

High Glucose Inhibits the Aspirin-Induced Activation of the Nitric Oxide/cGMP/cGMP-Dependent Protein Kinase Pathway and Does Not Affect the Aspirin-Induced Inhibition of Thromboxane Synthesis in Human Platelets

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Since hyperglycemia is involved in the “aspirin resistance” occurring in diabetes, we aimed at evaluating whether high glucose interferes with the aspirin-induced inhibition of thromboxane synthesis and/or activation of the nitric oxide (NO)/cGMP/cGMP-dependent protein kinase (PKG) pathway in platelets. For this purpose, in platelets from 60 healthy volunteers incubated for 60 min with 5–25 mmol/L D-glucose or iso-osmolar mannitol, we evaluated the influence of a 30-min incubation with lysine acetylsalicylate (L-ASA; 1–300 μ mol/L) on 1) platelet function under shear stress; 2) aggregation induced by sodium arachidonate or ADP; 3) agonist-induced thromboxane production; and 4) NO production, cGMP synthesis, and PKG-induced vasodilator-stimulated phosphoprotein phosphorylation. Experiments were repeated in the presence of the antioxidant agent amifostine. We observed that platelet exposure to 25 mmol/L D-glucose, but not to iso-osmolar mannitol, 1) reduced the ability of L-ASA to inhibit platelet responses to agonists; 2) did not modify the L-ASA-induced inhibition of thromboxane synthesis; and 3) prevented the L-ASA-induced activation of the NO/cGMP/PKG pathway. Preincubation with amifostine reversed the high-glucose effects. Thus, high glucose acutely reduces the antiaggregating effect of aspirin, does not modify the aspirin-induced inhibition of thromboxane synthesis, and inhibits the aspirin-induced activation of the NO/cGMP/PKG pathway. These results identify a mechanism by which high glucose interferes with the aspirin action. *Diabetes* 61:2913–2921, 2012

Atherothrombotic cardiovascular events are the leading cause of morbidity and mortality in type 2 diabetes (1). Platelet hyperactivity, a critical factor in the pathogenesis of atherothrombosis (2), plays a major role in this phenomenon (3). Thus, an aggressive antiaggregating treatment with aspirin is strongly recommended for diabetic patients (4). The aspirin-induced prevention of cardiovascular events, however, is lower in diabetes than in the general population (5–7). Among the mechanisms involved in aspirin resistance are the accelerated half-life of circulating platelets, the occurrence of a proinflammatory and prothrombotic status, the presence of an increased oxidative stress responsible

for platelet activation through isoprostanes, and the use of inadequate aspirin doses (8–10).

Type 2 diabetes is frequently associated with obesity and insulin resistance; the role of these two conditions on platelet dysfunction and on aspirin resistance has been reviewed (9,11,12). The main feature of diabetes, however, is hyperglycemia, which is involved in the pathogenesis of vascular complications (13); the direct role played by high glucose on platelet sensitivity to aspirin has not been fully investigated thus far. Experimental and clinical investigations have mainly focused on chronic hyperglycemia (14–16); a few studies have evaluated the effects of acute platelet exposure to high glucose in *in vitro* models (17–19).

Since viability of platelets *in vitro* is limited to a few hours, studies *in vitro* are designed only to evaluate whether acute increases in glucose concentrations influence platelet responses to aspirin. This is a relevant topic because the so-called “stress hyperglycemia” worsens the prognosis of acute coronary syndromes (20), and postprandial glycemic excursions are considered a strong cardiovascular risk factor (21); among the pathophysiologic mechanisms involved, the role of oxidative stress has been underlined (21). In type 2 diabetes, postprandial hyperglycemia predicts cardiovascular morbidity and all-cause mortality even when glycated hemoglobin is taken into account (22).

The classic mechanism by which aspirin interferes with platelet responses is the prevention of thromboxane A₂ (TXA₂) formation by an irreversible acetylation of the catalytic site of cyclooxygenase-1 (23,24). Besides this pivotal action, aspirin increases platelet bioavailability and synthesis of nitric oxide (NO) (25–27) via acetylation of the constitutive nitric oxide synthase (cNOS) (28). As previously reviewed (29), NO is deeply involved in platelet antiaggregation through the synthesis of cGMP and the consequent activation of the cGMP-dependent protein kinase (PKG); the cGMP/PKG system modulates basic mechanisms of platelet activation, such as agonist-induced increases of cytosolic calcium and cytoskeleton protein contraction. A relevant target of PKG is the focal adhesion protein vasodilator-stimulated phosphoprotein (VASP), which PKG preferentially activates via phosphorylation at serine 239 (30); thus, VASP is a mediator of cGMP action involved in platelet inhibitory pathways. Impaired NO synthesis and/or sensitivity plays a crucial role in the platelet dysfunction described in diabetic patients (31).

Short-term incubations of platelets with high glucose *in vitro* decrease the effects of aspirin on platelet GPIIb-IIIa and P-selectin expression (18) and reduce the aspirin-induced

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Received 12 January 2012 and accepted 23 May 2012.

DOI: 10.2337/db12-0040

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inhibition of platelet aggregation (17,19). It has not been evaluated thus far, however, whether high glucose impairs the ability of aspirin to inhibit thromboxane synthesis or activate the NO pathway; the second phenomenon could be hypothesized because NOS inhibition prevents the effects of high glucose on the aspirin antiaggregating properties (19).

The aims of this study are therefore to investigate whether a short-term platelet exposure to high glucose *in vitro* modifies the aspirin effects on thromboxane synthesis and/or on the NO/cGMP/PKG pathway activation.

RESEARCH DESIGN AND METHODS

Chemicals. ADP, amifostine, sodium arachidonate (NaA), trisodium citrate, D-glucose, L-monomethylarginine (L-NMMA), methylene blue (MB), HEPES-Na, PBS, Tween-20, indomethacin, and mannitol were obtained from Sigma-Aldrich (St. Louis, MO). L-[2,3,4,5-³H]Arginine monohydrochloride was obtained from GE Healthcare Europe GmbH (Milan, Italy). The source of the specific antibodies for Western blotting is shown in the section concerning Western blot analysis. Lysine acetylsalicylate (L-ASA) was provided by sanofi-aventis (Milan, Italy). We preferred L-ASA to ASA because it is soluble in water instead of in DMSO or ethanol, which influence platelet function; L-ASA is a soluble salt of acetylsalicylic acid (ASA) that is converted to ASA and is widely used in clinical practice because it presents the powerful antiplatelet effect of ASA with fewer gastrointestinal side effects (32).

