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Prevention of pulmonary thromboembolism by NCX 4016, a nitric oxide-releasing aspirin

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Abstract

We studied the antithrombotic activity of 2-acetoxybenzoate 2-[1-nitroxy-methyl]-phenyl ester (NCX 4016), a novel nitric oxide (NO)-releasing aspirin derivative, in vivo in different animal models of platelet-dependent and independent pulmonary thromboembolism and compared it with that of aspirin. NCX 4016 protected mice from death induced by the intravenous (i.v.) injection of collagen plus epinephrine, of 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) and of thrombin while aspirin was only active against collagen plus epinephrine. The drop in platelet count and number of lung emboli were reduced by NCX 4016 more effectively than aspirin. NCX 4016 protected mice also from mechanical pulmonary embolism (i.v. injection of hardened rat red blood cells) while aspirin was ineffective. In rabbits, NCX 4016 significantly reduced the accumulation of [^{111}In]oxine-labeled platelets in the pulmonary vasculature induced by collagen and by thrombin while aspirin produced reductions which were significant only versus collagen. In conclusion, NCX 4016 exerts a more pronounced antithrombotic activity than aspirin in vivo in two different animal species, largely due to a deeper inhibitory effect on platelets. NCX 4016 may represent a better antithrombotic agent than aspirin.

Keywords: Endothelial factor; Hemostasis; Nitric oxide (NO); Platelet; Thrombosis

1. Introduction

Aspirin is an effective antithrombotic agent for the secondary prevention of ischaemic cardiovascular disorders (Antiplatelet Trialists' Collaboration, 1994; Patrono et al., 1998). Aspirin inhibits platelet aggregation by acting as a cyclo-oxygenase inhibitor and thus suppressing the production of thromboxane A_2, an important agonist of platelet activation and a vasoconstrictor (Gresele et al., 1991; Patrono et al., 1998).

However, the suppression of only one pathway of platelet activation, albeit important, may represent a limitation for the effectiveness of this antiplatelet agent (Gresele et al., 1991; Meyer, 1998). Indeed, aspirin does not suppress platelet aggregation induced by several agonists in vitro, including physiologic mediators such as thrombin, thromboxane A_2, high dose collagen, or the combination of collagen plus epinephrine (Larsson et al., 1994). In addition, some aspects of platelet activation important for the development of thrombosis, like adhesion under flow conditions or the activation induced by high shear forces, those which develop in stenosed coronary arteries, are not significantly inhibited by aspirin in vitro (Moake et al., 1988).

Aspirin is poorly effective in some animal models of thrombosis in which high shear stress and/or the simultaneous presence of several platelet agonists play a pathogenic role (Folts et al., 1999) and these observations have been taken as an explanation for the high residual incidence of ischaemic cardiovascular events in patients with acute coronary syndromes treated with aspirin (Meyer, 1998; Theroux, 1998). These considerations have led to a great effort in the search for new drugs to suppress platelet

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activation more extensively than aspirin is able to do. Currently, the preferred approach is that of combining the antiplatelet properties of aspirin with those of another drug class, such as an ADP receptor antagonist (ticlopidine, clopidogrel) or a glycoprotein IIb/IIIa antagonist (Born and Collins, 1997; Theroux, 1998). This approach has the advantage of keeping the positive antithrombotic properties of aspirin, mainly the suppression of thromboxane A_2 which is a powerful amplification signal for other agonists, a vasoconstrictor and a smooth muscle cell proliferative agent, and simultaneously of widening the range of platelet stimuli to which platelets are made unresponsive.

Nitric oxide (NO) plays an important role in preventing vasoconstriction stimulated by platelet-released mediators at localized sites of vessel wall damage in coronary arteries (Noll and Lusher, 1998; Bing et al., 1999) and is also a powerful platelet inhibitor able to suppress platelet adhesion and activation induced by a wide range of agonists (Riddel and Owen, 1999).

2-acetoxybenzoate 2-[1-nitroxy-methyl]-phenyl ester (NCX 4016) is a novel nitroaspirin derivative able to release NO and to suppress a wide range of platelet functions in vitro (Del Soldato et al., 1999; Mezzasoma et al., 1999).

Only one study until now has addressed the antithrombotic effectiveness of NCX 4016 in an animal model of thrombosis in a extracorporeal circuit in rats with limited results (Wallace et al., 1999).

The aim of the present study was to investigate the antithrombotic activity of NCX 4016 in vivo in comparison with that of aspirin in different animal models of platelet-dependent and independent pulmonary thromboembolism.

