NCX-4016, a nitric oxide-releasing aspirin, protects endothelial cells against apoptosis by modulating mitochondrial function

Stefano Fiorucci,* Andrea Mencarelli,* Roberta Mannucci,* Eleonora Distrutti,* Antonio Morelli,* Piero del Soldato,† and Salvador Moncada‡

*Dipartimento di Medicina Clinica e Sperimentale, Clinica di Gastroenterologia ed Epato logia, Università degli Studi di Perugia, 06122 Perugia, Italy; †NicOx S. A., 06906 Sophia Antipolis, France; ‡The Wolfson Institute for Biomedical Research, University College London, London WC1E 6BT, UK

Corresponding author: Stefano Fiorucci, Dipartimento di Medicina Clinica e Sperimentale, Clinica di Gastroenterologia ed Epato logia, Policlinico Montelu ce, Via E. dal Pozzo, 06122 Perugia, Italy. E-mail: fiorucci@unipg.it

ABSTRACT

We investigated the effect of a nitric oxide (NO)-releasing derivative of aspirin (NCX-4016) on a mitochondria-dependent model of apoptosis in human umbilical endothelial cells (HUVEC). Exposure of HUVEC to staurosporine caused a progressive fall in mitochondrial membrane potential (Δψₘ) and apoptosis. Exposure to an NO donor, (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO), caused an early (1-3h) hyperpolarization of Δψₘ and reduction of apoptosis that was followed (at 4-8 h) by an accelerated collapse of Δψₘ and cell death. In contrast, treatment with NCX-4016, but not with aspirin or a non-NO-releasing analogue of NCX-4016, protected HUVEC against the apoptotic actions of staurosporine for up to 8 h. Confocal microscopy demonstrated that although NCX-4016 released NO in subcellular compartments, DETA-NO caused a generalized increase in cytosolic fluorescence. Exposure to DETA-NO resulted in a rapid and profound inhibition of cell respiration (78.3 ± 6.4%), whereas NCX-4016 caused a less pronounced reduction in oxygen consumption (43.5 ± 5.3%). Staurosporine caused a time-dependent activation of proapoptotic caspases. NCX-4016 prevented this activation, whereas DETA-NO failed to inhibit caspase activity. In contrast to DETA-NO, NCX-4016 did not increase mitochondrial oxidative stress. These data demonstrated that NCX-4016 conveys NO directly inside endothelial cells and modulates mitochondrial function.

Key words: caspase-8 • caspase-9 • cytochrome c • cell respiration

The endothelium plays a pivotal role in regulating recruitment of blood-borne cells during inflammation, atherosclerosis, and immune surveillance (1). Alteration of endothelial function may thus contribute to inflammatory and degenerative disorders. Disruption of
the endothelium in response to vascular injury is induced in part through activation of proapoptotic regulatory pathways. Indeed, an increased rate of apoptosis of endothelial cells is common in several human diseases including stroke, diabetes, ischemia, and hypertension (1).

Nitric oxide (NO), a ubiquitous signaling molecule (2), is increasingly recognized as a key mediator in regulating the fate of cells (3). It is now clear that NO can induce necrosis or apoptosis and can even protect cells from death (4-10). Several studies have shown that NO donor agents can either rescue cells from apoptosis or cause endothelial cell death by activating intracellular mediators in the apoptotic cascade (4-6). One aspect that has attracted considerable attention is the effect that NO exerts on mitochondria (7-12). Recent data have revealed that NO, by regulating mitochondrial respiration (12) and cytochrome c release (13, 14), triggers a defensive mechanism against cell death induced by proapoptotic stimuli. Two major sites of interaction of NO with mitochondria have been identified at complexes I and IV (7-9, 11, 12).

During cell respiration, electrons flow through the respiratory chain and are used to reduce oxygen at the level of complex IV. This process is coupled to the extrusion of $H^+$ from the mitochondrial matrix to the intermembrane space so as to generate an electrochemical gradient that is expressed as the mitochondrial membrane potential ($\Delta \psi_m$). Studies with NO donors such as (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NO), which releases NO slowly and continuously for a long period, have demonstrated that exposure of endothelial cells to physiologically relevant concentrations of NO (about 1-1.5 $\mu$M) causes an almost complete (85%) inhibition of cell respiration and reduction of the intracellular concentration of reduced glutathione (GSH), thus leading to cell damage by oxidative stress (8). In contrast, in endothelial cells exposed to proapoptotic agents, inhibition of cell respiration seems to contribute to the antiapoptotic effects of NO—an as yet unexplained paradox (12). Interestingly, these contradictory results are not observed when endogenous sources of NO are considered. Indeed, transfection of endothelial NO synthase (eNOS) to eNOS-deficient endothelial cells did not increase spontaneous cell death and protected against apoptosis (10).

