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The Antiplatelet Effects of a New Nitroderivative of Acetylsalicylic Acid – An In Vitro Study of Inhibition on the Early Phase of Platelet Activation and on TXA_2 Production

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Summary

We studied in vitro the antiplatelet activity of a new nitroderivative chemically related to acetylsalicylic acid: 2 acetoxybenzoate 2-[1-nitroxy-methyl]-phenyl ester (NCX 4016), in order to identify any effects due to the release of nitric oxide and the blockade of cyclo-oxygenase.

The effects of scalar doses of NCX 4016 on the early phase of platelet activation, platelet aggregation and thromboxane A_2 production were investigated. We observed inhibitory effects of NCX 4016 on platelet adhesion ($IC_{50} = 7.3 \times 10^{-5}$ M), platelet cytosolic calcium concentration, assayed by fluorescent probe Fura 2, and the expression of glycoprotein IIb/IIIa (CD41 / $\alpha_{IIb}\beta_3$) ($IC_{50} = 3.4 \times 10^{-5}$ M) and P-selectin (CD62 / GMP-140) ($IC_{50} = 4.9 \times 10^{-5}$ M) measured by flow cytometry. NCX 4016 also prevented thrombin-induced platelet aggregation ($IC_{50} = 3.9 \times 10^{-5}$ M). None of these parameters were affected by acetylsalicylic acid. These inhibitory activities of NCX 4016 were abolished by oxyhaemoglobin and methylene blue. Intracellular cyclic GMP observed during thrombin-induced aggregation was increased by incubation with NCX 4016. These results appear to be attributable to the release of nitric oxide, which activates soluble platelet guanylyl-cyclase and promotes intracellular cyclic GMP increase. NCX 4016 almost completely inhibited platelet thromboxane A_2 production and arachidonic acid-induced platelet aggregation. This also occurred in the presence of oxyhaemoglobin and methylene blue, indicating that its antiplatelet activity can be attributed not only to nitric oxide release but also to cyclo-oxygenase inhibition.

Introduction

In vitro and in vivo studies concerning the effects of nitroderivative compounds on platelet function have given conflicting results; their efficacy as antiaggregating drugs is, therefore, still a matter of debate (1, 2), and continues to elicit considerable interest.

A new class of nitroderivative non-steroidal anti-inflammatory drugs has been recently synthesized (3). These compounds exibit dual antiplatelet functionality by acting through cyclo-oxygenase (COX) and nitric oxide (NO) dependent mechanisms. In a previous study, we tested the antiaggregating activity of one of these compounds, which was chemically related to acetylsalicylic acid (NO-aspirin, NCX 4215) (4, 5), confirming its dual mechanism of action. The aim of the present study was to characterize in vitro the activity of a new formula of NO-aspirin, by defining both its overall antiaggregating activity and the relevant mechanisms of action. The molecule concerned is: 2 acetoxy benzoate 2-[1-nitroxy-methyl]-phenyl ester (NCX 4016, M.W. 335.3; NICOX, London, UK), with the following formula (Fig. 1):

This compound appears to be of potential clinical interest since two independent but synergistic antiplatelet activities are combined in the same molecule. Therefore, we sought to identify any effect due to NO, which acts on the early phase of platelet activation, and any effect mediated by the blockade of platelet COX. We tested the effects on platelet adhesion and aggregation as well as intracellular free calcium ($[Ca^{2+}]_i$) and alterations in cyclic GMP (cGMP) levels induced by thrombin, since concentrations of these intracellular mediators are modified by NO-donors and endothelium-derived NO. Study parameters also included the expression of glycoprotein IIb/IIIa (integrin) and P-selectin (CD62 / GMP-140), using flow cytometry (6) after stimulus with agonist. Finally, we sought to investigate whether NCX 4016 had antiplatelet activities similar to those of acetylsalicylic acid (ASA) and, in particular, if it could irreversibly inhibit thromboxane A_2 (TXA_2) production. Therefore, we studied the in vitro inhibition by NCX 4016 of maximally stimulated TXA_2 production, compared with that induced by ASA.

Materials and Methods

Platelet Preparation

Venous blood samples (40 ml) were collected from 10 healthy adult volunteers who had not previously been taking any drugs for at least 3 weeks. Acid-citrate-dextrose mixture (14 mM sodium citrate, 11.8 mM citric acid, 18 mM dextrose, Merck, Darmstadt, G) was used as an anticoagulant agent. Washed platelets were obtained, by further centrifugation (700 g for 15 min), from platelet-rich plasma. This was prepared according to Hallam (7) using low-

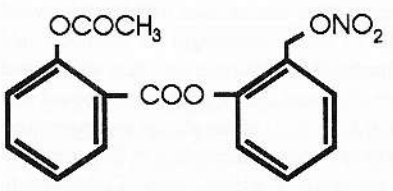

Fig. 1 Chemical structure of NCX 4016

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speed centrifugation (200 g for 10 min at room temperature) of 10 ml blood samples. Platelets were resuspended in 2-3 ml of NaCl 145 mM, KCl 5 mM, HEPES 10 mM (Sigma Chemical Co., St Louis, MO, USA), Na_2HPO_4 0.5 mM and glucose 6 mM, pH 7.4 at 37° C (HEPES buffer). Platelets were then counted using an automatic analyzer (model T-890, Coulter Co., Hialeah, FL, USA). A final concentration of 2×10^8 platelets/ml was obtained by dilution. The cells were kept at room temperature and used within 2 h.

