ANTIOXIDANTS & REDOX SIGNALING
Volume 9, Number 11, 2007

DOI: 10.1089/ars.2007.1603

# Original Research Communication

# Nitroaspirin (NCX-4016), an NO Donor, is Antiangiogenic Through Induction of Loss of Redox-Dependent Viability and Cytoskeletal Reorganization in Endothelial Cells

NARASIMHAM L. PARINANDI,[1] ASHISH SHARMA,[1] TIMOTHY D. EUBANK,[1] BRUCE F. KAUFMAN,[1] VIJAY KUMAR KUTALA,[1] CLAY B. MARSH,[1] LOUIS J. IGNARRO,[2] and PERIANNAN KUPPUSAMY[1]

## ABSTRACT

**We recently reported that NCX-4016, a derivative of aspirin containing a nitro moiety that releases nitric oxide (NO) in a sustained fashion in biologic systems, is a potent cytotoxic agent inhibiting the proliferation of cisplatin-resistant human ovarian cancer cells. Therefore, we hypothesize that NCX-4016 possesses antiangiogenic properties. Our study with the bovine lung microvascular endothelial cells (BLMVECs) revealed that NCX-4016 significantly induced the loss of redox-dependent cell viability in a dose- and time-dependent manner, as assayed by the redox-sensitive Alamar blue cell viability assay. Fluorescence microscopy of cells labeled with NO-specific fluorophore (DAF-FM) confirmed that NCX-4016 generated significant levels of intracellular NO. NO donors, including *S*-nitroso-*N*-acetylpenicillamine, spermine NONOate, and isosorbide dinitrite, were less effective in causing loss of cell viability. Thiol-protectant, *N*-acetylcysteine, significantly attenuated the NCX-4016-induced loss of cell viability, suggesting the role of alteration of thiol-redox status therein. NCX-4016 also suppressed oxygen consumption, decreased transendothelial electrical resistance (EC barrier dysfunction), and induced actin cytoskeletal reorganization in BLMVECs. The *in vitro* assay with human umbilical vein ECs and BLMVECs revealed that NCX-4016, in a dose-dependent manner, significantly inhibited angiogenesis with almost complete inhibition at a 100-$\mu M$ concentration, suggesting that NCX-4016 can act as an antiangiogenic drug.** *Antioxid. Redox Signal.* 9, 1837–1849.

## INTRODUCTION

Nitric oxide (NO), commonly known as the endothelium-derived relaxing factor in the vasculature and an important biologic signaling molecule, is generated by an endothelial nitric oxide synthase that uses L-arginine as the substrate (20, 21). The endogenously generated NO acts as a mediator of the vascular smooth muscle relaxation through which it regulates blood-vessel homeostasis (35). NO, a crucial biologically active molecule, is also potentially toxic in nature (49). As a free radical in nature, NO is a key player in mammalian cells in regulation of signal-transduction cascades in the vascular, nervous, and immune systems (45). Recently, NO also was recognized as an antioxidant molecule (38, 39). However, the nature of NO, with both toxic and protective features, depends on the chemical reactivity of the molecule and targets in the biologic systems (38, 45)

The cytoprotective actions of NO have been well documented. In this regard, several well-known NO donor com-

[1]Department of Internal Medicine, Divisions of Cardiology and Pulmonary, Critical Care, and Sleep Medicine, Dorothy M. Davis Heart and Lung Research Institute, College of Medicine, The Ohio State University, Columbus, Ohio.
[2]Department of Molecular and Medical Pharmacology, Center for the Health Sciences, University of California School of Medicine, Los Angeles, California.

pounds have been widely used to release NO in the biologic systems for its protective effects against pathologic or toxic phenomena (22, 30, 60). Protection against peroxide-mediated toxicity in lung fibroblasts and aortic endothelial cells (ECs), inhibition of oxidized low-density lipoprotein toxicity to aortic ECs, and protection against tumor necrosis factor-$\alpha$-induced vascular EC toxicity are a few but not an exhaustive list of examples of the cytoprotective nature of NO (28, 40, 50, 57, 58). Conversely, the toxic actions of NO in several cellular systems have been extensively documented. A relation between oxidative and nitrosative stress and cytotoxicity of NO has been established (59). NO as an effector of apoptosis and its role in the mitochondrial-mediation of apoptosis have been recognized (2, 4). The toxicity of NO, especially generated by NO donors, to vascular ECs has been well recognized (6, 19, 23, 26, 42).

NO has been shown to inhibit the proliferation of rat aortic smooth muscle cells in a cGMP-independent pathway, wherein the cytostatic action of NO has been attributed to its inhibitory effect on ornithine decarboxylase, which generates cell proliferation-mediating polyamines (20, 36). Apparently, the NO donor drugs are promising cytostatic agents against proliferating cells (3). The cytotoxic action of NO against tumor cells has been attributed to the inhibition of mitochondrial heme enzymes and tricarboxylic acid cycle (7). NO donors have been shown to modulate angiogenesis in myocardial infarction and solid tumors (60). The nitro derivatives of aspirin (nitroaspirins) have recently gained attention as safe and more potent gastrointestinal-sparing antithrombotic drugs (55). NCX-4016 [2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester], a derivative of aspirin containing a nitro moiety that releases nitric oxide (NO) in a sustained fashion in biologic systems, has also been shown to be an effective drug in diminishing restenosis (32). Nitroaspirin has been shown to correct immune dysfunction in the tumor-bearing hosts and to promote tumor eradication after cancer vaccination (10). Angiogenesis has been identified as one of the hallmarks of cancer, and several therapeutic strategies to inhibit vascularization in the neoplastic tissue have been introduced in clinical oncologic practice (33, 41). Cancer has been recognized as an inflammatory disease, and cyclooxygenase-2 (COX-2) plays a critical role in tumorigenesis (9). Because the nitro nonsteroidal antiinflammatory drugs (NO-NSAIDs), including NCX-4016, have been used to suppress COX activity and associated inflammatory events (8, 43), it is conceivable that nitro NSAIDs can act as antiangiogenic drugs. We recently reported that NCX-4016 is a potent cytotoxic agent inhibiting the proliferation of cisplatin-resistant human ovarian cancer cells (3). As we have shown that NCX-4016 selectively kills cisplatin-resistant human ovarian cancer cells and can be a potential anticancer chemotherapeutic agent, we hypothesize that NCX-4016 would also possess vascular EC-altering properties and angiogenesis modulatory actions. We have established the procedures for the redox-dependent cell viability and signaling studies in our laboratory by using the BLMVECs as the model EC system (53). To test our hypothesis, we chose bovine lung microvascular ECs (BLMVECs) as an ideal EC model to study the effects of NCX-4016 on cell viability, barrier function, and cytoskeletal alterations. Human umbilical vein ECs (HUVECs) are widely accepted as a standard model for *in vitro* angiogenesis studies with a pathophysiologic relevance to patients (13, 14). Hence, we used the *in vitro* angiogenesis assay with HUVECs and BLMVECs to investigate the effects of NCX-4016 on angiogenesis. The results of the current study revealed that NCX-4016 induced NO-mediated loss of redox-dependent cell viability, barrier dysfunction, and actin cytoskeletal reorganization in BLMVECs and inhibition of angiogenesis in HUVECs and BLMVECs.

## MATERIALS AND METHODS

### *Reagents*

Minimum essential medium (MEM), nonessential amino acids, aspirin, trypsin-EDTA, disodium ethylenediaminetetracetic acid ($Na_2$-EDTA), $H_2O_2$ (30%), *N*-acetylcysteine (NAC), isosorbide dinitrite, penicillin-streptomycin, and fetal bovine serum, were obtained from Sigma Chemicals (St. Louis, MO). The bovine lung microvascular ECs (BLMVECs) (passage 4) were purchased from Cell Systems (Kirkland, WA). EC growth supplement was obtained from Upstate Biotechnology (Lake Placid, NY). Alamar blue reagent was obtained from Biosource International (Camarillo, CA). DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) and rhodamine-phalloidin were purchased from Molecular Probes (Eugene, OR). Spermine NONOate, *S*-nitroso-*N*-acetylpenicillamine (SNAP), and 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) were obtained from Calbiochem (San Diego, CA). EC growth factor (ECGF) was obtained from Upstate Biotechnology (Lake Placid, NY). Fluoromount-G was purchased from Southern Biotechnology Associates, Inc (Birmingham, AL). Glass coverslips (22-$mm^2$ and 1-mm thick) were obtained from Harvard Apparatus (Holliston, MA). Gold electrodes were purchased from Applied Biophysics Inc. (Troy, NY). NCX-4016 [2-(acetyloxy)benzoic acid 3-(nitrooxymethyl) phenyl ester] was obtained from NicOx (Sophia, Antipolis, France).

### *EC culture*

The BLMVECs were cultured in MEM containing FBS (10%), antibiotics (1%), nonessential amino acids (1%), and endothelial growth supplement and maintained at 37°C in a humidified atmosphere of 95% air/5% $CO_2$ and grown to contact-inhibited monolayers with typical cobblestone morphology (53). Cells from each primary flask were detached with 0.05% trypsin, resuspended in fresh medium and subcultured in 35-mm and 60-mm sterile dishes in complete medium to ~95% confluence under 95% air/5% $CO_2$ for exposure to NCX-4016 and desired pharmacologic agents. BLMVECs from passages 6 to 18 were used in all the experiments. For *in vitro* angiogenesis assay, HUVECs from Cascade Biologicals (Portland, OR) were used. Cells used for the angiogenesis assay were always between passages 2 and 4. HUVECs were grown in RICH medium (M200 minimal medium) supplemented with 2% FBS, 1 $\mu$g/ml hydrocortisone, 10 ng/ml human epidermal growth factor (hEGF), 3 ng/ml basic fibroblast growth factor (bFGF), and 10 $\mu$g/ml heparin in a humidified atmosphere of 95% air/5% $CO_2$ at 37°C.

