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NCX4016 (NO-Aspirin) has multiple inhibitory effects in LPS-stimulated human monocytes

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1 NCX4016 (2 acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl ester, NicOx S.A., France) is an anti-thrombotic agent, chemically related to acetylsalicylic acid (ASA) and able to release NO.

2 We tested the effects of NCX4016 and ASA on the release of the thromboxane (TX) A_2 metabolite TXB_2, tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), expression and activity of tissue factor (TF) in stimulated, adherent human monocytes.

3 Both ASA and NCX4016 1–1000 μmol l^{-1} dose-dependently reduced TXB_2 concentration, measured by RIA in the supernatant of 10 μg ml^{-1} LPS-stimulated cells. NCX4016 activity was comparable to that of equimolar ASA when incubation lasted 6 h (NCX4016 30 μmol l^{-1}: $-86.0\pm10.1\%$, NCX4016 300 μmol l^{-1}: $-92.2\pm9.0\%$, ASA 30 μmol l^{-1}: $-92.3\pm7.5\%$, ASA 300 μmol l^{-1}: $-97.3\pm1.0\%$, $n=6$, M±s.d.). Most of the activity of NCX4016 up to 100 μmol l^{-1} was prevented by 10 μmol l^{-1} ODQ, inhibitor of cyclic GMP.

4 NCX4016 100–300 μmol l^{-1} reduced TNF-α (NCX4016 300 μmol l^{-1} = $-77.2\pm19.9\%$, $n=6$) and IL-6 (NCX4016 300 μmol l^{-1}: $-61.9\pm15.2\%$, $n=6$) in LPS stimulated monocytes while ASA had no significant effects.

5 TF activity (NCX4016 300 μmol l^{-1}: $53.7\pm39.9\%$, $n=4$) and immunoreactive TF (NCX4016 300 μmol l^{-1}: $-93.9\pm7.9\%$, $n=7$), measured in the supernatant of stimulated cells, were also dose-dependently inhibited by NCX4016 but not by ASA.

6 The present results indicate that NCX4016 inhibits TXA_2 generation as well as cytokine release and TF in human monocytes partly *via* NO-dependent mechanisms. NCX4016 may have a favourable profile of activities in the clinical setting of athero-thrombosis.
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Keywords: NCX4016; acetylsalicylic acid; thromboxane; nitric oxide; cyclo-oxygenase; interleukin-6; tumour necrosis factor-α; tissue factor; human monocytes; LPS

Abbreviations: ASA, acetylsalicylic acid; COX, cyclo-oxygenase; DMSO, dimethylsulphoxide; DPBS, Dulbecco's phosphate buffered saline; FCS, foetal calf serum; IL-6, interleukin-6; NO, nitric oxide; NSAIDs, non-steroidal anti-inflammatory agents; TF, tissue factor; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2

Introduction

NCX4016 is 2 acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl ester (NicOx S.A., France), chemically related to acetylsalicylic acid (ASA). This compound belongs to a new class of non-steroidal anti-inflammatory agents (NSAIDs) able to release nitric oxide (NO) and to inhibit cyclo-oxygenase (COX) (del Soldato *et al*., 1999; Lechi *et al*., 1996). Due to chemical similarity, NCX4016 and ASA may share anti-thrombotic properties. However, due to the presence of a NO-containing moiety in NCX 4016, these two drugs may have different pharmacological profiles.

Similarly to ASA, NCX4016 has been shown *in vitro* to prevent the release of thromboxane (TX) B_2 from activated human platelets through the irreversible inhibition of platelet COX (Lechi *et al*., 1996). When repeatedly administered in rat, NCX4016 reduced *ex vivo* platelet release of TXB_2 to an extent similar to that observed with ASA (Cuzzolin *et al*., 1996). However, the anti-platelet activity of NCX4016 is somehow broader and partly different from that of ASA. In fact, NCX4016, unlike ASA, has been shown to inhibit platelet adhesion as well as platelet aggregation induced by thrombin. These effects were entirely dependent on the release of NO, since not being achievable through the inhibition of platelet COX (Lechi *et al*., 1996). When tested *in vivo* NCX4016 proved to have anti-thrombotic activity even better than ASA, as observed in experimental animals (Momi *et al*., 2000; Wallace *et al*., 1999). In addition to these anti-platelet activities, NCX4016 has been recently shown to have cardioprotective effects and to reduce infarct size in experimental miocardial ischaemia and reperfusion. This activity was almost entirely dependent on NO, since ASA was much less active (Rossoni *et al*., 2001). NCX4016 also reduced restenosis after ballon angioplasty in hypercholesterolemic mice and this was associated with reduced vascular

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smooth muscle cell proliferation and macrophage deposition at the site of injury (Napoli *et al*., 2001). It is therefore plausible to speculate that, due to the release of NO, not only platelet but also leukocyte function may be the target of NCX4016 activity.

