NCX-4016 (NO-Aspirin) Inhibits Lipopolysaccharide-Induced Tissue Factor Expression In Vivo
Role of Nitric Oxide

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Background—NCX-4016 is an acetylsalicylic acid (ASA) derivative containing a nitric oxide–releasing moiety. Compared with ASA, NCX-4016 has a broader spectrum of antithrombotic and antiinflammatory activities. We hypothesized that NCX-4016 might inhibit in vivo lipopolysaccharide (LPS)-induced expression of tissue factor (TF).

Methods and Results—Rats were administered 90 mg/kg NCX-4016 orally for 5 days. Placebo, 50 mg/kg ASA, and 80 mg/kg isosorbide-5-mononitrate (ISMN) were used in control groups. On day 5, rats were injected intraperitoneally with 100 μg/kg LPS and killed 6 hours later. The expression of TF in monocytes was measured by flow cytometry and Western blot analysis. Reverse transcriptase–polymerase chain reaction was performed to assess expression of TF and cyclooxygenase-2 (COX-2) genes. Plasma concentrations of interleukin-1β and tumor necrosis factor-α were measured. Urine samples were collected to evaluate the excretion of the thromboxane metabolite 11-dehydro-thromboxane (TX)B2. Gastric mucosa was inspected. LPS injection was followed by synthesis TF and COX-2 mRNAs in circulating monocytes, which were blunted by NCX-4016 but not by ASA or ISMN. Both NCX-4016 and ISMN reduced TF expression on surface of circulating monocyte. LPS increased the excretion 11-dehydro-TXB2, and this was prevented by NCX-4016 and ASA. Unlike ASA, NCX-4016 reduced plasma interleukin-1β and tumor necrosis factor-α. In addition, NCX-4016 almost completely prevented mucosal damage, whereas ASA increased the extension of gastric lesions in LPS-injected rats.

Conclusions—NCX-4016 prevents monocyte TF expression; this is accompanied by inhibition of TX and cytokine biosynthesis. These additive effects of nitric oxide release and COX inhibition may help explain efficacy and tolerability of NCX-4016. (Circulation. 2002;106:3120-3125.)

Key Words: thromboxane ▪ aspirin ▪ platelets ▪ nitric oxide

Mechanistic and pharmacological investigations have identified expression of tissue factor (TF) as a key link between inflammation and thrombogenesis.1,2 TF is a transmembrane protein structurally related to cytokine type-2 receptor.3 Binding of factor VII to the extracellular domain of TF catalyzes the generation of factor Xa and IXa, triggering thrombin generation. TF expression is induced in circulating monocytes by bacterial lipopolysaccharides (LPS) and proinflammatory cytokines.2 Stimulated monocytes also release prostanoids, such as thromboxane (TX)A2, which induce platelet aggregation and promote inflammation.4,5

Drugs able to prevent both TF expression and TXA2 generation therefore may be effective in the clinical setting of atherothrombosis, where platelet and monocyte activation is observed.5,6 Low-dose acetylsalicylic acid (ASA) has been shown to be effective in preventing thromboembolic events, as it selectively inhibits platelet cyclooxygenase type 1 (COX-1). Higher doses of aspirin also have antiinflammatory effects by inhibiting inducible cyclooxygenase type 2 (COX-2) activity.7,8 However, to date, inconsistent data have been obtained on the effects of any dose of ASA (or different COX inhibitors) on TF expression.9,10

NCX-4016 (2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)-phenyl ester) is an antithrombotic and antiinflammatory agent and consists of acetylsalicylic acid, to which a nitroxbybutyl moiety has been added through an ether linkage.11 Part of NCX-4016 bioactivity, at least in vitro, depends on its ability to release nitric oxide (NO) in the proximity of target cells.11,12 When tested in vivo, NCX-4016 was more effective than ASA in preventing experimental pulmonary thrombosis, myocardial infarction, and restenosis after coronary artery angioplasty.13-15 Data obtained from in vitro experiments
may help to explain these results. In fact, not only does NCX-4016 inhibit platelet aggregation, but it also prevents the expression of adhesion molecules.\textsuperscript{16-18} In addition, NCX-4016 has also been shown to have multiple inhibitory activities on LPS-stimulated monocytes, being able to reduce the release of prostanoids and cytokines as well as the expression and the activity of TF.\textsuperscript{19,20} This suggests that NCX-4016 might blunt in vivo monocyte activation preventing induction of TF expression. We tested this hypothesis by comparing the effects of NCX-4016 with equimolar ASA and isosorbide-5-mononitrate (ISMN) in a rodent model of acute inflammation.