### *Cell-viability assay*

Alamar blue assay is an accepted and a widely used cell-viability assay. The assay is based on the bioreduction of the dye, resazurin, by nonspecific cellular dehydrogenases (diaphorase, NADH dehydrogenase, and plasma membrane electron-transport system) including those of the respiratory chain (44). Alamar blue assay has been considered a cellular redox indicator in addition to being nontoxic to the cells (31, 34). Previously, we successfully used this reagent to determine the vitamin C-induced loss of redox-dependent EC viability (53). Therefore, the Alamar blue reduction viability assay measures overall cellular redox-dependent cell viability. The BLMVECs, cultured in 35-mm dishes (~95% confluence; $5 \times 10^5$ cells/dish) were exposed to NCX-4016 or $H_2O_2$ or NCX-4016 + $H_2O_2$ or NO donors (SNAP, spermine NONOate, and isosorbide dinitrite, or aspirin) at desired concentration(s) for different time periods in the absence and presence of pharmacologic inhibitors wherever desired, after which the medium was removed, the cells were washed once with 1.0 ml of PBS (37°C), and incubated with 1 ml of fresh MEM containing 100 $\mu$l of the Alamar blue reagent (Alamar blue cell-viability assay kit; Biosource, Camarillo, CA) for 3 h under humidified 95% air/5% $CO_2$ atmosphere at 37°C. The metabolic reduction of the redox probe (resazurin) of the Alamar blue in the medium was measured spectrophotometrically at 570 and 600 nm on a Molecular Devices Spectra Max 190 UV-visible plate reader, according to the manufacturer's instructions. The extent of Alamar blue reduction was normalized to the corresponding untreated control cells and expressed as percentage of cell viability. NCX-4016 stock solutions were prepared in DMSO, and the final concentration of DMSO in the incubation medium was 0.05%. Vehicle-treated controls were also treated with MEM containing 0.05% DMSO.

### *Fluorescence microscopy of actin stress fibers*

Formation of actin stress fibers as an index of endothelial cytoskeletal reorganization was analyzed by fluorescence microscopy, as described earlier (54). BLMVECs grown on sterile glass coverslips (70% confluence) were treated with different concentrations of NCX-4016 (100, 250, 500 $\mu M$) for 2 h, rinsed twice with PBS, and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The cells were then permeabilized with 0.25% Triton X-100 prepared in Tris-buffered saline containing 0.01% tween-20 (TBST). Actin stress fibers were visualized by staining the cells with rhodamine-phalloidin (1 U/coverslip) for 20 min. The cells were then rinsed with deionized distilled water to remove excess stain. The cells were then stained with 1% DAPI to visualize the nuclei. Cells were finally examined under a Nikon Eclipse 800 fluorescence microscope at a magnification of 40× and 555-nm excitation, and the images were captured digitally.

### *NO detection*

Intracellular generation of NO, after the treatment of cells with NCX-4016, was determined by the NO-specific intracellular fluorophore, DAF-FM. BLMVECs grown on glass coverslips (70% confluence) were preloaded with DAF-FM (10 $\mu M$), after which, they were treated with chosen concentrations of NCX-4016 for the desired times. The cells were then rinsed with PBS to remove the excess fluorophore and examined under Nikon Eclipse 800 fluorescence microscope under a magnification of 40× at 490-nm excitation and 520-nm emission, and the images were captured digitally. Fluorescence intensity (as an index of intracellular NO generation) of the vehicle-treated control and NCX-4016-treated cells was quantified from the digital images by using the MetaMorph software.

### *Measurement of transendothelial cell electrical resistance*

Transendothelial cell electrical resistance (TER) was essentially measured as described earlier (48, 52). BLMVECs were cultured in complete MEM on gold electrodes (Applied Biophysics, Troy, NY) at 37°C in a humidified atmosphere of 95% air/5% $CO_2$ and grown to a contact-inhibited monolayer with typical cobblestone morphology. The TER of the BLMVEC monolayer on gold electrodes was measured on an electric cell substrate impedance-sensing system (ECIS; Applied Biophysics) after treatment of the cells in MEM with the chosen concentrations of NCX-4016 or $H_2O_2$ or NCX-4016 + $H_2O_2$ in a humidified atmosphere of 95% air/5% $CO_2$ at 37°C. The total endothelial electrical resistance, as measured across the EC monolayer, was determined by the combined resistance between the basal or cell matrix adhesion or both. TER measurements were done in triplicate and expressed as normalized resistance for each of the treatments.

### *Cellular oxygen-consumption measurement*

The cellular oxygen-consumption rate was measured by using the electron paramagnetic resonance (EPR) spectroscopy, as described previously (37). The EPR measurements were carried out at 37°C by using a Bruker ER-300 (X-band, 9.8 GHz) EPR spectrometer (Bruker Instruments, Karlsruhe, Germany) equipped with $TM_{110}$ cavity. The cavity was rotated 90° so that the capillary tube filled with the cell suspension could be kept horizontal to avoid settling of the cells. EPR spectra were acquired by using custom-developed data-acquisition software. Unless mentioned otherwise, the EPR line widths reported are peak-to-peak widths ($\Delta$Bpp) of the first derivative spectra. The oxygen sensitivity of the LiNc-BuO crystals was calibrated, after which BLMVECs ($5 \times 10^5$ cells/ml) were treated with the chosen concentrations of NCX-4016 or $H_2O_2$ or NCX-4016 + $H_2O_2$, and the cellular oxygen consumption was determined in triplicate by using the LiNc-BuO crystals. The cellular oxygen consumption was expressed as $\%O_2$ consumption normalized to untreated control cells obtained as a function of time from the cell suspension in sealed capillary tubes.

### *In vitro angiogenesis assay*

The effect of NCX-4016 on angiogenesis was studied by the widely used *in vitro* angiogenesis assay using HUVECs and BLMVECs, according to Eubank *et al*. (13, 14). The RICH medium was also used when growing cells, as well as the assay media and positive control. Growth factor-reduced Matrigel matrix (Discovery Labware) was thawed at 4°C overnight, and 120 $\mu$l was plated on the surface of 48-well plates, followed by

polymerization at 37°C for 1 h. HUVECs from passage 3 were starved in M200 minimal medium for 8 h in T-125 culture flasks, after which the cells were trypsinized, washed, and counted. BLMVECs from passage 2 were starved in MEM alone for 4 h in T-75 culture flasks, after which cells were trypsinized, washed, and counted. For the treatment of HUVECs, NCX-4016 was prepared in Rich medium (M200 media supplemented with fetal bovine serum, 2% vol/vol; hydrocortisone, 1 $\mu$g/ml; hEGF, 10 ng/ml; bFGF, 3 ng/ml; and heparin, 10 $\mu$g/ml; Cascade Biologics, Portland, OR) to 25, 50, and 100 $\mu M$. For the treatment of BLMVECs, NCX-4016 was prepared in the complete BLMVEC MEM containing FBS (10%), antibiotics (1%), nonessential amino acids (1%), and endothelial growth supplement. HUVECs or BLMVECs ($1.5 \times 10^4$ cells) were resuspended in minimal medium or Rich (complete) medium or Rich (complete) medium supplemented with 25, 50, or 100 $\mu M$ NCX-4016 and plated in 1 ml total volume/well. The cells were incubated at 37°C and 95% air/5% $CO_2$ in a humidified atmosphere for 10 h. The medium was carefully aspirated from the plates containing cells, and 1 ml 4% paraformaldehyde was added to all the wells for 15 min at room temperature to fix the tubes that were formed between cells. The paraformaldehyde was aspirated, and 1 ml PBS was added to the wells with cells. Four digital pictures were captured at a magnification of 10×, and the tube formation was counted in a blinded manner. Tube formation was quantified by counting tubes that extend from an individual cell.

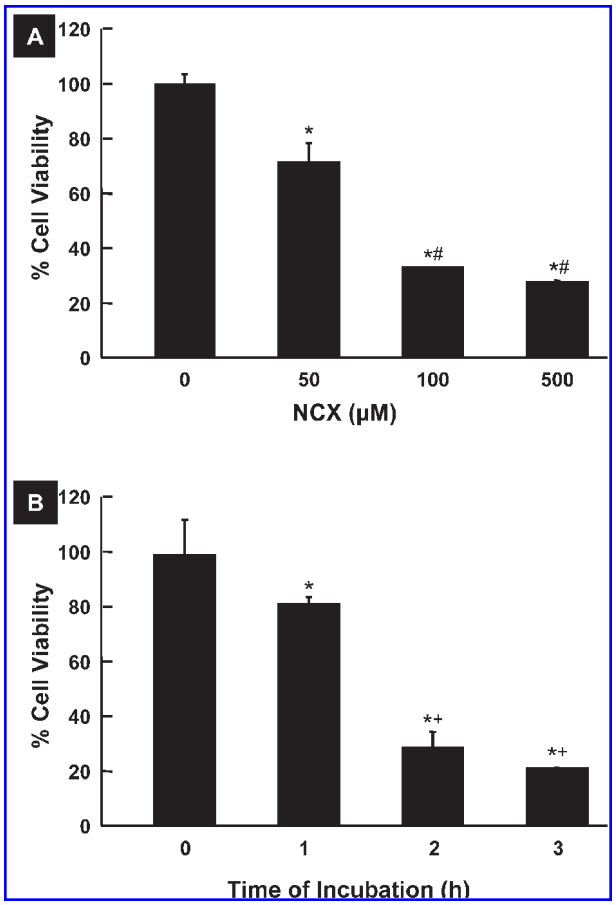


**FIG. 1. NCX-4016 induces loss of redox-dependent cell viability in a dose- and time-dependent manner.** BLMVECs (~95% confluence; $5 \times 10^5$ cells) grown in 35-mm dishes were treated with different concentrations (50, 100, and 500 $\mu M$) of NCX-4016 for 2 h (**A**) and with 100 $\mu M$ NCX-4016 for 1, 2, and 3 h (**B**) in MEM under a humidified atmosphere of 95% air/5% $CO_2$ at 37°C. At the end of exposure, the cell viability was determined spectrophotometrically by using the Alamar blue reagent, as outlined in Materials and Methods. Results are expressed as the mean ± SD from three independent experiments in triplicate. $*p < 0.05$ *versus* vehicle-treated controls. $\#p < 0.05$ *versus* NCX (50 $\mu M$)-treated cells. $+p < 0.05$ *versus* 1 h incubation.