2. Material and methods

2.1. In vivo thrombosis model in the mouse

2.1.1. Pulmonary thromboembolism

Pulmonary thromboembolism in mice was induced by a method described previously (Gresele et al., 1990; Paul et al, 1993). Briefly, male CD-1 mice (Charles Rivers, Calco, Como, Italy), weighing 20–25 g, were used. Mice were caged and fed a regular diet for at least 1 week before use. The drugs to be tested, or their vehicles, were administered intraperitoneally (i.p.) in a fixed volume of 100 μl, 2 min before the thrombotic challenge. In these experiments, the thrombotic challenge was induced by the rapid intravenous (i.v.) injection into a tail vein alternatively of a mixture of collagen (250 μg/ml) and epinephrine (1.5 μg/ml) (Gresele et al., 1990; Emerson et al., 1999), of 9,11-dideoxy-11α,9α epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) (0.2 mg/kg), a stable thromboxane A_2 analog (Gresele et al., 1990), or of thrombin (1000 U/kg) (Paul et al, 1993; Gresele et al., 1998). The doses of the agonists used were selected from a concentration/response curve as the minimal dose giving a reproducible 80–90% mortality in the control group. In each experimental session, at least five animals per treatment group were tested; control groups were run at the beginning and at the end of every experimental session. Mice were accustomed to handling by the investigators and the injections were carried out by skilled investigators with minimal disturbance to the animals. The total duration of each experiment was 15 min and all surviving animals were sacrificed by exposure to ether vapours. The animals which did not die within this time, or which were obviously distressed, were sacrificed after 15 min and were recorded as survivors. No anesthesia was used during the experiment because of the short duration and because anesthesia interferes with thromboembolism in this model (Paul et al., 1993). This study was approved by the Committee on Ethics of Animal Experiments of the University of Perugia and by the Italian Ministry of Public Health.

The evaluation of the effect of drug treatments on the i.v. challenge with collagen plus epinephrine was carried out as previously described (Gresele et al., 1990, 1998): the cumulative end point to be overcome was death of the animal or prolonged paralysis of the hind limbs (for more than 15 min).

The data are presented as number of animals dead/total number of animals tested or as percentage of total.

Protection against collagen plus epinephrine, thrombin or U46619-induced mortality was expressed as $(1\text{-TDRUG/TSAL}) \times 100$, where TDRUG is the mortality rate in treated mice, and TSAL is the mortality rate in controls.

Finally, in a few experiments, pulmonary thromboembolism was induced by a suspension of swollen, hardened rat red blood cells produced as previously described (Clement et al., 1983; Molinari et al., 1987; Gresele et al., 1990). Briefly, rat blood collected by heart puncture under ether anesthesia and anticoagulated with trisodium citrate 3.8% was centrifuged at $150 \times g$ for 10 min. The buffy coat was removed and chlorpromazine (Largactil, Carlo Erba, Milan, Italy) was added to the red blood cells at a final concentration of 0.2 mg/ml. The suspension was carefully mixed and incubated for 15 min at room temperature. Red cells were then centrifuged at $2000 \times g$ for 10 min, the supernatant was removed and the cell pellet was resuspended in glutaraldehyde 3% in Sorensen buffer pH 7.4 and incubated for 30 min at 4°C under continuous mixing. The cells were then washed three times in NaCl 0.154 M and finally resuspended in saline at a hematocrit of 12.5%. The evaluation of the effects of the i.v. challenge with hardened rat red blood cells was carried out as described above.

2.1.2. Platelet counts

Platelets were counted as described previously (Gresele et al., 1990; Emerson et al., 1999). Briefly, blood was

collected by cardiac puncture from mice under ether anesthesia and anticoagulated with 1:10 vol of tripotassium ethylenediaminetetraacetic acid (EDTA). After thorough mixing platelets were counted optically by the Brecher–Cronkite method (Brecher and Cronkite, 1950) by an operator unaware of the treatment groups. Platelet counts were carried out 2 min after the thrombotic challenge in saline pretreated (controls) or drug pretreated animals.

2.1.3. Lung histology

Two minutes after the i.v. injection of collagen plus epinephrine, mice were rapidly killed with ether vapours and the chest was opened, the trachea was cannulated and lungs were instillated with a fixing solution (formalin 10% buffered with calcium carbonate). The trachea was then ligated and removed together with the lungs, which were rinsed in cold saline and immediately fixed in 10% formalin for at least 24 h. The right-lower lobe was embedded in paraffin and several sections, 5–6 μm thick, were cut and stained with hematoxylin and eosin (Horobin and Walter, 1987) to reveal any platelet thrombi in tissues (Gresele et al., 1990; Paul et al., 1993). The specimens were examined under a light microscope (Wild-Leitz, Heerbrugg, West Germany) by a pathologist unaware of the treatment administered to the animals. At least 10 fields, at a magnification of $400 \times g$, were observed for every specimen. The total number of identifiable lung vessels per field was counted and the percentage of them occluded by platelet thrombi was annotated.

