 g . The platelets were sedimented by centrifugation of the supernatant at 1000 g for 10 minutes. The pellets were resuspended in pH 7.5 HEPES buffered saline. The resuspended pellets were incubated with fura-2/AM (2 μ mol/l final concentration) for 45 minutes at 37°C . Before the last centrifugation, apyrase was added to 20 μ g/ml. The platelets were then centrifuged at 350 g for 15 minutes, then resuspended in HEPES buffered saline and adjusted to a platelet count of 200×10^9 /l.

To measure the fluorescence, we used a Hitachi F-4000 spectrofluorometer (Hitachi Corp, Tokyo, Japan) according to the method of Grynkiewicz *et al.*¹² The excitation wavelength was 340 nm and emission wavelength 500 nm. In this study we determined the basal values for ionised calcium by using the dissociation constant and formula described by Grynkiewicz *et al.*

In vitro studies

Peripheral venous blood samples were collected from 10 patients with chronic stable angina (age 48–65 years) without use of a tourniquet. Patients affected by diabetes, atrial fibrillation, or haematological disease were excluded. None of the subjects had taken any platelet-active drugs in the previous 10 days. Whole blood (0.50 ml) was incubated with normal saline (diluent for diltiazem) or with diltiazem (50, 200, 500, 1000, 5000, 10 000 ng/ml final concentration) at 37°C for 1 minute. GP IIb/IIIa and P-selectin expression were analysed by whole blood flow cytometry described before.

Statistical methods

All data are shown as mean (SEM). The effects of coronary interventional procedures on the variables were compared by the paired t test or non-parametric (Wilcoxon) tests. One way analysis of variance was used to determine the significance of the differences between the two groups. A p value <0.05 was considered significant. Data were analysed on SPSS 10.0 for Windows software.

RESULTS

Clinical characteristics

Platelet counts were normal in all patients and were not significantly affected by coronary interventional procedures (data not shown). There were no differences in baseline characteristics and procedural characteristics between control patients and those receiving diltiazem therapy (table 1). In the diltiazem treated group, intravenous diltiazem produced a reduction in mean arterial pressure (from 110.6 (1.8) to 103.2 (1.5) mm Hg) that was associated with a decrease in heart rate (from 86.2 (1.6) to 80.6 (1.6) beats/min).

Platelet expression of GP IIb/IIIa and P-selectin

In blood samples from the coronary sinus, relative to the diltiazem treated group, platelet expression of GP IIb/IIIa significantly increased in the control group after first inflation, with a peak at 10 minutes after the inflation (fig 1A). Expression of P-selectin significantly increased relative to baseline in the control group five minutes and 10 minutes after the first inflation (fig 2A). In peripheral samples, expression of GP IIb/IIIa and P-selectin in the control group significantly increased 10 minutes after PTCA (fig 1B and 2B). These increases were not observed in diltiazem treated patients. Additionally, in the control group, at 24 hours and 48 hours after PTCA, expression of GP IIb/IIIa and P-selectin significantly decreased relative to 10 minutes after PTCA. However, in the diltiazem treated

Table 1 Patient characteristics

Characteristic	Control patients (n=20)	Diltiazem treated patients (n=15)
Mean (SEM) age in years	63.4 (2.3)	67.7 (1.5)
Males (%)	16 (80)	13 (87)
Unstable angina (%)	16 (80)	12 (80)
Previous myocardial infarction (%)	4 (20)	1 (6.7)
Risk factors (%)		
Hypertension	10 (50)	6 (40)
Diabetes	3 (15)	0
Procedural variables		
Number with three vessel disease (%)	5 (25)	3 (20)
Stenosis of culprit vessel in % (SEM)	84.4 (2.1)	85.7 (2.4)
Length of stenosis lesion in mm (SEM)	15.6 (1.2)	18.9 (1.5)
Number of stents (SEM)	1.3 (0.1)	1.1 (0.1)
Maximal pressure of inflation (atm) (SEM)	11.8 (0.7)	11.2 (0.4)
Duration of inflation in sec (SEM)	20.3 (1.6)	25.8 (3.0)
Dose of heparin in mg (SEM)	73.6 (3.9)	64.6 (3.3)

Figure 1 Platelet expression of GP IIb/IIIa in control group or diltiazem treated group during PTCA from coronary sinus samples (A) or peripheral samples (B). Values are mean (SEM). Difference between means is significant between baseline and time point (** $p < 0.01$, *** $p < 0.001$), and diltiazem treated group and control group (## $p < 0.01$, ### $p < 0.001$).

group, at 24 hours and 48 hours after PTCA, expression of GP IIb/IIIa and P-selectin significantly decreased relative to baseline (fig 1B and 2B).