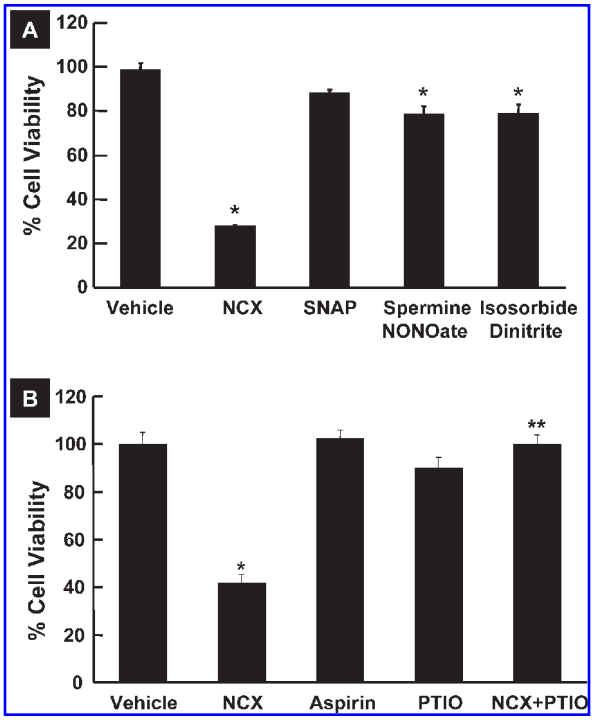


**FIG. 2. NCX-4016 and NO donors, but not aspirin, induce loss of cell viability.** BLMVECs (~95% confluence; $5 \times 10^5$ cells) grown in 35-mm dishes were treated with NCX-4016 (100 $\mu M$) or NO donors (SNAP, spermine NONOate, isosorbide dinitrite; 100 $\mu M$) (**A**) or aspirin (100 $\mu M$) for 2 h or PTIO (NO scavenger, 25 m$M$) 30 min before NCX (**B**) and then incubated for 2 h in MEM under a humidified atmosphere of 95% air/5% $CO_2$ at 37°C. At the end of exposure, the cell viability was determined spectrophotometrically by using the Alamar blue reagent, as outlined in Materials and Methods. Results are expressed as mean ± SD from three independent experiments in triplicate. $*p < 0.05$ *versus* vehicle-treated controls.

## *Statistical analysis*

All values were expressed as mean ± SD from three independent experiments. Data were subjected to one-way ANOVA, and the pair-wise multiple comparisons were done by Dunnett's method. A value of $p < 0.05$ was considered significant.

## RESULTS

### *NCX-4016 induces loss of redox-dependent cell viability in a dose- and time-dependent manner*

First, to establish whether NCX-4016 could induce the loss of cell viability in ECs in a dose-dependent fashion, we exposed BLMVECs to different concentrations of NCX-4016 for 2 h and assayed the cell viability. NCX-4016 at 50, 100, and 500 $\mu M$ concentrations significantly induced 28%, 67%, and 70% loss of cell viability, respectively, in a dose-dependent fashion in BLMVECs as compared with that observed in the vehicle-treated control cells (Fig. 1). Next, we determined the NCX-4016–induced time-dependent loss of redox-dependent cell viability in the ECs after the exposure of BLMVECs to 100 $\mu M$ NCX-4016 for 1, 2, and 3 h. As shown in Fig. 1B, NCX-4016 significantly caused the loss of cell viability at 1 h of treatment (19%); at 2 and 3 h of treatment of cells with the NO donor, the cell viability was significantly lower (72% and 74%, respectively) as compared with untreated cells. Also, NCX-4016 significantly decreased the viability in cells at 2 and 3 h of treatment as compared with that in cells exposed to NCX-4016 for 1 h. These results revealed that NCX-4016 induced the loss of redox-dependent cell viability in ECs in a dose- and time-dependent fashion.

### *NCX-4016 and NO donors, but not aspirin, induce loss of cell viability*

Here, we investigated whether the loss of cell viability caused by NCX-4016 was mediated by NCX-4016 or by its aspirin moiety. Along those lines, BLMVECs were treated with equimolar concentrations (100 $\mu M$) of NCX-4016, SNAP, spermine NONOate, isosorbide dinitrite, or aspirin for 2 h, after which the cell viability was determined. As shown in Fig. 2, only NCX-4016 was the most effective compound in causing a significant loss of cell viability (72%), whereas the other tested NO donors, although they caused a significant loss of the same, did not effectively decrease the viability in BLMVECs as compared with the same in the vehicle-treated control cells. However, aspirin alone did not show a significant effect on cell viability in BLMVECs (Fig. 2B). These results established that the NCX-4016 was the most effective compound, among the tested NO donors, in causing the loss of cell viability, which was not mediated by its aspirin moiety.

Next, we examined the effect of PTIO (a widely used intracellular NO scavenger) on the NCX-4016-induced loss of cell viability in the ECs to establish that NO generated by NCX-4016 was responsible for the loss of EC viability. After preloading of BLMVECs with PTIO (25 m$M$) for 30 min, the cells were then exposed to NCX-4016 for 2 h, and then the cell viability was determined. As shown in Fig. 2B, NCX-4016 significantly induced the loss of cell viability that was completely attenuated by PTIO. These results suggested that the NCX-4016-induced loss of cell viability was solely due to the NO generated by the donor.

### *NCX-4016 enhances $H_2O_2$-induced loss of cell viability*

As the interactions between NO and reactive oxygen species (ROS) and the protective effect of NO on $H_2O_2$-induced toxicity in cellular systems have been established, we investigated the effect of NCX-4016 on the $H_2O_2$-induced cell viability in the ECs. We established the dose-response effect of NCX-4016 on the $H_2O_2$-induced loss of cell viability in BLMVECs. $H_2O_2$ (100 $\mu M$) induced a significant dose-dependent loss of cell viability (50%) in BLMVECs at 2 h of treatment as compared with that in the vehicle-treated control cells. NCX-4016 caused a dose-dependent loss of cell viability (18%, 28%, and 47% at 25, 50, and 100 $\mu M$, respectively) as compared with the same in the vehicle-treated control cells (Fig. 3). When compared with an equimolar concentration of $H_2O_2$, NCX-4016 was significantly less effective in causing the loss of EC viability. However, the cell viability was significantly lower on co-treatment of cells with $H_2O_2$ (100 $\mu M$) and different concentrations of NCX-4016 (25, 50, and 100 $\mu M$), as opposed to that observed

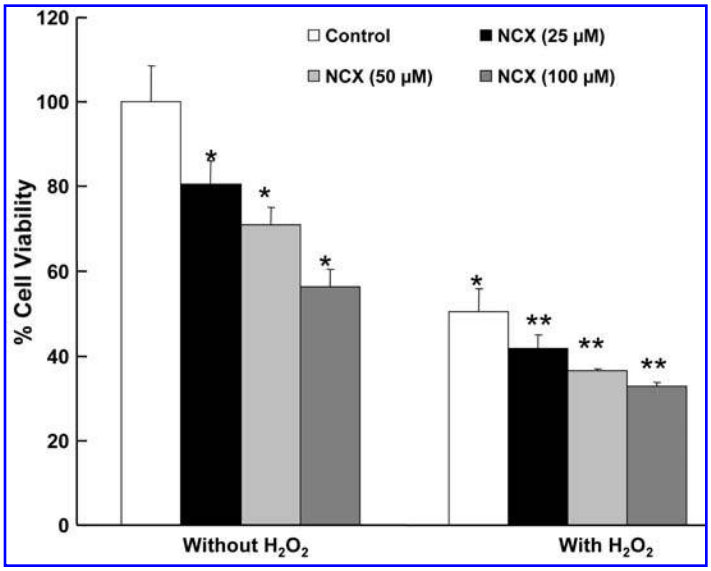


**FIG. 3. NCX-4016 enhances $H_2O_2$-induced loss of cell viability.** BLMVECs (~95% confluence; $5 \times 10^5$ cells) grown in 35-mm dishes were treated with different concentrations of NCX-4016 (25, 50, and 100 $\mu M$) alone or $H_2O_2$ (100 $\mu M$) alone or $H_2O_2$ (100 $\mu M$) + NCX-4016 (25, 50, and 100 $\mu M$) for 2 h in MEM under a humidified atmosphere of 95% air/5% $CO_2$ at 37°C. At the end of exposure, the cell viability was determined spectrophotometrically by using the Alamar blue reagent, as outlined in Materials and Methods. Results are expressed as mean ± SD from three independent experiments in triplicate. *$p < 0.05$ *versus* vehicle-treated controls. **$p < 0.05$ *versus* $H_2O_2$-treated cells.

in cells treated with either $H_2O_2$ alone or NCX-4016 alone (see Fig. 3). This clearly demonstrated that the combined treatment of cells with NCX-4016 and $H_2O_2$ was more potent in causing the loss of cell viability, as compared with the same caused by the exposure of cells to either NCX-4016 alone or $H_2O_2$ alone at the same concentrations. These results revealed that NCX-4016, in a dose-dependent manner, significantly enhanced the $H_2O_2$-induced loss of cell viability in BLMVECs.

### *NAC attenuates NCX-4016-induced loss of cell viability*

We further investigated whether thiol-protectants such as NAC would attenuate the NCX-4016-induced loss of cell viability in the ECs. BLMVECs were pretreated with 5 m*M* NAC for 2 h, after which they were exposed to NCX-4016 (100 $\mu M$) for 2 h, and then the cell viability was determined. As shown in Fig. 4, the NCX-4016 caused a significant loss of cell viability that was significantly and completely attenuated by NAC pretreatment in BLMVECs, as compared with the cells exposed to NCX-4016 alone. A slight increase (12%) in cell viability was noticed in the cells exposed to NAC (5 m*M*) + NCX-4016 (100 $\mu M$), which might be due to the NAC-mediated enhanced thiol protection of the redox system in the cells. The data suggest that the intracellular thiol-redox state was involved in the NCX-4016-induced loss of EC viability.

### *NCX-4016 releases NO in ECs*

To confirm that NCX-4016 was able to release NO in ECs, we tried to detect intracellular NO generation in BLMVECs after treatment of cells with NCX-4016 (100 $\mu M$) for 2 h as analyzed by fluorescence microscopy coupled with the NO-specific intracellular fluorophore, DAF-FM. As shown in Fig. 5, the fluorescence intensity of intracellular DAF-FM was strikingly greater in cells exposed to NCX-4016 as compared with that in the vehicle-treated control cells. Quantitative analysis of the fluorescence intensity of the NO-reactive fluorophore revealed a significant (4.7-fold) increase in the NO-enhanced fluorescence of the intracellular DAF-FM on exposure of the cells to NCX-4016 (Fig. 5B). These results confirm that NCX-4016 released intracellular NO in the BLMVECs.

