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# Influence of glucose concentration on the effects of aspirin, ticlopidine and clopidogrel on platelet function and platelet–subendothelium interaction

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**Abstract**

Clinical studies have shown that the ability of aspirin to prevent cerebrovascular accidents is weaker in patients with diabetes. The aim of this study was to determine whether high concentrations of glucose modified the effect of aspirin, ticlopidine and clopigodrel on platelet function and platelet–subendothelium interactions. This in vitro study tested three different concentrations of glucose. The effects were analyzed by comparing platelet aggregometry in whole blood, nitric oxide and prostacyclin production in cultures of human endothelial cells, and by quantitative analysis of morphological features of the platelet–subendothelium interaction under flow conditions. High concentrations of glucose increased platelet aggregation (13.9 Ω with 5 mM glucose vs. 21.6 Ω with 16.6 mM) and platelet–subendothelium interactions (28.9% with 5 mM glucose vs.35.2% with 16.6 mM), and decreased nitric oxide and prostacyclin production. In the presence of high concentrations of glucose, the antiaggregant effect of aspirin and its influence on nitric oxide production were diminished ($IC_{50}$ 54 μM with 5 mM glucose vs.556 μM with 16.6 mM glucose), and its effect on the platelet–subendothelium interaction was reduced (10.5% platelet occupancy with 5 mM glucose vs.23% with 16.6 mM glucose). The effects of ticlopidine and clopidogrel were not significantly modified.


*Keywords:* Aspirin; Ticlopidine; Clopidogrel; Platelet; Nitric oxide (NO); Platelet–subendothelium interaction.

## 1. Introduction

Hyperglucemia is clearly recognized as the main cause of vascular complications of diabetes. Initial stage changes affect biochemical and metabolic pathways and lead to alterations in endothelial function (Stout, 1989). As a result, the endothelium comes to function as a stimulus for thrombogensis, increased vasoconstrictor tone and neovascularization, a change that gives rise in turn to the angiopathy characteristic of diabetes (Stratton et al., 2000). One of the main biochemical mechanisms involved in this pathogenic process is diminished endothelial and leukocyte synthesis of prostacyclin and nitric oxide (NO) (De Vriese et al., 2000). Both changes interfere with platelet aggregation and favor vasodilation (Sadoshima et al., 1988; Moncada et al., 1991). In parallel with the resulting endothelial dysfunction, hyperglucemia stimulates platelet function, with the consequent appearance of proaggregant substances such as thromboxane $A_2$ (De La Cruz et al., 1997a). Together, these factors contribute to the appearance of thrombotic processes (Fuster et al., 1992).

Because of the imbalance between the endothelium and the platelets, antiplatelet aggregant drugs are believed to be of potential use in preventing vascular complications from diabetes. Traditionally, acetylsalicylic acid has been the antiplatelet agent used most widely to prevent arterial ischemic events (Antithrombotic Trialists' Collaboration, 2002).

Recent years have seen the appearance of new antiplatelet drugs such as ticlopidine and clopidogrel, whose biological effects differ in biochemical terms from those of aspirin (Weitz et al., 1995; Verstraete, 1995; Hirsh and

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Table 1
Effects of glucose on platelet aggregation (maximum intensity, in Ω), endothelial nitric oxide and 6-keto-prostaglandin $F_{1\alpha}$ production, and platelet–subendothelium interaction parameters ($n = 8–10$ samples in each experiment)

| | Glucose (mM) | | |
|---|---|---|---|
| | 5.0 | 8.3 | 16.6 |
| ADP (2.5 μM)-induced aggregation (Ω) | 13.94 ± 1.35 | 17.75 ± 1.5[a] | 21.59 ± 2.64[b] |
| Collagen (1 μg/ml)-induced aggregation (Ω) | 8.67 ± 0.90 | 12.80 ± 1.42[a] | 23.56 ± 1.40[c] |
| Nitric oxide (μM) | 6.23 ± 0.64 | 4.47 ± 0.40[a] | 2.42 ± 0.25[c] |
| 6-Keto-prostaglandin $F_{1\alpha}$ (nM) | 167 ± 16.48 | 111 ± 13.53[b] | 84.22 ± 8.41[b] |
| % Subendothelium covered with platelets | 28.97 ± 0.38 | 36.07 ± 1.64 | 35.24 ± 1.48 |
| % According to the size of the platelet structures | | | |
| ≤ 30 μm$^2$ | 15.38 ± 2.04 | 16.11 ± 1.97 | 43.86 ± 9.09 |
| 31–60 μm$^2$ | 12.00 ± 1.05 | 14.03 ± 2.37 | 19.26 ± 1.18 |
| ≥ 61 μm$^2$ | 72.61 ± 2.88 | 72.01 ± 1.90 | 37.11 ± 5.77 |
| % Subendothelium covered with platelets in presence of endothelium | 17.32 ± 0.54 | 25.50 ± 2.35 | 26.68 ± 3.47 |
| % According to the size of the platelet structures | | | |
| ≤ 30 μm$^2$ | 26.91 ± 2.33 | 70.22 ± 6.00 | 28.24 ± 3.47 |
| 31–60 μm$^2$ | 21.61 ± 0.47 | 16.10 ± 2.17 | 19.14 ± 0.91 |
| ≥ 61 μm$^2$ | 51.47 ± 2.79 | 13.68 ± 4.44 | 52.45 ± 4.40 |

[a] $P < 0.01$, [b] $P < 0.001$, [c] $P < 0.0001$, in comparison to glucose 5.0 mM.