Assay of Adhesion

Platelet adhesion was measured, as previously described (8), in culture microplates (Flow Laboratories, Amsterdam, Netherlands), precoated by means of overnight incubation at +4° C with 0.2 mg/ml human fibrinogen (Sigma Chemical Co., St Louis, MO, USA), in Dulbecco's phosphate buffer (Sigma Chemical Co., St Louis, MO, USA). Immediately before use, the plates were washed twice with 0.9% NaCl, using an automatic plate washer (Easy Washer 2, SLT Labs Instruments). The platelets were then suspended in HEPES buffer containing 0.2% of human albumin (Behring Institute, Marburg, Germany) and supplemented with 1 mM $CaCl_2$ and 1 mM $MgSO_4$.

NCX 4016 1×10^{-3} M was dissolved in dimethyl-sulfoxide (Sigma Chemical Co., St Louis, MO, USA) and then suitably diluted. The final volume of dimethyl-sulfoxide in the platelet suspension was always <0.1% of total volume; at these concentrations, dimethyl-sulfoxide does not interfere with cell metabolic activity (9). The suspension was incubated for 10 min with and without scalar doses of NCX 4016 using a humidified thermostat (37° C, 5% CO_2). After incubation, the adhesion measurement was carried out both in basal conditions and after the addition of thrombin, 0.05 U/ml (Calbiochem, San Diego, Ca). Each well contained 2.5×10^6 platelets and the final assay volume was 75 µl/well.

At the end of incubation, the plate was transferred to an automatic washer and subjected to two washing cycles with Dulbecco's phosphate buffer at room temperature. Adherent platelets were measured by acid phosphatase assay (8). The percentage of adherent cells was calculated on the basis of a standard curve, obtained with a known number of platelets of the same subject.

Platelet Intracellular Free Calcium

The cytosolic calcium concentration was measured in platelets with a fluorescent indicator, Fura 2 (Calbiochem, San Diego, Ca), as previously described by Pollock et al. (10). After loading with 2 µM Fura 2 AM in platelet-rich plasma (PRP), the platelets were collected by centrifugation and suspended ($2\text{-}4 \times 10^7$ cell/ml) in HEPES buffer. The external calcium and magnesium concentration was restored to 1 mM. NCX 4016 was incubated with the platelet suspension for 10 min at 37° C. Platelet $[Ca^{2+}]_i$ variations induced by thrombin (0.06 U/ml) were measured in the presence and absence of scalar doses of NCX 4016. Fluorescence variations were recorded at 37° C by F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), using excitation wavelength 340 nm and emission wavelength 500 nm. The effect of NCX 4016 on intracellular calcium mobilization was also evaluated in a medium containing EGTA 5 mM (Sigma Chemical Co., St Louis, MO, USA) to inhibit influx of extracellular calcium (11).

Expression of Glycoprotein IIb/IIIa (CD41/$\alpha_{IIb}\beta_3$) and P-selectin (CD62/GMP-140)

1 ml of platelet suspension (4.5×10^7/ml) was incubated for 10 min at 37° C in the presence and absence of scalar doses of NCX 4016. Then 4.5×10^6 unactivated platelets and thrombin (final concentration 0.05 U/ml)-activated platelets were incubated in HEPES buffer containing 0.2% albumin, 1 mM $CaCl_2$ and 1 mM $MgSO_4$. Thrombin-induced platelet activation was stopped after 3 min at 37° C, after which 10 µl were taken from each test tube and added to 50 µl of the same buffer at 4° C. 60 µl of this platelet suspension were incubated for 20 min at 4° C with saturating concentrations of fluorescein isothiocyanate (FITC)-labelled anti-human P-selectin (clone CLB-thromb/6; Immunotech, Birmingham, UK) and phycoerythrin (PE)-labelled anti-human GPIIb/IIIa (clone P2; Immunotech, Birmingham, UK) antibodies. All experiments included an isotype-matched non-specific conjugated mouse IgG1 FITC/IgG1 PE (Immunotech, Birmingham, UK) as negative control. The reaction was stopped with 1 ml of the HEPES buffer at 4° C. Samples were analyzed in duplicate in dual color fluorescence with a FAC Scan flow cytometer (Becton Dickinson, San Jose, California). The platelet population was identified on the basis of size and granularity (12).

ASA, if necessary, was dissolved in an ethanol-water mixture (1 : 10) at a concentration of 3×10^{-2} M. Final concentrations were achieved by suitable dilution.

The effect of ASA 3×10^{-5} M on $[Ca^{2+}]_i$, platelet adhesion and adhesion molecule expression after thrombin stimulation was preliminarily tested. None of these platelet responses were affected by ASA. The addition of ASA 1×10^{-5}, 5×10^{-5} and 1×10^{-4} M did not modify thrombin-induced platelet adhesion (3.24%), the observed percentages of adhesion being 3.08-3.31%. Similarly, the intensity of fluorescence (F) recorded when the adhesion molecules GMP-140 (F = 41) and GPIIb/IIIa (F = 29) were studied was not modified by 1×10^{-5}, 5×10^{-5} and 1×10^{-4} M ASA (F values ranging from 40 to 41 for GMP-140 and from 27 to 28 for GPIIb/IIIa), as previously described (13). In addition, the increase in $[Ca^{2+}]_i$ (522 nM intracellular calcium after thrombin addition) did not change in the presence of ASA 1×10^{-5}, 5×10^{-5} and 1×10^{-4} M (a concentration range of 525-534 nM was observed).

Platelet Aggregation

Platelet aggregation was monitored by a standard nephelometric technique in which 0.5 ml of platelet suspension (2×10^8 platelets/ml) were incubated at 37° C and stirred at 1.000 rpm in a four-channel aggregometer (Aggrecorder II, Daiichi, Tokyo, J). Agonist-induced aggregation was quantified by measuring the increase in light transmission (% T). Before the addition of an agonist, 1 mM $CaCl_2$ and 1 mM $MgSO_4$ were added to the platelet suspension.