Subjects and platelet preparation. After overnight fasting, we studied 60 healthy volunteers (34 men and 26 women; age = 23.8 ± 0.7 years; BMI = 22.4 ± 0.4 kg/m²), nonsmokers, who denied taking drugs in the previous 2 weeks and had normal fasting and 2-h plasma glucose concentrations after an oral glucose tolerance test, normal insulin sensitivity (HOMA IR = 1.7 ± 0.05), and arterial blood pressure values <140/90 mmHg. They gave informed consent before investigation, and the ethics committee of our hospital approved the study. Venous blood samples were withdrawn without stasis and anticoagulated with 3.8% trisodium citrate, pH 7.4 (volume for volume [v/v], 1/9), for aggregation studies or with citrate-dextrose solution (ACD; v/v, 1/6) for experiments on washed platelets (WPs). Platelet counts were performed on a hematology system of the Bayer Corporation (Tarrytown, NY). Platelet-rich plasma (PRP) was obtained by using the Platelet Function Centrifuge (Bio-Data Corporation, Horsham, PA), designed to provide a rapid separation of PRP by a centrifugation for 30 s and of platelet-poor plasma by a further centrifugation for 120 s. To prepare WPs, ACD-anticoagulated PRP was submitted to further centrifugation at 2,000 *g* for 10 min. The pellet was washed two times with HEPES-Na buffer (10 mmol/L HEPES Na, 140 mmol/L NaCl, 2.1 mmol/L MgSO₄, 5 mmol/L glucose, pH 7.4). WPs are usually used to measure the NO/cGMP/PKG signaling in platelets (33). They present some degree of activation; actually, the concentrations of the platelet activation marker sP-selectin (ELISA kit; Bender MedSystems GmbH, Vienna, Austria) were similar in PRP and in supernatants of WPs (24.2 ± 1.9 and 25.7 ± 1.7 ng/10⁹ platelets; $n = 7$; $P = \text{NS}$), indicating that WPs still release P-selectin after removal of the protein contained in the plasma fraction of PRP.

Study design. Whole blood and platelet samples (PRP and/or WPs) were exposed for 60 min to different D-glucose concentrations (5, 15, and 25 mmol/L) and then incubated for 30 min with L-ASA (1–300 μmol/L). Some experiments have been repeated with 20-min preincubations with the antioxidant agent amifostine (200 μmol/L), the NOS inhibitor L-NMMA (100 μmol/L), or the guanylate cyclase inhibitor MB (50 μmol/L). Some experiments have been carried out with the iso-osmolar control mannitol instead of glucose.

In the different samples, we measured 1) platelet reactivity in high shear-stress conditions in WB; 2) platelet aggregation in PRP in response to NaA (1 mmol/L) or ADP (10 μmol/L); 3) TXB₂ concentrations in response to NaA (1 mmol/L) or ADP (10 μmol/L) both in PRP at the end of each aggregation test and in resting WPs; 4) NOS activity in resting WPs; and 5) cGMP production in unstirred PRP and VASP phosphorylated at serine 239 in WPs.

Platelet reactivity in high shear-stress conditions. Platelet responses were evaluated in whole blood by a Platelet Function Analyzer (PFA-100; Siemens Healthcare Diagnostic Products GmbH, Marburg, Germany), which measures platelet responses under standardized high-shear conditions as a function of the time needed to form a platelet plug, named “closure time” (seconds), within an aperture cut into a platelet-reactive membrane coated with collagen and epinephrine (CEPI) (34).

Platelet aggregation studies. Platelet aggregation studies were carried out in PRP by following light-scattering changes as described by Born (35) using an eight-channel aggregation system (Platelet Aggregation Profiler, Model PAP-8; BioData Corporation). Platelet aggregation in response to agonists was reported

as maximal aggregation. Each aggregation test was recorded for 5 min after the addition of the agonist.

TXB₂ synthesis. In PRP, TXB₂ levels were measured at the end of the aggregation tests (i.e., 5 min after the addition of agonists) after blocking platelet responses with indomethacin (10 μmol/L) and 100 μL of ACD; each sample was then spun at 8,000 *g* for 2 min, and supernatants were stored and frozen at –80°C.

TXB₂ was also measured in WPs resuspended in HEPES buffer containing 0.38 mg/mL fibrinogen in the presence of a 60-min preincubation with 5 or 25 mmol/L glucose. WPs were then exposed to L-ASA (300 μmol/L for 30 min) and stimulated by 1 mmol/L NaA, or by 10 μmol/L ADP for 5 min at 37°C. The reaction was stopped by centrifugation at 2,300 *g* for 5 min at 4°C, and the supernatants were stored and frozen at –80°C. TXB₂ was measured by using the EIA kit (Cayman Chemical Company, Ann Arbor, MI).

Platelet NOS activity. NOS activity was measured by evaluating the conversion of L-[³H]arginine to L-[³H]citrulline (36). Actually, NOS induces the conversion of L-arginine to L-citrulline and to NO with a 1:1 stoichiometry (36). In brief, WPs were resuspended for 60 min in HEPES buffer in the presence of 5 and 25 mmol/L glucose and then exposed to L-ASA (300 μmol/L for 30 min) together with 1 μCi L-[³H]arginine (in HEPES-Na containing CaCl₂); platelet reactions were stopped by centrifugation at 2,000 *g* for 10 min; platelet lysates were mixed with Dowex cation exchange resin (Na⁺ form) to absorb L-arginine. L-[³H]citrulline in the supernatant was measured by liquid scintillation counting. Results were expressed as pmol L-citrulline/min/mg protein.

cGMP production. cGMP was measured in unstirred PRP samples (500 μL) incubated at 37°C for 60 min with 5 and 25 mmol/L glucose and then exposed to L-ASA (300 μmol/L for 30 min). Platelet reactions were stopped with 30% trichloroacetic acid (100 μL). Precipitated proteins were removed by 20-min centrifugation at 2,000 *g* at 4°C. After the addition of 1 mol/L of HCl (100 μL), the supernatant was submitted to 10 extractions with ethyl ether to remove trichloroacetic acid. Samples were then lyophilized and kept at –80°C until determination. cGMP measurement was carried out using a radioimmunoassay kit (Immuno Biological Laboratories, Hamburg, Germany). Data are expressed as pmol/10⁹ platelets.