One aspect that is poorly understood is how intracellular localization of NO affects endothelial cell function (10, 15). Indeed, in contrast to NO generated through NO donors, eNOS-derived NO is released in subcellular compartments where the enzyme is recruited and phosphorylated (10). There is evidence that in endothelial cells, after agonist stimulation, only specific subsets of eNOS are phosphorylated, an event that is associated with release of NO in specific cellular compartments at the perinuclear and plasma membrane regions (10).

In the last few years, we and others have described the generation of a class of NO-releasing agents obtained by coupling an anti-inflammatory drug with an NO-releasing moiety (16-19). One of these compounds, NCX-4016, generated by adding an NO-releasing moiety to aspirin, inhibits proapoptotic caspases in animal models of inflammation (17-20). In contrast to NO from conventional NO donors, NO released from NCX-4016 requires the presence of esterases that cleave the ester bridge between aspirin and the NO-releasing moiety of the molecule (20). Because the effects of NCX-4016 on mitochondria are unknown, we designed this study to investigate whether NCX-4016 modulates cell respiration and mitochondrial function and protects cells against death caused by staurosporine, a protein kinase C inhibitor (12). Because caspase-8 activation in staurosporine-treated cells occurs after mitochondrial depolarization and caspase-8-dependent Bid cleavage is required to amplify the mitochondrial damage caused by
this compound (21-24), staurosporine represents a useful model for testing the effect of NO on both caspase and mitochondria.

MATERIALS AND METHODS

Primary cultures of human umbilical endothelial cells (HUVEC) were from Istituto Zooprofilattico di Brescia (Brescia, Italy). Culture media and FBS were from GIBCO (Milan, Italy). AFC (7-amino-4-trifluoromethylcoumarin), Ac-IEDT-AFC (Ac-Ile-Glu-Thr-Asp-AFC), Ac-LEHD-AFC (Ac-Leu-Glu-His-Asp-AFC), DETA-NO, DCF-DA (dichlorofluorescin diacetate), and L-NIL (L-\(N^6\)-(1-iminoethyl)lysine) were from Alexis (San Diego, CA). DAF-DA (4,5-diaminofluorescein diacetate) was from Calbiochem (CN Biosciences, Darmstadt, Germany). JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) was from Molecular Probes (Eugene, OR). NCX-4016 (2-(acetyloxy) benzoic acid 3-(nitrooxymethyl)phenyl ester) and NCX-4017 (2-(acetyloxy) benzoic acid 3-(hydroxymethyl)phenyl ester), which is a non-NO-releasing analogue of NCX-4016, were from Nicox SA (Sophia Antipolis, France). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Cell culture

HUVEC were grown in endothelial basal medium supplemented with bovine brain extract (12 \(\mu\)g/ml), human epithelial growth factor (10 ng/ml), hydrocortisone (1 \(\mu\)g/ml), penicillin (100 U/ml), streptomycin (100 \(\mu\)g/ml), and gentamicin (5 \(\mu\)g/ml) at 37°C in a humidified atmosphere containing 5% CO\(_2\) and 2% FBS. Cells were used between passages 2 and 5. Cells were treated with 1-500 \(\mu\)M DETA-NO, NCX-4016, NCX-4017, or aspirin in the absence or presence of 100 nM staurosporine. At various times after the initiation of treatment, cells were removed so that different parameters could be measured as indicated. Parallel samples were incubated with vehicle alone.

Detection of intracellular NO formation by confocal microscopy

To assess the kinetics of NO formation, adherent HUVEC were incubated with 1-500 \(\mu\)M NCX4016, aspirin, or DETA-NO, and intracellular NO formation was assessed, by using DAF-DA, as previously described (25), according to the method of Kojima et al. (26). Briefly, adherent cells (5 \(\times\) \(10^3\)/ml) were preincubated with 1 mM L-NIL for 30 min to suppress endogenous NO generation, after which they were loaded by incubating them in PBS in the presence of 10 \(\mu\)M DAF-DA at 37°C for 30 min. Cells were then washed three times with PBS and were placed on a confocal microscopic stage (Bio-Rad 1024, Bio-Rad Laboratories, Milan, Italy), and an image was taken every 5 s as previously described. Image analysis was carried out by using the LaserSharp software (Bio-Rad Laboratories).

