

Hyperglycemia-Induced Platelet Activation in Type 2 Diabetes Is Resistant to Aspirin but Not to a Nitric Oxide–Donating Agent

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OBJECTIVE — Acute, short-term hyperglycemia enhances high shear stress–induced platelet activation in type 2 diabetes. Several observations suggest that platelets in type 2 diabetes are resistant to inhibition by aspirin. Our aim was to assess comparatively the effect of aspirin, a nitric oxide–donating agent (NCX 4016), their combination, or placebo on platelet activation induced by acute hyperglycemia in type 2 diabetes.

RESEARCH DESIGN AND METHODS — In a double-blind, placebo-controlled, randomized trial, 40 type 2 diabetic patients were allocated to 100 mg aspirin once daily, 800 mg NCX 4016 b.i.d., both of them, or placebo for 15 days. On day 15, 1 h after the morning dose, a 4-h hyperglycemic clamp (plasma glucose 13.9 mmol/l) was performed, and blood samples were collected before and immediately after it for platelet activation and cyclooxygenase-1 (COX-1) inhibition studies.

RESULTS — Acute hyperglycemia enhanced shear stress–induced platelet activation in placebo-treated patients (basal closure time 63 ± 7.1 s, after hyperglycemia 49.5 ± 1.4 s, -13.5 ± 6.3 s, $P < 0.048$). Pretreatment with aspirin, despite full inhibition of platelet COX-1, did not prevent it (-12.7 ± 6.9 s, NS vs. placebo). On the contrary, pretreatment with the NO donor NCX 4016, alone or in combination with aspirin, suppressed platelet activation induced by acute hyperglycemia (NCX 4016 $+10.5 \pm 8.3$ s; NCX 4016 plus aspirin: $+12.0 \pm 10.7$ s, $P < 0.05$ vs. placebo for both). Other parameters of shear stress–dependent platelet activation were also more inhibited by NCX 4016 than by aspirin, despite lesser inhibition of COX-1.

CONCLUSIONS — Acute hyperglycemia-induced enhancement of platelet activation is resistant to aspirin; a NO-donating agent suppresses it. Therapeutic approaches aiming at a wider platelet inhibitory action than that exerted by aspirin may prove useful in patients with type 2 diabetes.

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Type 2 diabetes is associated with a two- to fourfold increased incidence of ischemic cardiovascular events and markedly enhances the risk of stroke, amputation, and death (1). Not only long-term, continuous hyperglycemia but also transient, acute hyperglycemic spikes may contribute to the poor cardiovascular prognosis of patients with type 2 diabetes (2).

Platelet hyperreactivity has been identified as one of the mechanisms of enhanced arterial thrombosis in type 2 diabetes (3). We have previously shown that in type 2 diabetes an acute, short-term hyperglycemia enhances platelet activation, and, in particular, high-shear stress–induced activation, which is considered an important mechanism triggering arterial thrombosis (4). This

phenomenon is partly due to acute enhancement of the circulating levels of von Willebrand factor (vWF) (4) and indeed platelet-plasma interactions involving vWF have been previously suggested to cause increased platelet aggregability (5), and recent epidemiological data show that plasma vWF predicts cardiovascular events in patients with type 2 diabetes (6). High shear stress–induced platelet activation is hardly sensitive to inhibition by aspirin, and this has been advocated as one of the reasons for the high residual incidence of ischemic events in patients with acute coronary syndromes treated with aspirin (7). The effectiveness of aspirin as an antiplatelet agent in patients with type 2 diabetes is being increasingly questioned and aspirin nonresponsiveness, i.e., the incomplete inhibition of platelet aggregation upon chronic aspirin intake, has been documented in type 2 diabetes (3,8). In the antithrombotic trialists' collaboration overview in patients at risk of ischemic cardiovascular events, antiplatelet therapy did not reduce the odds of a vascular event in diabetes (-7%), different from the highly significant reduction produced in the overall population at risk (-25%) (9).

Nitric oxide (NO), a naturally occurring antiatherothrombotic mediator, inhibits the aggregation of platelets induced by all agonists, also suppressing aspirin-resistant pathways. The production of NO is defective in patients with type 2 diabetes (5). It seems thus logical to test NO-donating agents for their effect on platelet activation in type 2 diabetes.

NCX 4016 (2-(acetyloxy)benzoic acid-3-[(nitrooxy)methyl]phenyl ester), a NO-donating moiety linked to an acetylsalicylic acid backbone, is a prototype of a series of NO-donating hybrid drugs of potential use for cardiovascular disorders (10). NCX 4016 was shown to display a wide range of antiplatelet activities in vitro and in vivo (11) and to release biologically relevant amounts of NO after oral administration to humans (12,13).

Based on the above considerations, we have compared aspirin with the NO-donating agent NCX 4016 for their effects

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on the platelet hyperreactivity induced by acute, short-term hyperglycemia in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Forty patients with type 2 diabetes, as defined by the American Diabetes Association criteria, were enrolled in a randomized, double-blind, double-dummy, parallel groups, placebo-controlled study. Enrollment criteria were the following: male and female patients (age range 18–75 years) affected by type 2 diabetes (duration of disease ≤ 10 years) and having stable metabolic control (A1C in the range of 7–8%) with diet and oral antidiabetic treatment, and stable blood pressure control (< 130 – 80 mmHg) if hypertensive.

Concomitant treatments with antiplatelet drugs or nitrates were not allowed in the 10 days before randomization. ACE inhibitors, angiotensin II antagonists, and statins were allowed only if they had been taken regularly for > 3 months before enrollment and were continued throughout the study.