Weitz, 1999). In contrast to acetylsalicylic acid, whose mechanism of action consists of the preferential blockage of cyclooxygenase in both platelets and the endothelium via acetylation of its active center (Vane, 1975), clopidogrel and ticlopidine are thienopyridines whose mechanisms of action consists of blocking platelet ADP receptors (Foster et al., 2001). However, both acetylsalicylic acid and the thienopyridines increase NO production in neutrophils (Lopez-Farré et al., 1995; De La Cruz et al., 2000; Arrebola et al., 2002)) and the arterial wall (De La Cruz et al., 2002b).

Unlike ticlopidine (Sivenius et al., 1992) and clopidogrel (Bath et al., 2002), acetylsalicylic acid reportedly has a lower antithrombotic effect in persons with diabetes than in persons without this disease (Antithrombotic Trialists' Collaboration, 2002). The mechanism that underlies this difference is not well known, but in view of the role of glucose in the appearance of complications from diabetes, it may also have some influence on the differential effect of these two drugs. Accordingly, the aim of this study was to compare the in vitro behavior of acetylsalicylic acid, clopidogrel and ticlopidine on platelet function and platelet–vascular wall interactions in the presence of concentrations of glucose considered physiological and hyperglucemic.

## 2. Materials and methods

### 2.1. Methods

Whole blood for this in vitro study was obtained from healthy men (mean age 37.6 ± 1.5 years, range 19–47 years) who had not taken any medication for at least 15 days previously. One sample was obtained from each donor; all samples were collected with 3.8% sodium citrate at a proportion of 1/10 (v/v) as an anticoagulant. All blood samples were obtained between 9:00 and 10:00 h before the donor had anything to eat. Each subject gave his informed consent to participate in the study.

The experiments to investigate platelet–subendothelium interaction were carried out in a perfusion chamber; the subendothelium was obtained from human umbilical vein endothelial cells in culture. The umbilical cords were obtained from eutocic labors after maternal informed consent was obtained.

The experiments were carried out in samples with three glucose concentrations: (a) 4.4–5.5 mM (80–100 mg/dl) as physiological concentrations (mean value: 5.00 ± 0.23

Table 2
Concentrations of aspirin, ticlopidine and clopidogrel that reduced by 50% the maximum intensity of platelet aggregation in control samples ($IC_{50}$) induced with ADP or collagen after 20 min incubation in whole blood with different glucose concentrations ($n = 8–10$ samples in each experiment)

| $IC_{50}$ (μM) | | |
|---|---|---|
| GLUCOSE (mM) | ADP (2.5 M) | COLLN (1 g/ml) |
| *5.0* | | |
| Aspirin (60 μM) | 750 ± 68.12 (19.66%) | 24.09 ± 1.77 (85.58%) |
| Ticlopidine (10 μM) | 51.96 ± 6.20 (21.18%) | 608 ± 55.12 (0.00%) |
| Clopidogrel (10 μM) | 0.65 ± 0.07 (71.35%) | 133 ± 10.09 (14.02%) |
| *8.3* | | |
| Aspirin (60 μM) | 732 ± 75.23 (4.36%) | 257 ± 23.28 (19.76%) |
| Ticlopidine (10 μM) | 58.37 ± 6.15 (12.98%) | 666 ± 70.20 (5.18%) |
| Clopidogrel (10 μM) | 72.80 ± 9.15 (6.73%) | 221 ± 23.96 (8.38%) |
| *16.6* | | |
| Aspirin (60 μM) | 1593 ± 186 (1.27%) | 556 ± 60.23 (2.46%) |
| Ticlopidine (10 μM) | 947 ± 101 (7.02%) | 808 ± 91.15 (0.00%) |
| Clopidogrel (10 μM) | 178 ± 19.23 (11.06%) | 258 ± 26.26 (6.92%) |

Values in brackets are the percentages of inhibition for different concentrations of drugs used in the perfusion experiments, calculated from the concentration–effect curves.