2.2. In vivo thrombosis model in the rabbit

2.2.1. Preparation of animals

The study was carried out on male rabbits (New Zealand White, Highgate, UK) weighing 2.1–2.8 kg. Animals received a standard diet and water ad libitum. Animals were treated with a combination of diazepam (4 mg/kg, i.p.) and Hypnorm (0.4 ml/kg, i.m. containing 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone). Neuroleptanalgesia was maintained by intramuscle injection of Hypnorm (0.1 ml/kg) every 30 min. The i.v. injections were made via a cannula placed in a marginal ear vein.

2.2.2. [^{111}Indium]oxine-labeling of platelets

Rabbit platelets were labeled in vitro with [^{111}In]oxine. Details of the protocol have been described fully elsewhere (Page et al., 1982; May et al., 1990; Paul et al., 1993). A 9 ml of blood, obtained from a central ear artery, was collected into 1 ml of 3.8% (w/v) trisodium citrate and centrifuged at $200 \times g$ for 10 min to obtain platelet-rich plasma. The platelets were then washed by centrifugation at $640 \times g$ for 15 min in Ca^{2+}-free Tyrode solution containing prostaglandin E_1 (300 ng/ml). After removal of the supernatant, the surface of the platelet pellet was carefully rinsed three times with 1 ml of the same buffer. The platelets were resuspended gently in 1 ml of buffer and incubated for 4 min at 37°C with [^{111}In]oxine (25–50 μCi). After a further centrifugation at $640 \times g$ for 15 min, the supernatant containing free [^{111}In]oxine was removed and the platelet pellet was carefully rinsed three times with 1 ml of Ca^{2+}-free Tyrode solution containing prostaglandin E_1 (300 ng/ml) and then resuspended in the same buffer without prostaglandin E_1 (1 ml/recipient rabbit).

2.2.3. Measurement of in vivo platelet accumulation using an automated isotope monitoring system

[^{111}In]oxine-labeled platelets were administered via a marginal ear vein and allowed to equilibrate in the circulation for 45 min. Circulating [^{111}In]oxine-labeled platelets were continuously monitored in the thoracic and cerebral vascular beds by 1 in. crystal scintillation detectors placed over the thorax (to measure changes in the pulmonary circulation) and head (to measure changes in the cranial circulation). Counts were estimated with a dual channel spectrometer (Nuclear Enterprises NE 461) and logged with the aid of a special application interface (AIMS 8000, Mumed) by a microcomputer (Dell System 200).

Following administration of platelet agonists (ADP 20 μg/kg, collagen 50 μg/kg, thrombin 18 U/kg and N^G-nitro-L-arginine methyl ester 10 mg/kg plus thrombin 5 U/kg), changes in radioactivity from stable baseline values were continuously monitored and converted to percentage change in counts. Responses were expressed as (a) the peak response (the maximum percentage change from baseline) and (b) the trapezoidal area under the curve (AUC, arbitrary units) of the agonist-induced percentage increase in counts plotted against time (calculated by computer-assisted planimetry) to give a measure of the response over the monitored time course.

2.2.4. Study protocol

Aspirin and NCX 4016 were dissolved in methanol which was used as diluent control. In vitro experiments, using aspirin dissolved either in distilled water or in methanol, showed that the drug was equally effective in inhibiting the formation of thromboxane B_2 in serum, demonstrating that resuspension in methanol does not inactivate aspirin (data not shown). For i.p. treatment, drugs were administered in a volume of 2 ml/kg 30 min prior to injection of the platelet agonists (ADP, collagen or thrombin). For i.v. treatment, aspirin and NCX 4016 were dissolved in methanol and infused at a rate of 0.1 ml min^{-1} for 25 min, commencing 20 min prior to injection of thrombin.

2.3. Drugs

The sources of the drugs used were as follows: U46619, isosorbide 5 mononitrate, thrombin from human plasma and from bovine plasma, ADP, N^G-nitro-L-arginine methyl

ester, prostaglandin E_1 (Sigma Chemicals, St. Louis, MO); epinephrine bitartrate, a 5 mM solution in Tris buffer (Mascia Brunelli, Milan, Italy); diazepam (Valium, Roche, Welwyn Garden City, UK); equine tendon collagen in suspension (Hormon Chemie, Munich, Germany); Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml; Janssen Pharmaceuticals, Oxford), [^{111}In]oxine (Amersham International, UK) and 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylic acid methyl 2-[methyl-(phenylmethyl) amino] ethyl ester (nicardipine) (Novartis, Milan, Italy); acetyl salicylic acid or aspirin (Flectadol, Carlo Erba, Milan, Italy); sodium nitroprusside (Sigma-Aldrich, Milan, Italy); chlorpromazine (Largactil, Rhone-Poulenc Rorer Milan, Italy); NCX 4016 was a gift from Nicox.

All drugs were dissolved in 0.9% saline; NCX 4016 was dissolved in polyethylene glycole.