NO generation and oxygen consumption

NO formation was measured by using a 2-mm NO-sensitive electrode connected to the ISO-NO Mark II meter (World Precision Instruments, Sarasota, FL). The NO electrode was calibrated by addition of known concentrations of NaNO\(_2\) under reducing conditions (KI/H\(_2\)SO\(_4\)). Oxygen
consumption was measured by using a Clark-type oxygen electrode in a magnetically stirred, thermostatically regulated chamber at 30°C. HUVEC were suspended in a total volume of 0.5 ml of air-saturated isotonic buffer composed of 220 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH 7.2), 5 mM KH₂PO₄, and 1 mM EGTA (9, 11).

**Flow cytometric measurement of $\Delta \psi_m$**

To measure $\Delta \psi_m$ after exposure of cells to different agents, HUVEC (5 x 10⁵/ml) were incubated with 5 µg/ml JC-1 (12). This cyanine dye accumulates in the mitochondrial matrix under the influence of the $\Delta \psi_m$ and forms J aggregates that have characteristic absorption and emission spectra. After incubation for 20 min at room temperature in the dark, cells were washed once with PBS; cell suspensions were prepared for flow cytometry, and the 488-nm line of an argon ion laser was used for excitation. Orange and green emitted fluorescence was collected through 585/42-nm (FL2) and 530/30-nm (FL1) bandpass filters. Flow cytometry was performed with an Epics XL instrument (Coulter-Beckman, Milan, Italy). After eliminating small (i.e., noncellular) debris, 50,000 events were collected for each analysis. Results are expressed either as the mean aggregate fluorescence alone (red) or as the ratio of aggregate/monomer (red/green).

**Detection of apoptosis**

Apoptotic cells were detected by flow cytometry after staining with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI) by using a commercially available kit (Annexin V-FITC Kit, Immunotech, Marseille, France) as described previously (12). Cells were considered apoptotic when they were annexin V-positive and PI-negative. Staining of cells by PI was an indicator of the loss of plasma membrane integrity.

**Western blot analyses**

These analyses were performed according to standard protocols. Briefly, whole cell lysates, mitochondrial and cytosolic fractions, from a fixed quantity of cells (2.5 x 10⁵) were boiled and reduced and underwent 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the blots were transferred onto nitrocellulose membranes and were blocked, they were probed for 1 h with rabbit anti-human cytochrome c (clone H104, Santa Cruz Biotechnology, Santa Cruz, CA), caspase-8, p20 subunit (clone H134, Santa Cruz), or Bid (PharMingen, BD Bioscience, Milan, Italy) polyclonal antibody. After samples were washed, specific binding was detected with an appropriate horseradish peroxidase-conjugated second layer. Control blots were probed with isotype-matched primary reagents followed by an appropriate horseradish peroxidase-conjugated second layer. The blots were developed by use of an enhanced chemiluminescent technique (ECL, Amersham Biosciences Europe, Milan, Italy) according to the manufacturer’s instructions.

**Cellular concentration of reduced thiols**

After the cells were washed, they were harvested in 200 µl of buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 4 mM EGTA. An aliquot was used to
quantify both protein and nonprotein thiols, using a thiol quantification kit (Molecular Probes) according to directions provided by the manufacturer.

Caspase activity

Activities of caspase-8 and caspase-9 were monitored by cleavage of fluorescent peptide substrates (Ac-IETD-AFC and Ac-LEHD-AFC, respectively), as previously described (17-19).

Statistics

Data were analyzed with a two-tailed Student’s t test using Prism 3 (GraphPad Software, San Diego, CA) (27). Values are given as means ± se. A P value < 0.05 was considered to be statistically significant.

RESULTS

Exposure to NCX-4016 resulted in NO generation and $\Delta \psi_m$ hyperpolarization

To document intracellular formation of NO, HUVEC loaded with DAF-DA were exposed to 1-500 µM DETA-NO or NCX-4016, and intracellular fluorescence was monitored. DAF-DA is a dye that upon binding an oxidized species of NO results in irreversible fluorescence, thus permitting identification of the source of local NO formation in living cells. As illustrated in Fig. 1, exposure to 500 µM DETA-NO resulted in a generalized, time-dependent increase in cytosolic fluorescence. In contrast, confocal microscopic images obtained from HUVEC exposed to 500 µM NCX-4016 demonstrated “hot spots” of activity in specific subcellular compartments localized near the plasma membrane. This different pattern of intracellular formation of NO was maintained when cells were exposed to lower concentrations (10-100 µM) of NCX-4016 and DETA-NO. No significant increase in DAF-DA-related fluorescence was seen in cells treated with aspirin or NCX-4017, a non-NO-releasing analogue of NO-4016 (not shown).