The present study was aimed to investigate the effects of NCX4016 on activated monocytes. The rationale was that monocytic cells are involved in the initiation of thrombosis. In fact, stimulated monocytes and macrophages express tissue factor and release thromboxane (Hempel *et al*., 1994; Moreno *et al*., 1996; Toschi *et al*., 1997). Monocytes also contribute to the initiation and progression of atherosclerotic lesions by releasing cytokines, chemokines and by oxidizing low density lipoprotein (Ross, 1999; Tintut *et al*., 2000). Moreover, pro-inflammatory cytokines may be involved in the clinical manifestations of atherosclerosis (Ridker *et al*., 2000a, b). We hypothesized that NCX4016 might affect a broad spectrum of biological activities in activated monocytes both by releasing NO and by inhibiting COX. We tested this hypothesis by comparing in isolated human monocytes the effects of NCX4016 with those of ASA.

Methods

Preparation of isolated monocytes

Mononuclear cells were isolated from fresh human blood (drawn by venous puncture from healthy volunteers) by low speed centrifugation ($400 \times g$) using Histopaque 1077 (Sigma-Aldrich, Milan, Italy) to obtain a gradient separation. Cells were then washed twice with Hank's balanced salt solution. Suspended cells were further centrifuged after incubation with 2 mmol l^{-1} EDTA for 15 min at 37°C in RPMI HYBRI-MAX culture medium (Sigma-Aldrich) supplemented with 10% inactivated foetal calf serum (FCS) to remove contaminating platelets. Finally, mononuclear cells were suspended in culture medium without FCS to achieve a final concentration of 1×10^6 ml^{-1} and plated (1 ml) in multi-well plates. Mononuclear cells were allowed to adhere for 2 h in culture medium under controlled conditions (37°C, atmosphere: 95% O_2, 5% CO_2) to obtain adherent monocytes. Non-adherent cells were removed by washing the plates twice with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich).

When indicated, adherent monocytes were stimulated with 10 μg ml^{-1} *Escherichia coli* lipopolysaccharide (LPS, Serotype 026:B6, Sigma-Aldrich) added to culture medium supplemented with 5% FCS. The tested drugs were added before stimulation with LPS.

NCX4016 (NicOx), 2-(acetyloxy)benzoic acid 3-(idroxymethyl)phenil ester (NCX4017, NicOx), ASA (Sigma-Aldrich) and the guanylyl cyclase inhibitor H-(1,2,4) oxadiazolo(4,3-a) quinoxallin-1-one (ODQ, Tocris, Harlow, U.K.) were dissolved in dimethylsulphoxide (DMSO, Sigma-Aldrich) and diluted in DPBS. Equal amounts of DMSO were tested in the control samples. The final concentration of DMSO was lower than 3 μl ml^{-1} in the medium of cell cultures. The NO-donor 2,2′-hydroxynitrosohydrazino)*bis*-ethanamine (NOC-18 Calbiochem, Milan, Italy) was dissolved in water. LPS (Sigma-Aldrich) was dissolved in DPBS.

Measurement of TXB_2, tumour necrosis factor-α and interleukin-6

TXB_2, stable non-enzymatic metabolite of TXA_2, was assayed by radioimmunoassay using a commercial antiserum (Biomol Research Laboratories, Plymouth, PA, U.S.A.). Authentic TXB_2 (Cayman Chem. Co.) and $^3HTXB_2$ (Amersham, U.K.) were used in the assays. TXB_2 concentration was assayed in the centrifuged supernatant of monocyte cultures after dilution.

Both Tumor Necrosis Factor-α (TNF-α) and interleukin-6 (IL-6) were measured in centrifuged and diluted supernatants of cultured monocytes by enzyme-linked immunoadsorbent assay (Bender Medsystem Diagnostics, Vienna, Austria).

Measurement of intracellular cyclic GMP

Intracellular cyclic GMP was measured in cell extracts (5×10^6 cells ml^{-1}) obtained from monocytes stimulated with LPS and incubated for 6 and 16 h with NCX4016 300 μmol l^{-1} or ASA 300 μmol l^{-1}. At the end of the incubation period ice-cold ethanol (to give 65% ethanol) was added twice to suspended cells. Samples were centrifugated at $2000 \times g$ for 15 min at 4°C and the supernatant was dried under nitrogen. The dried extracts were dissolved in the assay buffer and measured by radioimmunoassay (I^{125} cyclic GMP, Amersham, U.K.).