The antiaggregant effects of NCX 4016, were tested incubating the drug with platelets at 37° C before the addition of the agonist. An incubation time of 10 min was used in most of the experiments. The effects of different incubation times, as well the effects of adding NCX 4016 at the same time of the agonist, were also studied.

For comparison, we used sodium nitroprusside (SNP), which has the capacity of releasing NO and therefore to inhibit aggregation. SNP $1 \times 10 - M$ (Carlo Erba, Milano, I) was dissolved in Dulbecco's phosphate buffer, and stored at 0° C wrapped in foil to shield it from light; fresh solutions were prepared at least every hour.

The effects of scalar doses of NCX 4016 on thrombin-induced platelet aggregation were tested. Thrombin was used as an agonist at relatively low concentrations (0.04-0.08 U/ml), to obtain submaximal aggregation in order to evaluate the drug's inhibitory effect (14). To evaluate any COX-independent activity, the effects of ASA 3×10^{-5} M were also preliminarely tested; this concentration, which totally inhibits TXA_2 production (15), did not affect thrombin-induced aggregation (4, 5). The mean of the differences observed in six experiments with and without ASA was +7% (95% CI = –18.5; +4.5; n.s.).

To ascertain whether any of the effects of NCX 4016 on thrombin-induced platelet aggregation was dependent on NO release from NCX 4016, aggregation tests and adhesion molecule measurements were performed in the presence of 2×10^{-5} M oxyhaemoglobin.

To evaluate whether nitric oxide is released from NCX 4016, we measured the nitrite/nitrate generation by incubating for 10 min NCX 4016 1×10^{-5} M and 1×10^{-4} M with buffer alone, platelet suspension (8×10^7/0.5 ml) and with platelet-poor plasma (PPP).

Nitrate/nitrite concentration was assayed using Griess reaction after conversion of nitrate to nitrite by means of bacterial nitrate reductase (Nitrate/Nitrite Assay Kit, Cayman Co, Ann Arbor, MI, USA).

To confirm the presence of a guanylyl-cyclase-dependent mechanism, in a different set of experiments the platelets were preincubated with 1×10^{-6} M methylene blue (Carlo Erba, Milano, I) for about 15 min at room temperature, and then incubated with NCX 4016 for 10 min at 37° C. When using oxyhaemoglobin and methylene blue, the calibrations were performed in the presence

of these agents to compensate for possible changes in light transmission (16).

We tested the effects of scalar doses of NCX 4016 on platelet aggregation and the duration of the lag phase induced by arachidonic acid 1×10^{-5} M (Menarini, Firenze, I). Also in this case, in a different set of experiments, we evaluated the effects of oxyhaemoglobin and methylene blue.

Finally, to exclude the possible effect of NCX 4016 on platelet NO-synthase, platelets were incubated for 1 h at 37° C with 3×10^{-4} M L-N^G-monomethyl-arginine (L-NMMA, Sigma, St. Louis, MO, USA), a competitive inhibitor of NO-synthase.

Measurement of Platelet TXA_2 Production

The activity of NCX 4016 on platelet TXA_2 synthesis was studied in vitro and compared with that of ASA ($1 \times 10^{-6} - 1 \times 10^{-4}$ M), by evaluating the production of TXB_2 in whole blood. This was measured as a reflection of maximally stimulated COX activity of platelet by endogenously formed thrombin (17). Whole blood aliquots were left to clot at 37° C for 60 min and then centrifugated at 3,000 rpm for 10 min. Serum was collected from the centrifuged aliquots and stored at -20° C. The concentration of TXB_2, the stable, non-enzymatic metabolite of TXA_2, was determined by radioimmunoassay in diluted samples using commercial antisera (Biomol, Plymouth, PA, USA).

In order to verify the irreversible inhibition of aggregation, 10 ml of whole blood were incubated with NCX 4016 1×10^{-4} M and then the platelets were isolated to test arachidonic acid-induced aggregation .

Measurement of Platelet Cyclic GMP

The intraplatelet cGMP concentration was determined under the same conditions as those in which platelet aggregation was assayed. Washed platelets (2×10^8 platelets/ml) were preincubated for 2 min with SNP 1×10^{-6} M and 1×10^{-4} M and 10 min with NCX 4016 1×10^{-5}, 5×10^{-5} and 1×10^{-4} M and then stimulated with thrombin (0.1 U/ml). After $1^1/_2$ min and $3^1/_2$ min ice-cold ethanol (to give 65% ethanol) was added twice. Samples were centrifugated at 2000 g for 15 min at 4° C and the supernatant was dried under nitrogen at 40° C in a vacuum oven. The dried extracts were dissolved in a 1 ml buffer. Concentrations of platelet cGMP were measured using a commercial radioimmunoassay kit (Amersham, UK). All determinations were performed testing duplicates of two aliquots of each sample.

Statistical Analysis

Data are expressed as Mean ± Standard Error of the Means (SEM). Means were compared using Student's t-test for paired data. Unpaired data analysis was sometimes used and P values <0.05 were taken as significant. The mean of the differences observed in some study end-points and the 95% confidence intervals of the differences (CI) are also indicated. IC_{50} and linear regression were calculated by a computer program (GraphPAD InPlot, San Diego, USA).

Results

Effects of NCX 4016 on the Early Phase of Platelet Activation

Thrombin (0.05 U/ml)-stimulated platelet adhesion on fibrinogen was reduced by NCX 4016 at concentrations ranging from 1×10^{-5} to 1×10^{-3} M ($IC_{50} = 7.3 \times 10^{-5}$ M). The effects of all tested concentrations of NCX 4016, except 5×10^{-6} M, were statistically significant when compared to basal values (n = 5; P <0.002 for all the tested concentrations) (Fig. 2).