The kinetics of NO formation was further investigated by use of an NO-specific electrode. In these experiments, HUVEC monolayers were incubated with 1-500 µM NCX-5016 or DETA-NO, and NO formation was monitored continuously for 3 h. Exposure to NCX-4016 or DETA-NO resulted in a concentration- and time-dependent formation of NO (Fig. 2a). At steady state, a concentration of 500 µM DETA-NO released about 1.5 µM NO ($n = 11$). As illustrated in Fig. 2a, NCX-4016 released approximately three times less NO than did equimolar concentrations of DETA-NO. Exposure to 500 µM NCX-4016 released about 600 nM NO ($n = 11$, $P<0.05$ versus DETA-NO).

Flow cytometry of $\Delta \psi_m$ in the absence of apoptogenic stimuli revealed that NCX-4016 and DETA-NO could induce a concentration-dependent $\Delta \psi_m$ hyperpolarization. However, not only was the $\Delta \psi_m$ hyperpolarization induced by NCX-4016 (10-500 µM) more pronounced but the effect was maintained over time (Fig. 3a). In contrast, although both NCX-4016 and DETA-NO caused a concentration-dependent hyperpolarization of $\Delta \psi_m$ (Figs. 2b) the effect of DETA-NO was biphasic (Fig. 3a), with a transitory (1-3 h) $\Delta \psi_m$ hyperpolarization that was followed by significant depolarization, so that at 8 h $\Delta \psi_m$ was significantly lower than that of control cells ($n$
As illustrated in Fig. 2, exposure to aspirin and NCX-4017 did not result in detectable NO formation; however, both compounds caused a slight (about 20%) reduction of $\Delta \psi_m$ (Figs. 2b and 3a). This effect was concentration- and time-dependent ($n = 8, P<0.05$ versus control cells).

Because the biphasic effect of DETA-NO on $\Delta \psi_m$ has been linked to oxidative stress (8, 9), we next examined the effect of both DETA-NO and NCX-4016 on the total level of cellular reduced thiols and DCF-DA fluorescence, a marker of mitochondrial oxidative stress (Fig. 3b, c). Exposure to 500 µM DETA-NO for 3 h resulted in a time-dependent reduction of the level of total (protein and nonprotein) reduced thiols, which became significant at 3 h ($n = 8, P<0.01$ versus basal value). Furthermore, exposure to DETA-NO increased DCF-DA fluorescence ($n = 7, P<0.01$ versus basal value and NCX-4016). NCX-4016, NCX-4017, and aspirin had no effect on these parameters.

As shown in Fig. 4, exposure to 500 µM DETA-NO resulted in a rapid (5 min) and profound inhibition of cell respiration (78.3 ± 6.4% inhibition, $n = 6$). In contrast, 500 µM NCX-4016 caused a less pronounced reduction of oxygen consumption (43.5 ± 5.3% at 15 min, $n = 8, P<0.05$ versus DETA-NO). With both compounds, the degree of inhibition was constant for the duration of the experiment (data not shown). Inhibition of cell respiration caused by NCX-4016 was reversed by incubating the cells with 10 µM hemoglobin (Fig. 4, $n = 8, P<0.05$), whereas the NO scavenger only delayed the phenomenon in cells exposed to DETA-NO (Fig. 4, $n = 8$). Thus, NCX-4016 caused a reversible inhibition of cell respiration.

**NCX-4016 protected HUVEC from apoptosis: effect on $\Delta \psi_m$ and cytochrome c release**

Because the above-mentioned results indicated that DETA-NO and NCX-4016 modulate mitochondrial function, we then examined the effect of these agents on a mitochondria-dependent model of apoptosis. Exposure of HUVEC to staurosporine resulted in a concentration- and time-dependent reduction of cell viability (Fig. 5a, c, d). Exposure of staurosporine-treated cells to 500 µM DETA-NO (Fig. 5c, d) resulted in an early (1-3 h) reduction of apoptotic death rate ($n = 8, P<0.05$ versus staurosporine). Thereafter, at 3-8 h, DETA-NO was unable to counteract the effect of staurosporine. In contrast, protection exerted by 500 µM NCX-4016 was maintained over time ($n = 6-8, P>0.01$ versus staurosporine and DETA-NO). Analysis of cell death revealed that staurosporine-treated cells were dying from apoptosis at an early phase (1-3 h), whereas necrotic features were observed in the following period (3-8 h). Exposure of staurosporine-treated HUVEC to NCX-4016 significantly reduced the number of apoptotic cells (i.e., HUVEC that were annexin V+/PI-) and reduced the transition from apoptosis to necrosis (annexin V+/PI). In contrast, exposure to 100-500 µM DETA-NO resulted in a slight reduction of apoptotic cell death, in the early phase, that was followed by a rapid transition from apoptosis to necrosis. Incubation of HUVEC with aspirin and NCX-4017 exerted no effect on staurosporine-induced cell death (data not shown).