Measurement of tissue factor antigen and activity

Tissue Factor (TF) activity was assayed by measuring the peptidyl activity of the TF/factor VII complex using a specific chromogenic substrate (American Diagnostica, Greenwich, CT, U.S.A.). TF/factor VII complex was obtained by incubating the centrifuged supernatant of cell cultures with human factor VII (American Diagnostica). Immunoreactive TF was measured in the supernatant of monocyte cultures by enzyme-linked immunoassay (American Diagnostica).

Study protocols

Experiments were performed to investigate the effects of NCX4016, on prostanoid biosynthesis. In all these experiments NCX4016 was compared with equimolar amounts of ASA. TXB_2 concentration was measured in the supernatant of cell cultures as an index of TXA_2 biosynthesis and COX activity.

In a first set of experiments adherent monocytes were incubated with NCX4016 or ASA (1 – 1000 μmol l^{-1}), added to cell cultures immediately before stimulation with LPS. The incubation period was prolonged for 6 or 16 h. Preliminary experiments were performed with 1, 2 and 24-h incubation periods. The effects of NCX4017 10 – 300 μmol l^{-1} and NOC-18 10 – 100 μmol l^{-1} on TXB_2 release were also tested. NCX4017 has the same chemical structure of NCX4016 but does not contain the NO-releasing moiety. NOC-18 is a NO-donor able to gradually release NO.

To test the hypothesis that NCX4016 might affect thromboxane biosynthesis *via* a NO-dependent mechanism, intracellular cyclic GMP was measured after 6 and 16 h of incubation. In addition, experiments were performed using adherent monocytes treated with ODQ, inhibitor of cyclic

GMP generation or the NO scavenger and guanylyl cyclase inhibitor methylene blue (Ignarro, 1989; Schrammel *et al*., 1996). NCX4016 or ASA (10–300 μmol l^{-1}) were added to cell cultures in the presence of 10 μmol l^{-1} ODQ or 10 μmol l^{-1} methylene blue before stimulation with LPS and the incubation period lasted 6 h.

The effects of NCX4016 and ASA on cytokine release were also investigated. In these experiments the release of TNF-α and IL-6 was assayed in the supernatant of cell cultures. NCX4016 10–300 μmol l^{-1} or ASA 10–300 μmol l^{-1} were added before stimulation and incubation lasted 6 h. These experiments were performed also in the presence of 10 μmol l^{-1} ODQ.

The effects of NCX4016 10–300 μmol l^{-1} and ASA 10–300 μmol l^{-1} on TF concentration and TF activity were also investigated. In these experiments adherent monocytes were incubated for 6 h with the tested drugs, added before stimulation with LPS.

Statistical analysis

The effects of different concentrations of the studied drugs were analysed using Kruskal-Wallis test. The Mann–Whitney test was used to compare two variables (computer program SPSS9, Bologna, Italy). $P<0.05$ was assumed as statistically significant. Data are presented as mean and standard error of the mean.

Results

Effects of NCX4016 and ASA on TXB_2, release in stimulated monocytes

The amount of TXB_2 released in the supernatant of non-stimulated monocytes was lower than 0.1 ng ml^{-1} h^{-1}. When monocytes were stimulated with 10 μg ml^{-1} LPS the amount of TXB_2 released in the supernatant increased up to 1.6 ± 0.2 ng ml^{-1} h^{-1} during the first 16 h (Figure 1A) and then declined to 0.8 ± 0.1 ng ml^{-1} h^{-1} during the following 6 h.

NCX4016 and ASA dose-dependently inhibited TXB_2 release when added to cell cultures before stimulation with LPS and incubation lasted 6 h. (Figure 1A). The effects of 1–300 μmol l^{-1} NCX4016 were similar to that of ASA (no differences between NCX4016 and ASA were observed at any of the tested concentration except for concentration 100 μmol l^{-1}, Figure 1A) and similar were the maximum inhibitory effects (1 mmol l^{-1} ASA: $97.6\%\pm0.9$, 1 mmol l^{-1} NCX4016: $95.9\%\pm0.9$, $n=6$).

No differences between NCX4016 and ASA were observed in the maximum inhibitory activity also when incubation lasted 16 h (1 mmol l^{-1} ASA: $99.0\%\pm0.3$, 1 mmol l^{-1} NCX4016: $98.0\%\pm0.6$, $n=7$). However, in these experimental conditions the effects of NCX4016 10–300 μmol l^{-1} proved lower than that of ASA (Figure 1B).

Finally, we observed that also NCX4017, which is similar to NCX4016 but does not release NO, dose-dependently reduced the concentration of TXB_2 in the supernatant of monocyte cultures when added to cell cultures before stimulation with LPS and incubation lasted 6 h (maximum inhibition: $-78.1\%\pm7.4$, $n=4$, Figure 1C).