Patients were randomly assigned to one of the following treatments: 100 mg aspirin once daily plus placebo, 800 mg NCX 4016 b.i.d. plus placebo, aspirin plus NCX 4016, or placebo for 15 days. A treatment using the combination of aspirin and NCX 4016 seemed appropriate because most of the new antiplatelet agents that more recently entered clinical use have been tested along with aspirin and because previous studies have shown that thromboxane (Tx) A_2 synthesis inhibition after oral NCX 4016 is less than optimal (11).

Patients were instructed to take one sachet (NCX 4016 or placebo) and one tablet (aspirin or placebo) in the morning after breakfast, and one sachet (NCX 4016 or placebo) in the evening after dinner. The last study drug medication was taken in the morning of day 15, 1 h before the start of the clamp procedure. A 4-h hyperglycemic clamp was performed as described previously (4). In brief, glucose was infused at a variable rate, based on plasma glucose measured every 5 min, to maintain steady-state plasma glucose levels (250 mg/dl, 139 mmol/l). Plasma glucose was measured with a glucose analyzer (Glucose Analyzer II; Beckman Instruments, Fullerton, CA), plasma insulin and C-peptide were measured by assays described previously (4), and A1C was measured by high-performance liquid chromatography, using an H1-

AutoA_{1c} HA 8121 apparatus (DIC, Kyoto Daiichi, Kogaku Co., Japan). Blood for platelet function studies was collected before and at the end of the clamp procedure. A bleeding time test was performed before blood sampling preclamp and again after blood sampling postclamp. Urine samples were collected for the following periods: the 24 h ending at the start of the clamp (preclamp), the 4 h of the clamp procedure (clamp), and the 24 h starting from the beginning of the clamp (postclamp). The study was approved by our institutional review board (Comitato Etico delle Aziende Sanitarie dell'Umbria), and all patients gave informed, witnessed, written consent before enrollment.

Laboratory methods

Bleeding time. A standardized bleeding time test was performed using an automatic template device (Simplate II) as described previously (14). The blood emerging from the skin wound was collected on a chilled antiplatelet-anticoagulant mixture (citrate, theophylline, adenosine, and dipyridamole) for the quantification of platelet activation, as described (14).

Shear-induced platelet activation. Shear-induced platelet activation was assessed with the O'Brien filtration test (15) as described previously (4). Filter closure time and percentage of platelets retained were measured (4).

Platelet adhesion to collagen under flow conditions. Platelet adhesion to a collagen-coated surface was studied in a perfusion chamber as described previously (4). In brief, citrated blood (5 ml) was passed through a rectangular parallel plate perfusion chamber over a plastic coverslip sprayed with collagen from equine tendon (~ 30 $\mu\text{g}/\text{cm}^2$; Mascia Brunelli, Milano, Italy), at a wall shear rate of $3,000$ s^{-1} . The coverslip was then harvested, gently washed with 10 mmol/l HEPES, placed in 400 μl of a lytic buffer for 1 h, and then frozen and thawed three times. The supernatants were stored at -80°C until assayed for β -thromboglobulin by an ELISA assay (Asserachrom β -TG; Roche Diagnostics, Monza, Italy) (16). The total number of platelets deposited on the coverslip was calculated by dividing the amount of β -thromboglobulin present in the lysate by that present in a known number of nonactivated platelets of the same patient. Results are expressed as number of platelets deposited per square micrometer (16).

Light transmission platelet aggregometry. Platelet aggregation induced by arachidonic acid (0.3, 0.4, and 1 mmol/l) and by collagen (1 $\mu\text{g}/\text{ml}$) was studied with the photometric method as described previously (4), and the maximal amplitude of aggregation was recorded at 3 min.

Platelet activation by flow cytometry. Flow cytometric analysis of activated platelets was performed in samples from peripheral venous blood and from the whole blood emerging from the bleeding time wounds by assessing the expression on platelets of the activation antigen P-selectin (4) and the binding of monoclonal antibody PAC-1, which recognizes activated glycoprotein IIb/IIIa (4,14). The presence of circulating platelet-leukocyte aggregates in venous blood was assessed by flow cytometry as described previously (17).

von Willebrand factor. vWF activity was measured in citrated plasma with a system (vWF Activity Kit; Shield Diagnostic, Dundee, U.K.) that uses a monoclonal antibody recognizing an epitope of vWF involved in the interaction with glycoprotein Ib α . Results are expressed as percentage of control (4).

Plasmatic nitrite plus nitrate. Plasma levels of NO degradation products, nitrites plus nitrates, were measured by the Griess reagent method (12).

Platelet-derived Tx. Serum TxB₂ was measured by radioimmunoassay as described previously (18). The urinary excretion of 11-dehydro-TxB₂ was evaluated by a previously validated radioimmunoassay technique as described (4).

Statistical analysis

The sample size was calculated, based on the results of a previous study (4), using the filter closure time in the O'Brien filtration test as a primary end point and assuming that a mean \pm SD difference of 6.5 ± 6 s between the active treatment (NCX 4016) and placebo would occur with a power of 80% and a one-sided $\alpha = 0.05$. Based on these assumptions, 10 patients per group had to be enrolled. Patients were randomly assigned to one of the four treatment groups using a computer-generated random scheme.

Statistical analysis was performed using SAS system software. ANOVA was performed to test differences between different time points and between treatments. Data are expressed as means \pm SEM. $P < 0.05$ was considered as significant.