2.4. Statistical analysis

The Chi-square test was applied to the studies on mortality, using the Bonferroni's correction (statistically significant $P < 0.05$/number of comparisons). One-way analysis of variance (ANOVA), followed by Dunnet's test for multiple comparisons, was used for the other studies. Unless otherwise stated data are expressed as mean ± S.E.M.

3. Results

3.1. In vivo thrombosis model in the mouse

3.1.1. Pulmonary thromboembolism

The i.v. injection of collagen plus epinephrine (100 μl) led to death or prolonged paralysis of 85.7% of control mice (156/182 dead or paralyzed/tested). Pretreatment of animals with NCX 4016 i.p., 30 min before the i.v. injection of collagen plus epinephrine, exerted a dose-dependent protective effect against mortality. The first dose that significantly reduced the mortality was 60 mg/kg (44% mortality, 11/25, $P < 0.001$) with the highest tested dose (120 mg/kg) protecting 71.5% of animals from death (4/14, $P < 0.0001$)(Fig. 1).

From the dose–response curve, the estimated dose protecting 50% of animals from death (IC_{50}) was 69.3 mg/kg. Under the same experimental conditions, aspirin protected significantly mice from thromboembolism induced by collagen plus epinephrine only at the highest dose tested (300 mg = 44.4% mortality, 16/36 dead/tested, $P = < 0.001$; IC_{50} = 260 mg/kg)(Fig. 1).

Pretreatment with NCX 4016 (60 mg/kg i.p.) protected animals from the lethality provoked by the i.v. injection of the thromboxane A_2-analog U46619 (0.2 mg/kg) (8/35, $P < 0.001$) while aspirin up to 300 mg/kg did not prevent U46619-induced mortality (Fig. 2A).

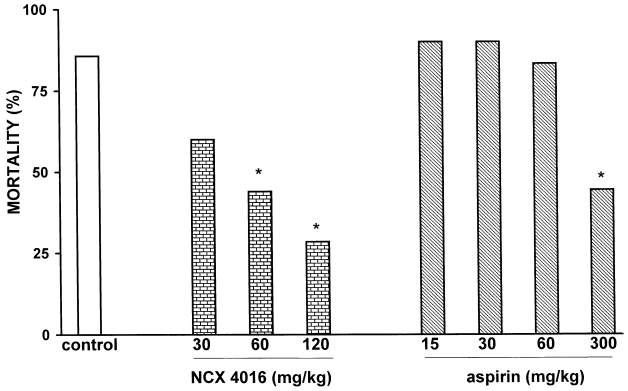

Fig. 1. Effects of NCX 4016 or aspirin upon collagen and epinephrine-induced death in mice. Drugs were given by i.p. route 30 min before i.v. injection of the thrombogenic stimulus and the mortality rate was evaluated as reported under Section 2. Asterisks indicate a statistically significant reduction of mortality as compared with controls ($P < 0.05$, at least).

When the thrombogenic stimulus was thrombin, at a dose inducing 85% mortality (1250 U/kg) neither NCX 4016 (up to 120 mg/kg) nor aspirin (up to 300 mg/kg) exerted any protective effect (data not shown). However, when the dose of thrombin was lowered (1000 U/kg), reducing the mortality to 52.2%, NCX 4016 exerted a dose-dependent protection (NCX 60 mg/kg = 32.6% mortality, 16/49, $P < 0.05$) while aspirin (15–300 mg/kg) was not effective (Fig. 2B).

Finally, NCX 4016 reduced, dose-dependently, the lethal consequences of the i.v. injection of a 12.5% suspension of swollen, hardened rat red blood cells, a model of non-thrombotic, disseminated pulmonary microembolism, while aspirin was ineffective (Fig. 2C). In the latter model, nicardipine (100 mg/kg i.p.), a vasodilator calcium-antagonist, and isosorbide mononitrate (10 mg/kg, i.v.), a NO-donor, were both effective (Fig. 2C).

The bioavailability of aspirin given by i.p. route in our studies is confirmed by more than 97% suppression of thromboxane B_2 formation in serum prepared from blood taken from mice pretreated with 300 mg/kg or rabbit pretreated with 33 mg/kg of the drug given i.p. 30 min before (data not shown).