Staurosporine-induced cell death was associated with mitochondrial damage, as demonstrated by the early collapse of $\Delta \psi_m$ observed in cells incubated with 100 nM staurosporine (Fig. 5b). Addition of NCX-4016 protected against mitochondrial depolarization caused by staurosporine; indeed, exposure of staurosporine-treated cells to 500 µM NCX-4016 caused a significant
hyperpolarization of $\Delta \psi_m$ that remained higher than basal values for 8 h (Fig. 5b, $n = 8$, $P<0.001$ versus staurosporine alone). The effect exerted by DETA-NO on $\Delta \psi_m$ was biphasic: after an initial hyperpolarization, $\Delta \psi_m$ gradually declined to the value seen in cells treated with staurosporine alone ($n = 8$, NS versus staurosporine alone).

To investigate whether the effects exerted by NCX-4016 were dependent on the aspirin carrier, staurosporine-treated HUVEC were coincubated with 500 µM aspirin and NCX-4017. However, both compounds were unable to protect the cells from $\Delta \psi_m$ depolarization caused by staurosporine. Furthermore, the combination of 500 µM DETA-NO plus 500 µM aspirin failed to reproduce the hyperpolarizing effect of DETA-NO (Fig. 6), thereby suggesting that the effect of NCX-4016 was unrelated to the aspirin part of the molecule but was instead related to the NO moiety.

Analysis of DCF-DA fluorescence, a measure of oxidative stress, demonstrated that exposure to staurosporine for 3 h increased the fluorescence. This effect was markedly reduced by 500 µM NCX-4016, from 30.2 ± 6.3 (arbitrary units) to 8.4 ± 4.3 ($P<0.01$, $n = 8$). In contrast, 500 µM DETA-NO exerted no effect on oxidative stress caused by staurosporine (36.5 ± 5.3, NS versus staurosporine alone). Thus, NCX-4016 protected against oxidative stress caused by staurosporine.

Cytochrome $c$ translocation from the mitochondrial inner space to the cytosol is required for the intrinsic pathway of apoptosis to progress. As illustrated in Fig. 7, although 100 nM staurosporine caused a time-dependent translocation of cytochrome $c$ from the mitochondrial to the cytosolic fraction, this process was inhibited by NCX-4016 and, in the early phase, also by DETA-NO at concentrations of 100 (data not shown) and 500 µM. Again, neither aspirin nor NCX-4017 could reproduce this effect. Interestingly, the time course of cytochrome $c$ release could almost be superimposed on the time course of $\Delta \psi_m$ loss.

**NCX-4016 inhibited caspase activation**

Exposure of staurosporine-treated cells to NCX-4016 (1-500 µM) for up to 8 h caused a concentration-dependent inhibition of caspase-8 and caspase-9 activity (Fig. 8). These effects were not reproduced by DETA-NO. Aspirin and NCX-4017 also had no effect on caspase activation induced by staurosporine (data not shown). Incubation of cell lysates with 20 mM DTT, an agent that effectively removes NO from thiol groups, resulted in an approximate 50% recovery of caspase-8 activity in cells treated with NCX-4016, whereas it had no effect on caspase-8 activity measured in lysates obtained from DETA-NO-treated cells.