Table 1—Demographic data of the patients enrolled

	ASA	NCX 4016	ASA + NCX 4016	Placebo	All patients
Age (years)	62.1 ± 9.2	56.2 ± 7.7	60.1 ± 6.9	60.0 ± 8.9	59.4 ± 8.4
Male sex (%)	90	70	70	70	75
BMI (kg/m ²)	29.8 ± 3.7	30.6 ± 4.1	29.3 ± 2.3	28.8 ± 2.4	29.6 ± 3.1
Fasting plasma glucose (mg/dl)	113.6 ± 10	116.9 ± 14	105.6 ± 5.4	124.1 ± 16.2	115 ± 13.2
Plasma insulin (μU/ml)	13 ± 1.0	12.3 ± 2.2	15.3 ± 2.2	12 ± 1.2	13.1 ± 0.9
C-Peptide (ng/ml)	2.7 ± 1.6	1.9 ± 1.2	2.1 ± 1	1.9 ± 1	2.2 ± 1.2
A1C (%)	7.2 ± 0.9	7.7 ± 0.8	7.8 ± 0.4	7.4 ± 0.4	7.5 ± 0.6
Arterial hypertension (%)	40	80	70	70	65
ACE inhibitors (n)	5	6	4	5	20
ATII antagonists (n)	4	0	1	2	7
Doxazosine (n)	0	0	1	1	2
Ca Channels Blockers (n)	4	4	2	1	11
Diuretics (n)	1	4	4	2	11
Hypertriglyceridemia (%)	40	40	50	70	50
Antidiabetic therapy (n)					
Diet	1	0	2	1	4
Insulin	6	5	2	5	18
Metformin	2	7	4	5	18
Repaglinide	1	1	3	0	5
Sulfonylureas	1	2	1	0	4
Statins (n)	2*	1*	1†	1*	5

No significant differences were found between the four groups for any of the indicated parameters. *Atorvastatin, †simvastatin.

RESULTS

Patients

The demographic data of the patients enrolled in the study are reported in Table 1. No significant differences in any of the demographic parameters were evident between the treatment groups. Fasting plasma glucose and insulin concentrations before the clamp were not different in the four study groups. After the beginning of glucose infusion, plasma glucose reached the target of 250 mg/dl by 31.6 ± 2.2 min, with no differences between groups (Fig. 1A). Plasma insulin increased in response to hyperglycemia (baseline = 12.1 ± 0.8 μU/ml and steady-state = 28.0 ± 4.2 μU/ml) with no significant differences between the groups, although with a trend to higher insulin levels upon hyperglycemia in the NCX 4016-treated groups. Platelet count was similar before the clamp in the four study groups and decreased slightly, but not significantly, after the clamp. Hematocrit values were similar at baseline in all study groups and remained unchanged after the clamp.

Bleeding time

Average baseline bleeding time (before clamp) was 415.1 ± 30.0 s (n = 40), with no significant differences among the four

groups, although a trend toward a longer bleeding time was observed in the aspirin plus NCX 4016 group (535 ± 32.5 s vs. placebo = 373.5 ± 30.9 s, NS). Four hours of hyperglycemia shortened slightly, but significantly, the bleeding time in the placebo group (325.5 ± 20 s, P = 0.02), confirming previous data (4). In the NCX 4016 and NCX 4016 plus aspirin groups the bleeding time was unchanged after the clamp procedure. In the aspirin group, a prolongation of the bleeding time was found after the clamp (from 369.5 ± 34.5 to 439 ± 45.2 s, P = 0.03).

Shear-induced platelet activation

Platelet activation in the O'Brien test did not differ among the four different treatment groups at baseline (before clamp) (average filter closure time 61.9 ± 6.3 s; retained platelets 78 ± 6.3%, n = 40). The hyperglycemic clamp significantly shortened the filter closure time and enhanced the percentage of platelets retained in the placebo group (Fig. 1B and C). Aspirin treatment did not affect the enhancing action of acute, short-term hyperglycemia on shear stress-induced platelet activation, with a persistence of the shortening of the filter closure time after the clamp and of the increase of the percentage of retained platelets. Treat-

ment with NCX 4016 or with NCX 4016 plus aspirin, instead, reversed the effect of hyperglycemia on both the filter closure time and on the percentage of retained platelets with a statistically significant difference compared with both the aspirin and the placebo groups (P = 0.038 and P = 0.043, respectively) (Fig. 1B and C).

Comparing the overall NCX 4016-treated groups, i.e., cumulating the two groups who received NCX 4016 alone or in combination, with the groups not receiving NCX 4016 (placebo and aspirin), differences were statistically highly significant for both filter closure times (P = 0.0046) and percentage of platelets retained (P = 0.0169), indicating a NCX 4016-related effect on shear-induced platelet activation (Fig. 1B and C).