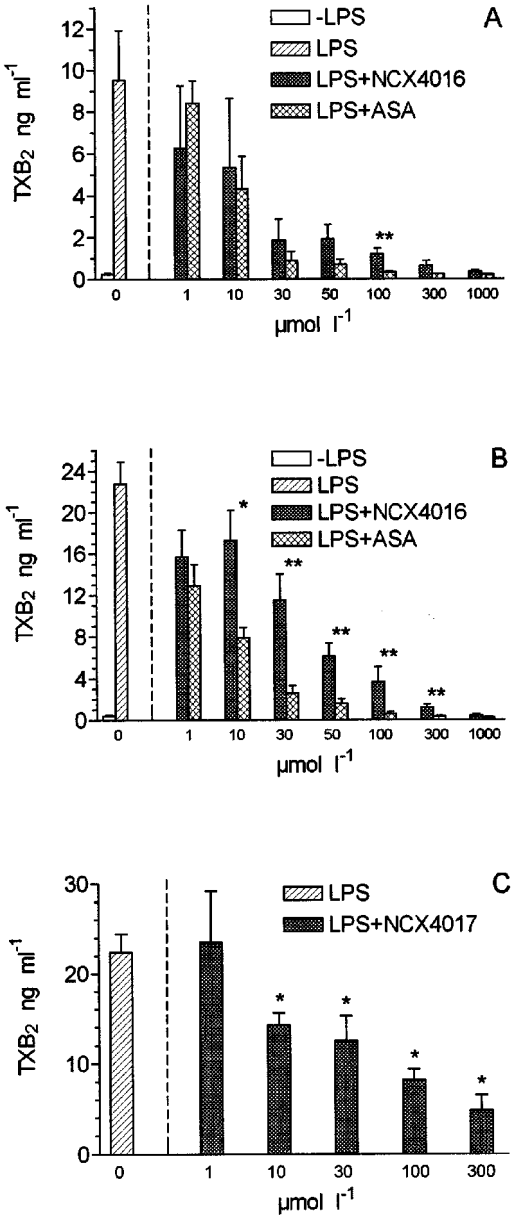

Figure 1 (A) Effects of NCX4016 and ASA 1–1000 μmol l^{-1} on TXB_2 release. Adherent monocytes were incubated with the tested drugs and LPS for 6 h ($n=6$). No differences are observed between NCX4016 and ASA except for concentration 100 μmol l^{-1}. (B) Effects of NCX4016 and ASA 1–1000 μmol l^{-1} on TXB_2 release from monocytes incubated with drugs and LPS for 16 h ($n=7$). Significant differences are observed between NCX4016 and ASA 10–300 μmol l^{-1}; $^*P<0.05$, $^{**}P<0.001$. LPS- indicates non-stimulated cells. The inhibition in TXB_2 release is statistically significant ($P<0.01$) when 30–1000 μmol l^{-1} of tested drugs are compared with the 0 concentration (LPS). (C) Effects of NCX4017 1–300 μmol l^{-1} on TXB_2 release ($n=4$). NCX4017 is chemically related to NCX4016 but does not release NO. $^*P<0.05$ when 10–300 μmol l^{-1} NCX4017 are compared with the 0 concentration (LPS).

NO-dependent and NO-independent mechanisms in the inhibitory effects of NCX4016 on TXB2 release

We performed some experiments to investigate the role of NO in the inhibitory activity of NCX4016. We observed that,

in the presence of ODQ, the release of TXB_2 was reduced by NCX4016 only at concentrations exceeding 30 μmol l^{-1}. In these conditions NCX 4016 10–100 μmol l^{-1} was significantly less effective than ASA (Figure 2). The inhibition in TXB_2 release observed with 300 μmol l^{-1} NCX4016 or equimolar ASA exceeded 90% also in the presence of ODQ and no differences between these two drugs were observed (Figure 2).

Intracellular cyclic GMP measured after 6-h incubation was higher with 300 μmol l^{-1} NCX4016 (46.4 $\pm$ 2.57 pmol l^{-1}, $n=4$) than with 300 μmol l^{-1} ASA (37.8 $\pm$ 0.06 pmol l^{-1}, $n=4$, $P<0.02$) and LPS-alone (38.1 $\pm$ 0.27 pmol l^{-1}, $n=4$, $P<0.02$). This increase with NCX4016 was not observed after 16 h of incubation (NCX4016: 25.6 $\pm$ 0.55 pmol l^{-1}; ASA: 28.2 $\pm$ 3.22 pmol l^{-1}; LPS: 29.6 $\pm$ 1.66 pmol l^{-1}, $n=4$).

