Nitric Oxide–Releasing Aspirin Derivative, NCX 4016, Promotes Reparative Angiogenesis and Prevents Apoptosis and Oxidative Stress in a Mouse Model of Peripheral Ischemia

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Background—Recently, nitric oxide (NO) donors have been developed that mimic the physiological intracellular release of NO. We evaluated whether one of these new compounds, consisting of aspirin coupled to an NO-releasing moiety (NCX 4016), would protect limbs from supervening arterial occlusion.

Methods and Results—Mice were assigned to receive regular chow or chow containing NCX 4016 or aspirin (both at 300 μmol/kg body weight, daily) throughout the 3-week experimental period. One week after randomization, they underwent surgical excision of the left femoral artery. Limb blood flow recovery (laser Doppler flowmetry) was accelerated by NCX 4016 as compared with aspirin or vehicle (P<0.05). In controls, histological analysis revealed a 35% increase in the capillary density of ischemic muscles compared with contralateral ones, indicative of spontaneous angiogenesis. Neovascularization was enhanced by NCX 4016 (91%; P<0.05 versus vehicle), but not by aspirin (51%; P=NS versus vehicle). Furthermore, NCX 4016 reduced endothelial cell (EC) apoptosis (4.3±1.0 versus 8.7±2.0 in aspirin and 12.6±3.3 ECs/1000 cap in vehicle; P<0.05 for either comparison) as well as caspase-3 mRNA levels in ischemic muscles ([caspase-3/GAPDH]*100 = 0.09±0.04 versus 2.30±0.44 in aspirin and 2.30±0.32 in vehicle; P<0.05 for either comparison). Nitrite levels and the ratio of reduced to oxidized glutathione were selectively increased in ischemic muscles by NCX 4016. Vascular endothelial growth factor-A expression was reduced by aspirin, with this effect being blunted by NCX 4016.

Conclusions—Pretreatment with the new oral NO-releasing aspirin derivative stimulates reparative angiogenesis and prevents apoptosis and oxidative stress, thereby alleviating the consequences of supervening arterial occlusion. (Arterioscler Thromb Vasc Biol. 2004;24:2082-2087.)

Key Words: angiogenesis □ ischemia □ peripheral vascular disease □ apoptosis □ endothelial cell
NO donors, NCX 4016 releases NO intracellularly at a rate similar to that observed with NO generation by endogenous endothelial NO synthase (eNOS). Noteworthy, NCX 4016 proved to be more effective as an antithrombotic agent than aspirin. The compound also exerts greater protective effects than aspirin in focal cerebral ischemia, myocardial infarction, arterial restenosis, and diabetes-induced endothelial dysfunction. The vasoprotective action of NCX 4016 extends beyond thrombosis to include local vasodilatation, reduction of oxidative stress, and inflammation (via inhibition of cytokine release and downregulation of iNOS and tissue factor expression). The compound also exerts greater protective effects than aspirin in focal cerebral ischemia, myocardial infarction, arterial restenosis, and diabetes-induced endothelial dysfunction. The vasoprotective action of NCX 4016 extends beyond thrombosis to include local vasodilatation, reduction of oxidative stress, and inflammation (via inhibition of cytokine release and downregulation of iNOS and tissue factor expression). The compound also exerts greater protective effects than aspirin in focal cerebral ischemia, myocardial infarction, arterial restenosis, and diabetes-induced endothelial dysfunction.

Methods

All procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, 1996).

Effects of NCX 4016 on Postischemic Hemodynamic Recovery

The experimental protocol was aimed at determining whether pre-treatment with NCX 4016 would improve the recovery from acute limb ischemia as compared with the parent compound aspirin.

Eight-week-old male CD1 mice (Charles River, Calco, Italy) were assigned randomly to regular chow (n=7) or the same chow containing NCX 4016 (n=8) or aspirin (n=7). Drugs were added to the food by the vendor (Mucedola) so that the animals would receive 300 µmol/kg body weight per day of NCX 4016 or aspirin. The chosen dosage of NCX 4016 produces significant increases in plasma nitrate levels without affecting systemic blood pressure of normotensive animals. One week after randomization, unilateral hind limb ischemia was induced by surgically excising the left femoral artery under 2,2,2-tribromoethanol anesthesia (880 mmol/kg body weight intraperitoneally). Animals were maintained on the assigned treatment over the 3-week experimental period and individual food consumption was calculated every day. Tail-cuff systolic blood pressure, heart rate, and body weight were measured in conscious mice under basal conditions and then at weekly intervals. Superficial hind limb blood flow was measured in anesthetized mice by laser Doppler flowmetry (Lisca Inc) immediately after surgery and weekly thereafter. The ischemic to nonischemic blood flow ratio was then calculated.