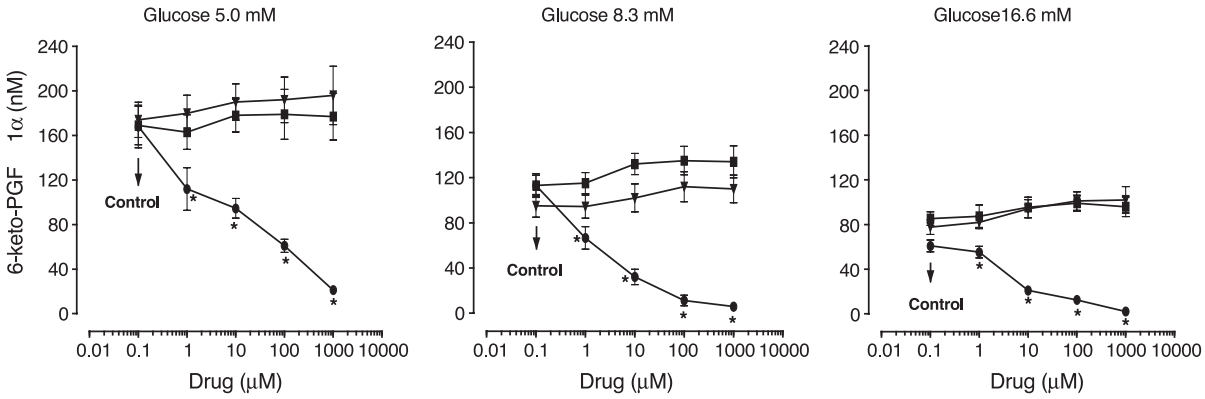


Fig. 1. Concentration-dependent curves of inhibition of 6-keto-prostaglandin $F1_{\alpha}$ production in calcium (1 μM calcium ionophore A 23187)-induced human endothelial cells after 20 min of incubation with aspirin, ticlopidine or clopidogrel, with different glucose concentrations ($n = 8-10$ samples in each experiment). *$P < 0.0001$ with respect to ticlopidine and clopidogrel. —●— Acetylsalicylic acid —■— Ticlopidine —▼— Clopidogel.

mM); (b) 8.3 mM (150 mg/dl) as a model for the situation in patients with metabolically moderately well controlled diabetes; (c) 16.6 mM (300 mg/dl) as a model for the situation in patients with poorly controlled diabetes. In cultured cell experiments the physiological glucose concentration used was 5.0 mM. Blood samples and endothelial cells were incubated with glucose for 1 h before the experiment.

### 2.2. Drugs

Acetylsalicylic acid (Sigma, St. Louis, IL, USA), ticlopidine and clopidogrel (Sanofi-Synthelabo, Barcelona, Spain) were incubated at different concentrations. Eight to ten different samples were run in each of the experiments detailed below.

### 2.3. Analytical procedures

#### 2.3.1. Platelet aggregometry

Platelet aggregation was measured in whole blood with electronic impedance method described by Cardinal and Flower (1980). We used a Chrono-Log 540 aggregometer (Chrono-Log, Haverton, PA, USA), with ADP (2.5 μM) and collagen (1 μg/ml) (Menarini Diagnostica, Barcelona, Spain) to induce aggregation. Drugs were incubated at 37 °C for 5 or 20 min before the aggregation inducer was added, and aggregation was recorded for 10 min. Maximum intensity of aggregation was quantified as the maximum change in electronic impedance in samples without the drug or with a given concentration of each drug.

The concentrations of the aggregating agent were chosen according to previous experiments in wich $EC_{50}$ values were $2.10 \pm 0.37$ μM for ADP ($n = 10$) and $1.10 \pm 0.14$ μg/ml for collagen ($n = 10$).

#### 2.3.2. Endothelial production of nitric oxide

Nitric oxide production was induced with the calcium A 23187 ionophore (1 μM) and quantified in cultured human umbilical vein endothelial cells. The amount of NO was quantified with an electrochemical method (Shibuki, 1992) that used a specific electrode coupled to an ISO-NO detector (Word Precision Instruments, Aston, Stevenage, Hertsforshire, UK).

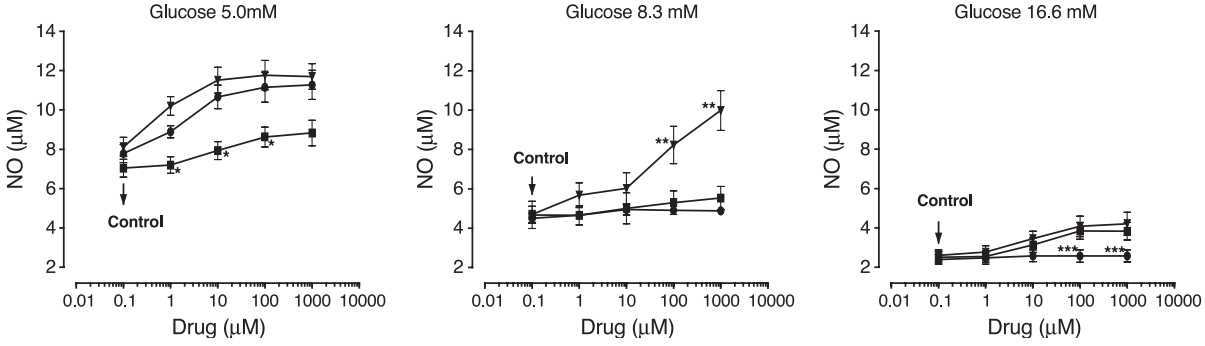


Fig. 2. Concentration-dependent curves of nitric oxide (NO) production induced in endothelial cells with 1 μM calcium ionophore A 23187 after 20 min of incubation with aspirin, ticlopidine or clopidogrel, with different glucose concentrations ($n = 8-10$ samples in each experiment). *$P < 0.05$ with respect to aspirin and clopidogrel; **$P < 0.005$ with respect to aspirin and ticlopidine; ***$P < 0.05$ with respect to ticlopidine and clopidogrel. —●— Acetylsalicylic acid —■— Ticlopidine —▼— Clopidogel.