**DISCUSSION**

The central findings of this study are that exposure of human endothelial cells to NCX-4016, an NO-releasing derivative of aspirin, hyperpolarizes $\Delta \psi_m$ and that this effect is due to the NO moiety of the molecule. Confocal microscopic analysis demonstrated that incubation of HUVEC with NCX-4016 caused a time- and concentration-dependent increase in the intracellular concentration of NO. However, although NO formation in cells exposed to DETA-NO was associated with the appearance of diffuse cytosolic fluorescence, NCX-4016 induced a
characteristic pattern of hot spots of activity localized at specific subcellular compartments (25). The predominant locus of activity was near the endothelial cell plasma membrane. Although the physiological relevance of this pattern of NO generation is still unclear, it is noteworthy that a similar pattern of NO formation has been documented in endothelial cells in response to eNOS activation (10). Indeed, Paxinou et al. recently demonstrated that, after stimulation with vascular endothelial growth factor (VEGF), only membrane-anchored eNOS was phosphorylated, as indicated by the finding that serine-1179 phosphorylated eNOS coprecipitated with calveolin-1-enriched membranes (10). Confocal microscopic analysis of formation of eNOS-derived NO in response to VEGF in endothelial cells loaded with DAF-DA demonstrated the appearance of hot spots of activity in the perinuclear region and plasma membrane of these cells (15). Our data, therefore, indicate that the aspirin moiety of NCX 4016 may be “transporting” NO to membrane sites, which may coincide with those at which eNOS is located.

The demonstration that HUVEC can convey NCX-4016 to specific intracellular compartments at which NO is generated may account for the difference between the activity of this compound and that of DETA-NO. Indeed, although NCX-4016 released approximately one-third of the NO generated by an equimolar concentration of DETA-NO, it was more potent than DETA-NO in modulating ∆ψm (12): ∆ψm hyperpolarization caused by NCX-4016 was more pronounced and lasted longer than that caused by DETA-NO. Furthermore, the effect of DETA-NO on ∆ψm, as demonstrated previously (12), was typically biphasic: after an early (1-3 h) hyperpolarization, continuous exposure of endothelial cells to this agent resulted in rapid collapse of ∆ψm that was associated with the appearance of cellular markers of oxidative stress. Furthermore, this biphasic pattern was observed when staurosporine-treated cells were exposed to DETA-NO, so that after an early hyperpolarization, DETA-NO caused a collapse of ∆ψm that was associated with a rapid progression from apoptosis to necrosis (12). The finding that exposure to NCX-4016 increased ∆ψm and prevented cytochrome c release induced by staurosporine suggests that NCX-4016-derived NO stabilizes the mitochondrial membrane and prevents the opening of the permeability transition pore (11, 13, 14). Finally, because the activity of NCX-4016 was not reproduced by aspirin (28), NCX-4017 (a non-NO-releasing analogue of NCX-4016), or the combination of DETA-NO and aspirin, these data strongly support the concepts that not only are the effects of NCX-4016 mediated by NO but the specificity of NCX-4016 activity is due to the release a NO at specific intracellular targets.

It is interesting that our study demonstrated that NCX-4016, unlike DETA-NO, inhibited the activation of proapoptotic caspases (29-32). Although the subject of caspase inhibition by NO remains controversial (see ref 33), our results also suggest that it may be due to S-nitrosoylation, because the process was reversible by DTT. The reason for two different NO donors having different S-nitrosoylation patterns are not clear at present. However, one possible explanation is that caspase S-nitrosoylation by NO depends on the formation of NO+—like (nitrosonium) species (32-34). For NO to generate NO+ species, it must release an electron. Potential electron acceptors in cells are oxygen and nonheme iron (32-34). Although we have not measured nonheme iron content in staurosporine-treated cells, we have documented that exposure to DETA-NO results in approximately an 80% reduction of oxygen consumption. Thus, it is likely that inhibition of cell respiration contributes to the inability of DETA-NO to carry out a nitrosative reaction on caspases. Our data suggest that the capacity of NO to S-nitrosylate caspases could dictate whether cells are protected from NO and whether cells, if not protected, undergo apoptosis or
necrosis. In contrast to DETA-NO, NCX-4016 caused only about a 40% reduction of oxygen consumption, suggesting that the presence of respiring mitochondria is required for NO to protect against apoptotic damage (8, 9, 12).

Inhibition of caspase activity may contribute to the potent antiapoptotic effect observed with NCX-4016. It is now recognized that caspase-8 activation occurs early in staurosporine-treated cells and precedes ΔΨ_m collapse (35). Once activated, caspase-8 shortens the execution time of apoptosis by activating an autoamplifying loop. Indeed, activated caspase-8 cleaves Bid, a proapoptotic member of the Bcl-2 family, leading to Bid-dependent ΔΨ_m depolarization (21-24). The finding that NCX-4016 prevents caspase-8-dependent Bid cleavage (Fiorucci et al., manuscript in preparation) demonstrates that this compound acts on multiple targets in the apoptosis chain.