Plasma thromboxane B_2

Plasma thromboxane B_2 significantly increased 10 minutes after the first inflation in the control group. In diltiazem treated patients, plasma thromboxane B_2 was significantly less than that in control patients five minutes and 10 minutes after first inflation (fig 3A). In the control group, plasma thromboxane B_2 significantly increased 10 minutes after PTCA. This increase was not observed in diltiazem treated patients. In the control group, at 48 hours after PTCA, plasma

Figure 2 Platelet expression of P-selectin in control group or diltiazem treated group during PTCA from coronary sinus samples (A) or peripheral samples (B). Values are mean (SEM). Difference between means is significant between baseline and time point (** $p < 0.01$).

thromboxane B_2 significantly decreased relative to 10 minutes after PTCA (fig 3B).

Cytosolic calcium concentration

Platelet $[Ca^{2+}]_i$ significantly increased after first inflation in the control group, with a peak at 10 minutes after the inflation. Relative to aspirin/ticlopidine therapy alone, platelet $[Ca^{2+}]_i$ was significantly lowered with combined diltiazem and aspirin/ticlopidine therapy five minutes and 10 minutes after first inflation (fig 4A). Only in the control group, platelet $[Ca^{2+}]_i$ significantly increased 10 minutes after PTCA. In both groups, at 24 hours and 48 hours after PTCA, platelet $[Ca^{2+}]_i$ significantly decreased relative to 10 minutes after PTCA (fig 4B).

In vitro studies

Expression of GP IIb/IIIa and P-selectin was not significantly inhibited by diltiazem in the concentration of 50 ng/ml, but in the concentrations of 200 ng/ml or 500 ng/ml (data not shown). A representative experiment is shown in fig 5. Higher concentrations ($\geq 1 \mu\text{g/ml}$) of diltiazem almost inhibited their expression completely.

DISCUSSION

We have shown a time dependent increase of the parameters of platelet activation after PTCA; the increase of platelet expression of GP IIb/IIIa and P-selectin peaked 10 minutes after first inflation in coronary sinus samples; expression of GP IIb/IIIa and P-selectin and plasma thromboxane B_2 increased 10 minutes after PTCA in peripheral samples, with a decrease at 24 hours and 48 hours.

These data demonstrate that systemic and local platelet activation occurs after coronary interventional procedures despite conventional aspirin/ticlopidine treatment. In contrast to our present findings, Fredrickson *et al* showed no increased platelet activation in peripheral blood samples from patients

Figure 3 Plasma thromboxane B₂ in control group or diltiazem treated group during PTCA from coronary sinus samples (A) or peripheral samples (B). Values are mean (SEM). Difference between means is significant between baseline and time point (**p<0.01), and diltiazem treated group and control group (#p<0.05, ##p<0.001).

undergoing elective PTCA, but the number of subjects in the study was too small and samplings were performed much later, at a time when acute changes might have been attenuated by the rapid clearance of activated platelets from the circulation.¹³ Differences in outcomes of studies may also be due to our standardised protocol, local sampling techniques, and frequent sampling.

Although there is no any evidence of a direct effect of ticlopidine on the GP IIb/IIIa complex, previous studies reported that the addition of ticlopidine for ≥3 days to aspirin effectively inhibited expression of GP IIb/IIIa.^{14, 15} Our results would therefore agree with most of these studies, in that administration of initial dose of ticlopidine 12–24 hours before PTCA failed to prevent an acute rise of the parameters of platelet activation as a consequence of the vascular injury after elective PTCA.¹⁶ Since continuing platelet activation contributes to an adverse outcome, this suggests that additional antiplatelet therapy is necessary. However, all parameters fell to close to or below baseline values 24 hours and 48 hours after PTCA, suggesting the slow effect of ticlopidine on platelet activation and systemic blood dilution to the local plaque event.