3.1.2. Platelet count

In control animals, basal platelet count in circulating blood was 1,075,000 ± 181,000/μl ($n = 69$). The i.v. injection of collagen plus epinephrine (100 μl) in control animals caused a fall in platelet count to the value of 121,000 ± 59,900/μl (89% drop, $n = 41$, $P < 0.001$ vs. control). Pretreatment with NCX 4016 (60 mg/kg) i.p. 30 min before the stimulus, reduced significantly the drop of circulating platelets induced by collagen plus epinephrine which attained the value of 310,000 ± 13,000/μl ($n = 10$,

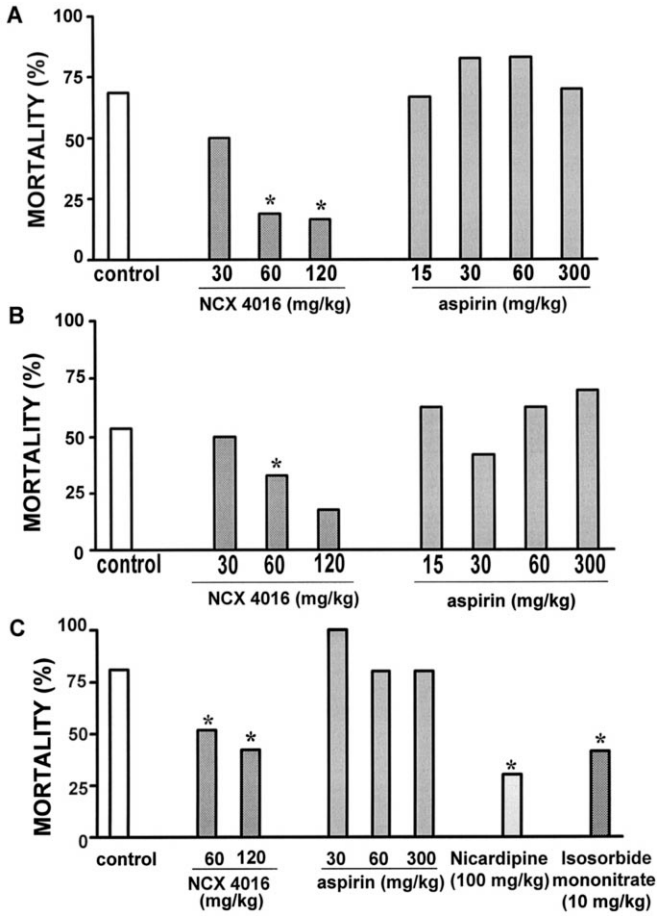

Fig. 2. Effects of NCX 4016 or aspirin on the mortality induced by the i.v. injection of: (A) U46619; (B) thrombin; (C) hardened rat red blood cells. In the latter experimental setting, nicardipine and isosorbide mononitrate were also tested. All drugs were given by i.p. route 30 min before i.v. challenge with the agonists, except for isosorbide mononitrate that was given 10 min before challenge. Asterisks indicate a statistically significant reduction of mortality as compared with control ($P < 0.05$, at least).

$P < 0.001$), while aspirin, at an equivalent dose (30 mg/kg), was not effective in counteracting the drop in platelet count (platelets fell to 190,800 ± 22,000/μl, $n = 6$, P = NS vs.control) and significantly less active than NCX 4016 60 mg/kg ($P < 0.03$)(Fig. 3). Aspirin at 300 mg/kg, an effective antithrombotic dose, limited the drop

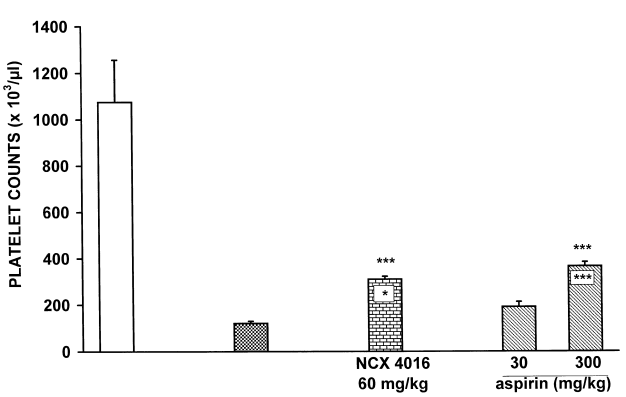

Fig. 3. Number of circulating platelets before (basal) and 2 min after the i.v. injection of collagen plus epinephrine (control). Drugs were administered, at the indicate dosages, by i.p. route 30 min before i.v. challenge. Asterisks outside the columns indicate a significant difference as compared with controls; asterisks inside the columns indicate a significant difference as compared with aspirin 30 mg/kg. * $P < 0.03$; * * $P < 0.01$; * * * $P < 0.001$.