VASP phosphorylation at serine 239. For detection of VASP phosphorylated at serine 239, WPs were incubated with 300 μmol/L L-ASA for 30 min in the presence of a 60-min preincubation with 5 and 25 mmol/L glucose and processed as previously described (37). After Western blot, membranes were incubated with a monoclonal antibody recognizing VASP phosphorylated at serine 239 (1:1,000; Merck KGaA, Darmstadt, Germany) and then with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA). After additional washes, membranes were submitted to chemiluminescence (GE Healthcare Europe GmbH), and the density of bands was analyzed with Kodak 1D Image Analysis software.

Statistical analysis. Values in the text and figures are means ± SEM. Statistical analysis was performed with different approaches in the different protocols according to the scale type of data: 1) when only two measures had to be compared, we used Student *t* test for paired data when appropriate; 2) results concerning PFA-100 have been evaluated with the nonparametric Wilcoxon signed rank test because closure time is a harmonic variable with a maximum of 300 s, which does not fluctuate proportionally within the scale; 3) results concerning platelet aggregation responses and agonist-induced thromboxane synthesis have been evaluated with a parametric generalized linear model two-factor within subject ANOVA for repeated measures (*n* glucose concentration × *m* L-ASA concentration). All data analyses were performed using SPSS Statistics version 17.0 (SPSS 2008).

RESULTS

Platelet function evaluated by PFA-100. Figure 1 shows the L-ASA effects on PFA-100 CEPI closure time in the presence of three different glucose concentrations ($n = 34$). L-ASA increased time closure (Wilcoxon signed rank test $P < 0.0001$ between every L-ASA concentration), but glucose levels did not modify closure time responses in the presence of the different L-ASA concentrations.

Platelet aggregation

In response to NaA. Glucose 25 mmol/L attenuated the inhibitory effect of L-ASA on NaA-induced platelet aggregation ($n = 24$). In particular, a two-factor within subject ANOVA 3×5 (glucose concentration [5, 15, and 25 mmol/L] × L-ASA concentration [0, 1, 5, 50, and 100 μmol/L]) shows that 1) in the absence of L-ASA, high glucose did not modify platelet aggregation; 2) the main effect (i.e., on

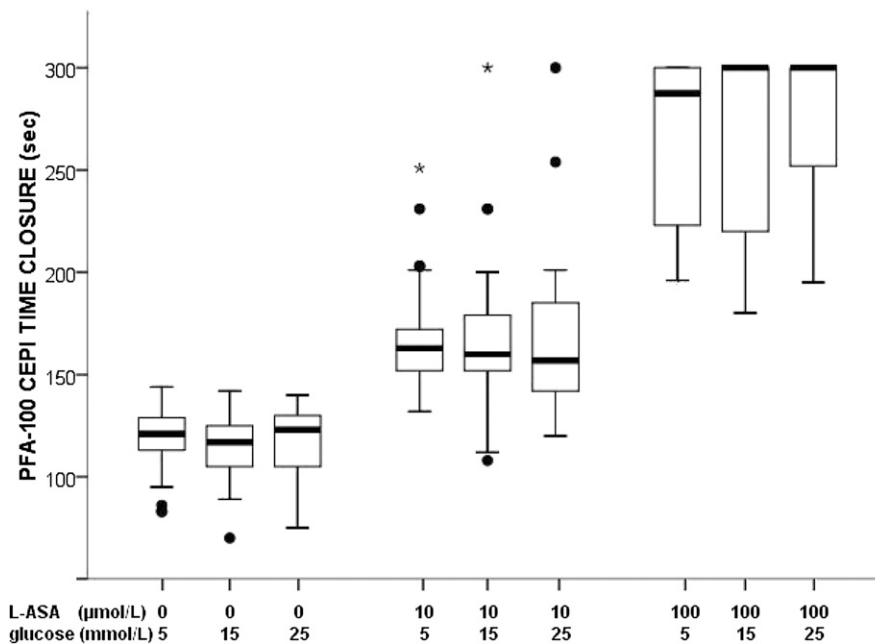


FIG. 1. Effect of platelet exposure to different L-ASA concentrations on closure time of PFA-100 CEPI in the presence of different glucose concentrations. Box plots range from the first to the third quartile; bold line in the boxes represents the median. Whiskers range from the minimum to the maximum of the measured values in the absence of outliers. Dots represent outliers with values between 1.5 and 3 (interquartile range); asterisks represent extreme outliers. Statistical analysis, carried out by Wilcoxon signed rank test, shows that glucose (5, 15, and 25 mmol/L; $n = 34$) did not modify closure time responses in the presence of the different L-ASA concentrations.

the whole sample) of L-ASA was significant starting from the concentration of 5 $\mu\text{mol/L}$ ($P < 0.0001$ for all the concentrations vs. baseline); 3) the inhibitory effect of L-ASA was significant starting from the concentration of 1 $\mu\text{mol/L}$ in the presence of 5 mmol/L glucose (the worst P value = 0.009 vs. baseline) and starting from 5 $\mu\text{mol/L}$ L-ASA in the presence of 15 and 25 mmol/L glucose ($P < 0.0001$ for all concentrations vs. baseline); and 4) the inhibitory effects of 5 and of 50 $\mu\text{mol/L}$ L-ASA did not differ in the presence of 5 vs. 15 mmol/L glucose, whereas it differed in the presence of 5 vs. 25 mmol/L glucose ($P < 0.0001$) and 15 vs. 25 mmol/L glucose ($P < 0.0001$) (Fig. 2A).