Methods

Study Protocol
The study was approved by the Animal Study Committee of the University of Perugia. Male Wistar rats (weight, 200 to 250 g) were purchased from Charles River Italia (Milan, Italy) and housed in mesh cages maintained at 25°C and illuminated in 12:12-hour light-dark cycles. Rats (6 to 8 per group) were orally administered a single daily dose of placebo (500 µL PBS), 50 mg/kg ASA (molecular wt, 180.2), 90 mg/kg NCX-4016 (molecular wt, 335), or 80 mg/kg ISMN (molecular wt, 200,1) for 5 days. The dosages and timing were chosen to allow absorption of equimolar concentration of ASA and NO and on the basis of previous study demonstrating that at least 3 days were required by NCX-4016 to obtain full inhibitory activity on TXA\textsubscript{2} production.\textsuperscript{21,22} Drugs were dissolved in carboxy-methyl-cellulose and administered by gavage at a final volume of 500 µL. On day 5, rats were fasted overnight; 3 hours after oral administration of the last dose of the indicate drug, the rats were given an intraperitoneal injection of 100 µg/kg of bacterial endotoxin (LPS) (\textit{Escherichia coli} Serotype 055:B5 (Sigma)). Animals were killed 6 hours later, and blood samples were taken by cardiac puncture. Urine samples were collected 4 hours before and after LPS injection by using metabolic cages. To examine gastric mucosal injury, stomachs were rapidly removed, opened by an incision along the greater curvature, and pinned out on a wax platform. An investigator, unaware of the treatment the rats had received, scored the damage, measured the length of each erosive lesion with digital calipers, and summed the data for each stomach.

Blood Cells, Prostanoid, and Cytokine Analysis
Blood samples were collected with the use of Vacutainer tubes containing sodium heparin for hemochromatometry analysis (Cells counter FF3000 Dasit). To determine the urinary excretion of the TXA\textsubscript{2} metabolite 11-dehydro-TXB\textsubscript{2}, urine samples were subjected to purification procedures and 11-dehydro-TXB\textsubscript{2} was measured with commercial EIA kits (Cayman Chemicals). Plasma concentrations of interleukin (IL)-1β and tumor necrosis factor (TNF)-α were measured by commercial ELISA kits (Endogen).

Flow Cytometry Analysis
Blood samples were collected in tubes containing sodium citrate, blood samples were diluted in lysis buffer for red cells (155 mmol/L NH\textsubscript{4}Cl, 10 mmol/L KH\textsubscript{2}CO\textsubscript{3}, and 0.1 mmol/L EDTA). White blood cells were then incubated with anti-CD11b/c phycoerythrin (FITC)-conjugated monoclonal antibody for 30 minutes at 4°C (Cedarlane), washed, and stained with goat anti-human anti-tissue factor polyclonal antibody\textsuperscript{23} (American Diagnostica, catalog No. 4501) for 30 minutes at 4°C followed by addition of a secondary rabbit anti-goat, FITC-conjugated, antibody (PharMingen). The anti-TF antibody has previously been demonstrated to cross-react with rat TF.\textsuperscript{24} Freshly stained cells were analyzed by single-color or double-color fluorescence distribution on an EP IX XL cytofluorometer (Beckman Coulter) and the number of CD11 b/c-TF positive cells counted.

Reverse Transcriptase–Polymerase Chain Reaction
Total RNA was isolated from circulating lymphomonocytes by using the TRizol reagent, following the manufacturer’s instructions (Life Technologies, Inc). This yielded 10 to 12 µg of total RNA from 5x10\textsuperscript{6} cells. RNA samples were diluted to a final concentration of 1 µg/µL in RNase-free water and stored at ~80°C until use. Synthesis of cDNA was performed with 1 µg of total RNA. The 20-µL reverse transcription (RT) reaction consisted of 5× first-strand buffer, 0.5 mmol/L dNTP, 50 mmol/L random primers, and 20U Superscript reverse transcriptase (all reagents from Life Technologies, Inc). Primer and conditions of polymerase chain reaction (PCR) analysis are described elsewhere.\textsuperscript{12} For the quantitative SYBR Green real-time PCR, 250 ng of cDNA was used per reaction. Each 25-µL SYBR Green reaction consisted of 5 µL of cDNA (50 ng/µL), 12.5 µL of 2× Universal SYBR Green PCR Master Mix (PE Biosystems), and 4 µL of 50 mmol/L forward and reverse primers. Quantitative PCR was performed onBioRad I Cycler. Specificity of the produced amplification product was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that single DNA sequence was amplified during PCR. In addition, end-reaction products were visualized on ethidium bromide–stained 1.4% agarose gels. Appearance of a single band of the correct molecular size confirmed specificity of the PCR. Each sample was tested in triplicate with quantitative PCR, and samples obtained from at least 3 independent experiments were normalized against β-actin and control and used to calculate the means and standard errors.