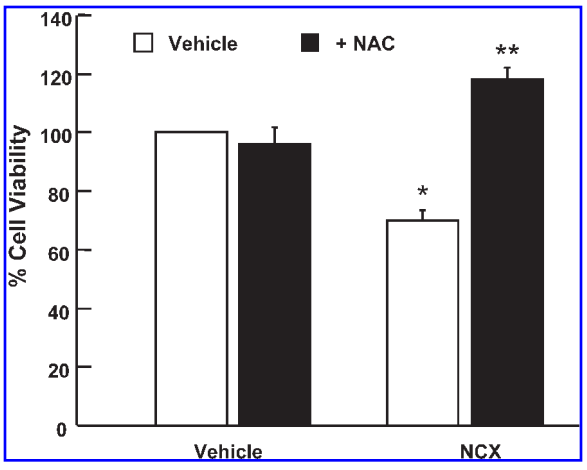


**FIG. 4. NAC attenuates NCX-4016-induced loss of cell viability.** BLMVECs (~95% confluence; $5 \times 10^5$ cells) grown in 35-mm dishes were pretreated with MEM without or with NAC (5 m*M*) for 2 h, after which they were treated with NCX-4016 (100 $\mu M$) for 2 h in MEM under a humidified atmosphere of 95% air/5% $CO_2$ at 37°C. At the end of exposure, the cell viability was determined spectrophotometrically by using the Alamar blue reagent, as outlined in Materials and Methods. Results are expressed as mean ± SD from three independent experiments in triplicate. *$p < 0.05$ *versus* vehicle-treated controls; **$p < 0.05$ *versus* NCX-4016-treated cells.

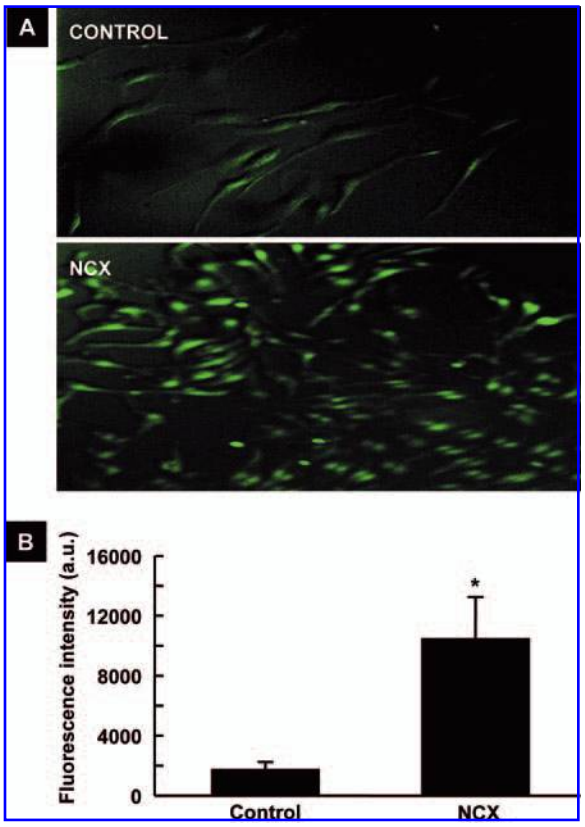


**FIG. 5. NCX-4016 releases NO in ECs.** BLMVECs grown on glass coverslips (~70% confluence) were preloaded with DAF-FM (10 $\mu M$) for 30 min, after which, they were treated with NCX-4016 (100 $\mu M$) for 2 h. At the end of incubation, cells were examined under fluorescence microscope (490-nm excitation and 520-nm emission), as described in Materials and Methods, to visualize intracellular NO generation. Each picture is a representation of three independent experiments conducted under identical conditions (**A**). Fluorescence intensity (as an index of NO generation) of control and NCX-4016-treated cells was quantified as described in Materials and Methods (**B**). Results are expressed as mean ± SD from four independent experiments in triplicate. *$p < 0.05$ *versus* vehicle-treated controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

### *NCX-4016 suppresses EC oxygen consumption*

As oxygen consumption is an important metabolic index of cell viability, we measured the oxygen consumption by

BLMVECs treated with NCX-4016 (100 $\mu M$) or $H_2O_2$ (100 $\mu M$) or a combination of equimolar (100 $\mu M$) NCX-4016 + $H_2O_2$ using the EPR oximetry method. Measurement of oxygen consumption by cells would also be a complementary assay of cell viability to the Alamar blue redox-dependent cell viability assay. As shown in Fig. 6, oxygen consumption by the cells immediately after the treatment with NCX-4016 was marginally, but significantly, lower as compared with that in the vehicle-treated control cells. However, oxygen consumption by BLMVECs after 1- and 2-h treatments with NCX-4016 was markedly and significantly suppressed as compared with that in the vehicle-treated control cells. Treatment of cells with $H_2O_2$ for 2 h significantly suppressed oxygen consumption by the ECs that was further significantly lowered by co-treatment of cells with NCX-4016. These results showed that NCX-4016, with increase in exposure time, suppressed EC oxygen consumption, and $H_2O_2$-induced suppression of the same was further exacerbated by the treatment of cells with NCX-4016.

### *NCX-4016 induces EC barrier dysfunction*

Dysfunction of the EC barrier property is often measured by TER in cultured EC monolayers. Decrease in TER reflects the loss or dysfunction of the barrier property, which can also be used as an index of loss of cell viability and cytoskeletal alterations. Therefore, we measured the TER in BLMVEC monolayers after treatment with NCX-4016 (100 $\mu M$) or $H_2O_2$ (100 $\mu M$) or an equimolar mixture (100 $\mu M$) of NCX-4016 + $H_2O_2$ by using the ECIS technique. As illustrated in Fig. 7, NCX-4016, with an increase in exposure time, markedly and significantly decreased the endothelial TER. Although $H_2O_2$ marginally caused a decrease in TER of the BLMVEC monolayer with time, it was further reduced by the co-treatment with NCX-4016. These results revealed that NCX-4016 caused a significant decrease in endothelial TER and further enhanced $H_2O_2$-induced decrease of TER, suggesting that NCX-4016 induced endothelial barrier dysfunction. Furthermore, the observed decrease in TER in BLMVEC monolayer confirmed our findings of this study that NCX-4016 induced loss of cell viability and suppression of cellular oxygen consumption as an additional index of cell-viability measurement. The observed NCX-4016-induced loss of endothelial TER is suggestive of cytoskeletal alterations.

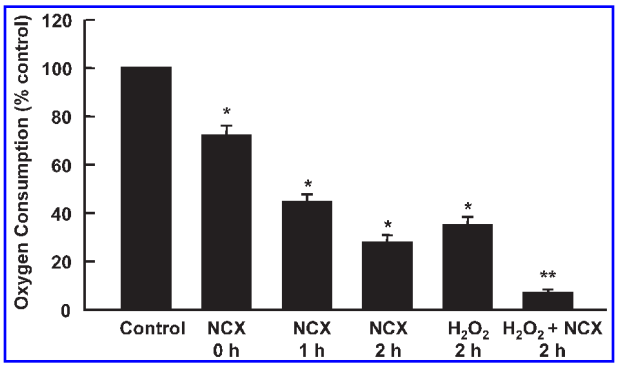


**FIG. 6. NCX-4016 suppresses EC oxygen consumption.** BLMVECs ($5 \times 10^5$ cells) were treated with NCX-4016 (100 $\mu M$) for 1 and 2 h or with $H_2O_2$ (100 $\mu M$) for 2 h or with NCX-4016 (100 $\mu M$) + $H_2O_2$ (100 $\mu M$) for 2 h, after which cellular oxygen consumption was measured by EPR oximetry by using LiNc-BuO probe, as described in Materials and Methods. Under identical conditions, oxygen consumption by cells without treatment and immediately after treatment with NCX-4016 (100 $\mu M$) was measured. Cellular oxygen consumption was expressed as $\%O_2$ consumption, normalized to untreated control cells. Results are expressed as mean ± SD from three independent experiments in triplicate. $^*p < 0.05$ *versus* vehicle-treated controls; $^{**}p < 0.05$ *versus* NCX-4016-treated cells.

### *NCX-4016 induces endothelial actin cytoskeletal reorganization*

As was revealed from this study, NCX-4016 caused a decrease in TER in BLMVEC monolayer (an index of barrier dysfunction). We examined the formation of actin stress fibers that would reflect the cytoskeletal reorganization. As depicted in Fig. 8, NCX-4016 induced the formation of actin stress in BLMVECs in a dose-dependent fashion as compared with the vehicle-treated cells. It also was evident that NCX-4016 induced changes in cell morphology with distinctive paracellular gaps, indicative of EC barrier dysfunction. The data clearly showed that NCX-4016 induced actin stress formation, actin cytoskeletal reorganization, and barrier property alteration in BLMVECs.

### *NCX-4016 inhibits angiogenesis in vitro*

As the aforementioned studies revealed that NCX-4016 induced loss of viability, suppressed cellular oxygen consumption, barrier dysfunction, and actin cytoskeletal rearrangement in BLMVECs, we investigated the effect of NCX-4016 on the growth factor-mediated angiogenesis in HUVECs and BLMVECs *in vitro*. After starvation, HUVECs ($1.5 \times 10^4$ cells/plate) were plated onto Matrigel matrix and cultured for 10 h in M200 medium alone; in Rich M200 medium supplemented with FBS, bFGF, heparin, EGF, and PSA; or Rich M200 medium additionally supplemented with NCX-4016 (25, 50, or 100 $\mu M$). BLMVECs ($1.5 \times 10^4$ cells/plate), after starvation, were plated onto Matrigel matrix and cultured for 10 h in MEM alone; complete BLMVEC MEM containing FBS, antibiotics, nonessential amino acids, and endothelial growth supplement; or complete BLMVEC MEM containing NCX-4016 (25, 50, or 100 $\mu M$). Afterward, tubes that grew from individual cells were blindly counted. Both qualitative observation (Figs. 9 and 10) and quantitative analysis (Figs. 9B and 10B) showed that NCX-4016 significantly inhibited the tube-formation ability (angiogenesis) of both HUVECs and BLMVECs *in vitro* at 25, 50, and 100 $\mu M$ as compared with that exhibited by HUVECs and BLMVECs cultured in Rich medium or complete BLMVEC MEM alone, respectively. These results suggested the antiangiogenic property of NCX-4016.