The NO-donor NOC-18 10–100 μmol l^{-1}, when added to cell cultures before LPS, reduced TXB_2 release with 6-h incubation periods (LPS: 28.8 $\pm$ 1.4 ng ml^{-1}; NOC-18: 10 μmol l^{-1} 19.3 $\pm$ 0.8* ng ml^{-1}, NOC-18: 100 μmol l^{-1}: 18.8 $\pm$ 1.7* ng ml^{-1}; $n=4$; *$P<0.05$ versus LPS).

To investigate the effects of NCX4016 independent of NO release, NCX4016 1–300 μmol l^{-1} was tested in the presence of methylene blue, used to prevent the intracellular effects of NO. In these experimental conditions NCX4016 reduced the amount of TXB_2 released from LPS-stimulated monocytes (Figure 3). Interestingly, in the presence of methylene blue the amount of TXB_2 released from LPS-stimulated monocytes was lower than that observed in the previous sets of experiments.

Effects of NCX4016 on cytokines and TF

NCX4016 at concentrations exceeding 30 μmol l^{-1} significantly decreased the release of TNF-α (Figure 6A) and IL-6 (Figure 4B), while ASA up to 300 μmol l^{-1} had no significant effects. When the effects of the two tested drugs on IL-6 release were compared, NCX4016 100 μmol l^{-1} (inhibition: 37.8% $\pm$ 7.8, $n=6$) and 300 μmol l^{-1} (inhibition: 61.9% $\pm$ 6.2, $n=6$) proved more effective than equimolar ASA. No differences were observed in the effects of 300 μmol l^{-1} ASA (inhibition: 43.6% $\pm$ 16.9) and 300 μmol l^{-1} NCX4016 (inhibition: 77.2% $\pm$ 8.2, $n=6$) on TNF-α release. The effects of NCX4016 300 μmol l^{-1} on IL-6 and TNF-α were not altered by ODQ (Table 1).

NCX4016 100 μmol l^{-1} (−65.7% $\pm$ 15.9, $n=4$) and 300 μmol l^{-1} (−93.9% $\pm$ 3.9, $n=4$) reduced in a dose-dependent manner the concentration of immunoreactive TF in the supernatant of LPS-stimulated monocytes (Figure 5A). Similarly, TF activity was reduced in the presence of 300 μmol l^{-1} NCX4016 (−53.7% $\pm$ 15.1, $n=7$, Figure 5B). Significant differences were observed in TF activity and TF concentration between 300 μmol l^{-1} NCX4016 and 300 μmol l^{-1} ASA (Figure 5A,B).

No differences in cell viability were observed at any dose of the tested drugs (NCX4016, NCX4017 and ASA) compared to LPS as assessed by the Trypan blue exclusion test (viability exceeding 90%) and the measurement of lactate dehydrogenase in the supernatant of cultured cells.

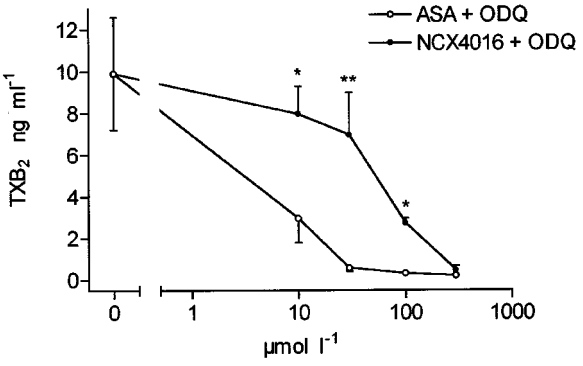

Figure 2 Effects of NCX4016 or ASA on TXB_2 release. Adherent monocytes are incubated with and LPS up to 6 h in the presence of 10 μmol l^{-1} ODQ plus NCX4016 or ASA ($n=6$). NCX4016 100 μmol l^{-1} ($P<0.05$) and 300 μmol l^{-1} ($P<0.005$) inhibits TXB_2 release. A significant inhibition is observed with ASA 10 μmol/L ($P<0.05$) and ASA 30–300 μmol l^{-1} ($P<0.005$). *$P<0.05$, **$P<0.005$ when equal concentrations of NCX4016 and ASA are compared.

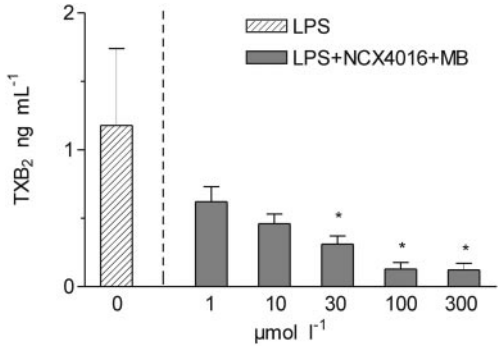

Figure 3 Effects of NCX4016 1–300 μmol l^{-1} on TXB2 release in the presence of methylene blue, added to prevent the intracellular effects of NO ($n=4$). *$P<0.05$ when 30–300 μmol l^{-1} NCX4016 are compared with the 0 concentration (LPS).