Western Blot Detection of Tissue Factor
Cell lysis and Western blot analysis were carried out as described.\textsuperscript{23} Circulating lymphomonocytes (1x10\textsuperscript{6}) were lysed in 100 µL of lysis buffer (50 mmol/L Tris, 150 pmol/L NaCl, 10 mmol/L EGTA, 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/L sodium vanadate, 50 mmol/L NaF, 2 mmol/L EDTA (pH 8.0), 1 mmol/L phenylmethylsulfonyl fluoride, and 5 g/mL of leupeptin/pepsatin A/proteinin for 15 minutes at 4°C. Protein concentration in the supernatants was determined by the Bradford method (Bio-Rad). Equal amounts of proteins were denatured and separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybnd-C extra; Amersham). Membranes were blocked in 5% milk powder in PBS and probed with a monoclonal anti-TF antibody (American Diagnostica) at a 1:2500 dilution. A secondary antibody conjugated with horseradish peroxidase (Sigma Chemical Co) and enhanced chemiluminescent kit (Amersham Pharmacia Biotech) were used to visualize TF-immunoreactive bands. The protein bands were then scanned (Wathman, Biometa) and relative intensities quantified with the use of a specific software (Delta Sistemi). The relative expression of TF was normalized to controls on the same blot with the same time of exposure.

TF Activity Assay
Cell lysates or standard authentic TF were incubated at 37°C in a microtiter plate for 4 minutes in the presence of pooled normal human plasma. The chromogenic substrate of factor Xa (S-2765 Chromogenix) was added and the plate incubated at 37°C for 30 minutes. Changes in optical density at 405 nm were quantified. The amount of TF was calculated from a standard curve of authentic TF.

Statistical Analysis
Data are presented as mean±SEM. ANOVA and Student’s \emph{t} test were routinely used. Probability values <0.05 were considered significant.

Results
The administration of LPS was followed by a significant drop in the number of circulating platelets and leukocytes (Figure 1). While the prior administration for 5 days of NCX-4016, ASA, or ISMN partially prevented the effect of LPS on platelet concentration, the reduction in leukocyte count caused by LPS was not altered (Figure 1). Furthermore, no
Effect was documented in the counts of lymphocytes, granulocytes, and monocytes (data not shown). Inspection of the gastric mucosa 6 hours after LPS injection revealed a marked increase in the extension of lesions in rats treated with ASA compared with rats treated with vehicle alone, ISMN, or NCX-4016 (Figure 2).

NCX-4016 Modulates TF Expression and Activity
The percentage of CD11b/c-positive leukocytes (identified as monocytes) expressing TF was increased by LPS. Administration of NCX-4016 and ISMN but not ASA prevented this change (Figure 3). TF mRNA as assessed by real-time RT PCR was significantly enhanced by LPS (Figure 4, A and B). Pretreatment with NCX-4016 but not with ISMN and ASA reduced the expression of TF mRNA in LPS-treated rats (Figure 4, A and B). In addition, exposure to NCX-4016 but not to ISMN and ASA reduced total TF protein content in monocytes obtained from rats challenged with LPS (Figure 5). TF activity was 1.05 ± 0.28 nmol/100 mg protein in LPS-treated rats and dropped to 0.33 ± 0.05 nmol/100 mg protein in rats treated with LPS plus NCX-4016 (n=4; P<0.05). No change in TF activity was demonstrated in animals treated with LPS in combination with ASA (0.58 ± 0.19 nmol/100 mg protein) and ISMN (0.57 ± 0.17 nmol/100 mg protein).