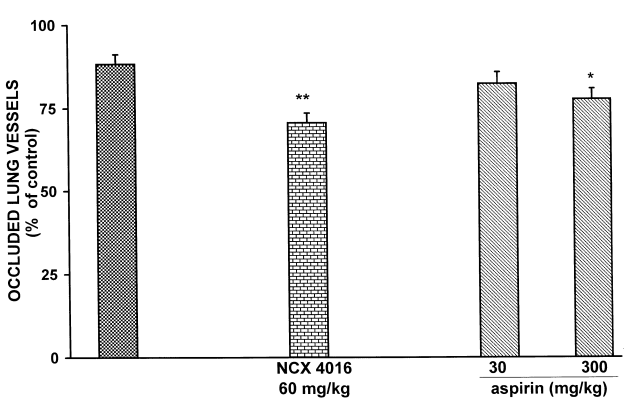

Fig. 4. Effects of NCX 4016 or aspirin on the percentage of lung vessels occluded by platelet emboli. Number of vessels counted in each group: control = 194; NCX 4016 = 240; aspirin 30 mg/kg = 152; aspirin 300 mg/kg = 99. Asterisks indicate a significant difference as compared with controls. * $P < 0.04$; * * $P < 0.001$.

Table 1
Effect of NCX 4016 (60 mg/kg i.p.) compared to an equimolar dose of aspirin (33 mg/kg i.p.) on ADP-induced platelet accumulation in the pulmonary vasculature of the rabbit
Results are expressed as maximum percentage change in counts above stable baseline values following ADP (20 μg/kg, i.v.). Neither drug-treated group was significantly different from saline treated animals: one-way ANOVA.

Treatment	Time after drug admistration (30 min)
	Max% increase counts
Vehicle ($n = 5$)	33.9 ± 5.2
Aspirin ($n = 5$)	38.5 ± 1.7
NCX 4016 ($n = 5$)	32.2 ± 1.7

of the platelet count provoked by collagen plus epinephrine to $365{,}000 \pm 18{,}000/\mu l$ ($n = 22$, $P < 0.01$).

3.1.3. Lung histology

The microscopic observation of lung slices prepared from control animals injected 2 min before sacrifice with collagen plus epinephrine (100 μl) showed that $88.3 \pm 2.9\%$ (171/194) of lung vessels were totally or partially occluded by platelet thrombi. In NCX 4016-pretreated (60 mg/kg, i.p.) animals, the number of lung vessels occluded by emboli was slightly, but significantly, reduced ($70.5 \pm 2.5\%$, $P < 0.001$, 169/240) while aspirin at an equivalent dose (30 mg/kg) was not effective in reducing the number of pulmonary thromboemboli ($82.3 \pm 3.5\%$, $P =$ NS vs.control, 125/152) (Fig. 4). Aspirin at 300 mg/kg reduced platelet lung microemboli to $77.7 \pm 3.2\%$ ($P = 0.038$ vs. control, 77/99).

In a previous study, we showed that collagen plus epinephrine injection did not induce formation of microthrombi in brain, spleen, liver or kidney (Emerson et al., 1999).

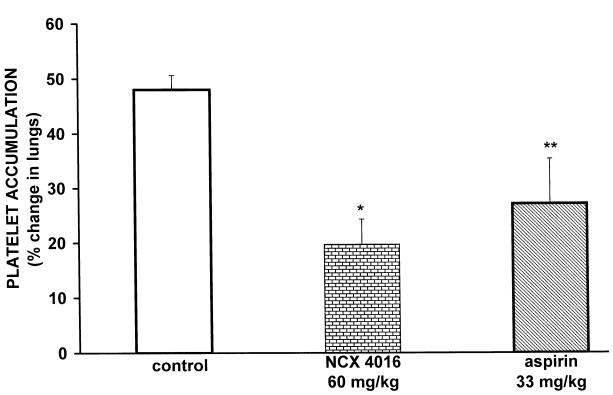

Fig. 5. Effects of equimolar doses of NCX 4016 and aspirin on collagen-induced [^{111}In]oxine-labeled platelet accumulation in the pulmonary vasculature of rabbits. Results are expressed as maximum percentage increase in counts from stable baseline values. Drugs were administered i.p. 30 min before collagen-challenge. Asterisks indicate a significant difference as compared with control. $^{*}P < 0.05$; $^{**}P < 0.01$.

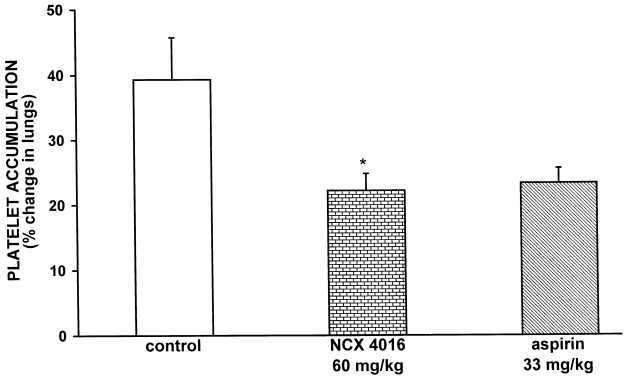

Fig. 6. Effects of equimolar doses of NCX 4016 or aspirin on thrombin-induced platelet accumulation in the pulmonary vasculature of rabbits. Results are expressed as in Fig. 5. Drugs were administered i.p. 30 min before thrombin-challenge. Asterisks indicate a significant difference as compared with control. $^{*}P < 0.05$.