Platelet deposition on collagen at high shear rate

Platelet deposition on collagen did not differ among the four treatment groups at baseline (before clamp) (average = 99.4 ± 6.3 platelets/μm², n = 40). Acute hyperglycemia significantly enhanced platelet deposition in the placebo-treated group (+7.1 ± 3.2 platelets/μm²) (P = 0.01 vs. preclamp). Both NCX 4016 and aspirin, although the latter to a smaller extent, reversed the enhancing activity of acute hyperglycemia on platelet deposi-

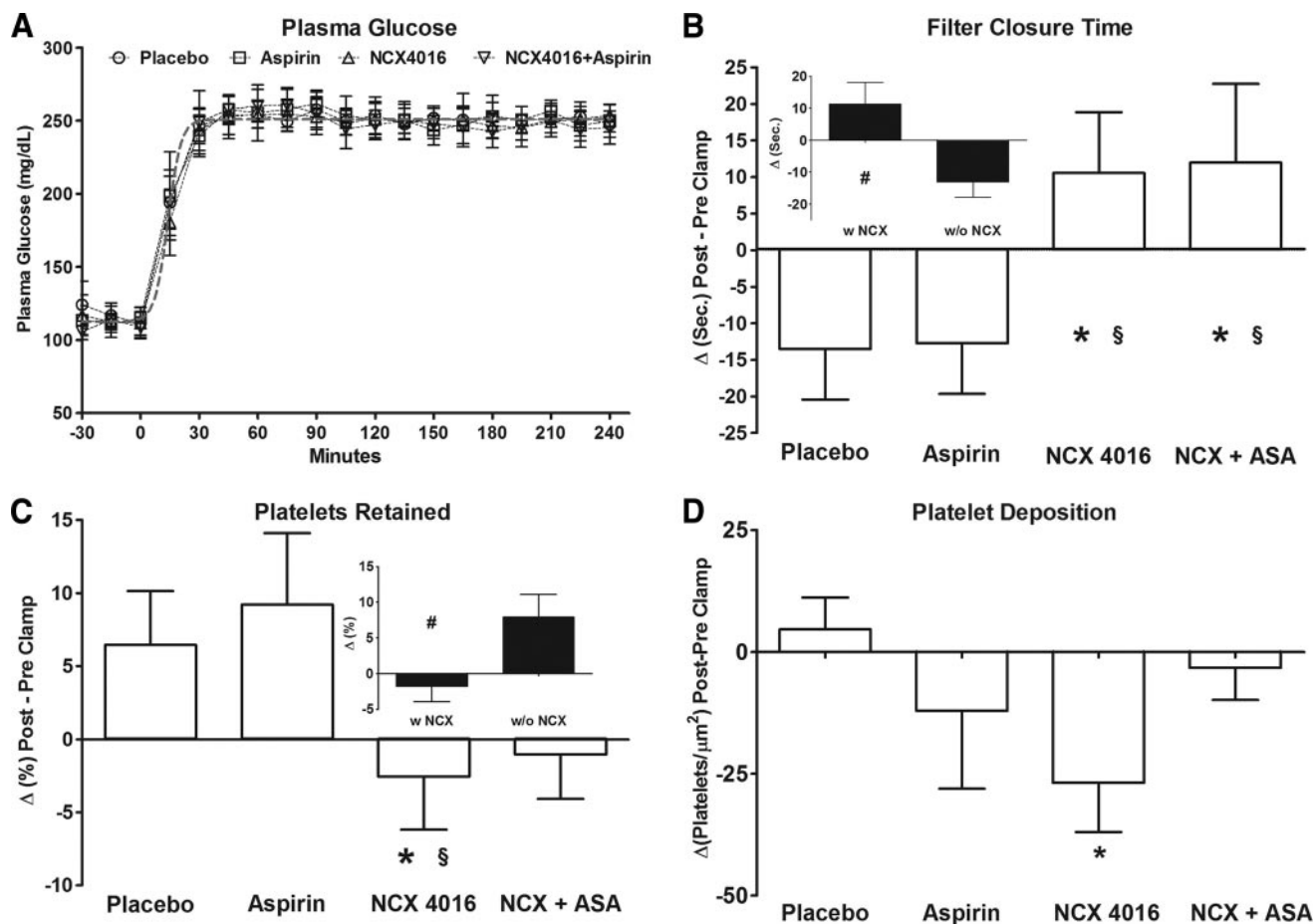


Figure 1—A: Plasma glucose concentrations before and during the 4-h hyperglycemic clamp in the four treatment groups. No differences between groups were observed at any of the observation times. B and C: Shear stress–induced platelet activation using the O'Brien filtration test. Data are expressed as differences (Δ) between the values observed after the 4-h hyperglycemic clamp (post) and the values observed before (pre). B: Filter closure time expressed in seconds. C: Platelets retained between 20 and 40 s, expressed as a percentage of the platelet count before filtration. In the insets are reported results cumulating the two groups receiving NCX 4016 (w NCX) and the two groups not receiving it (w/o NCX). Data are expressed as means \pm SEM. * $P < 0.05$ vs. placebo; $\S P < 0.05$ vs. aspirin (ASA); # $P < 0.05$ vs. w/o NCX 4016. D: Platelet deposition on a collagen-coated surface under high shear rate ($3,000 \text{ s}^{-1}$). Data are expressed as differences (Δ) between the values observed after the 4-h hyperglycemic clamp (post) and the values observed before (pre). * $P < 0.001$ versus placebo.

tion, with a significant difference compared with placebo for NCX 4016 (Fig. 1D). Combined treatment with aspirin and NCX 4016, although producing a trend toward a reversal of the platelet deposition—enhancing effect of acute hyperglycemia, did not modify it significantly compared with placebo (Fig. 1D).

Platelet aggregation

Arachidonic acid induced a dose-related increase of platelet aggregation in the placebo group, which was not significantly affected by the hyperglycemic clamp, confirming previous data (4) (supplementary Fig. 1, available in an online appendix at <http://care.diabetesjournals.org/cgi/content/full/dc09-2013/DC1>). Arachidonic acid–induced platelet aggregation at baseline (preclamp) was significantly inhibited in the three active treatment groups

compared with placebo, although significantly less in the NCX 4016 group when the highest concentration of arachidonic acid (1 mmol/l) was used (supplementary Fig. 1A).