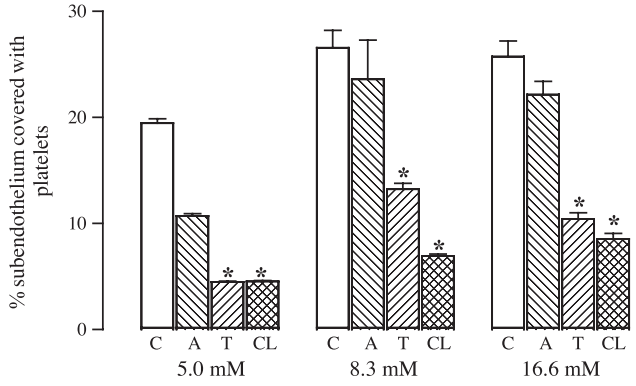


Fig. 3. Percentages of subendothelial matrix covered with platelets after 10 min of blood perfusion (shear rate 800 $s^{-1}$) in control samples and blood samples incubated for 20 min with aspirin, ticlopidine or clopidogrel, with different glucose concentrations ($n = 8–10$ samples in each experiment). *$P < 0.0001$ with respect to the control group and aspirin group. Black bars: control; white bars: aspirin; shaded bars: ticlopidine; hatched bars: clopidogrel.

Nitric oxide production was initially measured under basal conditions with the drug under study or saline solution (control sample) for 20 min at 37 °C. After this step, calcium A 23187 ionophore was added, and the increase in NO production induced by activation of constitutive or calcium-dependent NO synthase was recorded.

### 2.3.3. Endothelial production of 6-keto-prostaglandin $F_{1\alpha}$

The production of prostacyclin was measured as the synthesis of its stable metabolite (6-keto-prostaglandin $F_{1\alpha}$). Endothelial cells harvested from human umbilical veins were stimulated with 1 μM calcium ionophore A 23187 for 3 min at 37 °C. The amount of 6-keto-prostaglandin $F_{1\alpha}$ in the supernatant of cultured endothelial cells was determined with an enzyme immunoassay (Biotrak® RPN 220, Little Chalfont Buckinghmshire, England). The sensitivity of this method was 3.1 pg/ml; within-assay variability for duplicate determinations was 2.8% and between-assay variability was 9.7%.

### 2.3.4. Platelet–vascular subendothelium interaction

To investigate platelet–vascular subendothelium interactions we used blood perfusion experiments with human subendothelial matrix preparations in a flat perfusion chamber (Sakariassen et al., 1983). The subendothelial matrix preparations were obtained from cultures of human umbilical vein endothelial cells basically as described by Jaffe et al. (1973).

The umbilical cord was canulated at both ends, and endothelial cells were obtained by flushing the cord with a collagenase solution (0.05 g in 10 ml Hank's solution, pH 7.2). The resulting liquid was centrifuged to obtain the cell pellet, which was resuspended in a culture dish with MEM 199 (Minimum Essential Medium) culture medium supplemented with 20% deproteinated human serum and 2% penicillin-streptomycin (Bio-Whittaker Europe, Verviers, Belgium). Cell cultures were maintained by washing with Hank's solution (pH 7.2) and adding supplemented MEM 199 (Minimum Essential Medium) medium. Once the desired concentration of cells was reached, the cells were detached with a trypsin solution (pH 7.4) and cultured on pretreated coverslips (30 min with 0.1% gelatin, 30 min with 0.5% glutaraldehyde) to obtain subendothelial matrix preparations. To harvest the subendothelial matrices the cell growths were detached with 3% EGTA (pH 7.4).

For perfusion studies the blood samples were incubated for 5 or 20 min at 37 °C with different concentrations of the drugs under study. The perfusion chamber was coupled to a peristaltic pump such that the incubated blood with or

Table 3
Percentage of subendothelial matrix covered by platelets in experiments without endothelium in the perfusion chamber. Blood was perfused at 37 °C for 20 min, at a shear rate of 800 $s^{-1}$, with different glucose concentrations ($n = 8–10$ samples in each experiment)