## DISCUSSION

The results of the current study showed that NCX-4016 induced the loss of redox-dependent cell viability in a dose- and

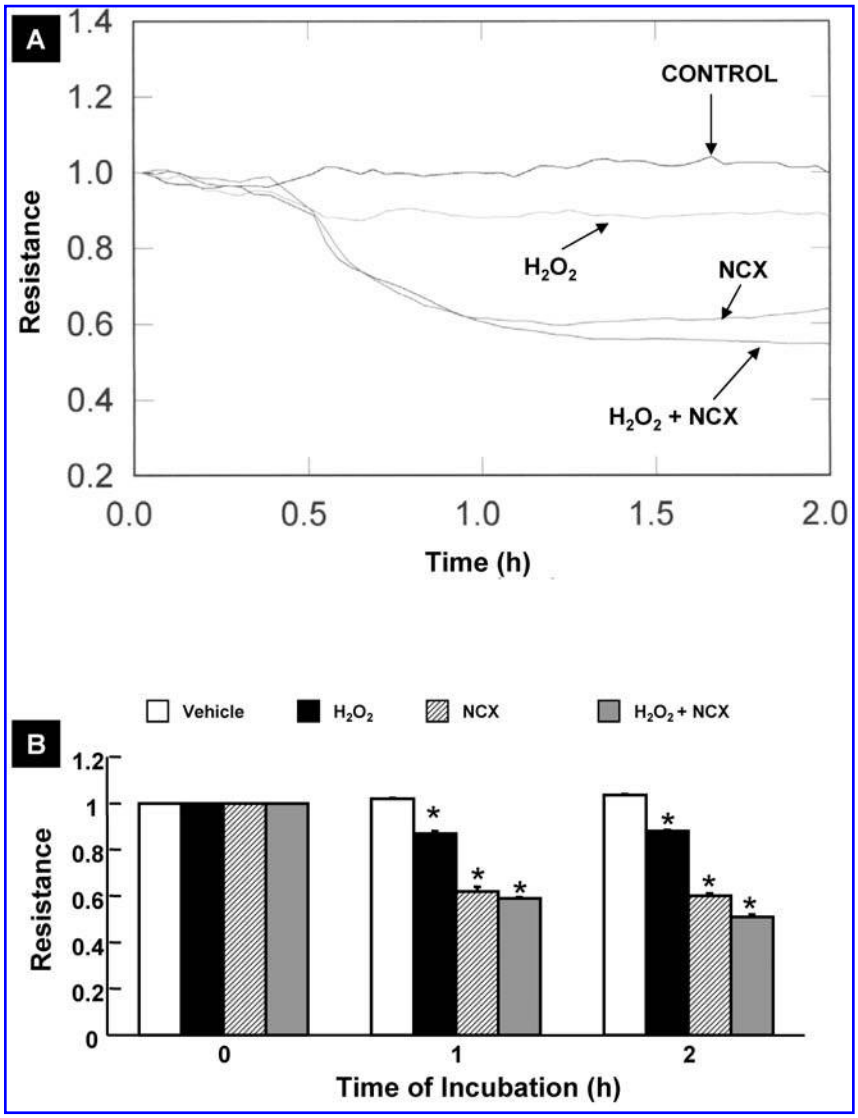


**FIG. 7. NCX-4016 induces EC barrier dysfunction.** BLMVEC monolayers were cultured in complete MEM on gold electrodes and exposed to NCX-4016 (100 $\mu M$) or $H_2O_2$ (100 $\mu M$) or NCX-4016 (100 $\mu M$) + $H_2O_2$ (100 $\mu M$) for desired length of time in a humidified atmosphere of 95% air/5% $CO_2$ at 37°C, and the TER was measured continuously in an ECIS system, as described in Materials and Methods. Shown is a representative tracing of three independent experiments conducted under identical conditions (**A**). Normalized resistance values of vehicle-treated control and NCX-4016-, $H_2O_2$-, and NCX-4016 + $H_2O_2$-treated monolayers at 0, 1, and 2 h of treatment were mean ± SD from three independent ECIS experiments in triplicate (**B**). *$p < 0.05$ versus vehicle-treated controls.

time-dependent fashion, suppression of cellular oxygen consumption, decrease in TER (barrier dysfunction), actin cytoskeletal reorganization, and inhibition of angiogenesis in the vascular ECs in culture. NCX-4016 belongs to a new class of NSAIDS capable of releasing NO (3). Esterases *in vivo* have been involved in cleaving the aspirin moiety from the substituted benzene spacer–NO complex of NCX-4016, thus causing a slow release of NO from the NO-releasing group of the NO donor (27). NCX-4016 is metabolized by esterases in the hepatocytes and plasma to salicylic acid and 3-(nitroxymethyl)phenol, which is rapidly metabolized to 3-hydroxybenzylalcohol and NO (5). Although the *in vivo* metabolism of these NO-NSAIDS has been reasonably well established in a rat model, the precise mechanism by which NO is released from the nitro moiety has yet to be disclosed (3, 18). Earlier, with the EPR spectroscopy, we showed that NO is released from NCX-4016 in the human ovarian cancer cell line (3). Generation of NO from NCX-4016 in HUVECs has been shown (16, 56). By using fluorescence probe, we also detected NO generation in BLMVECs treated with NCX-4016, further substantiating that NO is released intracellularly in the vascular ECs from NCX-4016.

Based on the reports made by several investigators in both *in vitro* and *in vivo* systems, we chose the 50, 100, and 500 $\mu M$ concentrations of NCX-4016. The $IC_{50}$ of NCX-4016 to attain 50% growth inhibition of human colon adenocarcinoma cell lines has been reported to be in the range of 165–250 $\mu M$ (51). In the investigation of the protective action of NCX-4016 against endothelial apoptosis by modulating mitochondrial function, endothelial cells have been treated with NCX-4016 up to 500 $\mu M$ from 1 to 8 h (16). In our recent study to establish reversal of cisplatin sensitivity in recurrent human ovarian cancer cells by NCX-4016, we used NCX-4016 at concentrations ranging from 1 to 500 $\mu M$ for 6 h (3). Therefore, the concentrations of NCX-4016 used in our current study were in the ranges used by other investigators.

Cytotoxicity of NO to vascular ECs is well established (6, 19, 23, 26, 42). The current study demonstrated that NCX-4016

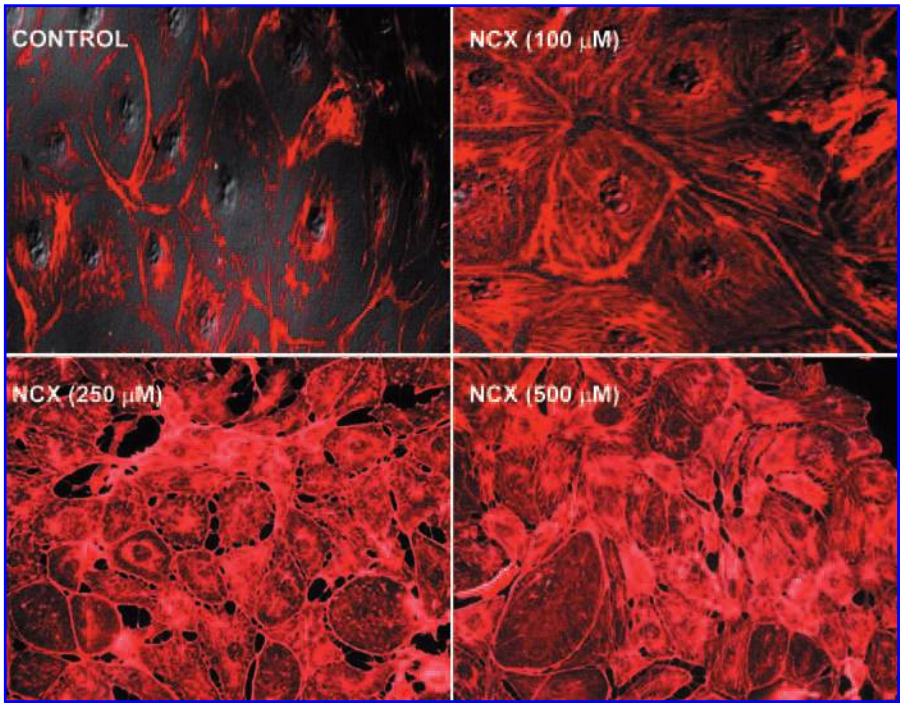


**FIG. 8. NCX-4016 induces endothelial actin cytoskeletal rearrangement.** BLMVECs grown on glass coverslips (~70% confluence) were treated with different concentrations of NCX-4016 (100, 250, and 500 $\mu M$) for 2 h and then stained with rhodamine-phalloidin, and examined under fluorescence microscope (555-nm excitation) to visualize actin stress fibers, as described in Materials and Methods. Each micrograph is a representative of three independent experiments conducted under identical conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars)