The inhibition of platelet aggregation in the three active treatment groups was unaffected by the hyperglycemic clamp, except for the NCX 4016 group in which the inhibition of aggregation induced by the highest concentration of arachidonic acid (1 mmol/l) after the clamp was no longer less than that in the other two active treatment groups (supplementary Fig. 1A). The lack of effect of NCX 4016 on platelet aggregation induced by high concentrations of arachidonic acid in the sample taken before the clamp, but no longer evident in the sample taken after the clamp, can be explained by the fact that NCX 4016 has a slower absorption

rate than aspirin (13), and this may translate in incomplete inhibition of TxB_2 production before the clamp, but not after. Collagen-induced platelet aggregation was significantly inhibited in the aspirin and NCX 4016 plus aspirin groups but not in the NCX 4016 group (supplementary Fig. 1B). Acute hyperglycemia did not affect collagen-induced platelet aggregation in any of the treatment groups, confirming previous data (4). The observation that collagen-induced light transmission aggregometry was not significantly inhibited by NCX 4016 whereas it was by aspirin and, on the contrary, that platelet deposition on collagen at a high shear rate was inhibited more extensively by NCX 4016 than by aspirin can be explained by the fact that low-dose collagen-induced aggregation is largely dependent on TxA_2 production, which is

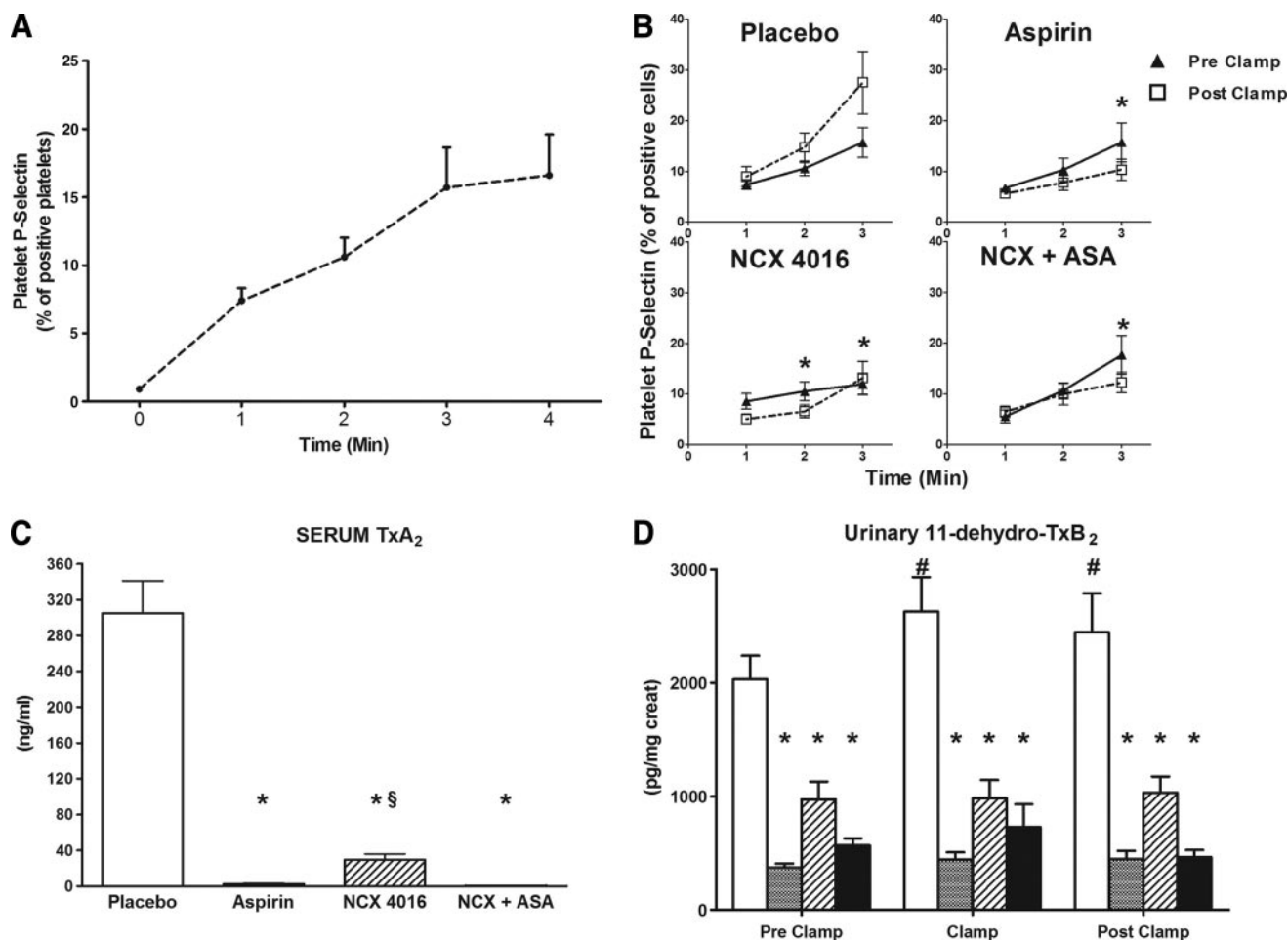


Figure 2—A: Platelet activation in bleeding-time blood as assessed by the expression of P-selectin on the surface of platelets in the blood oozing from the skin wound in the placebo group before the clamp. Time 0 indicates the levels detected in venous blood (not activated platelets). B: P-selectin expression on platelets in the four treatment groups before (▲) and after (□) the clamp. * $P < 0.05$ vs. placebo. C and D: Platelet-derived thromboxane in the four treatment groups. C: Serum TxB₂. D: Urinary excretion of 11-dehydro-TxB₂. * $P < 0.05$ vs. placebo; § $P < 0.05$ vs. aspirin; # $P < 0.05$, at least, vs. preclamp; § $P < 0.05$ vs. aspirin.

incompletely inhibited by NCX 4016 (see below), whereas platelet adhesion at a high shear rate is very sensitive to NO inhibition and hardly to aspirin (7,11).