The concentration of NO is also known to play a major role in modulating apoptosis (32). Indeed, although antiapoptotic effects are generally associated with exposure to low concentrations (10 nM to 1 µM) of NO, apoptosis and necrosis are observed in response to high concentrations (>1 µM) of NO (29). In the present study, we demonstrated that DETA-NO released approximately three times more NO than did NCX-4016, generating intracellular NO levels of about 1.5 µM. Although these concentrations can be measured in response to activation of cytokine-inducible NOS, they cause a significant consumption of reduced thiols and the appearance of markers of oxidative stress (8, 9, 12). NO toxicity depends largely on the redox environment of target cells, and there is evidence that NO-induced apoptosis, in certain cell types, can be reversed by increasing the cellular redox potential. Thus, it appears that by acting as a low-output source of NO, NCX-4016 protects HUVEC against oxidative stress.

In the present study, we demonstrated that exposure to NCX-4016, similar to exposure to DETA-NO, inhibits oxygen consumption by endothelial cells. However, although DETA-NO at the concentration of 500 µM caused a virtually complete inhibition of cell respiration (8,9), exposure to equimolar concentrations of NCX-4016 caused only an approximately 40% reduction of oxygen consumption. Interestingly, Paxinou et al. (10) demonstrated that transfection of eNOS-deficient endothelial cells with eNOS reduced oxygen consumption, further suggesting that intracellular NO may function as a oxygen sensor (8, 9). Inhibition of cell respiration caused by DETA-NO and NCX-4016 was reversed by hemoglobin, consistent with an NO-dependent mechanism. However, in contrast to NCX-4016-induced inhibition, inhibition caused by DETA-NO became irreversible after 4 h. Because inhibition of cell respiration impairs energy generation by mitochondria (8, 9), complete and prolonged inhibition of oxygen consumption is likely to contribute to the transition from protection to necrosis in cells exposed to high concentrations of DETA-NO.

In conclusion, our results support the view that NCX-4016 penetrates the endothelial cell membrane and releases NO at compartments near the plasma membrane. By dynamically regulating mitochondrial function, NCX-4016 provides protection against cell injury. Our confocal microscopic analysis demonstrated that intracellular distribution NO is an important, previously unrecognized, factor in determining NO activity on specific targets. Finally, we demonstrated that two different sources of NO can exert divergent effects on the same cell function. This observation should be taken into consideration when “physiological” effects of
NO are measured using potent NO donors and may provide an explanation for some of the reported paradoxical actions of NO.

ACKNOWLDEGMENTS

This study was supported in part by a Grant from Ministero della Istruzione e della Ricerca Scientifica (MIRST) n. 9906275238 to S. F.