NCX-4016 Modulates COX-1 and COX-2 Expression and Activity
Since we obtained evidence of inhibitory effects of NCX-4016 on TF expression that were not shared by ASA, we investigated the effects on COX activity and expression. The urinary excretion of 11-dehydro-TXB2, measured as an index of in vivo TXA2 biosynthesis, was increased by the administration of LPS (Figure 6). ASA and NCX-4016 showed similar inhibitory effects on TX-metabolite excretion, both before and after LPS (Figure 6). We also explored the effects of these drugs on the expression of COX-1 and COX-2. No changes were found on COX-1 expression. COX-2 mRNA was undetectable in untreated rats, but administration of LPS

Figure 1. A, Blood count of platelets dropped after LPS injection (*P<0.01 vs control, upper panel); this was partially prevented by the tested drugs (***P<0.01 vs LPS alone). B, Reduction in white cell count observed after LPS was not altered by treatments (*P<0.01 vs control, lower panel). Data are mean±SEM of 6 to 8 rats per group.

Figure 2. Administration of LPS in aspirin-treated rats is associated with excess damage of gastric mucosa (scored as indicated in Methods, *P<0.05) as compared with rats receiving NCX-4016 or ISMN before LPS. Data are mean±SEM of 6 to 8 rats per group.

Figure 3. Flow cytometry analysis showed that the number of CD11b/c-positive cells bearing TF epitopes increase after LPS plus placebo or LPS plus ASA (*P<0.05 vs control) and is downregulated by ISMN and NCX-4016 (***P<0.01 vs LPS alone). Data are mean±SEM of 6 to 8 rats per group.
induced the synthesis of COX-2 mRNA. This LPS-induced expression was prevented by NCX-4016, whereas ASA and ISMN had no effect (Figure 4, A and B).

**Effects on Cytokine Release**

To further investigate the spectrum of NCX-4016 activities, we measured the plasma concentration of IL-1β and TNF-α. As shown in Figure 7, NCX-4016 but not ASA prevented the LPS-induced increase in plasma levels of IL-1β and TNF-α.

**Discussion**

In the present study, we have demonstrated that NCX-4016 but not ASA prevents upregulation of TF expression in circulating monocytes challenged with LPS, establishing a new mechanism of action of NCX-4016. This effect is associated with an inhibitory activity of NCX-4016 on cytokine release and prostanoid generation. Consistent with the well-defined effects of LPS, we observed an activation of circulating monocytes in treated animals, which was identified by the release of prostanoids and proinflammatory cytokines as well as by the expression of TF. We characterized the expression of TF in circulating monocytes by evaluating levels of mRNA and protein expression in the whole cell and also by measuring the surface expression of TF at flow cytometry, demonstrating that both

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**Figure 4.** A, RT-PCR analysis of TF, COX-1 and COX-2, and β-actin mRNA revealed gene transcription after LPS. Treatment with NCX-4016 resulted in a reduction in COX-2 and TF transcripts. Lane 1, Control; lane 2, LPS+placebo; lane 3, LPS+ISMN; lane 4, LPS+ASA; and lane 5, LPS+NCX-4016. One representative of 4 experiments. B, Real-time PCR analysis of TF and COX-2 expression in monocytes obtained from LPS-treated rats ("P<0.05 vs lane 1; **P<0.01 vs lanes 2, 3, and 4). Lane 1, Control; lane 2, LPS+placebo; lane 3, LPS+ISMN; lane 4, LPS+ASA; and lane 5, LPS+NCX-4016.

**Figure 5.** TF expression is detectable by Western blot analysis in mononuclear cell lysates 6 hours after injection of LPS ("P<0.05 vs control). Treatment of rats with NCX-4016 but not ASA or ISMN reduced LPS-induced TF expression (""P<0.05). M indicates markers; lane 1, control; lane 2, LPS+placebo; lane 3, LPS+ISMN; lane 4, LPS+ASA; and lane 5, LPS+NCX-4016. One representative of 4 experiments. Molecular weight of TF is indicated.

**Figure 6.** Urinary excretion of 11-dehydro-TXB2 is reduced in rats after repeated administration of either aspirin or NCX-4016 ("P<0.05 vs day 5). The increase in 11-dehydro-TXB2 excretion observed after LPS is prevented by treatment with ASA or NCX-4016 (""P<0.01 vs control; ""P<0.05 vs LPS alone). Data are mean±SEM of 6 to 8 rats per group.
transcription and translation of the TF gene is induced by LPS. TF protein expression is increased in LPS-stimulated monocytes, mostly through de novo protein synthesis and the subsequent expression of the assembled protein on cell surface. All of the steps leading to the formation of a catalytically active TF factor–VIIa complex are inhibited by NCX-4016. Only surface expression of TF was reduced by ISMN, which did not significantly alter LPS-induced TF synthesis.