The increase of intracellular free calcium induced by thrombin (0.06 U/ml), measured by fluorescent probe Fura 2 AM, was dose-dependently reduced by NCX 4016 in a concentration range from 2.5×10^{-5} to $1 \times^5 10^{-3}$ M. This was assessed by linear regression analysis of the means, both in the presence (r = -0.894, n = 7, P <0.01) and absence (r = -0.968, n = 4, P <0.05) of extracellular calcium (Fig. 3).

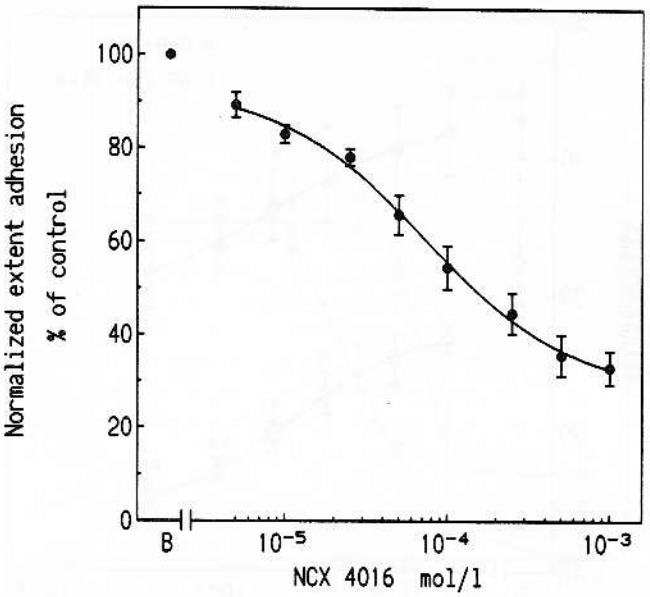

Fig. 2 Inhibition of thrombin (0.05 U/ml)-induced platelet adhesion by increasing concentrations of NCX 4016. Platelets were treated with ASA 3×10^{-5} M. For all concentrations tested, except 5×10^{-6} M: P < 0.002 versus basal values (n = 5). Mean ± SEM

Mean values of $[Ca^{2+}]_i$ determined with and without extracellular calcium in the medium were also correlated (r = 0.929, n = 5, P <0.05). In the presence of extracellular calcium, the reduction of calcium response to thrombin at different concentrations of NCX 4016 is closely correlated to the inhibition of aggregation (r = 0.927, n = 8, P <0.001).

Surface expression of the adhesion molecules glycoprotein IIb/IIIa ($CD41/\alpha_{IIb}\beta_3$) and P-selectin (CD62/GMP-140) on thrombin (0.05 U/ml)-stimulated platelets was inhibited by NCX 4016 at concentrations ranging from 2.5×10^{-5} to 2.5×10^{-4} M (Fig. 4). IC_{50} were respectively 3.4×10^{-5} M and 4.9×10^{-5} M.

The inhibitory effect of NCX 4016 between 1×10^{-5} and 1×10^{-4} M on GPIIb/IIIa and P-selectin expression was completely removed by the

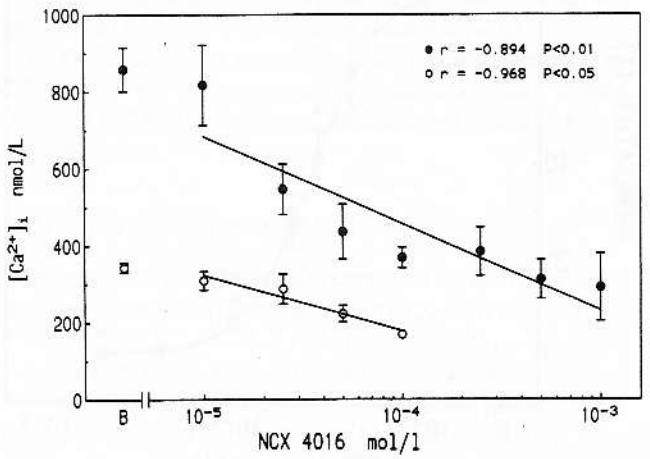

Fig. 3 Effect of different concentrations of NCX 4016 on thrombin (0.06 U/ml)-induced platelet $[Ca^{2+}]_i$ increase in presence of extracellular calcium (●) and absence of extracellular calcium (○). Platelets were treated with ASA 3×10^{-5} M. For each concentrations of the drug at least 4 experiments were performed in duplicate. Mean ± SEM

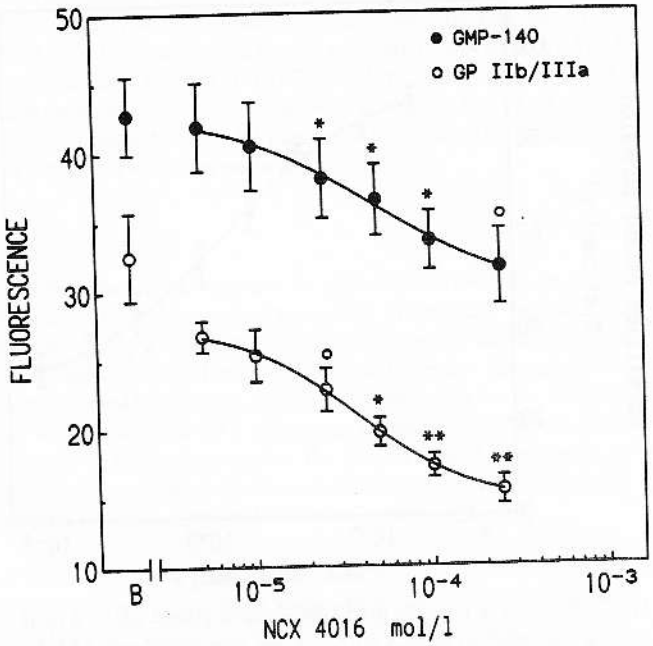

Fig. 4 Effects of increasing concentrations of NCX 4016 on the expression of adhesion molecules on platelets stimulated with thrombin (0.05 U/ml). Platelets were treated with ASA 3×10^{-5} M. For each concentrations of the drug at least 6 experiments were performed in duplicate. Mean ± SEM. ** $P < 0.001$, * $P < 0.01$, ° $P < 0.02$ versus basal values