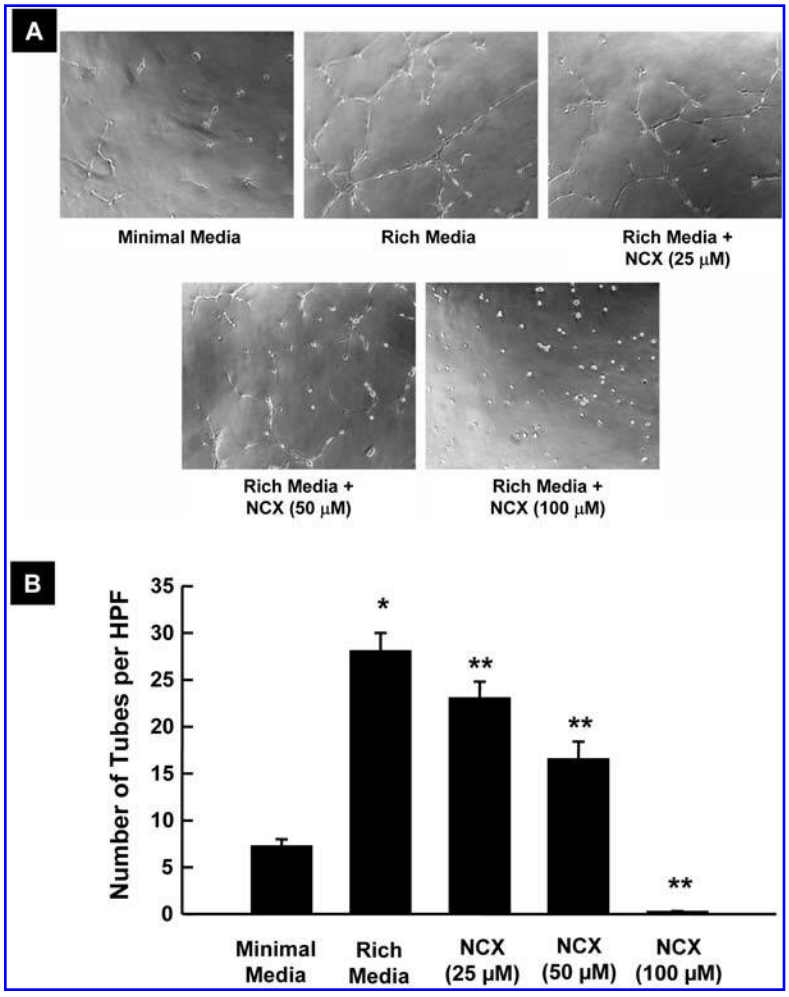


**FIG. 9. NCX-4016 inhibits angiogenesis *in vitro*.** HUVECs ($1.5 \times 10^4$ cells) were starved for 8 h, plated onto growth-factor-reduced Matrigel matrix, and cultured in M200 media alone (minimal medium), Rich M200 medium supplemented with FBS, bFGF, heparin, EGF, and PSA (Rich media), or Rich M200 medium additionally supplemented with different concentrations of NCX-4016 (25, 50, and 100 $\mu M$) for 10 h in a humidified atmosphere of 95% air/5% $CO_2$ at 37°C. Extent of angiogenesis *in vitro* was examined, as described in Materials and Methods. Tubes that formed from individual HUVECs were photographed (**A**) and (**B**), blindly counted, and quantified. Those cells cultured in Rich medium alone had significantly greater number of tubes formed than did those among cells cultured in minimal medium (*$p < 0.001$), whereas HUVECs cultured in presence of 50 or 100 $\mu M$ NCX-4016 had tube formation inhibited relative to those grown in Rich medium alone (**$p < 0.01$ and **$p < 0.001$, respectively).

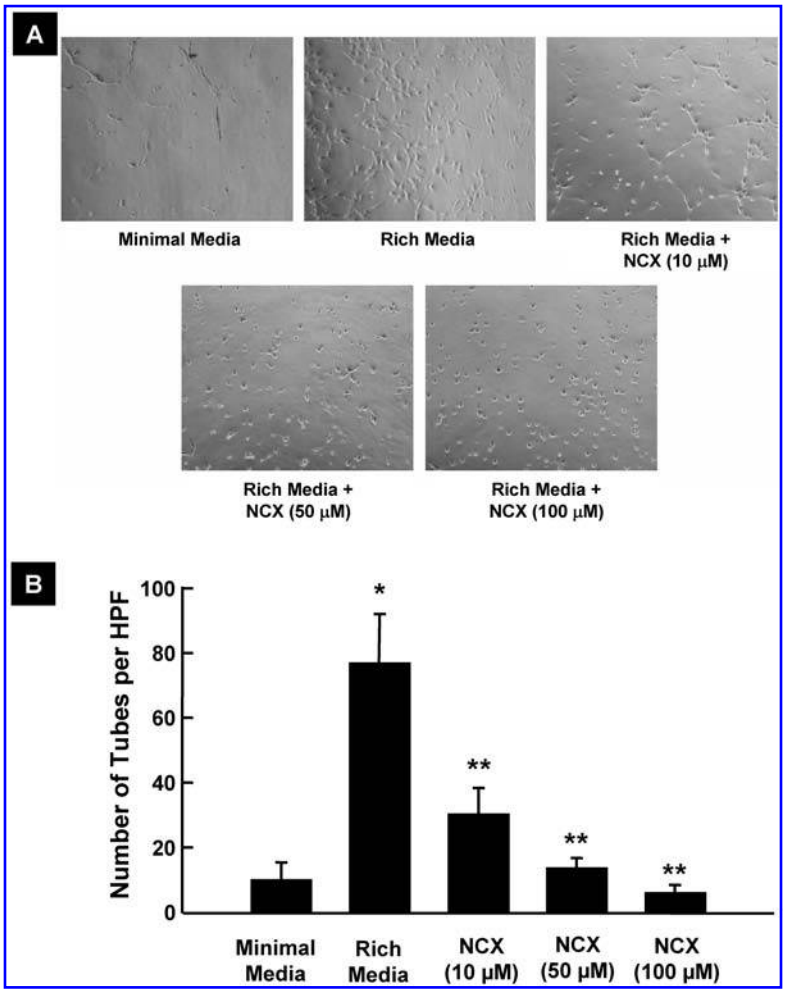


**FIG. 10. NCX-4016 inhibits angiogenesis *in vitro*.** BLMVECs ($1.5 \times 10^4$ cells) were starved for 4 h, plated onto growth-factor-reduced Matrigel matrix, and cultured in MEM alone, complete BLMVEC medium supplemented with FBS, antibiotics, nonessential amino acids, and endothelial growth supplement, or complete BLMVEC medium additionally supplemented with different concentrations of NCX-4016 (25, 50, and 100 $\mu M$) for 10 h in a humidified atmosphere of 95% air/5% $CO_2$ at 37°C. Extent of angiogenesis *in vitro* was examined as described in Materials and Methods. Tubes that formed from individual BLMVECs were photographed (**A**) and (**B**), blindly counted, and quantified. Those cells cultured in complete BLMVEC medium alone had significantly greater numbers of tubes formed than did those among cells cultured in minimal medium ($^*p < 0.001$), whereas BLMVECs cultured in the presence of 50 or 100 $\mu M$ NCX-4016 had tube formation inhibited relative to those grown in complete BLMVEC medium alone ($^{**}p < 0.01$ and $^{**}p < 0.001$, respectively).

induced the loss of viability in BLMVECs, and aspirin alone failed to cause such an effect, further suggesting that the aspirin moiety of NCX-4016 was not responsible for the observed loss of cell viability. The attenuation of loss of cell viability in conjunction with the detection of NO generation in ECs on treatment with NCX-4016, as noticed in the present study, clearly demonstrates the role of NO generated from the NO donor in exerting the loss of viability in ECs. Our present data reveal that different NO donors (SNAP, spermine NONOate, and isosorbide dinitrite) significantly decrease the viability of BLMVECs but are less effective in causing the same when compared with NCX-4016 at the same concentration. This could be attributed to the slow intracellular NO release by NCX-4016, on enzymatic conversion in ECs, rendering the cells exposed to sufficient doses of biologically reactive NO, whereas the NO released instantaneously in bolus concentrations immediately after addition of the NO donors (SNAP and spermine NONOate) in the extracellular environment, might have diffused out without efficiently reaching the intracellular compartments. Furthermore, the attenuation of NCX-4016-mediated loss of viability in BLMVECs by the thiol-protectant, NAC, as noticed in the current study, emphasized the involvement of nitrosative stress in NCX-4016-mediated loss of redox-dependent cell viability. In our study, the cells were preloaded with NAC for 2 h, the NAC-containing medium was removed, and then cells were exposed to NCX-4016. NAC is believed to enhance the cellular soluble thiols such as GSH. Some intracellular NAC also may be present. Therefore, it is conceivable that NAC may (a) offer protection by replenishing loss of cellular thiols (*e.g.*, GSH) caused by NCX-4016-generated NO, and also (b) by reacting with NO to form *S*-nitrosothiols, thus quenching NO and protecting the cells from NCX-4016-released NO. Both of these mechanisms could have operated in our model. This is further supported by the reports that NO induces nitrosative stress in biologic systems by attacking the thiol-redox system (59). In our study, we did not observe any effect of NAC on the intracellular NO release from NCX-4016 (data not shown), which further suggests that the effect of NAC could be at specific cellular NO targets (*e.g.*, thiol protection and regeneration) as opposed to its NO-quenching property. This was further supported by our observation that NCX-4016 did not alter the levels of GSH in ECs (data not shown). It is important to emphasize that the formation of peroxynitrite in ECs is not ruled out. However, it has been shown that NCX-4016, when orally administered, promotes reparative angiogenesis and prevents apoptosis and oxidative stress in an *in vivo* mouse model of peripheral ischemia (12). NCX-4016 has also been shown to protect endothelial cells against apoptosis through modulation of

mitochondrial function (16). Both of these studies show the endothelial protective actions of NCX-4016 in contrast to our current findings, which revealed that the NO donor induced loss of EC viability. Nevertheless, it should be emphasized that NCX-4016 may act as a cytoprotective or cytotoxic agent in ECs either *in vitro* or *in vivo*.

Reports have been made that NO offers protection against peroxide-mediated toxicity in lung fibroblasts and aortic ECs, and inhibition of oxidized low-density lipoprotein toxicity to aortic ECs (11, 40, 50, 57, 58). However, our current findings demonstrate that $H_2O_2$-induced loss of redox-dependent EC viability was not attenuated but enhanced by the NO donor, NCX-4016. Several mechanisms have been proposed to explain the cytotoxicity of NO generated by the NO donors; it has been proposed that NO enhances the cytotoxicity of ROS (*e.g.*, $H_2O_2$), which is a strong oxidant (58). Adhering to this proposal, we surmise that the NCX-4016-mediated increase of $H_2O_2$-induced loss of redox-dependent EC cell viability, as demonstrated by the current study, might possibly be due to the formation of cytotoxic reactive species generated from the intracellular reactions between the NO released from NCX-4016 and $H_2O_2$. Nevertheless, in our current study, we did not observe the protection of peroxide-induced loss of cell viability by the NO donor, NCX-4016, but observed the exacerbation of $H_2O_2$-induced loss of cell viability by the combined treatment with NCX-4016. This is further supported by the suggestion that the toxicity and protection exhibited by NO will be dictated by myriad intracellular factors such as the target sites and relative concentrations and diffusion distances of each reactive species (45).

In the present study, we observed that NCX-4016 diminished oxygen consumption by ECs and also further reduced the $H_2O_2$-induced decrease of oxygen consumption by the cells. In a study with HUVECs, it was demonstrated that the NO donors, including NCX-4016, caused a significant reduction in cellular oxygen consumption and modulation of mitochondrial function (16). Taken together, we suggest that NCX-4016 might have acted at the mitochondrial level in ECs, thus leading to decreased cellular oxygen consumption, as observed in our current study. Hence, the NCX-4016–induced decrease in EC oxygen consumption could have contributed to the loss of cell viability in our present study. NO generated by NCX-4016 apparently caused the decrease in EC oxygen consumption.

