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

STEFANO FIORUCCI,² ANDREA MENCARELLI, ROBERTA MANNUCCI, ELEONORA DISTRUTTI, ANTONIO MORELLI, PIERO DEL SOLDATO,* AND SALVADOR MONCADA†

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

SPECIFIC AIMS

The endothelium plays a pivotal role in regulating recruitment of blood-borne cells during inflammation and immune surveillance. An increased rate of apoptosis of endothelial cells is a common event in several human diseases and plays a pathogenetic role in stroke, atherosclerosis, diabetes, ischemia, and hypertension. Nitric oxide (NO) modulates endothelial integrity and exerts anti-apoptotic activity. In the present study we have examined the effect of NCX-4016, a NO-releasing derivative of aspirin, on endothelial apoptosis induced by staurosporine.

PRINCIPAL FINDINGS

1. Exposure to NCX-4016 resulted in NO generation

Exposure of human endothelial cells (HUVEC) loaded with DAF-DA, a dye that on binding an oxidized species of NO results in irreversible fluorescence permitting identification of the source of local NO formation in living cells, to 1–500 μM(2)-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2 diolate (DETA-NO) resulted in a generalized time- and concentration-dependent increase of cytosolic fluorescence (Fig. 1). In contrast, confocal microscope images obtained from HUVEC exposed to 500 μM NCX-4016 demonstrated ‘hot spots’ of activity in specific subcellular compartments localized near the plasma membrane. No significant increase in DAF-DA-related fluorescence was seen in cells treated with aspirin or NCX-4017, a non-NO-releasing analog of NO aspirin (not shown). Exposure to DETA-NO (500 μM) caused a rapid (5 min) and profound inhibition of cell respiration (78.3±6.4% inhibition, n=6). In contrast, NCX-4016 (500 μM) induced a less pronounced reduction of oxygen consumption (43.5±5.3%, n=8, P<0.05 vs. DETA-NO). Inhibition of cell respiration caused by NCX-4016, but not by DETA-NO, was reversed by addition of 10 μM hemoglobin (n=8, P<0.05).

Figure 1. Time course of intracellular NO formation in DAF-DA-loaded HUVEC. Cells were incubated with no agent (1, 5) or with 500 μM DETA-NO for 5 min (2) and 10 min (10) or 500 μM NCX-4016 for 5 min (6) and 10 min (7); intracellular fluorescence was recorded by confocal microscopy. Original magnification ×400. 4, 8) Higher magnification of cells demonstrating subcellular distribution of NO-related fluorescence in HUVEC exposed to 500 μM DETA-NO or NCX-4016 for 10 min; original magnification ×600. 9–12) Single cell analysis of NO-related fluorescence. 9, 11) Control HUVEC, original magnification ×600. 10) The effect of 100 μM DETA-NO after 10 min of incubation. Original magnification ×600. 12) Effect of 100 μM NCX-4016 after 10 min of incubation. Original magnification ×600.

1 To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.02–0297fje; to cite this article, use FASEB J. (August 7, 2002) 10.1096/fj.02–0297fje

2 Correspondence: Clinica di Gastroenterologia ed EpatoLOGIA, Policlinico Monteluce, Via E. dal Pozzo, 06100 Perugia, Italy. E-mail: fiorucci@unipg.it
2. NCX-4016 protects HUVEC from apoptosis

Staurosporine caused a time-dependent reduction of cell viability. Exposure of staurosporine-treated cells to 500 μM DETA-NO (Fig. 2) resulted in an early (1–3 h) reduction of apoptotic death rate (n=8, P<0.05). Thereafter (3–8 h), DETA-NO was unable to counteract the effect of staurosporine. In contrast, protection exerted by NCX-4016 (500 μM) was maintained over time (n=6–8, P>0.01). Analysis of cell death revealed that staurosporine-treated cells were dying from apoptosis at an early phase (1–3 h) while necrotic features were observed in the ensuing period (3–8 h). Exposure of staurosporine-treated HUVEC to NCX-4016 significantly reduced the amount of apoptotic cells and reduced the transition from apoptosis to necrosis. Exposure to DETA-NO resulted in a slight reduction of apoptotic cell death only in the early phase.