addition of oxyhaemoglobin 2×10^{-5} M. The expression of P-selectin decreased to 14.8 ± 1.4 F (n = 4) in the presence of NCX 4016 5×10^{-4} M, with the addition of oxyhemoglobin it increased to 40.4 ± 0.88 F (n = 4; P for unpaired data <0.001). A similar phenomenon was observed when GP IIb/IIIa was studied.

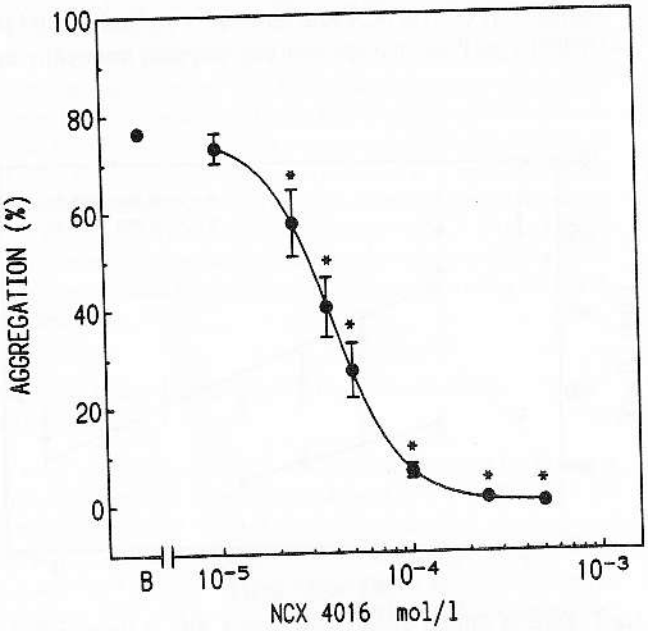

Fig. 5 Effects on thrombin (0.06 U/ml)-induced platelet aggregation by increasing concentrations of NCX 4016. Platelets were treated with ASA 3×10^{-5} M. For each concentrations of the drug at least 7 experiments were performed in duplicate. Mean ± SEM. * P<0.01 versus basal values

Effects of NCX 4016 on Platelet Aggregation

Thrombin-induced aggregation of platelets, treated with ASA 3×10^{-5} M to prevent TXA_2 production, was tested after 10 min incubation with NCX 4016 at 37° C. Platelet aggregation was almost totally inhibited by NCX 4016 at concentrations ranging from 2.5×10^{-5} to 5×10^{-4} M ($IC_{50} = 3.9 \times 10^{-5}$ M) (Fig. 5) after 10 min of incubation at 37° C. We observed that NCX 4016 did not affect platelet aggregation, when added at the same time of the agonist.

When the incubation times were prolonged, a time-dependent reduction in the effects of NCX 4016 on thrombin-induced aggregation was observed; the mean of the difference in platelet aggregation with and without NCX 4016 at the dose of 5×10^{-5} M was -70.4% at 10 min (95% CI = -79.6; -71.1; n = 7; P <0.001), -52.2% at 30 min (95% CI = -71; -33.3; n = 5; P <0.002), -27% at 60 min (95% CI = -47.2; -6.7; n = 3; P <0.05).

The inhibitory effects of increasing doses of NCX 4016 on the lag phase and the aggregation induced by arachidonic acid, compared with those produced by ASA, are shown in Table 1. At concentrations of 1×10^{-4} and 5×10^{-5} M NCX 4016 displayed inhibitory effects similar to ASA, while at concentrations of 2.5×10^{-5} and 1×10^{-5} M had significantly less effects than acetylsalicylic acid on lag phase and aggregation.

The irreversibility of the effect on arachidonic acid-induced platelet aggregation was tested by adding the drug (1×10^{-4} M) directly to whole blood and then testing aggregation on washed platelets obtained

Table 1

	AGGREGATION (%)				LAG PHASE (sec)	
	NCX 4016	n	ASA	n	NCX 4016	ASA
Basal	80.7 ± 0.4	11	81.6 ± 1.0	14	6 ± 0.9	4 ± 0.5
5×10^{-6} M	81.3 ± 0.7	3	80.9 ± 2.7	7	6 ± 0.5	22° ± 5
1×10^{-5} M	79.0 ± 0.6	4	52.7°° ± 14.0	7	11°° ± 0.5	46° ** ± 0.9
2.5×10^{-5} M	25.4° ± 6.8	6	0° *	7	38° ± 6.3	149° ** ± 76
5×10^{-5} M	6.0° ± 5.2	7	0°	5	187° ± 77	173° ± 59
1×10^{-4} M	1.7° ± 1.2	7	0°	5	185° ± 52	183° ± 17

Comparison between the effects of scalar doses of NCX 4016 and ASA on lag-phase and arachidonic acid-induced platelet aggregation. Mean ± SEM.
* P<0.001 ** P<0.05 (ASA vs NCX 4016)
* P<0.001 ** P<0.01 (ASA and NCX 4016 vs Basal).