Oxidants have been shown to cause decrease in TER and loss of barrier function in ECs, which are tightly intertwined with the oxidant-mediated cytoskeletal reorganization, including the actin stress fiber formation (48, 52, 54). Decrease in TER and barrier dysfunction can also be used as markers for stress-induced morphologic alterations (alterations in tight junctions) and loss of cell viability. Peroxynitrite ($ONOO^-$) and 3-morpholinosydnonimine (SIN-1) have been shown to induce barrier dysfunction, increased paracellular gaps, and actin cytoskeletal reorganization (stress fiber formation) in pulmonary artery EC monolayers (25). This study clearly established that reactive nitrogen species ($ONOO^-$) and NO are potent candidates in inducing EC barrier dysfunction and actin cytoskeletal reorganization. Along these lines, our findings of the current study demonstrated that the NO donor, NCX-4016, induced loss of barrier function and actin cytoskeletal reorganization in ECs, further suggesting that NO released from NCX-4016 could be responsible for the observed events. In the current study, NAC treatment did not offer a protective effect on either NCX-4016-induced barrier dysfunction or on NCX-4016-induced inhibition of angiogenesis in both HUVECs and BLMVECs (data not shown). However, NAC treatment alone resulted in a marked decrease in the TER in EC monolayers and inhibition of tube formation (angiogenesis) in both HUVECs and BLMVECs, which could be attributed to the prooxidant nature of NAC, leading to the formation of reactive species on prolonged incubation of cells with the redox-active thiol antioxidant. Mechanical connections and cellular polarities are maintained by cell-to-cell junctions of ECs, which are crucial players in processes such as angiogenesis (46). Actin cytoskeletal dynamics mediate the cellular motility and migration (15). Therefore, barrier property reflects the vigor of EC tight junctions, as tight junctions are essential for angiogenesis. Proliferation of ECs is vital for angiogenesis, and agents that can cause endothelial cytotoxicity have the ability to either inhibit or arrest angiogenesis. Hence, from the present study, it could be suggested that the loss of viability caused by NCX-4016 in ECs could have contributed to the inhibition of angiogenesis. Drugs such as rosaglitazone have been shown to cause inhibition of endothelial proliferation and angiogenesis, which apparently is associated with disorganization of the actin cytoskeleton (47). These studies have clearly demonstrated that the actin cytoskeleton of ECs plays an important role in cell migration and angiogenesis, and reorganization of the actin cytoskeletal elements will lead to disruption of angiogenesis. Inflammation has been identified as a key process in cancer development, and inflammatory cells and regulators have been speculated to participate in angiogenesis during tumorigenesis (29). Angiogenesis is also featured as a prime requirement for the growth of solid tumors (24). COX-2 is emerging as a key modulator in tumorigenesis and angiogenesis (17). NCX-4016 has been shown to act as an antiinflammatory agent (1), further suggesting that the NO-NSAID may very well effectively interrupt angiogenesis in solid tumors, in addition to acting as an anticancer agent. Therefore, the NCX-4016-induced actin cytoskeletal reorganization, as observed in the current study, could have caused profound effects on the EC motility and cell-to-cell adhesion that are vital for processes including vascular permeability and angiogenesis.

Having observed the NCX-4016-induced loss of cell viability, decrease in oxygen consumption, barrier dysfunction, and actin cytoskeletal reorganization in ECs, we studied the effect of NCX-4016 on the growth factor-mediated angiogenesis in ECs *in vitro*. Our results clearly demonstrated that NCX-4016 was an inhibitor of angiogenesis. Therefore, NCX-4016 is a potential antiangiogenic NO-NSAID to arrest vascularization of tumors, in addition to acting as a cytotoxic agent against neoplastic cells. Nevertheless, NCX-4016 can also be cytotoxic to the normal endothelium when used to arrest tumor angiogenesis and in the treatment of cardiovascular diseases. This limitation could be addressed if the sensitivities of endothelia of normal and tumor blood vessels to NCX-4016, route of delivery of NCX-4016 to the tumor endothelium, and safer and effective pharmacologic doses of NCX-4016 are established for tumor antiangiogenic and cardiovascular therapies.

## ACKOWLEDGMENTS

This work was supported by NIH grant CA102264. V. K. Kutala was on a sabbatical from Nizam's Institute of Medical Sciences, Hyderabad, India.

## ABBREVIATIONS

bFGF, bovine fibroblast growth factor; BLMVECs, bovine lung microvascular endothelial cells; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein; DAPI, 4,6-diamidino 2-phenyl-indole dihydrochloride; EPR, electron spin resonance spectroscopy; $H_2O_2$, hydrogen peroxide; hEGF, human epidermal growth factor; HUVECs, human umbilical vein endothelial cells; LiNc-BuO, lithium octa-*n*-butoxy-naphthalocyanine; NCX-4016, [2-(acetyloxy)benzoic acid 3-(nitrooxymethyl) phenyl ester]; NO, nitric oxide; NSAIDs, nonsteroidal antiinflammatory drugs; $ONOO^-$, peroxynitrite; PSA, prostate specific antigen; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; ROS, reactive oxygen species; TER, transendothelial cell electrical resistance.

## REFERENCES

1. Bolla M, Momi S, Gresele P, and Del Soldato P. Nitric oxide-donating aspirin (NCX 4016): an overview of its pharmacological properties and clinical perspectives. *Eur J Clin Pharmacol* 62(suppl 13): 145–154, 2006.
2. Boyd CS and Cadenas E. Nitric oxide and cell signaling pathways in mitochondrial-dependent apoptosis. *Biol Chem* 383: 411–423, 2002.
3. Bratasz A, Weir NM, Parinandi NL, Zweier JL, Sridhar R, Ignarro LJ, and Kuppusamy P. Reversal to cisplatin sensitivity in recurrent human ovarian cancer cells by NCX-4016, a nitro derivative of aspirin. *Proc Natl Acad Sci U S A* 103: 3914–3919, 2006.
4. Brune B, von Knethen A, and Sandau KB. Nitric oxide (NO): an effector of apoptosis. *Cell Death Differ* 6: 969–975, 1999.
5. Carini M, Aldini G, Orioli M, and Maffei Facino R. In vitro metabolism of a nitroderivative of acetylsalicylic acid (NCX4016) by rat liver: LC and LC-MS studies. *J Pharm Biomed Anal* 29: 1061–1071, 2002.
6. Chung HY, Yokozawa T, Kim MS, Lee KH, Kim KW, Yang R, and Choi JH. The mechanism of nitric oxide and/or superoxide cytotoxicity in endothelial cells. *Exp Toxicol Pathol* 52: 227–233, 2000.
7. Cifone MG, Cironi L, Meccia MA, Roncaioli P, Festuccia C, De Nuntiis G, D'Alo S, and Santoni A. Role of nitric oxide in cell-mediated tumor cytotoxicity. *Adv Neuroimmunol* 5: 443–461, 1995.
8. Corazzi T, Leone M, Roberti R, Del Soldato P, and Gresele P. Effect of nitric oxide-donating agents on human monocyte cyclooxygenase-2. *Biochem Biophys Res Commun* 311: 897–903, 2003.
9. Dalgleish AG and O'Byrne K. Inflammation and cancer: the role of the immune response and angiogenesis. *Cancer Treat Res* 130: 1–38, 2006.
10. De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, Del Soldato P, Melani C, Guiducci C, Colombo MP, Iezzi M, Musiani P, Zanovello P, and Bronte V. Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. *Proc Natl Acad Sci U S A* 102: 4185–4190, 2005.
11. Degnim AC, Morrow SE, Ku J, Zar HA, and Nakayama DK. Nitric oxide inhibits peroxide-mediated endothelial toxicity. *J Surg Res* 75: 127–134, 1998.
12. Emanueli C, Van Linthout S, Salis MB, Monopoli A, Del Soldato P, Ongini E, and Madeddu P. Nitric oxide-releasing aspirin derivative, NCX 4016, promotes reparative angiogenesis and prevents apoptosis and oxidative stress in a mouse model of peripheral ischemia. *Arterioscler Thromb Vasc Biol* 24: 2082–2087, 2004.
13. Eubank TD, Galloway M, Montague CM, Waldman WJ, and Marsh CB. M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. *J Immunol* 171: 2637–2643, 2003.
14. Eubank TD, Roberts R, Galloway M, Wang Y, Cohn DE, and Marsh CB. GM-CSF induces expression of soluble VEGF receptor-1 from human monocytes and inhibits angiogenesis in mice. *Immunity* 21: 831–842, 2004.
15. Fenteany G and Zhu S. Small-molecule inhibitors of actin dynamics and cell motility. *Curr Top Med Chem* 3: 593–616, 2003.
16. Fiorucci S, Mencarelli A, Meneguzzi A, Lechi A, Morelli A, del Soldato P, and Minuz P. NCX-4016 (NO-aspirin) inhibits lipopolysaccharide-induced tissue factor expression in vivo: role of nitric oxide. *Circulation* 106: 3120–3125, 2002.
17. Gately S and Kerbel R. Therapeutic potential of selective cyclooxygenase-2 inhibitors in the management of tumor angiogenesis. *Prog Exp Tumor Res* 37: 179–192, 2003.
18. Grosser N and Schroder H. A common pathway for nitric oxide release from NO-aspirin and glyceryl trinitrate. *Biochem Biophys Res Commun* 274: 255–258, 2000.
19. Hashida K, Sasaki K, and Makino N. Interactions of nitric oxide and oxygen in cytotoxicity: proliferation and antioxidant enzyme activities of endothelial cells in culture. *Free Radic Res* 33: 147–156, 2000.
20. Ignarro LJ, Buga GM, Wei LH, Bauer PM, Wu G, and del Soldato P. Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. *Proc Natl Acad Sci U S A* 98: 4202–4208, 2001.
21. Ignarro LJ, Buga GM, Wood KS, Byrns RE, and Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 84: 9265–9269, 1987.
22. Ignarro LJ, Napoli C, and Loscalzo J. Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: an overview. *Circ Res* 90: 21–28, 2002.
23. Ioannidis I, Batz M, Paul T, Korth HG, Sustmann R, and De Groot H. Enhanced release of nitric oxide causes increased cytotoxicity of *S*-nitroso-*N*-acetyl-DL-penicillamine and sodium nitroprusside under hypoxic conditions. *Biochem J* 318: 789–795, 1996.
24. Kanda S, Miyata Y, and Kanetake H. Current status and perspective of antiangiogenic therapy for cancer: urinary cancer. *Int J Clin Oncol* 11: 90–107, 2006.
25. Nepler JL Jr, Taher LN, Gupta MP, Patterson C, Pavalko F, Ober MD, and Hart CM. Peroxynitrite causes endothelial cell monolayer barrier dysfunction. *Am J Physiol Cell Physiol* 281: C1064–C1075, 2001.
26. Law A, Wu J, Zeng LH, and Wu TW. Aortic endothelial cells damaged by a nitric oxide donor and protected by flavonoids. *Life Sci* 64: PL199–PL204, 1999.
27. Levin RI. Theriac found? Nitric oxide-aspirin and the search for the universal cure. *J Am Coll Cardiol* 44: 642–643, 2004.
28. Liu L and Stamler JS. NO: an inhibitor of cell death. *Cell Death Differ* 6: 937–942, 1999.
29. Lu H, Ouyang W, and Huang C. Inflammation, a key event in cancer development. *Mol Cancer Res* 4: 221–233, 2006.
30. Muscara MN and Wallace JL. Nitric oxide; V: therapeutic potential of nitric oxide donors and inhibitors. *Am J Physiol* 276: G1313–G1316, 1999.
31. Nakayama GR, Caton MC, Nova MP, and Parandoosh Z. Assessment of the Alamar Blue assay for cellular growth and viability in vitro. *J Immunol Methods* 204: 205–208, 1997.
32. Napoli C, Aldini G, Wallace JL, de Nigris F, Maffei R, Abete P, Bonaduce D, Condorelli G, Rengo F, Sica V, D'Armiento FP, Mignogna C, de Rosa G, Condorelli M, Lerman LO, and Ignarro LJ. Efficacy and age-related effects of nitric oxide-releasing aspirin on experimental restenosis. *Proc Natl Acad Sci U S A* 99: 1689–1694, 2002.