In the presence of 20 mmol/L mannitol, the inhibitory effect of L-ASA on NaA-induced platelet aggregation was not different from that observed in the presence of 5 mmol/L glucose ($n = 8$; $P = \text{NS}$ for all L-ASA concentrations). In particular, the inhibition exerted by 50 $\mu\text{mol/L}$ L-ASA was $52.44 \pm 2.85\%$ with 20 mmol/L mannitol vs. $54.25 \pm 1.42\%$ with 5 mmol/L glucose.

In response to ADP. Glucose (25 mmol/L) attenuated the inhibitory effect of L-ASA on ADP-induced platelet aggregation ($n = 24$). In particular, a two-factor within subject ANOVA 3×4 (glucose concentration [5, 15, and 25 mmol/L] \times L-ASA concentration [0, 75, 150, and 300 $\mu\text{mol/L}$]) shows that 1) in the absence of L-ASA, high glucose did not modify platelet aggregation; 2) the concentration-dependent inhibitory main effect (i.e., on the whole sample) of L-ASA on ADP-induced aggregation was significant starting from the concentration of 75 $\mu\text{mol/L}$ ($P < 0.0001$ for all the concentrations vs. baseline); 3) the inhibitory effect of L-ASA was significant starting from the concentration of 75 $\mu\text{mol/L}$ in the presence of 5 and 15 mmol/L glucose ($P < 0.0001$ for all concentrations vs. baseline) but only from 150 $\mu\text{mol/L}$ in the presence of 25 mmol/L glucose ($P < 0.0001$ for 150 and 300 $\mu\text{mol/L}$ L-ASA vs. baseline); and 4) the inhibitory effects of 75,

150, and 300 $\mu\text{mol/L}$ L-ASA did not differ in the presence of 5 vs. 15 mmol/L glucose, whereas they differed in the presence of 5 vs. 25 mmol/L glucose ($P < 0.0001$) and 15 vs. 25 mmol/L glucose ($P < 0.0001$) (Fig. 2B).

In the presence of 20 mmol/L mannitol, the inhibitory effect of L-ASA on ADP-induced platelet aggregation was not different from that observed in the presence of 5 mmol/L glucose ($n = 8$; $P = \text{NS}$ for all L-ASA concentrations). In particular, the inhibition exerted by 150 $\mu\text{mol/L}$ L-ASA was $35.10 \pm 1.98\%$ with 20 mmol/L mannitol vs. $36.25 \pm 2.42\%$ with 5 mmol/L glucose.

The influence of amifostine. The results are shown in Fig. 3. In the presence of the radical oxygen scavenger amifostine, the L-ASA-induced inhibition on platelet aggregation of NaA and ADP did not differ in experiments performed at 5 vs. 25 mmol/L glucose ($n = 9$). Amifostine, therefore, reversed the inhibitory effect exerted by 25 mmol/L glucose on the L-ASA effects, without modifying platelet aggregation in the absence of L-ASA or the L-ASA effect in the presence of 5 mmol/L glucose.

Thromboxane synthesis

In PRP. As shown in Fig. 4A and B, L-ASA induced a concentration-dependent reduction of TXB_2 measured at the end of both NaA- and ADP-elicited aggregation ($n = 24$, main effect of L-ASA; $P < 0.0001$ for both agonists); TXB_2 values did not differ between 5 and 25 mmol/L glucose either in the absence of L-ASA or in the presence of each L-ASA concentration.

In WPs. In unstirred WPs ($n = 8$), baseline TXB_2 concentrations were 27.9 ± 0.9 and 27.03 ± 0.31 ng/mL in the presence of 5 and 25 mmol/L glucose, respectively ($P = \text{NS}$); high glucose failed to modify the TXB_2 response to NaA and ADP, both in the presence and in the absence of L-ASA. In particular, 1) after NaA stimulation, TXB_2 concentrations in the presence of 5 and 25 mmol/L glucose were 56.8 ± 3.9 and 56.9 ± 3.3 ng/mL without L-ASA ($P = \text{NS}$), 27.9 ± 2.8