Table 2

	Basal	SNP 10^{-8} M	SNP 10^{-7} M	SNP 10^{-6} M
Aggregation AA (10^{-5} M)	75.2 ± 5.1 n=6	24.5 ± 12 n=4 P<0.01*	3 ± 0.6 n=6 P<0.001*	3.25 ± 0.5 n=4 P<0.002*
Aggregation Thr (0.06 U/ml)	71.8 ± 1.3 n=4	39.9 ± 3.9 n=27 P<0.001*	9.4 ± 2.9 n=20 P<0.001*	0.6 ± 0.4 n=10 P<0.001*

Effect of increasing concentrations of sodium nitroprusside (SNP) on arachidonic acid and thrombin-induced platelet aggregation. Mean ± SEM. * SNP versus Basal.

from the same sample. A complete inhibition of aggregation was observed (0% aggregation in all tests, n = 7).

A different NO-donor, SNP, tested for comparison at the concentrations from 1×10^{-8} to 1×10^{-6} M significantly inhibited both the aggregation induced by thrombin in ASA-treated platelets and that induced by arachidonic acid (Table 2).

Effects of NCX 4016 Mediated by NO Activity, Examined as Follows:

(I) The possible release of NO by NCX 4016 1×10^{-5} and 1×10^{-4} M was assessed by measuring nitrates/nitrites concentration in the medium. An increase in nitrate/nitrite was observed; this increase was observed when NCX 4016 (1×10^{-4} M) was incubated for 10 min with both washed platelets (from 1.4×10^{-5} M to 2×10^{-5} M) and PPP (from 1.9×10^{-5} M to 2.6×10^{-5} M). Nitrite/nitrate concentration did not change in the presence of the buffer alone ($<2 \times 10^{-6}$ M with and without NCX 4016).

(II) To test whether the effects of NCX 4016 on thrombin-induced platelet aggregation were NO-mediated, oxyhaemoglobin 2×10^{-5} M was added to the platelet suspension 1 min before thrombin. The inhibition of thrombin-induced platelet aggregation by NCX 4016 5×10^{-5} M was for the most part removed. Aggregation values were: in the presence of NCX 4016 7% ± 0.9, with NCX 4016 + oxyhaemoglobin 62.5% ± 0.9; the mean of the differences was +55.5% (95% CI = +50.6; +60.4; n = 4; P <0.001).

Oxyhaemoglobin 2×10^{-5} M was also able to reduce the inhibition of arachidonic acid-induced aggregation produced by increasing concentration of NCX 4016 (n = 4) (Fig. 6).

(III) Incubation of the platelets in the presence of scalar concentrations of NCX 4016 significantly increased the intraplatelet concentration of cGMP. The values of cGMP concentration were 0.11 ± 0.005 pmol/10^8 platelets (n = 4) in basal conditions, 0.12 ± 0.006 pmol and 0.11 ± 0.006 ($1^1/_2$ min and $3^1/_2$ min respectively), after stimulus with thrombin. In the presence of NCX 4016 1×10^{-4} M, cGMP concentration was 0.19 ± 0.017 pmol/10^8 platelets $1^1/_2$ min after thrombin (P <0.02 vs thrombin alone) and 0.21 ± 0.004 pmol after $3^1/_2$ min (P <0.02). With lower concentrations of NCX 4016 the increase in cGMP was minor. SNP 1×10^{-6} and 1×10^{-4} M induced a dose-dependent increase in cGMP concentration, ranging from 1.98 to 4.76 pmol/10^8 platelets.

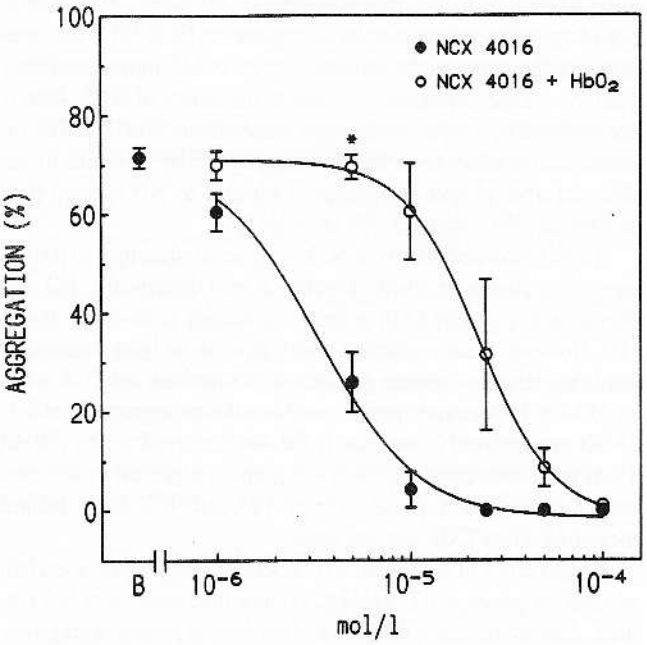

Fig. 6 Effect of 2×10^{-5} M oxyhaemoglobin on inhibition of arachidonic acid (1×10^{-5} M)-induced platelet aggregation by increasing concentrations of NCX 4016. Mean ± SEM. * P <0.02 (NCX 4016 plus oxyhaemoglobin versus NCX 4016)