3.2. In vivo thrombosis model in the rabbit

3.2.1. Effects in rabbit pulmonary vasculature after i.p. administration

Neither aspirin (33 mg/kg) nor NCX 4016 (60 mg/kg), administered i.p. 30 min before the stimulus, had any significant effect ($n = 5$) on responses to ADP (20 μg/kg) (Table 1).

Responses to collagen (50 μg/kg) were significantly reduced ($n = 4$–5) by both aspirin (33 mg/kg i.p.; $P < 0.05$) and NCX 4016 (60 mg/kg i.p.; $P < 0.01$) (Fig. 5).

Responses to thrombin (18 U/kg) were inhibited ($n =$ 5–6) by aspirin (33 mg/kg i.p.) and NCX 4016 (60 mg/kg i.p.), although only NCX 4016 achieved a statistically significant ($P < 0.05$) reduction of platelet accumulation in lungs (Fig. 6).

3.2.2. Effects in rabbit pulmonary vasculature after i.v. infusion

Pretreatment of animals with N^{G}-nitro-L-arginine methyl ester (10 mg/kg) 5 min before the stimulus strongly potentiated peak platelet accumulation in lungs induced by thrombin (5 U/kg), as previously reported (Paul et al.,

Table 2
Effect of NCX 4016 and aspirin after N^{G}-nitro-L-arginine methyl ester (10 mg/kg i.v.) and thrombin (5 U/kg i.v.) injection

Treatment	Max% increase counts	AUC[a]
Methanol[b]	11.4 ± 1.7	46.9 ± 10.5
ASPIRIN (0.25 mg/kg min^{-1})	8.4 ± 1.4	18.3 ± 5.7
	$P =$ ns[c]	$P =$ ns
NCX 4016 (0.5 mg/kg min^{-1})	7.9 ± 1.1	9.7 ± 2.3
	$P =$ ns	$P < 0.05$

[a]AUC = area under the curve.
[b]Treatments were administered by continuous i.v. infusion over 25 min, commencing 20 min before thrombin.
[c]Significances refer to the comparison with control platelet accumulation (methanol-pretreated animals).

1993). Responses to thrombin (5 U/kg) in N^G-nitro-L-arginine methyl ester (10 mg/kg)-pretreated animals, were reduced by 25 min infusion (commencing 20 min before thrombin) of either aspirin (0.25 mg/kg/min) or NCX 4016 (0.5 mg/kg/min). Neither drug had a significant effect when responses were expressed as Max. Percentage increase in lung counts but NCX 4016 had a significant effect ($P < 0.05$) when responses were expressed as AUC (Table 2).

4. Discussion

Our data show that NCX 4016, a nitroderivative of aspirin, is effective in preventing platelet pulmonary embolism in vivo in two different animal species, after single-dose administration. When comparing the effect of NCX 4016 with those of the parent compound aspirin, it was clear that NCX 4016 is superior to the latter in terms of effective dose and of range of models in which the drug was found to be active.

In mice, when the thrombogenic stimulus used was collagen plus adrenaline, NCX 4016 was significantly more active, on a molar basis, than aspirin which is efficacious, in this model, only at high doses (≥ 300 mg/kg) (Gresele et al., 1990). Similarly, in the rabbit, [^{111}In]oxine-labeled platelet accumulation in lungs induced by i.v. collagen was significantly reduced by aspirin and NCX 4016 at equimolar doses, although somewhat more effectively by the latter drug.

More interestingly, NCX 4016 protected mice from mortality induced by the i.v. injection of a stable thromboxane A_2 analogue, U46619, a model not influenced by aspirin (Gresele et al., 1990). Moreover, although no protective effects whatsoever were seen with either NCX 4016 or aspirin when the thrombogenic stimulus used was high-dose thrombin, a condition in which thromboembolism is completely mediated by fibrin formation and which is not influenced by antiplatelet agents (Momi et al., 1992; Paul et al., 1993; Gresele et al., 1998), when the dose of thrombin was reduced NCX 4016 turned out to be significantly more effective than equimolar doses of aspirin. The latter observation was confirmed in the rabbit in which the pulmonary accumulation of [^{111}In]oxine-labeled platelets induced by thrombin was inhibited significantly only by NCX 4016.

Moreover, when the rabbits were pretreated with N^G-nitro-L-arginine methyl ester, an inhibitor of the endogenous production on NO (May et al., 1991), platelet accumulation in lungs induced by i.v. thrombin was significantly reduced only by NCX 4016 and not by aspirin.