Discussion

The major finding from the present study is that NCX4016 dose-dependently inhibits the release of prostanoid, namely TXB_2, as well as TF, TNF-α and IL-6 from stimulated human monocytes. Part of this inhibitory activity is NO-dependent.

Adherent human monocytes when stimulated with LPS start releasing prostanoids. This is dependent on cell activation, since not observed in non-stimulated cells, and is accompanied by increased expression of COX-2 (Hempel *et al*., 1994). The inhibitory activity of NCX4016 on platelet COX has been shown to be irreversible since not reduced when the drug is washed away from the cell (Lechi *et al*., 1996). In the present study, while the effects of NCX4016 and ASA are similar when these drugs are added to adherent monocytes before stimulation and incubation with LPS lasts 6 h, the effects of 10–100 μmol l^{-1} NCX4016 appear weaker than ASA after a more prolonged period of incubation (16 h), thus suggesting that the anti-COX activity of NCX4016 might be reversible. We hypothesized that part of the observed inhibitory effects could be NO-dependent.

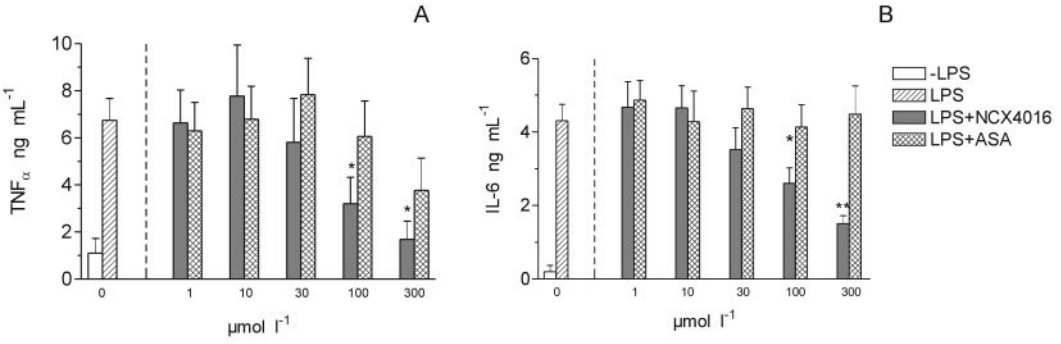

Figure 4 (A) Effects of NCX4016 and ASA on TNF-α release from LPS-stimulated monocytes ($n=6$). (B) Effects of NCX4016 and ASA on IL-6 release from LPS-stimulated monocytes ($n=6$). -LPS indicates non-stimulated cells. The effects of 100 and 300 μmol l^{-1} NCX4016 on TNF-α are statistically significant (*$P<0.05$). The effects of NCX4016 100 μmol l^{-1} (*$P<0.05$) and 300 μmol l^{-1} (**$P<0.005$) on IL-6 are significant and different from that of ASA ($P<0.005$).

Table 1 IL-6 and TNF-α release in the presence of ODQ

		IL-6 (ng ml^{-1}) n = 7	*TFN-α* (ng ml^{-1}) n = 4
−LPS		0.20 ± 0.31	1.10 ± 1.09
+LPS		10.55 ± 3.19	10.17 ± 4.68
LPS + ODQ		10.74 ± 4.43	9.30 ± 4.01
NCX4016 + ODQ	10 μmol l^{-1}	12.51 ± 5.92	10.09 ± 4.90
NCX4016 + ODQ	30 μmol l^{-1}	11.55 ± 4.55	9.04 ± 6.48
NCX4016 + ODQ	300 μmol l^{-1}	4.19 ± 2.04**	2.01 ± 1.21*
ASA + ODQ	10 μmol l^{-1}	12.33 ± 5.85	10.20 ± 4.91
ASA + ODQ	30 μmol l^{-1}	11.67 ± 6.35	9.88 ± 6.14
ASA + ODQ	300 μmol l^{-1}	12.35 ± 6.18	5.97 ± 3.63

Effects of NCX4016 and ASA 10 − 300 μmol l^{-1} on TNF-α and IL-6 in the presence of the guanylyl cyclase inhibitor ODQ 10 μmol l^{-1}. The reduction in the release of IL-6 (**$P<0.01$) and TNF-α (*$P<0.05$) with 300 μmol l^{-1} NCX4016 is statistically significant when compared with LPS. The differences in IL-6 concentration observed between NCX4016 300 μmol l^{-1} and ASA 300 μmol l^{-1} are also statistically significant ($P<0.05$).