| Size of the platelet structures | Control | Aspirin (60 μM) | Ticlopidine (10 μM) | Clopidogrel (10 μM) |
|---|---|---|---|---|
| *Glu: 5.0 mM* | | | | |
| 0–30 $\mu m^2$ | 15.38 ± 2.05 | 46.39 ± 2.48 | 38.21 ± 4.91 | 57.17 ± 2.63 |
| 31–60 $\mu m^2$ | 12.01 ± 1.05 | 24.06 ± 2.34 | 22.95 ± 3.07 | 32.14 ± 2.07 |
| >60 $\mu m^2$ | 72.61 ± 2.88 | 27.94 ± 4.57 | 38.83 ± 5.96 | 10.68 ± 1.51 |
| *Glu: 8.3 mM* | | | | |
| 0–30 $\mu m^2$ | 16.11 ± 1.97 | 33.05 ± 4.06 | 61.02 ± 6.11 | 86.03 ± 3.79 |
| 31–60 $\mu m^2$ | 14.03 ± 2.37 | 22.18 ± 1.81 | 22.42 ± 2.41 | 9.19 ± 0.91 |
| >60 $\mu m^2$ | 72.01 ± 1.90 | 44.76 ± 7.41 | 17.29 ± 2.55 | 4.36 ± 0.66 |
| *Glu: 16.6 mM* | | | | |
| 0–30 $\mu m^2$ | 43.86 ± 9.09 | 59.87 ± 6.77 | 72.57 ± 7.10 | 83.96 ± 5.08 |
| 31–60 $\mu m^2$ | 19.26 ± 1.18 | 21.30 ± 0.59 | 18.42 ± 3.68 | 10.68 ± 3.69 |
| >60 $\mu m^2$ | 37.11 ± 5.77 | 18.82 ± 0.85 | 9.00 ± 0.64 | 4.51 ± 0.58 |

Glu = glucose.

without (control samples) the drug circulated for 5 min at a shear rate of 800 $s^{-1}$ in contact with the subendothelial matrix. The matrices were perfused in the presence and in the absence of the endothelium to test the possible influence of this part of the vessel wall on the platelet–vascular subendothelium interaction.

After perfusion each coverslip with the subendothelial matrix was washed with phosphate-buffered saline solution (pH 7.4) and platelet structures were fixed with an 0.5% solution of glutaraldehyde for 24 h, then stained with 0.25% toluidine blue for morphometric analysis.

In each perfused matrix we calculated the total percentage of subendothelium covered by platelets and the percentages of different platelet structures. For each perfused matrix we examined 20 fields, and for each concentration of drug we examined matrices from at least four different perfusions. Version 5.0 of the Visilog program (Noesis, Orsay-Cedex, France) was used with an inverted microscope to which a Sony B/W CCD camera was coupled.

In these experiments drugs were incubated at concentrations near therapeutic concentrations in the plasma reported in humans: 10–15 μg/ml (60–85 μM) for acetylsalicylic acid (Hart and Huskisson, 1984), 3–6 μg/ml (10–17 μM) for ticlopidine (Knudsen et al., 1992) and 3–6 μg/ml (11–19 μM) for clopidogrel (McEwen et al., 1999). We chosen the lower limit of these intervals (60 μM for acetylsalicylic acid and 10 μM for ticlopidina and clopidogrel).

### 2.4. Statistical analysis

All data in the text, tables and figures are the mean ± standard error of the mean (S.E.M.) of all values for each experiments. The results were tested with one-way analysis of variance followed by the least significant difference test. All analyses were done with version 10.0 of the SPSS program (SPSS, Chicago, IL, USA). The minimum value used to establish statistical significance was $P < 0.05$.

## 3. Results

### 3.1. Platelet aggregation in whole blood

At a concentration of 8.3 mM, glucose increased platelet aggregation induced with ADP (27.33%) and with collagen (47.63%) in comparison to blood samples with 5.0 mM glucose. At a concentration of 16.6 mM glucose the increases were greater: 54.87% when ADP was the inducer, and 171.74% with collagen (Table 1).

All three drugs inhibited platelet aggregation induced by ADP and by collagen in a concentration-dependent way, and for the concentrations usually attained in human plasma during chronic treatment with aspirin (60 μM), ticlopidine (10 μM) or clopidogrel (10 μM). Aspirin had the lowest $IC_{50}$ (concentration that inhibited platelet aggregation by 50%) when platelets were stimulated with collagen, whereas the two thienopyridines had the lowest $IC_{50}$ when platelets were stimulated with ADP. Their maximum effect was seen after 20 min of incubation. The effect of clopidogrel was greater than that of ticlopidine regardless of whether aggregation was induced with collagen or ADP (Table 2).

When higher concentrations of glucose were used in blood samples, the effect of acetylsalicylic acid on platelet aggregation was reduced significantly in samples induced with collagen, and to a lesser degree in samples induced with ADP. The effect of both thienopyridines on platelet aggregation was reduced in samples induced with ADP, but their effect was not modified in samples induced with collagen (Table 2).

### 3.2. Endothelial nitric oxide and prostacyclin production

Endothelial NO production was reduced by 28.25% and prostacyclin production by 33.57% in experiments with 8.3 mM glucose in comparison to cultures with 5.0 mM glucose. When glucose was tested at 16.6 mM, the reduction was