The increased antithrombotic activity of NCX 4016 over aspirin was exerted through a more pronounced inhibitory effect on platelets, as suggested by the studies with [^{111}In]oxine-labeled platelets in rabbits and by the reduction of the drop in the number of circulating platelets and the lower number of platelet pulmonary emboli, as detected by lung histology, in mice. These data are in agreement with previous observations demonstrating the important role of NO as an inhibitor of platelet aggregation in the pulmonary vasculature in vivo (Emerson et al., 1999).

On the other hand, it appears that NCX 4016 may exert its protective activity against pulmonary embolism also through a vasodilatory activity on lung blood vessels. This is suggested by the experiments in which pulmonary embolism was mechanical (injection of hardened rat red blood cells) and not thrombotic (Clement et al., 1983; Guarneri et al., 1988; Gresele et al., 1990). In this model, NCX 4016 was active, as was the calcium channel antagonist nicardipine, while aspirin, similarly to other antiplatelet agents (Molinari et al., 1987; Guarneri et al., 1988; Gresele et al., 1990), was totally ineffective. It is interesting to observe that another NO-donor, isosorbide mononitrate (Berenger et al., 1987; Plotkine et al., 1991), was also effective in this model. This observation suggests that NCX 4016 exerts a vasodilatory action on lung vessels; indeed, vasospasm appears to contribute to the noxious consequences of the i.v. injection of platelet aggregating stimuli (Di Minno and Silver, 1983; De Clerck et al., 1985; Molinari et al., 1987; Gresele et al., 1990; Emerson et al., 1999). On the other hand, the vasodilatory activity of NCX 4016 does not appear to be a generalized phenomenon, such as to induce hypotension, as shown by the lack of any significant blood redistribution in the pulmonary circulation of rabbits injected with [^{111}In]oxine-labeled platelets (data not shown). Indeed, an increase of radioactivity over lungs is typically detectable when a measurable hypotensive effect follows the administration of a drug (May et al., 1990, 1991; Emerson et al., 1999).

This implies that NCX 4016 exerts its activity mainly on platelets and/or on lung vessels without provoking systemic vasodilation, in agreement with previous studies. Indeed, the administration of NO-aspirin or other NO-nonsteroidal antinflammatory agents, even by i.v. route, fails to cause any significant hypotensive effect (Wallace et al, 1994, 1995; Del Soldato et al., 1999).

If the in vivo antithrombotic effects of NCX 4016 are due to NO-release, it is still conceivable that this activity is displayed without a significant hypotensive reaction because it seems possible to achieve selective antiplatelet effects with NO donors (de Belder et al., 1994). In vivo hydrolysis of nitro aspirin is not a fast phenomenon, it requires metabolism, probably at a cellular level, and produces discrete but relatively long lasting, plasma levels of NO (Del Soldato et al., 1999).

The potency of NCX 4016 in releasing NO as compared to classic NO-donors has not been quantified, but it is known that NCX 4016 in vitro increases intraplatelet cyclic GMP at higher concentrations that those of sodium nitroprusside and that its activity as a cyclooxygenase type-1 inhibitor is exerted at lower concentrations than

those required for cyclic GMP stimulation (Lechi et al., 1996; Gresele et al., unpublished).

NCX 4016 has been shown in vitro to exert broader antiplatelet effects than aspirin (Lechi et al., 1996; Mezzasoma et al., 1999) but only one study until now has assessed its potential antithrombotic activity in vivo (Wallace et al., 1999). The study of Wallace et al. has shown that NCX 4016 inhibits thrombus formation in an extracorporeal circuit in rats after prolonged administration, but not after one single dose, and to an extent which was not different from that obtained with an equimolar dose of aspirin (Wallace et al., 1999). It is conceivable that the model used, in particular the lack of endothelium at the level of the thrombogenic stimulus, may have prevented the detection of the full antithrombotic potential of NCX 4016 (Wallace et al., 1999).

The aim of the present study was to compare the antithrombotic activity of NCX 4016 with that of aspirin after acute administration in different animal models of platelet-dependent or -independent thromboembolism. Further studies aimed at analyzing mechanistically the reason for the broader antithrombotic action of NCX 4016 over aspirin are ongoing.

NCX 4016 has been reported to produce minimal gastrointestinal lesions (Takeuchi et al., 1998) and it is obvious that the availability of an aspirin derivative able to exert an effective antithrombotic activity with reduced gastrointestinal side effects may allow safe treatment of a considerably larger group of patients with ischemic cardiovascular disorders than those currently treated with aspirin (Wolfe et al., 1999). Moreover, if the better antithrombotic profile of NCX4016 over aspirin is indeed due to in vivo NO-release, this may represent an additional theoretical advantage for use in patients with coronary syndromes in view of the positive effects of NO in the coronary circulation (Noll and Lusher, 1998; Bing et al., 1999).

Further studies aimed at fully characterizing the mechanism of the antithrombotic activity of NCX 4016, as well as the demostration of its antiplatelet effects upon administration to humans, are warranted.

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