Flow cytometry

Platelet activation markers in venous blood. P-selectin expression on platelets in venous blood was similar in the four groups at baseline (preclamp) (average $1.5 \pm 0.2\%$ of positive platelets) and was not modified by the hyperglycemic clamp in any of the four groups, confirming previous results (4). Similar data were obtained concerning the expression of PAC-1 on platelets circulating in venous blood (data not shown).

Platelet activation in bleeding time blood. At baseline, preclamp, the expression of P-selectin on platelets in bleeding time blood increased progressively over time, consistent with ongoing

platelet plug formation, as reported previously (4,14) (Fig. 2A). The size of this increase at baseline did not differ among the four treatment groups.

P-selectin expression on platelets in bleeding time blood was significantly enhanced after the hyperglycemic clamp in the placebo group, confirming previous data (4), whereas in all the active treatment groups it was unchanged or decreased postclamp, with no substantial difference between NCX 4016 and aspirin (Fig. 2B).

Circulating platelet-leukocyte aggregates. Circulating platelet-leukocyte aggregates did not differ among the four groups at baseline. Four hours of hyperglycemia induced a significant increase in circulating platelet-leukocyte aggregates in the placebo-treated group (from 3.75 ± 0.7 to $5.5 \pm 1.0\%$, $P < 0.05$). The hyperglycemia-induced increase in circu-

lating platelet-leukocyte aggregates was not affected by aspirin ($+1.89 \pm 2.09\%$, NS vs. placebo), whereas it was significantly reduced by treatment with NCX 4016 ($+0.50 \pm 0.38\%$, $P = 0.039$ vs. placebo). Treatment with NCX 4016 plus aspirin also reduced the increase of circulating platelet-leukocyte aggregates induced by hyperglycemia, but not significantly compared with placebo ($+0.38 \pm 0.60\%$, NS vs. placebo).

vWF activity. vWF activity was similar in the four groups at baseline, and it increased significantly after clamp in the placebo group (preclamp $77.6 \pm 4.1\%$, postclamp $91.4 \pm 5.7\%$, $+13.8 \pm 4.5\%$, $P = 0.014$). Treatment with NCX 4016 and with aspirin produced a slight decrease of vWF activity after the clamp ($-13.8 \pm 7.9\%$, $P < 0.01$ and $-10.4 \pm 12.1\%$, NS), whereas the combination of NCX 4016 and aspirin did not affect the

increase of vWF activity observed after the hyperglycemic clamp ($+12.4 \pm 10.6\%$, NS).

Plasmatic nitrite plus nitrate. Plas-matic nitrite plus nitrate (NOx) levels before the clamp in the four treatment groups were: placebo $49.1 \pm 4.6 \mu\text{mol/l}$, aspirin $55.3 \pm 11.5 \mu\text{mol/l}$ (NS vs. placebo), NCX 4016 $66.9 \pm 11.6 \mu\text{mol/l}$ ($P = 0.05$ vs. placebo), and NCX + aspirin $97.7 \pm 7.2 \mu\text{mol/l}$ ($P = 0.002$ vs. placebo).

Platelet-derived TxB₂. Baseline serum TxB₂ (before clamp) was significantly and strikingly inhibited in all treatment groups compared with that treated with placebo, although significantly less in the NCX 4016-treated group than in the aspirin-treated group (Fig. 2C). Urinary 11-dehydro-TxB₂ excretion in the placebo group before the clamp was $2,090 \pm 209.9$ pg/mg creatinine, a value clearly enhanced compared with that in healthy subjects studied simultaneously in our laboratory ($200\text{--}500$ pg/mg creatinine) and increased significantly during the clamp ($2,264 \pm 282.8$ pg/mg creatinine, $P < 0.05$ vs. preclamp) and in the 24 h after the beginning of the clamp ($2,445.1 \pm 344.8$ pg/mg creatinine, $P = 0.001$ vs. preclamp). Preclamp urinary 11-dehydro-TxB₂ excretion was significantly lower in all active treatment groups than that in the placebo group (368.4 ± 39.4 pg/mg creatinine for the aspirin group, 876.8 ± 121 pg/mg creatinine for the NCX 4016 group, and 567.7 ± 61 pg/mg creatinine for the NCX 4016+aspirin group): and was unaffected by the clamp procedure (Fig. 2D).

Safety. Blood pressure and heart rate were not significantly changed at day 15 compared with those at day 0 in any of the treatment groups and laboratory tolerability markers were not significantly changed. One duodenal ulcer was recorded in the aspirin group after 14 days of treatment.

CONCLUSIONS— This study shows that acute short-term hyperglycemia induces a rapid and significant increase of high shear stress-induced platelet activation in patients with type 2 diabetes, confirming previous results (4). This result is documented by the enhanced shear stress-induced platelet activation, enhanced platelet deposition to collagen at high shear rate, increased expression of an activation antigen on platelets in the bleeding time blood, and increased urinary excretion of 11-

dehydro-TxB₂, a marker of in vivo platelet activation. Parameters of platelet function, which are not shear-dependent like light transmission aggregometry or the expression of activation antigens on platelets circulating in venous blood, did not change after short-term hyperglycemia (4).