Table 4
Percentage of subendothelial matrix covered by platelets in experiments with endothelium in the perfusion chamber. Blood was perfused at 37 °C for 20 min, at a shear rate of 800 $s^{-1}$, with different glucose concentrations ($n = 8–10$ samples in each experiment)

| Size of the platelet structures | Control | Aspirin (60 μM) | Ticlopidine (10 μM) | Clopidogrel (10 μM) |
|---|---|---|---|---|
| *Glu: 5.0 mM* | | | | |
| 0–30 $\mu m^2$ | 26.91 ± 2.33 | 45.99 ± 3.22 | 98.54 ± 0.53 | 72.98 ± 4.03 |
| 31–60 $\mu m^2$ | 21.61 ± 0.47 | 24.32 ± 1.72 | 1.45 ± 0.55 | 19.63 ± 2.51 |
| >60 $\mu m^2$ | 51.47 ± 2.79 | 29.93 ± 3.82 | 0 ± 0.00 | 7.37 ± 0.69 |
| *Glu: 8.3 mM* | | | | |
| 0–30 $\mu m^2$ | 70.22 ± 6.00 | 99.43 ± 0.46 | 88.67 ± 1.26 | 72.47 ± 4.73 |
| 31–60 $\mu m^2$ | 16.10 ± 2.17 | 0.71 ± 0.06 | 7.34 ± 2.89 | 21.03 ± 2.78 |
| >60 $\mu m^2$ | 13.68 ± 4.44 | 0 ± 0.00 | 3.98 ± 0.25 | 6.10 ± 1.62 |
| *Glu: 16.6 mM* | | | | |
| 0–30 $\mu m^2$ | 28.24 ± 3.47 | 85.74 ± 5.33 | 82.97 ± 6.63 | 92.19 ± 1.90 |
| 31–60 $\mu m^2$ | 19.14 ± 0.91 | 10.33 ± 1.23 | 12.56 ± 1.06 | 6.92 ± 0.84 |
| >60 $\mu m^2$ | 52.45 ± 4.40 | 4.01 ± 0.28 | 4.46 ± 0.51 | 0.87 ± 0.06 |

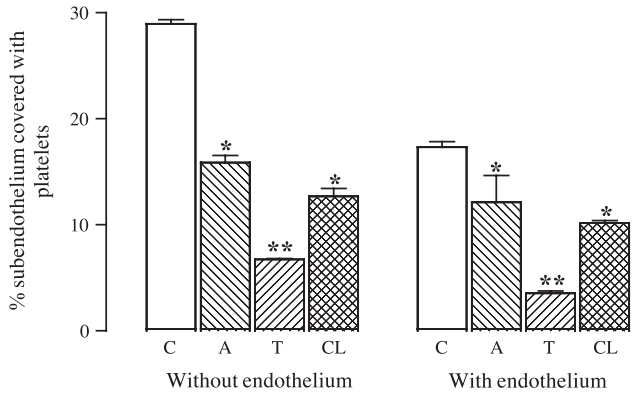


Fig. 4. Percentage of subendothelial matrix covered with platelets after 10 min of blood perfusion (shear rate 800 $s^{-1}$) with or without the endothelial cell monolayer in control samples and blood samples incubated for 20 min with aspirin, ticlopidine or clopidogrel, with 5.0 mM glucose. *$P<0.001$, **$P<0.0001$, with respect to control group ($n=8-10$ samples in each experiment). C: control; A: aspirin; T: ticlopidine; CL: clopidogrel.

greater in both NO (61.15%) and prostacyclin production (49.56%) (Table 1).

The endothelial production of 6-keto-prostaglandin $F_{1\alpha}$ was inhibited in a concentration-dependent way by aspirin, whereas ticlopidine and clopidogrel did not affect production, or even showed a tendency to increase production at high concentrations (Fig. 1).

All three drugs increased endothelial production of NO in a concentration-dependent way; however, the effect of aspirin and clopidogrel was greater than ticlopidine at concentrations similar to those found in human plasma during chronic treatment (Fig. 2).

The effect of acetylsalicylic acid on endothelial NO production was significantly reduced in the presence of high concentrations of glucose, while the effect of the thienopyridines was reduced to a lesser extent (Fig. 2). No effect was seen on the influence of any of the three drugs on endothelial prostacyclin production (Fig. 1).

### 3.3. Platelet–subendothelium interaction

The surface occupied with platelets was increased by 32.2% when 8.3 and 16.6 mM glucose was the concentrations in blood with respect to 5 mM, and by 58.94% when glucose was incubated in endothelial culture (Table 1).

In blood perfusion experiments with subendothelial matrix preparations, all three drugs significantly reduced the surface occupied by platelets in comparison to control samples (Fig. 3). This effect was seen mainly in the platelets structures >60 $\mu m^2$ (Tables 3 and 4).

The presence of endothelium in the flow experiment preparations reduce the subendothelial surface occupied by platelets. In control samples (incubated with no drugs) the area was $28.97 \pm 0.3\%$ without endothelium, and $17.32 \pm 0.54\%$ with endothelium ($P=0.0001$). The effect of all three drugs was modified only slightly by the endothelium (Fig. 4) (Table 1).

In blood samples incubated with high concentrations of glucose, the effect of acetylsalicylic acid on platelet–vascular subendothelium interactions was more markedly reduced ($-72\%$) than the effect of ticlopidine ($-23.38\%$) or clopidogrel ($-11.57\%$). Similar reductions were seen in endothelial cell cultures.

Fig. 5 shows representative examples of platelet–vascular subendothelium interactions observed with different drugs and concentrations of glucose.