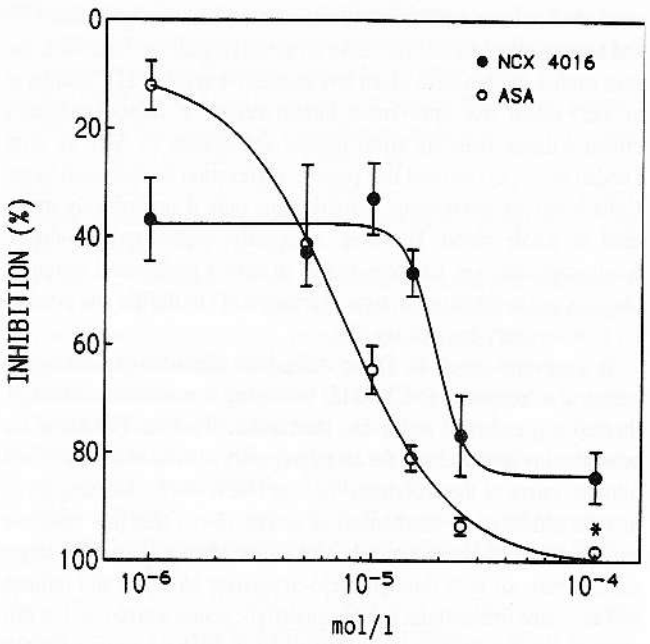

Fig. 7 Effects of scalar doses of NCX 4016 (IC_{50} = 1.9×10^{-5} M) and ASA (IC_{50} 7.3×10^{-6} M) on serum TXA_2 concentration, expressed as percent of inhibition of serum TXB_2 concentrations. Mean ± SEM. * P for unpaired data <0.001 (NCX 4016 versus ASA)

(IV) Methylene blue 1×10^{-6} M significantly decreased the effect of NCX 4016 5×10^{-5} M on thrombin-induced platelet aggregation. The mean of the observed differences in light transmission with and without methylene blue was +28.3% (95% CI = +8.4; +48.2; n = 7; P <0.02).

Methylene blue did not inhibit the effects of the highest tested doses of the drug (5, 2.5 and 1×10^{-5} M) on arachidonic acid-induced aggregation; methylene blue removed the effects of NCX 4016 (1×10^{-6} M) only when the inhibition in platelet aggregation was partial.

(V) The inhibition of thrombin-induced platelet aggregation by NCX 4016 5×10^{-5} M was not modified by the presence of L-NMMA 3×10^{-4} M (NCX 4016 19.8% ± 6.8, NCX 4016 + L-NMMA 14.6% ± 6.7; n = 5; P n.s.).

Effects of NCX 4016 on Platelet TXA_2 Production

The release of TXA_2 by platelets, evaluated by measuring serum TXB_2 concentration, was reduced by both NCX 4016 (IC_{50} = 1.9×10^{-5} M) and ASA (IC_{50} = 7.3×10^{-6} M). Compared to control values, these inhibitory effects were statistically significant at the doses of 2.5×10^{-5} and 1×10^{-4} M (P <0.01). The effect of NCX 4016 1×10^{-4} M was lower than that of ASA 1×10^{-4} M (P <0.001) (Fig. 7).

Discussion

The effect of organic nitrates on platelet function is complex, and probably differs according to the compound concerned (1, 2, 18). In

vitro studies have consistently demonstrated that nitroglycerine (19) and sodium nitroprusside (20) have an antiaggregating effect, while human studies in vivo have given less conclusive results (21). Stamler et al. (22) report that intravenous nitroglycerine, at hemodynamically effective doses, does not affect platelet aggregation by ADP ex vivo. Diodati et al. (23) showed that platelet aggregation is attenuated in patients receiving intravenous nitroglycerine only if immediately measured in whole blood. However, a recently synthesized NO-donor, S-nitrosoglutathione, has been shown to have a preferential action on platelets and to inhibit in vivo the expression of GP IIb/IIIa and P-selectin after coronary angioplasty (24).

In a previous report (4, 5), we studied the effects on platelet aggregation of a compound (NCX 4215) belonging to a new class of drugs, theoretically endowed with a dual mechanism of action. The aim of the present study is to evaluate the antiaggregatory activity of a more recent nitroderivative of the acetylsalicylic acid (NCX 4016), focusing on its in vitro efficacy and mechanism of action. Given that this molecule may have two pharmacologically active sites when it reaches the target cells, it may act both through cyclo-oxygenase blockade and through NO-mediated intracellular soluble guanylyl-cyclase activation. For this purpose, we investigated the effects of NCX 4016 on various platelet activation tests, including some specific to the study of NO-dependent action.

The mechanisms first explored in this study, which are involved in the early phase of cellular activation, are the following: increase in cytosolic calcium, platelet adhesion and expression of membrane glycoproteins (P-selectin and GP IIb/IIIa). These are specifically antagonized by NO through the activation of soluble guanylyl-cyclase (25).

It is now recognised that the activation of soluble guanylyl-cyclase by NO or NO-donors determines an increase in intracellular cGMP which, in turn, stimulates the calcium efflux through the membrane calcium-ATPase and calcium uptake by intracellular stores. The main action of cGMP is the inhibition of phospholipase C, thus decreasing production of diacylglycerol and inositol trisphosphate. The net overall result is a decrease in protein kinase C activity and $[Ca^{2+}]_i$ reduction (18, 26). In the present study we observed that in the presence of extracellular calcium, NCX 4016 dose-dependently reduces the increase in thrombin-induced $[Ca^{2+}]_i$ and that calcium mobilization from intracellular stores is also reduced in the absence of extracellular calcium. Since these calcium movements are not affected by ASA, the hypothesis that NCX 4016-induced inhibition depends on the released NO is strengthened. Increase in $[Ca^{2+}]_i$ is not completely prevented by NCX 4016; this could depend on stimulated calcium influx, partly occurring through NO-insensitive mechanisms (27, 28).