Histological Assessment of Angiogenesis and Apoptosis

Mice were euthanized at 21 days after ischemia induction. On the day of euthanasia, the limbs of anesthetized mice were perfusion-fixed and both adductors were harvested and processed for histology. Hemodynamic recovery was calculated every day.

Ischemia-induced apoptosis was assessed by calculating the number of TUNEL-positive endothelial cells (ECs) per 1000 capillaries and of TUNEL-positive myofibers per mm² of muscular section, respectively.

Quantification of VEGF-A, eNOS, and Caspase-3 mRNA Expression Levels in Skeletal Muscles

Quantitative real-time polymerase chain reaction (PCR) (ABI PRISM 7000 Sequence Detection System software version 1.0; Perkin Elmer) was used to determine VEGF-A and eNOS mRNA content in muscles (n=6 samples per group) harvested 5 days after induction of ischemia. Total RNA was isolated using TRIzol Reagent (Invitrogen), treated with DNase (Qiagen), and subsequently transcribed using M-MLV reverse-transcriptase (Invitrogen). VEGF-A, eNOS, and GAPDH primer sequences were described previously. Conventional PCR products of murine VEGF-A (111 bp), eNOS (105 bp), and GAPDH (156 bp) were obtained with the primers designed for the real-time PCR and were cloned into pGEM-T Easy vector (Promega) to be used as DNA standards.

Caspase-3 mRNA content was determined on the same samples used for the VEGF-A and eNOS experiment. Caspase-3 primers (designed on Genebank NM_009810) generate a 69-bp fragment and were the following: 5'-AGC TGT ACG CGC ACA AGC TA-3' (forward) and 5'-CCG TTG CCA CCT TCG TA-3' (reverse). Also in this case, the PCR product was cloned into a pGEM-T Easy vector to be used as DNA standard. VEGF-A, eNOS, and caspase-3 cDNA levels were normalized to GAPDH cDNA level.

Measurements of NO Degradation Products, Myeloperoxidase, and Indicators of Oxidative Stress

Separate experiments were performed to quantify the NO release in plasma and muscles of NCX 4016-treated or aspirin-treated mice (n=6 mice per group). In addition, we evaluated whether the same agents are able to affect systemic and local indicators of inflammation and oxidative stress.

NO metabolites were measured in plasma and muscle homogenates using a colorimetric nonenzymatic assay (Oxford Biomedical Research) after in vitro conversion of nitrates to nitrates. An aliquot of homogenate supernatant was used to determine tissue protein content by Lowry's method. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured in muscle homogenates by a colorimetric assay (Oxis Research). The GSH/GSSG ratio was then used to assess the effectiveness of treatment in reducing ischemia-induced oxidative stress.

Plasma myeloperoxidase, an inflammatory marker, was measured by enzyme-linked immunosorbent assay (EIA; Immunodiagnostik AG). On the same plasma samples, nitrotyrosine, an indicator of oxidative stress, was determined by EIA (Oxis Research).

Statistical Analysis

All results are expressed as mean±SEM. Multivariate repeated-measures ANOVA was performed to test for interaction between time and grouping factor. Differences within and between groups were determined using paired or unpaired Student t test, respectively. P<0.05 was interpreted to denote statistical significance.

Results

NCX 4016 Accelerates Postischemic Hemodynamic Recovery

No significant treatment-related effect was observed with regard to body weight, systolic blood pressure, and heart rate (data not shown).

Ischemic-to-contralateral limb blood flow ratio was reduced in all groups immediately after surgery (Figure 1A and 1B). During the next 2 weeks, the recovery rate was accelerated in mice given NCX 4016 as compared with aspirin or vehicle (P<0.05). Representative images of laser Doppler...
measurements document the improved limb blood flow of a NCX 4016-treated mouse compared with control at 2 weeks from surgery (Figure 2).

**NCX 4016 Stimulates Reparative Neovascularization and Inhibits Apoptosis**

In ischemic muscles of NCX 4016-treated mice, capillarization was significantly increased as compared with vehicle or aspirin ($P<0.01$ for either comparison; Figure 3A), whereas no treatment-related effect was observed with regard to the capillary density of contralateral muscles. NCX 4016-treated mice therefore showed an ischemic to contralateral capillary ratio ($1.92\pm0.19$) greater than that of vehicle-treated or aspirin-treated ones ($1.35\pm0.19$ and $1.51\pm0.11$, respectively; $P<0.05$ for either comparison). The difference was confirmed after normalization of capillary density by myofiber density (Figure 3B).