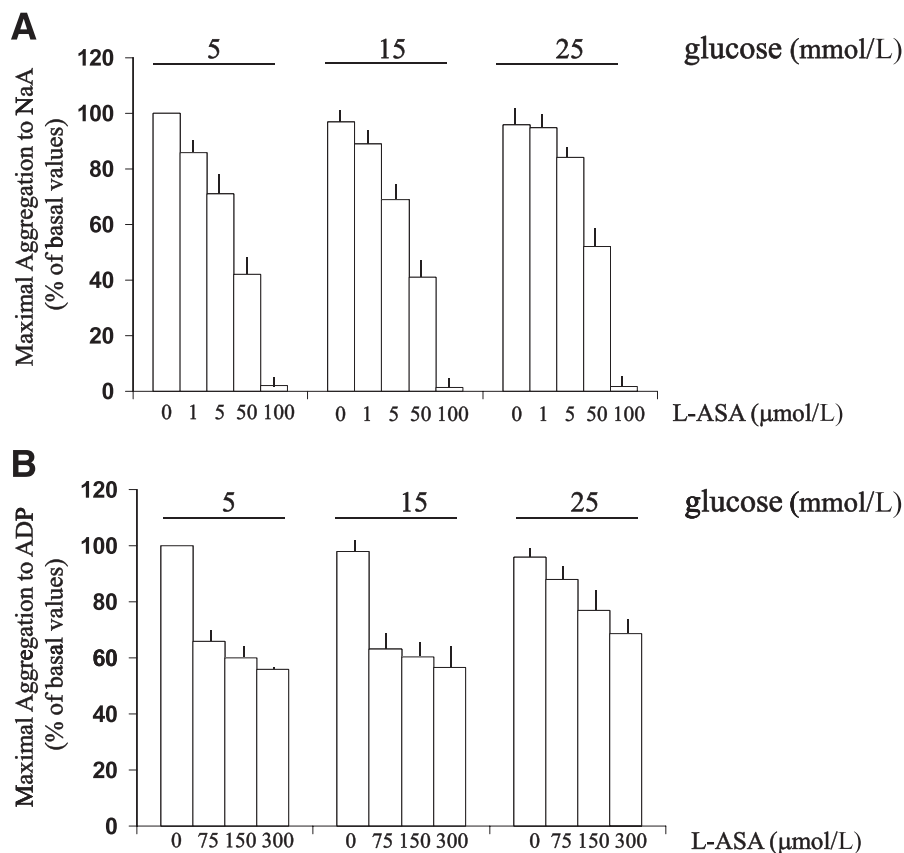


FIG. 2. Effect of platelet exposure to different glucose concentrations on the L-ASA-induced inhibition of platelet aggregation responses to NaA and ADP. Statistical analysis, carried out by two-factor within subject ANOVA for repeated measures, shows that the response to L-ASA on platelet aggregation induced by NaA (A) and ADP (B) did not differ between 5 and 15 mmol/L glucose, whereas it differed between 5 and 25 mmol/L glucose ($P < 0.0001$ for both agonists) and between 15 and 25 mmol/L glucose ($P < 0.0001$ for both agonists; $n = 24$).

and 29.8 ± 2.5 ng/mL with L-ASA ($P = \text{NS}$); 2) after ADP stimulation, TXB₂ concentrations in the presence of 5 and 25 mmol/L glucose were 31.9 ± 2.5 and 31.7 ± 2.7 ng/mL without L-ASA ($P = \text{NS}$), 17.5 ± 1.4 and 17.1 ± 1.7 ng/mL with L-ASA ($P = \text{NS}$).

Activation of the NO/cGMP/PKG pathway

Platelet NOS activity. The effect of L-ASA on platelet NOS activity in the presence of 5 and 25 mmol/L glucose is shown in Fig. 5 ($n = 12$). In experiments carried out at 5 mmol/L glucose, 300 μmol/L L-ASA increased platelet synthesis of NO ($P < 0.0001$), whereas in experiments carried out at 25 mmol/L glucose, the NO values without and with 300 μmol/L L-ASA did not differ. Amifostine restored the L-ASA ability to increase NO synthesis in experiments carried out at 25 mmol/L glucose ($P < 0.04$ vs. amifostine alone), without modifying NO synthesis in the absence of L-ASA or the L-ASA effect at 5 mmol/L glucose.

In experiments carried out at 5 mmol/L glucose in the presence of the NOS inhibitor L-NMMA, NO values without and with 300 μmol/L L-ASA did not differ (12.5 ± 1.3 and 14.4 ± 1.4 fmol [³H]citrulline/min/mg protein, respectively) ($n = 12$).

Finally, in experiments carried out in the presence of 20 mmol/L mannitol, 300 μmol/L L-ASA increased NO synthesis from 11.8 ± 2.8 to 25.3 ± 3.10 [³H]citrulline/min/mg protein ($P < 0.0001$), reaching values that did not differ from those measured at 5 mmol/L glucose, which were higher than those measured at 25 mmol/L glucose ($n = 12$; $P < 0.0001$).

cGMP production. Data are shown in Fig. 6. In the presence of 5 mmol/L glucose, 300 μmol/L L-ASA increased intraplatelet cGMP ($n = 12$; $P < 0.003$); this effect is attributable to the NO-induced activation of guanylate cyclase, as it was completely prevented by both L-NMMA and MB.

The L-ASA effect on cGMP was absent in experiments carried out at 25 mmol/L glucose ($n = 12$). The inhibitory effect of high glucose was independent of its osmotic action because in the presence of 20 mmol/L mannitol L-ASA induced a significant increase of cGMP from 13.1 ± 2.5 to 27.5 ± 2.4 pmol/10⁹ platelets ($P < 0.0001$); the cGMP values reached after L-ASA incubation in the presence of 20 mmol/L mannitol did not differ from those reached in the presence of 5 mmol/L glucose and were significantly higher than those reached in the presence of 25 mmol/L glucose ($P < 0.0001$).

VASP phosphorylation at serine 239. As shown in Fig. 7, in the presence of 5 mmol/L glucose, a 30-min platelet exposure to 300 μmol/L L-ASA caused a significant increase of VASP phosphorylated at serine 239 ($n = 6$; $P < 0.001$); this effect was absent in the presence of 25 mmol/L glucose ($n = 6$; $P = \text{NS}$ vs. without L-ASA).

DISCUSSION

This study shows that in vitro exposure to high glucose (i.e., 25 mmol/L) of platelets from healthy subjects reduces the antiaggregating action of aspirin, an effect blunted by