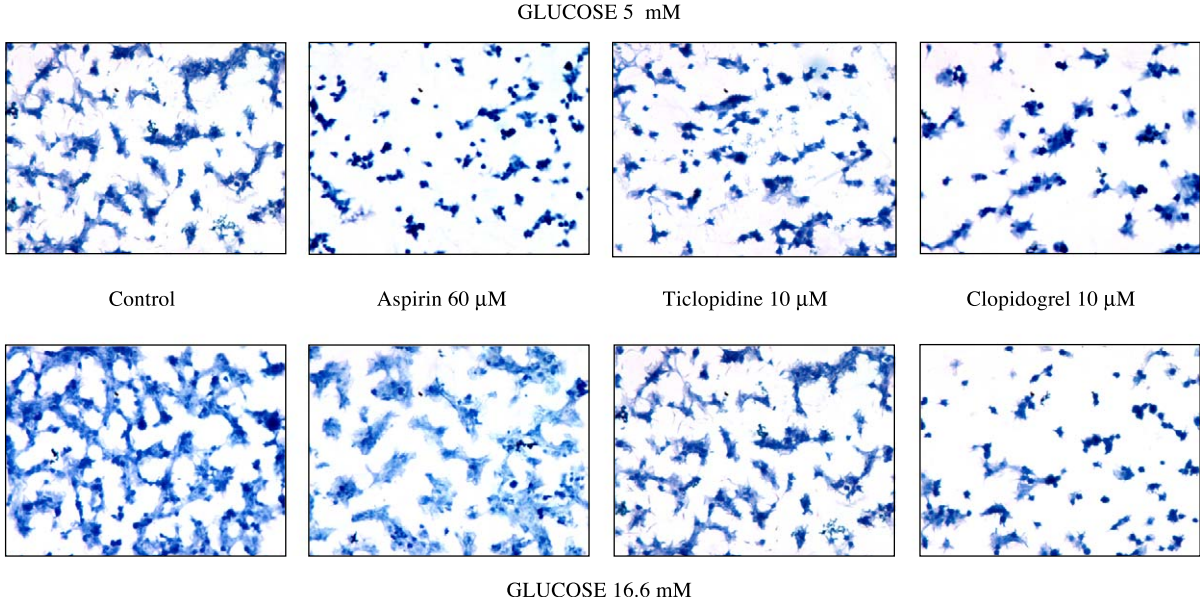


Fig. 5. Representative examples of subendothelial matrix after 10 min of blood perfusion (shear rate 800 $s^{-1}$) in control samples (C) and after incubation for 20 min with aspirin (A), ticlopidine (T) or clopidogrel (CL) with different glucose concentrations.

## 4. Discussion

Hyperglucemia is clearly recognized as the main cause of endothelial dysfunction in diabetes (Stout, 1989), because it leads to biochemical and metabolic alterations that impair functional and structural elements of the vasculature in patients with this disease (Stratton et al., 2000). Endothelial dysfunction leads, in turn, to diminished synthesis and functioning of prostacyclin, and to reduced NO production and effectiveness. The latter, together with increased platelet aggregation, gives rise to vascular conditions that curtail tissue blood flow and favor the tendency for intravascular thrombi to form (Guerci et al., 2001). Some hypothesis have been postulated to explain the mechanisms of endothelial damage after short term glucose exposition, mainly through the induction of oxidative stress (Peiro et al., 2001) or by the formation of advanced glycated end products (Rosen et al., 2001; Tooke, 2000): but is possible a combination of several mechanism interacting each others.

This study yields several key observations: (1) Platelet aggregation increased steadily regardless of which inducer was used. (2) Biochemical substances that limit platelet aggregation in the endothelium (prostacyclin and NO) were reduced in a concentration-dependent manner. (3) Glucose increased platelet–subendothelium interactions by 32.20% in the blood flow system, and by 58.94% in the endothelial cell culture system. These findings are compatible with the recognized importance of endothelial dysfunction in diabetes (Chakrabarti et al., 2000; De Vriese et al., 2000), and with results obtained in most studies of platelet function in this disease (Amado et al., 1981; Davi et al., 1990; Halushka et al., 1997; De La Cruz et al., 1997b). Moreover, our results in flow experiments with whole blood from patients with diabetes were also similar to earlier observations on the effect of glucose on platelet–subendothelium interactions (Knobler et al., 1998).

Our in vitro studies showed that clopidogrel had an antiaggregant action in whole blood. We found no earlier reports of this effect in whole blood, although one earlier study found an antiaggregant effect on washed platelets, albeit at concentrations much higher than those reached in the plasma of patients on long-term treatment with clopidogrel (Weber et al., 1999). It has thus been hypothesized that clopidogrel needs to undergo biotransformation in the liver to become effective, but our results show that this transformation is not necessary to value an antiplatelet effect in whole blood.

The results of this study show that unlike aspirin, both ticlopidine and clopidogrel spared endothelial prostacyclin synthesis. The main undesired effects of acetylsalicylic acid result from unspecific inhibition of cyclooxygenase and the consequent inhibition of prostacyclin synthesis, which can lead to gastrointestinal lesions and gastric mucosa ulcers (Roderick et al., 1993). Although acetylsalicylic acid has traditionally been the antiplatelet aggregant agent used most widely to prevent arterial ischemic events, new drugs such as clopidogrel, which act via different biological pathways, have appeared in recent years (Weitz et al., 1995; Verstraete, 1995; CAPRIE Steering Committee, 1996).