NCX4016 has been shown able to release NO. In fact, in isolated platelets or human plasma increased nitrite and nitrate concentration was observed after a 20 min incubation with NCX4016 (Lechi *et al*., 1996). Increase in plasma nitrate concentration was also found up to 6 h after the oral administration of NCX4016 in rats (Wallace *et al*., 1999). Additional evidence is given by the reduction in the antiaggregatory activity of NCX4106 in the presence of oxyhaemoglobin, a NO scavenger, or methylene blue (Lechi *et al*., 1996). Confirming previous studies, we found that intracellular cyclic GMP is increased in adherent human monocytes up to 6 h after incubation with NCX4016 (Fiorucci *et al*., 2000b). The absence of any increase in this NO-induced intracellular signal may explain the reduction in NCX4016 activity after more prolonged incubation. This evidence prompted us to investigate the effects of ODQ under the same experimental conditions in which ASA and NCX4016 induced similar inhibition in TXB_2 release (6-h incubation periods). The reduction in NCX4016 activity observed in the presence of ODQ strongly suggest that NO accounts for part of the inhibition in COX activity signalling through cyclic GMP-dependent kinases. A NO-donor able to gradually release NO, NOC-18, also reduced TXB_2 generation, further confirming that exogenous NO modulates COX activity. Although we did not investigate the mechanism of COX inhibition, this finding is in agreement with the observation that large amounts of NO inhibit the degree of COX expression and activity in LPS-stimulated cultured macrophages (Swierkosz *et al*., 1995).

Although NO plays a role in the reduction of TXB_2 release, direct COX inhibition is involved in NCX4016 activity. In fact, NCX4016 dose-dependently reduces TXB_2 also in the presence of ODQ or the NO-scavenger and guanylyl cyclase inhibitor methylene blue. Moreover, NCX4017 which is similar to NCX4017 but is deprived of the NO-releasing moiety, dose-dependently decreases TXB_2 generation. It is worth noting that NCX4017 was shown to be cytotoxic in human monocytes after prolonged incubation, but this is not observed after a 6-h incubation period (Fiorucci *et al*., 2000b). In all the conditions where NO activity is prevented, NCX4016 proves less effective than ASA. Since there is no evidence of a different metabolism between ASA and NCX4016 (Tagliaro *et al*., 1997), this is consistent with the results of previous *in vitro* studies comparing the antiplatelet effects of NCX4016 and ASA and may be related to steric hindrance due to differences in the chemical structure of NCX1016 and ASA (Lechi *et al*., 1996).

The results from the present study indicate that apart from TXA_2 biosynthesis, NCX4016 affects a number of additional activation-dependent phenomena in cultured human monocytes. According to our observations NCX4016 reduces the release of cytokines from LPS-stimulated monocytes and this activity does not seem to be related to COX inhibition since ASA up to 300 μmol l^{-1} has no significant effects. This is in agreement with the observation that only 5 − 10 mmol l^{-1} ASA inhibits TNF-α release from LPS-stimulated monocytes (Osnes *et al*., 1996).

NCX4016 may therefore affect cytokine secretion through NO-dependent mechanisms. There is evidence showing that NO can inhibit IL-6 and IL-8 release from cultured endothelial cells (De Caterina *et al*., 1995).