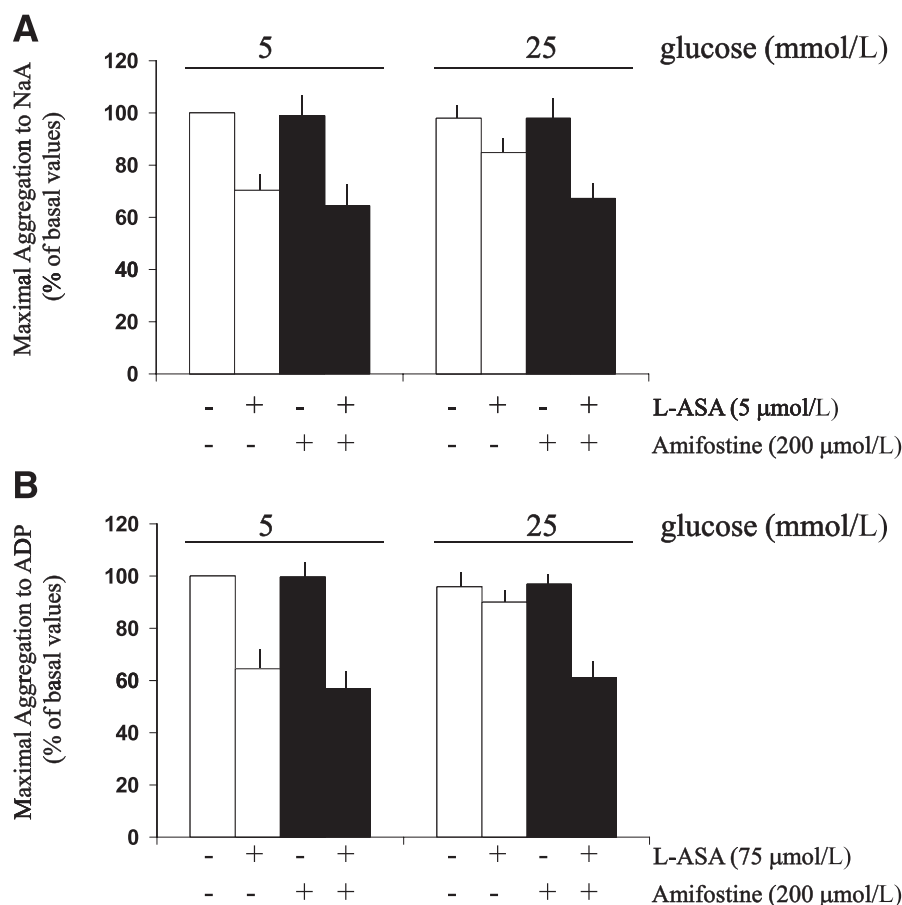


FIG. 3. Effect of preincubation with amifostine on the L-ASA–induced inhibition of platelet aggregation in response to NaA and ADP in the presence of different glucose concentrations. At 5 mmol/L glucose ($n = 9$): amifostine vs. baseline and amifostine + L-ASA vs. L-ASA alone, $P = \text{NS}$ for platelet aggregation induced by both NaA (A) and ADP (B). At 25 mmol/L glucose ($n = 9$): amifostine vs. baseline, $P = \text{NS}$ for platelet aggregation induced by both NaA (A) and ADP (B); amifostine + L-ASA vs. L-ASA alone, $P = 0.002$ (Student paired t test) for platelet aggregation induced by NaA (A) and $P < 0.0001$ (Student paired t test) for platelet aggregation induced by ADP (B).

the antioxidant agent amifostine. It also shows that high glucose does not affect the ability of aspirin to inhibit thromboxane synthesis but impairs the ability of aspirin to activate the NO/cGMP/PKG pathway. Furthermore, it demonstrates that high glucose per se does not influence platelet aggregation in response to agonists, thromboxane synthesis, and the NO/cGMP/PKG pathway.

Thus, high glucose reduces the antiaggregating properties of aspirin only at very high concentrations; the extent of inhibition, although significant, is modest. In our experimental conditions, we did not observe the dramatic dose-dependent inhibition of platelet sensitivity to aspirin described by other authors (17,19).

Is it possible to translate results obtained in vitro to in vivo conditions? It is interesting to observe that the lack of effects on platelet aggregation and platelet sensitivity to aspirin exerted by a 15 mmol/L glucose incubation in our in vitro study is in agreement with the results of studies carried out in vivo; actually, when acute hyperglycemia (~14 mmol/L) was induced by the hyperglycemic clamp in type 2 diabetic patients, glucose did not affect platelet sensitivity to agonists and did not modify the antiaggregating effects of aspirin (38).

In our study, however, in vitro exposure to high glucose failed to increase the shear stress–induced platelet activation evaluated by PFA-100 even at 25 mmol/L, whereas

acute hyperglycemia induced in vivo by the hyperglycemic clamp at ~14 mmol/L increased the shear stress–induced platelet activation (39); this discrepancy is likely attributable to the fact that the results obtained by the hyperglycemic clamp correlated with the increase of circulating levels of von Willebrand factor, which reflects the effects of high glucose on vascular endothelium in vivo (38).

Thus, the translation of results obtained with glucose incubated in vitro to in vivo conditions of acute hyperglycemia is not immediate. Actually, the so-called “hyperglycemic spikes” may affect other cells in vivo (such as endothelial cells and leukocytes), which can influence platelet function.

In our study, glucose impairs the L-ASA effects at 25 mmol/L. We observed that when PRP from aspirin-sensitive nondiabetic subjects on a chronic aspirin treatment is incubated in vitro with this glucose concentration, there is an increase of platelet responses to agonists, showing that high glucose reduces the aspirin action also when the drug is assumed in vivo (40). No in vivo study, however, has thus far evaluated the influence on platelets of a short-term increase of glucose, reaching the concentration of 25 mmol/L. This phenomenon occurs in clinical practice in type 1 and, more rarely, type 2 diabetes in high-stress conditions (infections, acute coronary syndromes, etc.), and therefore in states of severe insulin deficiency and