3. NCX-4016 maintains mitochondrial integrity

Staurosporine-induced cell death was associated with mitochondrial damage as demonstrated by the early collapse of mitochondrial membrane potential (ΔΨm) observed in cells incubated with 100 nM of this compound. Addition of 500 μM NCX-4016 to staurosporine-treated cells caused a significant hyperpolarization of ΔΨm that remained higher than basal values for 8 h (Fig. 2b n=8, P<0.001). The effect exerted by DETA-NO on ΔΨm was biphasic: after an initial hyperpolarization, ΔΨm gradually declined to the value seen in cells treated with staurosporine alone. Although staurosporine (100 nM) 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 500 μM DETA-NO. Exposure of staurosporine-treated cells to NCX-4016 (1–500 μM) for up to 8 h caused a concentration-dependent inhibition of caspase-3, -8, and -9. These effects were not reproduced by DETA-NO.

CONCLUSIONS AND SIGNIFICANCE

Alteration of endothelial function might contribute to inflammatory and degenerative disorders. The central findings of this study are that NCX-4016, a NO-releasing derivative of aspirin, protects endothelial cells from apoptosis induced by staurosporine via a mechanism that involves ΔΨm hyperpolarization and regulation of O2 consumption (Fig. 3). We demonstrated that this effect was associated with a significant reduction of cell respiration as indicated by a rapid, although reversible, decline in O2 consumption. By in vivo measuring of NO formation, O2 consumption, and oxygen radical generation, we documented that inhibition of cell respiration caused by DETA-NO results in early glutathione consumption and free radical accumulation into mitochondria. This ‘metabolic anoxia’ caused by NO was not observed in cells exposed to NCX-4016, likely as a consequence of the reversible inhibition of cell respiration. We also demonstrated that NCX-4016, unlike DETA-NO, inhibits the activation of preapoptotic caspases (caspase-3, -8, and -9) by a mechanism that involves S-nitrosylation of cysteine residue(s) in the enzyme catalytic core. The inability of DETA-NO to
cause caspase S-nitrosylation may be the result of the profound inhibition $O_2$ consumption caused by this compound. Collectively, our results indicate that inhibition of cell respiration represents a first line defense mechanism against apoptogenic stimuli. The ‘metabolic hypoxia’ induced by NO increases mitochondrial membrane potential, interrupting the progression of apoptosis. However, if the inhibition of cell respiration persists, mitochondria become more vulnerable to the deleterious effect of reactive oxygen species, precipitating cell damage. Furthermore, by pointing out on the biphasic activity of NO on $\Delta \psi_m$, our data shed light on the apparent paradox of NO being both pro- and anti-apoptotic in different cell systems. The intracellular site of NO formation seems to be critical in determining the ‘type’ of action of NO.

Finally, by using NCX-4016 as a tool to generate low intracellular amount of NO, we have established a hierarchy between the different anti-apoptotic mechanisms of NO. Indeed, $\Delta \psi_m$ hyperpolarization seems to be more effective in blocking apoptosis progression than caspase S-nitrosylation. In fact, progression toward apoptotic changes became detectable only when $\Delta \psi_m$ collapsed, whereas activated caspases are found in the absence of marker of apoptosis (i.e., annexin V negative cells). The finding that exposure to NCX-4016 increased $\Delta \psi_m$ 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. Because maintenance of $\Delta \psi_m$ delays apoptotic changes as effectively as caspase inhibitors, the results of this study establish that mitochondria may be the target for developing anti-apoptotic strategies using NO-based compounds.

Figure 3. Mechanism of anti-apoptotic activity of NO in staurosporine-treated endothelial cells. Staurosporine causes early activation of caspase-8 (1) and collapse of $\Delta \psi_m$ (2). Caspase-8-cleaved Bid contributes to an auto-amplifying loop of apoptotic pathways, causing the release of caspase-9 and -3 from mitochondria (3). NO aspirin inhibits activation of caspase-3 and -8 (4a, b) and spares $\Delta \psi_m$ (5) by modulating $O_2$ consumption.