It has been shown that 100 to 300 μmol/L NCX-4016 is able to reduce in vitro both the expression of TF and its activity in human monocytes stimulated with LPS. Although ASA lacks in vivo activity, an inhibitory effect has been observed at millimolar concentrations in vitro. This is the first evidence that NO donors prevent TF expression in vivo. Previous studies indicated that the NO precursor L-arginine is able to reduce glomerular thrombosis in LPS-challenged rats, which is worsened by the administration of NO synthase inhibitors. L-Arginine was also shown reduce TF expression in monocytes collected from hypercholesterolemic rabbits in which arterial endothelium was removed. Recent in vitro data confirm that L-arginine reduces TF expression and activity by reducing mRNA synthesis and whole-cell protein expression in human microvascular endothelial cells.

TF expression is under the control of nuclear factor (NF)-κB, Sp-1, and AP-1 transcription factors in LPS-stimulated monocytes. Exogenous NO has been shown to inhibit COX-2, and there is also evidence of an inhibitory activity on NF-κB activation by NO donors. ASA itself has also been found to inhibit NF-κB, even though the concentrations required are in the millimolar range. Therefore, inhibition of transcription factors may explain the multiple inhibitory effects of NCX-4016.

NCX-4016 may also induce posttranslational downregulation of TF surface expression. This is suggested by the evidence that ISMN reduces immunodetected TF on circulating monocytes but does not significantly affect TF protein synthesis. This may indicate that signaling pathways implicated in TF surface expression are modulated by NO. The expression of tissue factor is greatly enhanced in vivo by the interplay of activated monocytes with platelets, mediated by adhesion molecules, mainly by the binding of P-selectin to its monocyte receptor. This interaction is sensitive to the inhibitory effects of NO. In fact, NO donors and NCX-4016 in particular have been shown to prevent the expression of P-selectin in stimulated platelets, whereas ASA is ineffective.

Inhibition of platelet-monocyte interaction may contribute to the effects of NCX-4016 and ISMN on TF expression. Although the primary aim of the present study was not the investigation of the effects of NCX-4016 on platelets, we nonetheless obtained evidence of antiplatelet activities of all the tested drugs. In fact, NCX-4016, ASA, and ISMN partially prevented the reduction in platelets, which is consistent with a reduction in platelet recruitment. We also measured the urinary excretion 11-dehydro TXB2 as an index of platelet TXA2 generation and in vivo platelet activation, although, after stimulation with LPS, monocyte and neutrophil contribute to TX production. In the present study, ASA and NCX-4016 were compared on equimolar basis, and similar effects on TX metabolite excretion were observed. This is consistent with previous evidence that ASA and NCX-4016 have similar inhibitory capacities on TX generation when tested in vitro in LPS-stimulated monocytes and ex vivo in thrombin-stimulated platelets. The observed reduction in TX metabolite excretion may imply that COX activity is the major target of NCX-4016. However, the reduction in urinary 11-dh-TxB2 may result also from inhibition of COX-2 expression.

The observed reduction in plasma IL-1β and TNF-α in rats treated with NCX-4016 indicates that this drug displays in vivo the same inhibitory effects on cytokine release observed in vitro, which are independent of transcriptional events. It has been shown that inhibition of cytokine release is associated with cysteine-nitration and inhibition of caspase-I. Therefore, suppression of IL-1β and IL-18 processing may explain the antiinflammatory effects of NCX-4016, and TF inhibition may contribute to NCX-4016 antiinflammatory activity. In fact, TF stimulates endothelial cells and leukocytes through proteolysis dependent (ie, through the stimulation of PAR receptors by factor Xa and thrombin) and proteolysis-independent mechanisms (signaling through the intracytoplasmic tail of tissue factor).

NCX-4016 appears to have a better gastric tolerability than ASA. In fact, NCX-4016 did not cause the gastric lesions that are observed when LPS and ASA are associated. This protective effect has been attributed to NO-mediated inhibition of leukocyte adhesion to microvascular endothelium and inhibition of caspase activity.

In conclusion, the present data may aid in the interpretation of the mechanisms of action of NCX-4016. Specifically, it would appear that monocyte function and platelet function are inhibited by this compound. The present results may have further implications for the treatment of clinical conditions wherein TF, thromboxane, and proinflammatory cytokines have a role in the progression of disease, such as the
development of atherosclerosis, acute coronary syndromes, and restenosis after balloon angioplasty. 6,11,35

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References
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