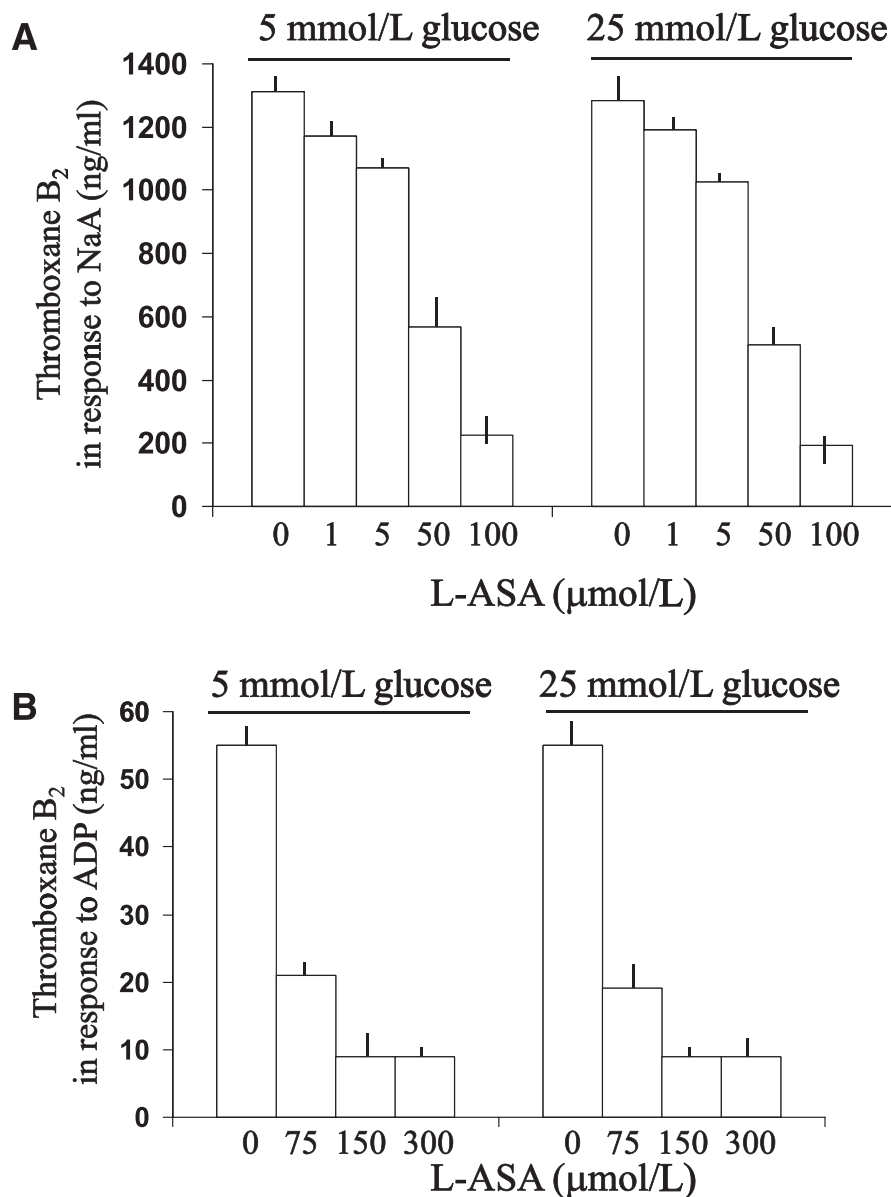


FIG. 4. Effect of platelet exposure to different glucose concentrations on the L-ASA-induced inhibition of thromboxane synthesis in response to NaA and to ADP. Statistical analysis, carried out by two-factor within subject ANOVA for repeated measures, shows that TXB₂ values did not differ between 5 and 25 mmol/L glucose either in the absence of L-ASA or in the presence of each L-ASA concentration for both NaA ($n = 24$) (A) and ADP ($n = 24$) (B).

resistance, and is accompanied by profound hormonal and metabolic abnormalities that can influence platelets and other cells in addition to high glucose. Further studies are needed to explore the changes in aspirin sensitivity occurring in these *in vivo* settings. The contribution of our *in vitro* investigation is to show the effects of high glucose isolated from those of other metabolic and hormonal molecules altered in acute hyperglycemic conditions and the effects on platelets isolated from those occurring in other cells able to influence platelet function.

From the biochemical point of view, our study originally shows that platelet exposure to high glucose does not modify either baseline thromboxane production or the ability of aspirin to inhibit agonist-induced thromboxane synthesis but blunts the aspirin-induced activation of the NO/cGMP/PKG pathway.

Furthermore, our study provides the first demonstration that in human platelets aspirin increases not only NO

synthesis and cGMP concentrations but also VASP phosphorylation at serine 239, and that high glucose inhibits the activating phosphorylation of VASP induced by aspirin.

The inhibitory effect of high glucose on the aspirin-induced activation of the NO pathway is not due to an osmotic mechanism, because it is not reproduced by iso-osmolar mannitol. On the other hand, the ability of the antioxidant agent amifostine to restore the aspirin-induced increase of NO production in the presence of high glucose strongly supports the role of oxidative stress, which is deeply involved in the reduction of NO synthesis/bioavailability in platelets (41). Amifostine, as is well known, is an organic thiophosphate prodrug that acts as a potent intracellular scavenger of free radicals (42). Since in our study high glucose does not influence the NO/cGMP/PKG pathway *per se*, in keeping with a previous observation (43), these results indicate that, by oxidative stress, it interplays with the mechanism by which aspirin activates the same pathway.

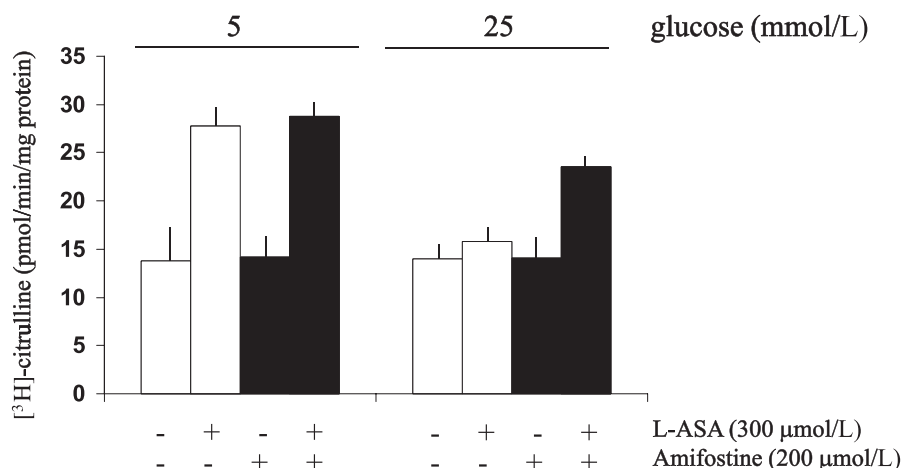


FIG. 5. Effect of L-ASA on platelet NOS activity in the presence of different glucose concentrations with and without preincubation with amifostine. At 5 mmol/L glucose ($n = 12$): L-ASA vs. baseline, $P < 0.0001$; amifostine vs. baseline and amifostine + L-ASA vs. L-ASA alone, $P = \text{NS}$ (Student paired t test). At 25 mmol/L glucose ($n = 12$): L-ASA vs. baseline, $P = \text{NS}$; amifostine vs. baseline, $P = \text{NS}$; amifostine + L-ASA vs. L-ASA alone, $P < 0.05$; amifostine + L-ASA vs. amifostine alone, $P < 0.04$ (Student paired t test).