High shear stress-induced platelet activation is considered to be a parameter relevant for arterial thrombosis more than light transmission aggregometry, and it has been reported to be insensitive to inhibition by aspirin in vitro (7). No studies, however, have addressed the effect of aspirin intake on high shear stress-induced platelet activation, especially in diabetic individuals. Because NO is able to inhibit high shear stress-induced platelet activation (19), we set up a study to compare aspirin and an NO donor, NCX 4016, for their capability to prevent the acute hyperglycemia-induced platelet activation in patients with type 2 diabetes.

We found that the enhancement of shear stress-induced platelet activation caused by hyperglycemia is not prevented by aspirin, despite adequate suppression of platelet cyclooxygenase-1 (COX-1), whereas it is completely suppressed by a NO-donating agent. In fact, although in patients treated with aspirin filter closure time was shortened and the percentage of retained platelets was increased by acute hyperglycemia to an extent similar to that observed in placebo-treated subjects, in diabetic individuals treated with NCX 4016 these parameters of platelet activation were unchanged or even reduced after acute hyperglycemia. Acute hyperglycemia also enhanced other parameters of platelet activation, such as platelet deposition on collagen at a high shear rate or the formation of platelet-leukocyte aggregates in circulating blood, and these were also consistently reduced by NCX 4016 but not by aspirin.

The baseline urinary excretion of 11-dehydro-TxB₂, a marker of in vivo TxA₂ generation in the placebo-treated diabetic subjects, was strikingly increased compared with that in our reference healthy control group. This result is consistent with the results of previous studies showing that diabetic patients excrete large amounts of urinary TxA₂ metabolites especially in the presence of poor glycemic control, most of which derive from platelets as an expression of increased in vivo platelet activation, being markedly reduced by low doses of aspirin (20). Short-term hyperglycemia further increased

urinary 11-dehydro-TxB₂ excretion, confirming increased activation of platelets exposed in vivo to high shear stress in diabetic individuals (4). Although all active treatments inhibited serum TxB₂ and urinary excretion of 11-dehydro-TxB₂ significantly compared with placebo, NCX 4016 did so significantly less than aspirin and not quite to the extent ($\geq 95\%$ inhibition of serum TxB₂) considered to be required for the effective suppression of in vivo platelet activation (21). The observation that despite incomplete suppression of platelet COX-1, NCX 4016 inhibited several parameters of ex vivo and in vivo platelet activation in diabetic individuals suggests that the antiplatelet activity exerted was largely due to the released NO. Indeed, NCX 4016, but not aspirin, enhanced plasma levels of NOx, demonstrating the delivery of NO in vivo (12). Considering that NCX 4016 in vitro inhibits platelet COX-1 irreversibly and with a potency similar to that of aspirin (18), the lower inhibition we found in vivo suggests that only a minor fraction of NCX 4016 is absorbed as such after oral administration (13).

Hyperglycemia may contribute to atherosclerotic complications in patients with type 2 diabetes not only when it is sustained but also when it occurs in spikes, as in the postprandial state (2): our findings suggest that the enhancement of in vivo platelet activation during acute hyperglycemia may be one of the effectors of cardiovascular complications. Indeed, intensive glycemic control in type 2 diabetes results in reduced mortality and myocardial infarction (22). Our study, using a model resembling the postprandial hyperglycemia of type 2 diabetes in terms of glucose and insulin plasma levels, suggests that platelet hyperactivity is involved in the detrimental cardiovascular effects of hyperglycemic spikes and shows that aspirin is not able to prevent it, supporting the concept that aspirin offers a less than optimal cardiovascular protection in diabetic patients (3,8,9). NO has been known for more than two decades as a very effective platelet function inhibitor, but the search for drugs able to release it in vivo in biologically relevant amounts without incurring in untoward effects has been so far unsuccessful elusive (23).

The present results with NCX 4016 show that it is possible to release NO in vivo in amounts sufficient to inhibit the platelet hyperactivity induced by acute hyperglycemia in diabetic patients with-

out provoking hypotension. Recent data showing that NCX 4016 stimulates glucose transport in adipocytes without negatively affecting insulin sensitivity and that it could therefore help in better controlling glycemia (24) further suggest that NCX 4016 may be suitable for patients with type 2 diabetes. However, NCX 4016 is poorly absorbed and has limitations of pharmaceutical formulation that prevent its further clinical development. Novel hybrid molecules able to exert a full aspirin effect and/or to produce larger or more sustained NO release may exert stronger antiplatelet action and thus represent ideal candidates for development in patients with type 2 diabetes. Agents potentially offering these characteristics are under study (25).