33. Nieder C, Wiedenmann N, Andratschke N, and Molls M. Current status of angiogenesis inhibitors combined with radiation therapy. *Cancer Treat Rev* 2006.
34. O'Brien J, Wilson I, Orton T, and Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem* 267(17): 5421–5426, 2000.
35. O'Donnell VB and Freeman BA. Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease. *Circ Res* 88: 12–21, 2001.
36. Ongini E, Impagnatiello F, Bonazzi A, Guzzetta M, Govoni M, Monopoli A, Del Soldato P, and Ignarro LJ. Nitric oxide (NO)-releasing statin derivatives, a class of drugs showing enhanced antiproliferative and antiinflammatory properties. *Proc Natl Acad Sci U S A* 101: 8497–8502, 2004.
37. Pandian RP, Kutala VK, Liaugminas A, Parinandi NL, and Kuppusamy P. Lipopolysaccharide-induced alterations in oxygen consumption and radical generation in endothelial cells. *Mol Cell Biochem* 278: 119–127, 2005.
38. Patel RP, Levonen A, Crawford JH, and Darley-Usmar VM. Mechanisms of the pro- and anti-oxidant actions of nitric oxide in atherosclerosis. *Cardiovasc Res* 47: 465–474, 2000.
39. Patel RP, McAndrew J, Sellak H, White CR, Jo H, Freeman BA, and Darley-Usmar VM. Biological aspects of reactive nitrogen species. *Biochim Biophys Acta* 1411: 385–400, 1999.
40. Polte T, Oberle S, and Schroder H. Nitric oxide protects endothelial cells from tumor necrosis factor-alpha-mediated cytotoxicity: possible involvement of cyclic GMP. *FEBS Lett* 409: 46–48, 1997.
41. Quesada AR, Munoz-Chapuli R, and Medina MA. Anti-angiogenic drugs: from bench to clinical trials. *Med Res Rev* 26: 483–530, 2006.
42. Ramachandran A, Moellering DR, Ceaser E, Shiva S, Xu J, and Darley-Usmar V. Inhibition of mitochondrial protein synthesis results in increased endothelial cell susceptibility to nitric oxide-induced apoptosis. *Proc Natl Acad Sci U S A* 99: 6643–6648, 2002.
43. Rao CV, Reddy BS, Steele VE, Wang CX, Liu X, Ouyang N, Patlolla JM, Simi B, Kopelovich L, and Rigas B. Nitric oxide-releasing aspirin and indomethacin are potent inhibitors against colon cancer in azoxymethane-treated rats: effects on molecular targets. *Mol Cancer Ther* 5: 1530–1538, 2006.
44. Raz B, Iten M, Grether-Buhler Y, Kaminsky R, and Brun R. The Alamar Blue assay to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) in vitro. *Acta Trop* 68: 139–147, 1997.
45. Rubbo H, Tarpey M, and Freeman BA. Nitric oxide and reactive oxygen species in vascular injury. *Biochem Soc Symp* 61: 33–45, 1995.
46. Schnittler HJ. Structural and functional aspects of intercellular junctions in vascular endothelium. *Basic Res Cardiol* 93(suppl 3): 30–39, 1998.
47. Sheu WH, Ou HC, Chou FP, Lin TM, and Yang CH. Rosiglitazone inhibits endothelial proliferation and angiogenesis. *Life Sci* 78: 1520–1528, 2006.
48. Shi S, Garcia JG, Roy S, Parinandi NL, and Natarajan V. Involvement of c-Src in diperoxovanadate-induced endothelial cell barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 279: L441–L451, 2000.
49. Stamler JS, Singel DJ, and Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms. *Science* 258: 1898–1902, 1992.
50. Struck AT, Hogg N, Thomas JP, and Kalyanaraman B. Nitric oxide donor compounds inhibit the toxicity of oxidized low-density lipoprotein to endothelial cells. *FEBS Lett* 361: 291–294, 1995.
51. Tesei A, Ricotti L, Ulivi P, Medri L, Amadori D, and Zoli W. NCX 4016, a nitric oxide-releasing aspirin derivative, exhibits a significant antiproliferative effect and alters cell cycle progression in human colon adenocarcinoma cell lines. *Int J Oncol* 22: 1297–1302, 2003.
52. Usatyuk PV, Vepa S, Watkins T, He D, Parinandi NL, and Natarajan V. Redox regulation of reactive oxygen species-induced p38 MAP kinase activation and barrier dysfunction in lung microvascular endothelial cells. *Antioxid Redox Signal* 5: 723–730, 2003.
53. Varadharaj S, Watkins T, Cardounel AJ, Garcia JG, Zweier JL, Kuppusamy P, Natarajan V, and Parinandi NL. Vitamin C-induced loss of redox-dependent viability in lung microvascular endothelial cells. *Antioxid Redox Signal* 7: 287–300, 2005.
54. Vepa S, Scribner WM, Parinandi NL, English D, Garcia JG, and Natarajan V. Hydrogen peroxide stimulates tyrosine phosphorylation of focal adhesion kinase in vascular endothelial cells. *Am J Physiol* 277: L150–L158, 1999.
55. Wallace JL, Del Soldato P, Cirino G, and Muscara MN. Nitric oxide-releasing NSAIDs: GI-safe antithrombotics. *IDrugs* 2: 321–326, 1999.
56. Wallace JL, Ignarro LJ, and Fiorucci S. Potential cardioprotective actions of no-releasing aspirin. *Nat Rev Drug Discov* 1: 375–382, 2002.
57. Wink DA, Cook JA, Krishna MC, Hanbauer I, DeGraff W, Gamson J, and Mitchell JB. Nitric oxide protects against alkyl peroxide-mediated cytotoxicity: further insights into the role nitric oxide plays in oxidative stress. *Arch Biochem Biophys* 319: 402–407, 1995.
58. Wink DA, Cook JA, Pacelli R, DeGraff W, Gamson J, Liebmann J, Krishna MC, and Mitchell JB. The effect of various nitric oxide-donor agents on hydrogen peroxide-mediated toxicity: a direct correlation between nitric oxide formation and protection. *Arch Biochem Biophys* 331: 241–248, 1996.
59. Wink DA, Miranda KM, and Espey MG. Cytotoxicity related to oxidative and nitrosative stress by nitric oxide. *Exp Biol Med (Maywood)* 226: 621–623, 2001.
60. Yamamoto T and Bing RJ. Nitric oxide donors. *Proc Soc Exp Biol Med* 225: 200–206, 2000.

Address reprint requests to:
*Periannan Kuppusamy, Ph.D.*
*Dorothy M. Davis Heart and Lung Research Institute*
*The Ohio State University*
*420 West 12th Avenue, Room 114*
*Columbus, OH 43210*

*E-mail:* kuppusamy.1@osu.edu

Date of first submission to ARS Central, February 2, 2007; date of acceptance, June 16, 2007.

**This article has been cited by:**


1. Jihoon Kim, Yanggy Lee, Kaushik Singha, Hyun Woo Kim, Jae Ho Shin, Seongbong Jo, Dong-Keun Han, Won Jong Kim. 2011. NONOates–Polyethylenimine Hydrogel for Controlled Nitric Oxide Release and Cell Proliferation Modulation. *Bioconjugate Chemistry* **22**:6, 1031-1038. [CrossRef]
2. Yu-Chiang Hung, Pei-Wen Wang, Tai-Long Pan. 2010. Functional proteomics reveal the effect of Salvia miltiorrhiza aqueous extract against vascular atherosclerotic lesions. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **1804**:6, 1310-1321. [CrossRef]
3. Sean M. Sliman, Timothy D. Eubank, Sainath R. Kotha, M. Lakshmi Kuppusamy, Shariq I. Sherwani, Elizabeth Susan O'Connor Butler, Periannan Kuppusamy, Sashwati Roy, Clay B. Marsh, David M. Stern, Narasimham L. Parinandi. 2010. Hyperglycemic oxoaldehyde, glyoxal, causes barrier dysfunction, cytoskeletal alterations, and inhibition of angiogenesis in vascular endothelial cells: aminoguanidine protection. *Molecular and Cellular Biochemistry* **333**:1-2, 9-26. [CrossRef]
4. Yu-Chiang Hung, Pei-Wen Wang, Tai-Long Pan, Grzegorz Bazylak, Yann-Lii Leu. 2009. Proteomic screening of antioxidant effects exhibited by Radix Salvia miltiorrhiza aqueous extract in cultured rat aortic smooth muscle cells under homocysteine treatment. *Journal of Ethnopharmacology* **124**:3, 463-474. [CrossRef]