Nitric oxide is related with enhanced effects of acetylsalicylic acid in whole blood (De La Cruz et al., 1987, 2000; Lopez-Farré et al., 1995) and has also shown a neuroprotective action (Moro et al., 2000). We have found that ticlopidine increases calcium-dependent NO production in neutrophils in vitro (De La Cruz et al., 2002a). In this connection, we report here that both ticlopidine and clopidogrel stimulated calcium-dependent NO production in cultured endothelial cells (80.8% for acetylsalicylic acid, 42.9% for ticlopidine, and 87.1% for clopidogrel) at concentrations that clearly inhibited platelet aggregation in vitro, and that are similar to the concentrations reached in human plasma during chronic oral treatment.

Although the in vitro antiaggregant effect of clopidogrel is greater than that of ticlopidine, experiments in the flow system used to investigate platelet–vascular subendothelium interactions showed that the effect of both thienopyridines was similar and greater than the effect of acetylsalicylic acid, as others have previously reported (Roald et al., 1994). This effect of the thienopyridines involved mostly larger platelet structures (>60 $\mu m^2$). The results of perfusions of endothelial cell cultures, used to investigate the influence of the endothelium on platelet–vascular subendothelium interactions in the presence of different drugs, lead us to conclude that the endothelium did not modify the antiaggregant action of any of the drugs tested here, but only facilitated the reduction in larger platelet–endothelium structures seen with the two thienopyridines.

The differences in the effect of clopidogrel and ticlopidina could be explained by the differences in their chemical structures; clopidogrel structure contains a methylcarboxy group, the structure responsible for the cyclooxygenase inhibition. In a recent study, we demonstrated that clopidogrel inhibits platelet thromboxane production stronger than ticlopidine, probably through this structural difference (Arrebola et al., 2003).

In experiments designed to study the influence of glucose on the action of the three drugs compared here, changes in the effects of acetylsalicylic acid were greatest when collagen was the inducer. This is similar to the results of earlier studies that found greater differences in platelet functioning in patients with diabetes than in persons without this disease when collagen was used to induce aggregation (De La Cruz et al., 1997b, 2002b). In experiments with the thienopyridines, their antiaggregant effect was reduced in the presence of high glucose concentrations when ADP was the inducer, whereas the differences were smaller when collagen was the inducer.

In blood perfusion experiments the percentage occupation of the subendothelium by platelets that was inhibited by acetylsalicylic acid was clearly reduced (by as much as

72%) at higher glucose concentrations. In contrast, the reduction in the effect of ticlopidine was only 23.38%, and the corresponding reduction in effect for clopidogrel was 11.57%. In tests of the effects of glucose on the endothelium, the percentage reductions were similar to those recorded in assays without endothelium: 69.07% for acetylsalicylic acid, 10.76% for ticlopidine, and 14.17% for clopidogrel.

Taken together, the findings showed that the aggregometric results paralleled the changes in platelet–vascular subendothelium interactions. In perfusion experiments the main platelet activator was collagen from the vascular subendothelium, and when higher concentrations of glucose were used, the drug whose action was most clearly curtailed was acetylsalicylic acid. These findings were reflected in the results of platelet aggregation studies with collagen as the inducer in the presence of high glucose concentrations. The explanation for these discrepancies is likely to be multifactorial, but one possible mechanism is NO synthesis. In fact, our data show that in the presence of high glucose concentrations, the effect of aspirin on NO production decreased markedly, whereas no such drastic decrease was seen in the action of thienopyridines.

Our findings also confirm that the antithrombotic effect of acetylsalicylic acid is weaker in persons with diabetes than in persons without this disease, in contrast to the findings for ticlopidine and for clopidogrel (present study). The action of the thienopyridines was only slightly diminished by high blood concentrations of glucose.

Earlier studies showed that in patients with diabetes, platelets are less sensitive to the antiaggregant action of endogenous substances such as prostacyclin, NO and adenosine (Gasser et al., 1993; De La Cruz et al., 2001, 2002c). Moreover, intraplatelet concentrations of cAMP and cGMP are lower in diabetes, indicating a lower activity of these cyclic nucleotides (De La Cruz et al., 2001, 2002c). These biochemical findings may explain the platelet hyperactivity reported earlier (De La Cruz et al., 1997a,b; Fuster et al., 1992) and observed in the present study in response to habitual stimuli such as collagen and ADP in the presence of high concentrations of glucose in blood. They might also account for the lesser effectiveness of acetylsalicylic acid in comparison to thienopyridines, in view of the fact that the latter favor the endothelial antithrombotic response by sparing NO and prostacyclin production regardless of glucemia levels.

In conclusion, we report that high concentrations of glucose in the medium appear to affect not only platelet and endothelial functioning per se, but also the action of antiplatelet aggregant drugs, albeit to different degrees. The drug most clearly affected by high glucose concentrations was acetylsalicylic acid, whereas the action of ticlopidine and clopidogrel was little affected. These findings should be taken into account when prophylaxis for thrombotic events is being considered in patients with diabetes.

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