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

References

- Coutinho M, Gerstein HC, Wang Y, Yusuf S. The relationship between glucose and incident cardiovascular events: a meta-regression analysis of published data from 20 studies of 95,783 individuals followed for 12.4 years. *Diabetes Care* 1999; 22:233–240
- Ceriello A. Postprandial hyperglycemia and diabetes complications: is it time to treat? *Diabetes* 2005;54:1–7
- Angiolillo DJ, Bernardo E, Sabaté M, Jimenez-Quevedo P, Costa MA, Palazuelos J, Hernández-Antolin R, Moreno R, Escaned J, Alfonso F, Bañuelos C, Guzman LA, Bass TA, Macaya C, Fernandez-Ortiz A. Impact of platelet reactivity on cardiovascular outcomes in patients with type 2 diabetes mellitus and coronary artery disease. *J Am Coll Cardiol* 2007; 50:1541–1547
- Gresele P, Guglielmini G, De Angelis M, Ciferri S, Ciofetta M, Falcinelli E, Lalli C, Ciabattini G, Davi G, Bolli GB. Acute, short-term hyperglycemia enhances shear stress-induced platelet activation in patients with type II diabetes mellitus. *J Am Coll Cardiol* 2003;41:1013–1020
- Colwell JA, Nesto RW. The platelets in diabetes: focus on prevention of ischemic events. *Diabetes Care* 2003;26:2181–2188
- Frankel DS, Meigs JB, Massaro JM, Wilson PW, O'Donnell CJ, D'Agostino RB, Tofler GH. Von Willebrand factor, type 2 diabetes mellitus, and risk of cardiovascular disease: the Framingham offspring study. *Circulation* 2008;118:2533–2539
- Barstad RM, Orvim U, Hamers MJ, Tjønnfjord GE, Brosstad FR, Sakariassen KS. Reduced effect of aspirin on thrombus formation at high shear and disturbed laminar blood flow. *Thromb Haemost* 1996;75:827–832
- Anfossi G, Russo I, Trovati M. Resistance to aspirin and thienopyridines in diabetes mellitus and metabolic syndrome. *Curr Vasc Pharmacol* 2008;6:313–328
- Antithrombotic Trialists' Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *Br Med J* 2002;324:71–86
- Martelli A, Breschi MC, Calderone V. Pharmacodynamic hybrids coupling established cardiovascular mechanisms of action with additional nitric oxide releasing properties. *Curr Pharm Des* 2009;15: 614–636
- Gresele P, Momi S. Pharmacologic profile and therapeutic potential of NCX 4016, a nitric oxide-releasing aspirin, for cardiovascular disorders. *Cardiovasc Drug Rev* 2006;24:148–168
- Gresele P, Migliacci R, Procacci A, De Monte P, Bonizzoni E. Prevention by NCX 4016, a nitric oxide-donating aspirin, but not by aspirin, of the acute endothelial dysfunction induced by exercise in patients with intermittent claudication. *Thromb Haemost* 2007;97:444–450
- Carini M, Aldini G, Orioli M, Piccoli A, Tocchetti P, Facino RM. Chemiluminescence and LC-MS/MS analyses for the study of nitric oxide release and distribution following oral administration of nitroaspirin (NCX 4016) in healthy volunteers. *J Pharm Biomed Anal* 2004; 35:277–287
- Falcinelli E, Giannini S, Boschetti E, Gresele P. Platelets release active matrix metalloproteinase-2 in vivo in humans at a site of vascular injury: lack of inhibition by aspirin. *Br J Haematol* 2007;138:221–230
- O'Brien JR, Salmon GP. Shear stress activation of platelet glycoprotein IIb/IIIa plus von Willebrand factor causes aggregation: filter blockage and the long bleeding time in von Willebrand's disease. *Blood* 1987;70:1354–1361
- Bossavy JP, Sakariassen KS, Barret A, Boneu B, Cadroy Y. A new method for quantifying platelet deposition in flowing native blood in an ex vivo model of human thrombogenesis. *Thromb Haemost* 1998;79:162–168
- Pitchford SC, Yano H, Lever R, Riffon-Vasquez Y, Ciferri S, Rose MJ, Giannini S, Momi S, Spina D, O'Connor B, Gresele P, Page CP. Platelets are essential for leukocyte recruitment in allergic inflammation. *J Allergy Clin Immunol* 2003;112:109–118
- Corazzi T, Leone M, Maucci R, Corazzi L, Gresele P. Direct and irreversible inhibition of cyclooxygenase-1 by nitroaspirin (NCX 4016). *J Pharmacol Exp Ther* 2005; 315:1331–1337
- de Graaf JC, Banga JD, Moncada S, Palmer RM, de Groot PG, Sixma JJ. Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. *Circulation* 1992;85:2284–2290
- Ferroni P, Basili S, Falco A, Davi G. Platelet activation in type 2 diabetes mellitus. *J Thromb Haemost* 2004;2:1282–1291
- Reilly IA, FitzGerald GA. Inhibition of thromboxane formation in vivo and ex vivo: implications for therapy with platelet inhibitory drugs. *Blood* 1987;69:180–186
- Ray KK, Seshasai SR, Wijesuriya S, Sivakumaran R, Nethercott S, Preiss D, Erqou S, Sattar N. Effect of intensive control of glucose on cardiovascular outcomes and death in patients with diabetes mellitus: a meta-analysis of randomised controlled trials. *Lancet* 2009;373:1765–1772
- Ignarro LJ, Napoli C, Loscalzo J. Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: an overview. *Circ Res* 2002;90:21–28
- Kaddai V, Gonzalez T, Bolla M, Le Marchand-Brustel Y, Cormont M. The nitric oxide-donating derivative of acetylsalicylic acid, NCX 4016, stimulates glucose transport and glucose transporters translocation in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 2008;295: E162–E169
- Lazzarato L, Donnola M, Rolando B, Marini E, Cena C, Coruzzi G, Guaita E, Morini G, Fruttero R, Gasco B, Biondi S, Ongini E. Searching for new NO-donor aspirin-like molecules: a new class of nitrooxy-acyl derivatives of salicylic acid. *J Med Chem* 2008;51:1894–1903