The inhibition of thrombin-stimulated platelet adhesion is again dose-dependent: the inhibition curve is very steep at drug concentrations ranging from 1×10^{-6} to 1×10^{-5} M, and forms a plateau at higher concentrations. Consistent with this finding, the surface expression of the glycoprotein IIb/IIIa and P-selectin on thrombin-stimulated platelets was also inhibited by NCX 4016. Prevention of the inhibitory effect of NCX 4016 by oxyhaemoglobin shows that this effect is NO-dependent.

Platelet surface expression of adhesion molecules is considered an index of platelet activation. P-selectin, an α-granule protein expressed on the surface of platelets only after activation, mediates the adherence of neutrophils and monocytes to activated platelets in a calcium-dependent manner (29). The binding of fibrinogen to glycoprotein IIb/IIIa is a major pathway in platelet aggregation (30). The effect of NCX 4016 on $[Ca^{2+}]_i$ increase and the expression of adhesion molecules seems to be due to the inhibition by NO of the "classic" signal transduction, mediated by soluble agonists (31, 32). These agonists probably do not act directly on the integrins, but bind to a cellular receptor leading to the engagement of the signal transduction system. However, some of the inhibitory effects of NCX 4016 on platelet activation could be due to direct interaction of the released NO with surface receptors and adhesion molecules, through a reaction with cell-surface thiols (33).

We observed antiaggregatory effects of NCX 4016 on thrombin-induced and arachidonic acid-induced aggregation. Both these tests of platelet aggregation are sensitive to the activity of NO-donors, as we also observed studying SNP. The inhibitory effect of NCX 4016 is evident only after incubation of platelets with the drug for 10 min at 37° C; this incubation period is probably necessary for NO release (34). When the incubation time is longer, the antiaggregatory effect of NCX 4016 on thrombin-induced aggregation decreases. This could be due to inactivation of NO, once released from the molecule. The effects of NCX 4016 on thrombin-induced platelet aggregation were studied in conditions of cyclo-oxygenase blockade in order to prevent TXA_2 production. The inhibitory effect of NCX 4016 is therefore attributable to a COX-independent mechanism: the removal of this effect by oxyhaemoglobin confirms that it depends on NO release (35). These experiments also suggest that the studied drug spontaneously releases NO in the extracellular medium where it can be captured by oxyhaemoglobin.

The release of NO is confirmed by the increase in nitrate/nitrite concentration after 10 min-incubation of NCX 4016 with washed platelets and PPP, but not with buffer alone.

The effects of oxyhaemoglobin on the inhibitory activity of NCX 4016 on arachidonic acid-induced platelet aggregation show the double action mechanism of this drug. Part of this effect seems to be dependent on NO release since oxyhaemoglobin, as methylene blue, can reduce this inhibition by shifting the dose-response curve to the right. However, it does not prevent the effects of high doses of NCX 4016. In these conditions the activity of NCX 4016 appears to depend mainly on the inhibition of platelet COX.

The experiments carried out with methylene blue (36) suggest that the effects of NCX 4016 are mediated by the stimulation of platelet guanylyl-cyclase. In fact, methylene blue is able to prevent the inhibition of thrombin-induced platelet aggregation by NCX 4016. Measurements of the intra-platelet concentration of cGMP further confirm a guanylyl-cyclase dependent mechanism: incubation of NCX 4016 is accompanied by a higher intraplatelet concentration of cGMP. This increase, though rather lower than that induced by SNP, is similar to that observed when platelet aggregation is inhibited by NO released from endothelial cells stimulated with thrombin (37).

The NO-mediated activity of NCX 4016 seems, therefore, to involve guanylyl-cyclase stimulation. However, at least theoretically, NO may directly affect platelet COX activity via binding to its heme moiety (38). However, quite conflicting results have so far been obtained on possible interaction between products of NO synthase and COX activity (39-42). In our experimental conditions the antiaggregating effects of NO may indirectly contribute to the observed reduction in platelet TXA_2 production caused by NCX 4016; this is suggested by the comparison of the dose-response curve of ASA and NCX 4016 obtained measuring serum TXB_2 concentration.

A direct effect of NCX 4016 on platelet NO synthase can be excluded since the presence of L-NMMA, a competitive inhibitor of NO-synthase, does not influence the effects of the drug on platelet aggregation.

The experiments described in this study also provide evidence concerning the effect of NCX 4016 on platelet COX. For this purpose, NCX 4016 was tested on arachidonic acid-induced aggregation, which, however, could not strictly be assumed as a specific test for COX activ-

ity, since it can be inhibited by NO. The direct effects of NCX 4016 and ASA on COX were therefore tested by measuring stimulated TXA_2 production as concentration of TXB_2 in whole blood. The inhibitory capacity of NCX 4016 proved lower than that of ASA, suggesting a lower affinity to the active site of platelet COX-1. However, the inhibition of arachidonic acid-induced aggregation remains hours after the removal of NCX 4016 from the medium, thus demonstrating that both drugs can irreversibly inhibit platelet COX activity. This result is in agreement with the observation that in rats, orally treated with NCX 4016, platelet TXA_2 production was almost completely inhibited even 48 h after the last drug administration (43).

The data from this study shows that NCX 4016 has significant antiaggregatory activity in vitro. It exhibits a dual mechanism of action on platelet function by acting via COX and NO dependent pathways. However it is less potent than aspirin and SNP with respect to COX inhibition and NO release respectively. We believe that the antiaggregating activity of this drug deserves further experimental study in vivo.

Acknowledgements

We gratefully acknowledge Dr. Piero Del Soldato of Nicox, UK. for providing NO-ASA and for his helpful criticism.

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Received July 25, 1995 Accepted after resubmission July 25, 1996