Immunohistochemical analysis was performed to evaluate the effects of treatment on ischemia-induced apoptosis. TUNEL-positive ECs averaged $12.6\pm3.3$ ECs/1000 cap in vehicle-treated mice. As shown in Figure 4A, the number of apoptotic ECs was significantly reduced by NCX 4016 ($4.3\pm1.0$ ECs/1000 cap; $P<0.05$ versus vehicle), whereas the effect of aspirin on apoptosis was negligible ($9.7\pm2.0$ ECs/1000 cap; $P=NS$ versus vehicle). Myofiber apoptosis was not influenced by treatments.

Quantitative real-time PCR analysis demonstrated that caspase-3 mRNA levels in ischemic muscles were 24-fold reduced by NCX 4016 ($P<0.01$ versus vehicle) but not altered after aspirin administration (see Figure 4B).

**NCX 4016 Increases Nitrite Levels in Plasma and Ischemic Muscles**

Consistent with previous reports, plasma concentration of NO metabolites was augmented in NCX 4016-treated mice ($239\pm43$ versus $120\pm14$ and $137\pm41$ μmol/L in vehicle and aspirin, respectively; $P<0.05$ for either comparisons). In addition, as shown in Figure 5, NCX 4016 treatment increased nitrite levels of ischemic muscles ($9.23\pm1.67$ versus $4.04\pm0.89$ μmol/mg protein in contralateral ones; $P<0.01$). In contrast, no significant change was observed between ischemic and contralateral limb muscles either in vehicle-treated ($5.68\pm0.98$ and $5.39\pm1.65$ μmol/mg protein, respectively; $P=NS$) or aspirin-treated animals ($4.05\pm1.07$ and $4.71\pm0.84$ μmol/mg protein, respectively; $P=NS$).

**NCX 4016 Reduces Oxidative Stress in Ischemic Muscles**

The impact of treatments on ischemia-induced oxidative stress was determined by measuring GSH-to-GSSG ratio in muscles harvested 5 days from induction of ischemia. We...
found that NCX 4016 increased the ratio by 3-fold ($91 \pm 12$) as compared with vehicle ($30 \pm 7$, $P<0.01$) or aspirin ($29 \pm 6$, $P<0.01$).

In contrast, circulating levels of nitrotyrosine (an indicator of systemic oxidative stress) or myeloperoxidase (a marker of inflammation) decreased below the detection limits of the assays, with no difference among groups ($P=N.S.$).

**NCX 4016 Blunts Aspirin-Induced Downregulation of VEGF-A Expression**

Finally, we evaluated the effect of treatments on the expression of angiogenic factors. In ischemic muscles of aspirin-treated mice, VEGF-A mRNA levels were 8.7-fold reduced as compared with contralateral adductor muscles, with this effect being significantly attenuated in NCX 4016-treated mice (4.9-fold reduction; $P<0.05$ versus aspirin).

eNOS expression was similarly increased (2.0-fold) in ischemic muscles of aspirin-treated or NCX 4016-treated mice ($P<0.05$ versus contralateral normoperfused muscles).

**Discussion**

Reduced NO bioavailability, as caused by various pathologic conditions and risk factors, not only contributes to the progression of atherosclerosis but also significantly dampens the angiogenic action of vascular GFs, thereby resulting in insufficient collateralization and delayed posts ischemic repair.$^{29,30}$ A possible remedy for addressing these liabilities consists in enhancing NO generation by supply side approaches. For instance, in preclinical models of limb ischemia, gene therapy with eNOS successfully promoted neovascularization and accelerated hemodynamic recovery, seemingly through local increase in VEGF expression.$^{31,32}$ However, concerns pertaining immunogenicity and safety of gene overexpression in combination with the technical difficulties of developing site-specific gene transfer presently limit the application of angiogenesis gene therapy for the treatment of chronic ischemic disease.$^{33}$ Moreover, eNOS gene therapy might fail under pathological conditions in which the availability of NOS cofactor tetrahydrobiopterin or substrate L-arginine is reduced. In fact, suboptimal concentrations of tetrahydrobiopterin favor eNOS uncoupling with consequent formation of superoxide anions instead of NO.