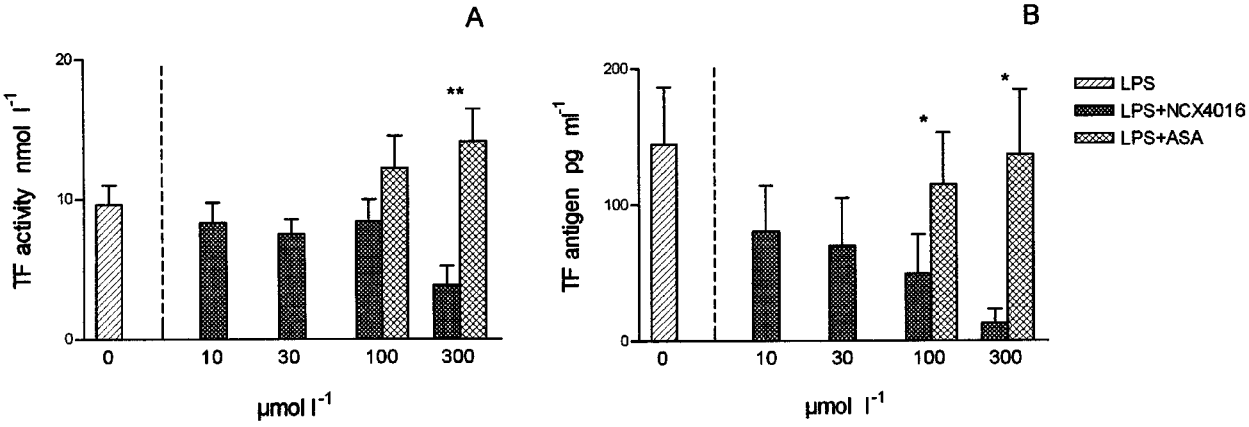

Figure 5 (A) Effects of NCX4016 and ASA 10–300 μmol l^{-1} on TF activity ($n=4$). A significant reduction is observed with 300 μmol l^{-1} NCX4016 when compared with both 0 concentration ($P<0.005$) and equimolar ASA ($P<0.05$). (B) NCX4016 300 μmol l^{-1} significantly reduces immunoreactive TF in the supernatant of LPS-stimulated monocytes ($P<0.05$ versus 0 concentration and 300 μmol l^{-1} ASA, $n=7$).

There is also evidence indicating that NCX4016 modulates cytokine release. The treatment with NCX4016 as well as with other NO-NSAIDs prevents the release of IL-1β from endotoxin-stimulated macrophages in rats (Fiorucci *et al*., 1999). Moreover, NCX 4016, but not ASA, at the oral dose of 100 mg kg^{-1} dose-dependently protects from concanavalin-A immuno-mediated liver damage and causes a 40–80% reduction of IL-1β, IL-12, IL-18, interferon-γ, and TNF-α production without affecting cytokine messenger RNA expression (Fiorucci *et al*., 2000a). Inhibition of caspase-1 activity and therefore suppression of IL-1β and IL-18 processing has been observed in human monocytes treated with NCX4016 (Fiorucci *et al*., 2000b).

We also observed that NCX1016 inhibits TF expression and activity. These data give information on the possible mechanisms responsible for the anti-thrombotic activity of NCX4106. TF expression occurs on the surface of monocytes stimulated with LPS or cytokines and requires the activation of transcription factors (Mackman, 1997). TF activity depends on the binding of the extracellular domain of TF with factor VII leading to the formation of the active complex able to catalyse the generation of factor Xa. This key event in thrombogenesis is tightly regulated (Mann *et al*., 1998).

NO has been shown to reduce TF expression and activity in human microvascular endothelial cells. In fact L-arginine, the substrate for NO synthases, reduces TF catalytic activity and this is accompanied by reduction in TF mRNA and protein expression (Yang & Loscalzo, 2000). This indicates that the inhibitory effect of NO occurs at transcriptional level.

Our data showing that cytokines and TF are reduced by NCX4016 are in agreement with previous observations indicating that exogenous NO, by acting at transcription level, modulates LPS and cytokine-induced cell activation, although there is some evidence of an activatory role of exogenous NO (Lander *et al*., 1993). NO-donors, and authentic NO, have been shown to inhibit LPS and IL-1β-induced NF-κB activation and its nuclear translocation, primarily acting on IκBα phosphorylation. The inhibition of transcription factors is accompanied by the inhibition of iNOS mRNA and protein expression in human microglia and in rat smooth muscle cells (Colasanti *et al*., 1995; Katsuyama *et al*., 1998). Interestingly, ODQ does not prevent these effects of NO, indicating that cyclic GMP may not be always involved as intracellular signal (Katsuyama *et al*., 1998). Although inhibition of cell activation may explain the observed differences between NCX4016 and ASA, there are additional explanations. Protein nitrosylation may be involved in NCX4016 activity. As recently observed, NCX4016 inhibits IL-1b and IL-8 release and this effect appears mediated by the S-nitrosylation of cysteine proteases required for cellular processing of proinflammatory cytokines (Fiorucci *et al*., 2000b). Moreover, it has been shown that TF procoagulant activity is modulated by peroxynitrite, possibly *via* protein nitrosylation (Adam *et al*., 1998). However, in our experimental conditions the parallel reduction in immunoreactive TF and TF activity suggests that protein expression may be primarily affected by NCX4016. Finally, a different pattern of eicosanoid may be generated in the monocytes in the presence of ASA or NCX4016. In fact, in a different experimental model a shift in arachidonic acid metabolism towards the synthesis of leukotrienes, thus increasing TF activity in LPS-stimulated mononuclear cells, was observed with ASA and this may not occur with NCX4016 (Lorenzet *et al*., 1986).

In conclusion, the present results confirm that NCX4016 has a different pharmacological profile from ASA. In our experimental conditions the effects of NCX4016 are dose-dependent and occur at concentrations comparable to those measured in plasma after the oral administration of NCX4016 in experimental animals (Momi *et al*., 2000; Tagliaro *et al*., 1997; Wallace *et al*., 1999). Therefore, the observed inhibition in thromboxane, tissue factor and cytokines in human monocytes support the hypothesis that NCX4016 may have *in vivo* anti-thrombotic and, possibly, anti-atherosclerotic properties by inhibiting COX activity and releasing NO.

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