Because aspirin activates cNOS by acetylation (28), it could be hypothesized that reactive oxygen species interfere with this acetylation process.

In conclusion, the current study demonstrates that a short-term platelet exposure to very high glucose concentrations modestly inhibits platelet responses to agonists by a mechanism likely attributable to oxidative stress; furthermore, it shows for the first time that high glucose does not modify the ability of aspirin to reduce thromboxane synthesis but inhibits the ability of aspirin to activate the antiaggregating pathway NO/cGMP/PKG. The short-term viability of platelets for in vitro studies does not allow long-term incubations; thus, our results provide information concerning mechanisms involved in the effects of “stress hyperglycemia” (20) or “postprandial spikes” (21), without excluding that smaller glucose concentrations could chronically affect platelet function playing

a role in the “aspirin resistance” described in diabetes, a condition in which an effective platelet antiaggregation is of crucial importance (44).

ACKNOWLEDGMENTS

This study was supported by a grant to M.T. from the European Foundation for the Study of Diabetes/sanofi-aventis European Programme (2008) entitled “Macrovascular Complications and Blood Glucose Abnormalities.”

No potential conflicts of interest relevant to this article were reported.

I.R., M.T., and G.A. conceived and designed the study, collected and interpreted data, and wrote the manuscript. M.V., C.B., L.M., and G.D. contributed to data collection and results interpretation. A.P. contributed to results interpretation and statistical analysis. F.C. contributed to

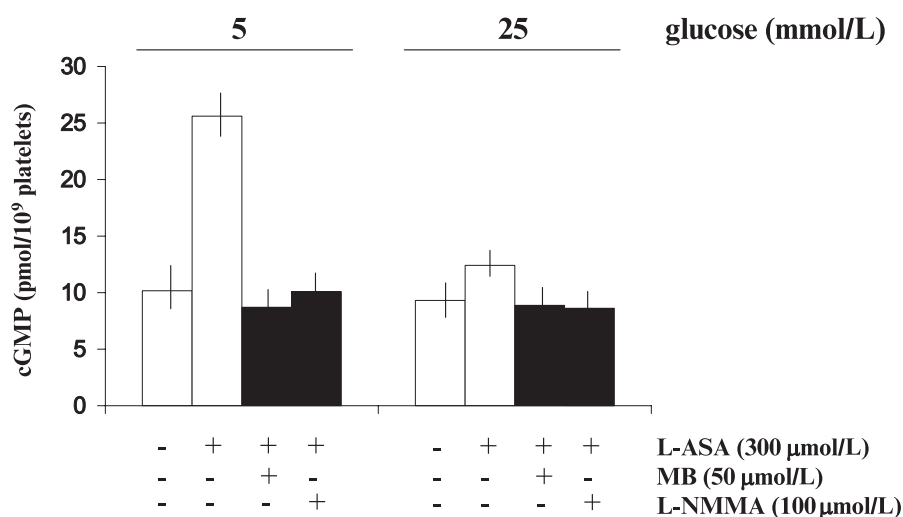


FIG. 6. Effect of L-ASA on platelet cGMP in the presence of different glucose concentrations without and with the guanylate cyclase inhibitor MB and the NOS inhibitor L-NMMA. At 5 mmol/L glucose ($n = 12$): L-ASA vs. baseline, $P < 0.003$; MB + L-ASA vs. baseline, $P = \text{NS}$; L-NMMA + L-ASA vs. baseline, $P = \text{NS}$ (Student paired t test). At 25 mmol/L glucose ($n = 12$): L-ASA vs. baseline, without or with MB or L-NMMA, $P = \text{NS}$ (Student paired t test).

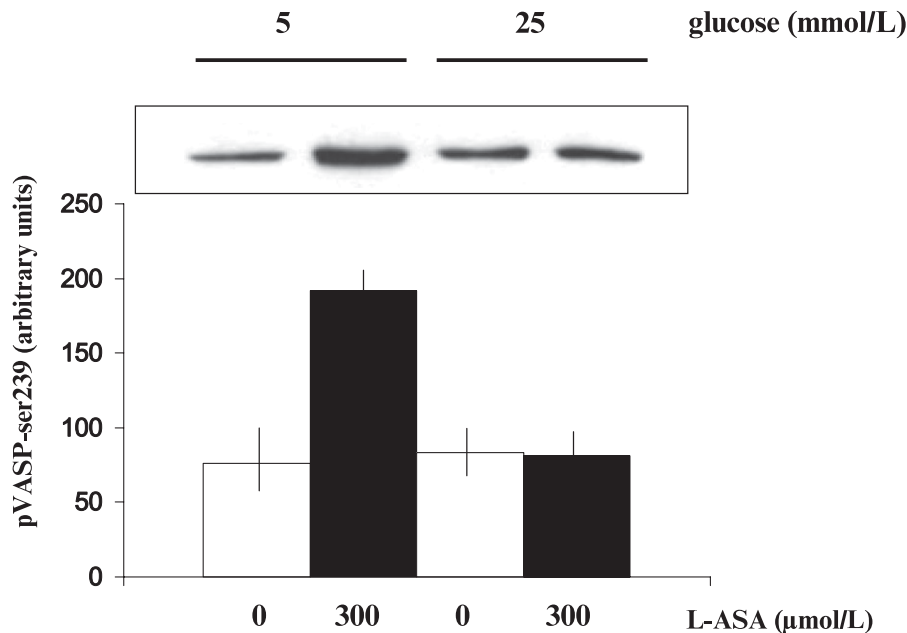


FIG. 7. Effect of L-ASA on platelet VASP phosphorylation at serine 239 in the presence of different glucose concentrations. At 5 mmol/L glucose ($n = 6$): L-ASA vs. baseline, $P < 0.001$ (Student paired t test). At 25 mmol/L glucose ($n = 6$): L-ASA vs. baseline, $P = NS$ (Student paired t test).

results interpretation and discussion. M.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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