One alternative approach to correct the endogenous deficit in NO may consist in the use of oral formulations containing NO adducts coupled to pharmacophores. NCX 4016, an NO-releasing aspirin derivative initially designed to overcome the side effects of nonsteroidal anti-inflammatory drugs on the gastrointestinal mucosa, proved to exert greater cardiovascular protection than the parent compound, apparently caused by improved antithrombotic and anti-inflammatory activity.$^{11-18}$ We considered the possibility that NCX 4016, besides providing a better way to prevent thrombotic events, could also ameliorate the reparative angiogenesis response that naturally occurs after arterial occlusion. Here, we document for the first time that NCX 4016 accelerates the rate of hemodynamic recovery of ischemic limbs. In addition, NCX 4016 increased the number of capillaries and nitrite levels in ischemic muscles. Increased NO levels may cause local vasodilation thereby leading to adaptive angiogenic response. The reasons for the increased release of NO limited to ischemic muscles are presently unknown and further studies are needed to clarify whether the hypoxic–acidic environment, either directly or through enzymatic activation, might have favored the release of NO from the linker. We observed a mild increase in plasma nitrite concentration after chronic NCX 4016 administration, yet not sufficient to alter systemic blood pressure, a result consistent with previous reports.$^{25}$

Therefore, systemic hemodynamics unlikely contribute to the observed angiogenic effects.

Apart from hemodynamic influences, NO may directly promote the formation of new microvessels by stimulating
the proliferation and migration of ECs and by inhibiting apoptosis.\textsuperscript{34} Thus, we focused on the possibility that NCX 4016 can improve neangiogenesis by blunting ischemia-induced EC apoptotic death. Results of the present study clearly demonstrate that NCX 4016 inhibits apoptosis in vivo. The consensus on whether NO can be pro-apoptotic or anti-apoptotic is not universal, being the final effect dependent on the source and concentration of NO as well as on environmental factors.\textsuperscript{34–36} Exposure of cultured ECs to high concentrations of NO, as obtained with conventional NO donors,\textsuperscript{19} causes an almost complete inhibition of cell respiration, enhances peroxynitrite generation, and depletes intracellular GSH stores.\textsuperscript{37} This is at variance with NCX 4016, which slowly releases NO intracellularly, thereby gently regulating mitochondrial membrane potential and causing only a partial and reversible inhibition of O\textsubscript{2}\textsuperscript{-} consumption.\textsuperscript{19}

The dual effect of NO on apoptosis might also depend on the redox environment of target cells, with anti-apoptosis action becoming evident under conditions of increased oxidative stress. In line with the possibility that NCX 4016 may exert antioxidant protection, we found that prevention of ischemia-induced apoptosis is accompanied by improvement of the GSH-to-GSSG ratio in limb muscles. At variance, circulating indicators of systemic inflammation or oxidative stress fell below the detection limits of the assays. However, Napoli et al\textsuperscript{16} documented that NCX 4016 inhibits oxidative modification of circulating LDL and reduces plasma isoprostane levels in hypercholesterolemic mice. Further research in atherosclerosis models is needed to determine whether these systemic effects are accompanied by additive improvements of reparative angiogenesis.

Another mechanism by which NO prevents apoptosis is the inhibition of caspase activity via S-nitrolysis of cystein residues in caspase-3 and caspase-8 catalytic cores.\textsuperscript{38} Conversely, aspirin reportedly induces apoptosis through mitochondrial cytochrome c release and activation of the caspase pathway.\textsuperscript{39} Here, we report that NCX 4016 reduces ischemia-induced upregulation of caspase-3 mRNA expression, thus adding the novel information that augmented NO availability may inhibit pro-apoptotic effector enzymes at transcriptional level. Whether NCX 4016 also affects caspase-3 activity via a S-nitrosylation–mediated mechanism requires additional experiments.

Finally, we explored whether NO-releasing aspirin may influence the expression of the master angiogenic factor, VEGF-A. This is a relevant issue because aspirin causes downregulation of pro-angiogenic factors and blunts VEGF-A release during myocardial ischemia in men.\textsuperscript{40,41} Furthermore, aspirin and salicylate inhibit VEGF-A–induced endothelial tube formation.\textsuperscript{42} However, the effect of NO on VEGF-A is controversial, with reports indicating induction\textsuperscript{43} and others inhibition of VEGF-A expression.\textsuperscript{44} Our results demonstrate that VEGF-A mRNA levels are reduced in ischemic muscles of mice given aspirin. Downregulation of VEGF-A expression was significantly less in animals treated with NCX 4016, an effect that may account for the improved cardiovascular profile of NO-releasing aspirin over the parent compound.

Long-term aspirin treatment represents a milestone in the prophylaxis of ischemic events. However, once arterial occlusion occurs, inhibition of prostacyclin and negative modulation of VEGF might be detrimental to reparative angiogenesis. The present study demonstrates that pretreatment with NCX 4016 ameliorates postischemic recovery by stimulating neovascularization and by reducing oxidative stress and EC apoptosis. Whether postponing NCX 4016 treatment after arterial occlusion might have similar therapeutic effects merits further investigation. Thus, at the present time, the major indication of NCX 4016 remains the prevention of ischemic events, with the important addition that prophylactic treatment with the new NO-releasing drug can significantly improve reparative collateralization in case of supervening arterial occlusion.

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