REFERENCES


*Received May 2, 2002; accepted May 22, 2002.*
Figure 1. Time course of intracellular NO formation in DAF-DA-loaded HUVEC exposed to DETA-NO or NCX-4016. Cells were incubated with no agent (shown in panels 1 and 5) or with 500 µM DETA-NO for 5 min (2) or 10 min (3) or with 500 µM NCX-4016 for 5 min (6) or 10 min (7), and intracellular fluorescence was recorded by confocal microscopy. Original magnification, 400 ×. Panels 4 and 8 show higher magnification of cells (panels 3 and 7) demonstrating subcellular distribution of NO-related fluorescence in HUVEC exposed to 500 µM DETA-NO or NCX-4016 for 10 min; original magnification 600 ×. Single-cell analysis of NO-related fluorescence is also shown (9-12): 9 and 11, control HUVEC, original magnification 600 ×; 10, the effect of 100 µM DETA-NO after 10 min of incubation, original magnification 600 ×; 12, the effect of 100 µM NCX-4016 after 10 min of incubation, original magnification 600 ×. Microphotographs represent at least three other similar experiments.
Figure 2. Exposure of HUVEC to NCX-4016 caused NO generation and ΔΨm hyperpolarization. A) NO generation in response to NCX-4016 or DETA-NO was concentration dependent. Data are means ± SE of 11 independent measures. NO formation was monitored for 3 h. * indicates $P < 0.05$ versus DETA-NO by ANOVA. B) Changes in ΔΨm as measured by JC-1 relative red fluorescence intensity. NCX-4017, a non-NO-releasing analogue of NCX-4016, failed to stimulate NO release. HUVEC were incubated for 3 h with the indicated compound. Data are means ± SE of eight experiments. * indicates $P < 0.05$ treated versus control cells by ANOVA.
Figure 3. Time course of $\Delta \psi_m$, GSH concentration, and MC-DCF-DA fluorescence in HUVEC exposed to NCX-4016, NCX-4017, or DETA-NO. In these experiments, HUVEC were incubated with each agent at 500 µM. A) NCX-4016 induced a long-lasting $\Delta \psi_m$ hyperpolarization, whereas DETA-NO caused hyperpolarization followed by depolarization. Data are means ± SE of eight experiments. * indicates $P < 0.01$ treated versus control cells. B) DETA-NO, but not NCX-4016, induced GSH consumption. Data are means ± SE of eight experiments. * indicates $P < 0.01$ versus control cells. C) DETA-NO, but not NCX-4016, triggered DCF-DA accumulation. Data are means ± SE of eight experiments. * indicates $P < 0.01$ versus control cells.
Figure 4. NCX-4016 reduced cellular oxygen consumption. HUVEC were incubated for 4 h with 500 µM NCX-4016 or DETA-NO with or without 10 µM hemoglobin (Hb) as an NO scavenger, and oxygen consumption was measured as described in Materials and Methods. Data are means ± SE of 12 experiments. * indicates $P < 0.05$ versus DETA-NO alone. ** indicates $P < 0.05$ versus NCX-4016 alone. ° indicates $P < 0.01$ versus DETA-NO alone.
Figure 5. NCX-4016 rescued cells from mitochondrial damage caused by staurosporine. A) Effect of increasing concentrations of NCX-4016 and DETA-NO on HUVEC apoptosis caused by exposure to 100 nM staurosporine for 3 h. The percentage of apoptotic cells (annexin V⁺/PI⁻) is shown. Data are means ± SE of six to eight experiments. * indicates \( P < 0.05 \) versus staurosporine alone. ** indicates \( P < 0.05 \) versus staurosporine alone and DETA-NO. B) Changes in \( \Delta \psi_m \) as measured by JC-1 relative red fluorescence intensity in HUVEC exposed to 100 nM staurosporine alone or in combination with 500 \( \mu \)M NCX-4016 or DETA-NO for a period of 8 h. Data are means ± SE of eight experiments. * indicates \( P < 0.01 \) versus staurosporine. C) Effect of 500 \( \mu \)M NCX-4016 or DETA-NO on apoptosis induced by 100 nM staurosporine. Cells were incubated for 1-3 h with different agents. The percentage of apoptotic cells (annexin V⁺/PI⁻) is shown. Data are means ± SE of six to eight experiments. * indicates \( P < 0.05 \) versus staurosporine alone. D) Effect of 500 \( \mu \)M NCX-4016 or DETA-NO on HUVEC necrosis (annexin V⁺/PI⁺) induced by staurosporine. Data are means ± SE of six to eight experiments. * indicates \( P < 0.01 \) versus staurosporine alone.
Figure 6. A combination of aspirin and DETA-NO failed to reproduce the effect of NCX-4016 on $\Delta\psi_m$. All agents were added to cell suspensions at a concentration of 500 µM except for staurosporine (100 µM). $\Delta\psi_m$ was measured after 3 h of incubation. * indicates $P < 0.01$ versus basal values ($n = 10$). ** indicates $P < 0.01$ versus staurosporine alone.
Figure 7. NCX-4016 protected against mitochondrial perturbation and prevented cytochrome c translocation. HUVEC were treated with 100 nM staurosporine with or without 500 µm NCX-4016 or DETA-NO for 1-8 h and were subsequently lysed as described in Materials and Methods. Lysates equivalent to $1 \times 10^7$ cells were subjected to 10-15% SDS-PAGE and were immunoblotted with the cytochrome c antibody. Each blot represents at least three other experiments.
Figure 8. NCX-4016 (500 μM) but not DETA-NO (500 μM) inhibited caspase-8 and caspase-9 activity in staurosporine-treated HUVEC. A, B) Time course of caspase-8 and caspase-9 activation in response to 100 nM staurosporine. Data are means ± SE of eight experiments. * indicates $P < 0.01$ versus staurosporine-treated cells. C, D) Concentration dependence of the inhibition of staurosporine-triggered caspases induced by NCX-4016. The black bars represent the effect of staurosporine alone. Data are means ± SE of eight experiments. * indicates $P < 0.01$ versus staurosporine alone or staurosporine plus DETA-NO. E) Dithiothreitol reversed the inhibition of caspase-8 activity induced by NCX-4016. Cells were incubated for 3 h with staurosporine alone or in combination with 500 μM NCX-4016 or DETA-NO. At the end of incubation, HUVEC were lysed and activity was measured, as described in Materials and Methods. Data are means ± SE of eight experiments. * indicates $P < 0.01$ versus staurosporine alone. ** indicates $P < 0.05$ versus staurosporine plus NCX-4016.