Elevation of intracellular calcium concentration, either as a result of calcium influx from the exterior or calcium release from intracellular stores, is a necessary and sufficient event for platelet activation.¹⁷ Previous studies showed that calcium antagonists have an inhibitory effect on platelet activation in vitro and ex vivo, but no clear consensus on concentrations that the inhibition requires has been reached from existing studies. We showed that, after incubation with higher than therapeutic concentrations of diltiazem (200–500 ng/ml) in vitro, expression of GP IIb/IIIa and P-selectin was significantly inhibited in whole blood samples from patients with chronic stable angina. The inhibition of platelet activation with diltiazem occurs in a concentration dependent fashion. Although diltiazem in therapeutic concentrations may exert

Figure 4 Platelet [Ca²⁺]_i in control group or diltiazem treated group during PTCA from coronary sinus samples (A) or peripheral samples (B). Values are mean (SEM). Difference between means is significant between baseline and time point (**p<0.01), and diltiazem treated group and control group (#p<0.05).

Figure 5 Effect of diltiazem on platelet expression of GP IIb/IIIa in vitro in a representative whole blood sample from a patient with chronic stable angina. A, no diltiazem; B, 200 ng/ml diltiazem (0.4 µM); C, 1 µg/ml diltiazem (2.2 µM).

modest inhibition on thromboxane B₂ generation and secondary aggregation induced by stimuli in subthreshold concentrations, our present study suggested that inhibition of fibrinogen binding and degranulation of α-granules can only be achieved in higher concentrations.

We added diltiazem by oral administration and infusion during PTCA and found that platelet activation occurring in the presence of standard antiplatelet treatment was prevented by this calcium antagonist. However, Knight *et al* reported that in healthy volunteers there was a rise in P-selectin expression and GP IIb/IIIa levels were not affected after oral administration of amlodipine for one week.¹⁸ The inconsistencies were not surprising and have several possible explanations. First, using a combination of oral administration and infusion, we obtained high blood drug concentrations of diltiazem during the interventional procedure that may exert a measurable effect on platelet function in vivo. But the regimen has been shown to cause a mild reduction of heart rate and mean arterial pressure with the starting infusion dose.

Second, patients with coronary artery disease, particularly those undergoing coronary interventional procedures, have abnormal platelet responsiveness resulting from functionally incompetent endothelium and unstable plaques.¹⁹ Their response to calcium antagonists may therefore differ from that observed in healthy subjects. For example, Sanguigni *et al* reported inhibition of exercise induced increase of expression of P-selectin after amlodipine in patients with chronic stable angina.²⁰

Our study, using the sensitive fluorescent probe for the first time to investigate changes in cytosolic calcium concentration of platelets during PTCA, found that platelet $[Ca^{2+}]_i$ significantly increased immediately after PTCA, with an obvious decrease 24 hours after PTCA. There was a statistical correlation between the changes in platelet $[Ca^{2+}]_i$ and those in the parameters of platelet activation measured. This substantiated the speculation that changes in $[Ca^{2+}]_i$ are fundamental to the platelet response to activation in clinical complicated settings as seen in vitro. We also observed that an angioplasty induced short term increase of platelet $[Ca^{2+}]_i$ was significantly reduced by diltiazem. In vitro studies, however, showed that diltiazem, verapamil, and nifedipine inhibited Ca^{2+} influx induced by ADP, platelet activating factor, and U46619 in concentrations far above those achieved with usual clinical use.²¹ Our results may relate to high concentrations of diltiazem obtained and relatively high platelet activation status in patients with coronary arterial disease. Unlike excitable cells, human platelets do not possess voltage dependent calcium channels and there is still lack of consensus on clear mechanisms of calcium influx.²²⁻²³ It has been proposed that calcium antagonists may exert their effects on platelet $[Ca^{2+}]_i$ by inhibition of calcium mobilization from intracellular stores, inhibition of GP IIb/IIIa complex, or interaction with receptor operated calcium channels such as α -adrenergic receptors.²⁴

Platelet activation after PTCA is generally associated with an adverse prognosis and is one of the primary targets of medication. We demonstrated that platelet activation that continued in the presence of conventional aspirin/ticlopidine treatment was inhibited by combined diltiazem and aspirin/ticlopidine treatment. Although accurate mechanisms of calcium influx are still unclear, inhibition of an increase of platelet $[Ca^{2+}]_i$ by calcium antagonists may represent a striking approach to the prevention of platelet activation after coronary interventional procedures. Future studies should include platelet specific and efficient calcium antagonists to evaluate their use.

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