# Participation of Nitric Oxide Pathway in the Relaxation Response Induced by *E*-cinnamaldehyde Oxime in Superior Mesenteric Artery Isolated From Rats

*Robson C. Veras, PhD,* Karoline G. Rodrigues,* Maria do C. Alustau, MD,* Islânia G.A. Araújo, PhD,* André Luis B. de Barros, PhD,† Ricardo J. Alves, PhD,† Lia S. Nakao, PhD,‡ Valdir A. Braga, PhD,§ Darizy F. Silva, PhD,¶ and Isac A. de Medeiros, PhD**

**Abstract:** For many years, nitric oxide (NO) has been studied as an important mediator in the control of vascular tone. Endothelial deficiencies that diminish NO production can result in the development of several future cardiovascular diseases, such as hypertension and arteriosclerosis. In this context, new drugs with potential ability to donate NO have been studied. In this study, 3 aromatic oximes [benzophenone oxime, 4-Cl-benzophenone oxime, and *E*-cinnamaldehyde oxime (*E*-CAOx)] induced vasorelaxation in endothelium-denuded and intact superior mesenteric rings precontracted with phenylephrine. *E*-CAOx demonstrated the most potent effect, and its mechanism of action was evaluated. Vascular reactivity experiments demonstrated that the effect of *E*-CAOx was reduced by the presence of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, 1*H*[1,2,4,]oxadiazolo[4,3-*a*]quinoxalin-1-one, and (Rp)-8-(*para*-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate, suggesting the participation of NO/sGC/PKG pathway. NO donation seems to be mediated through nicatinamide adenine dinucleotide phosphate-dependent reductases because 7-ethoxyresorufin decreased the effect of *E*-CAOx on vascular reactivity and reduced NO formation as detected by flow cytometry using the NO indicator diaminofluorescein 4,5-diacetate. Further downstream of NO donation, $K^+$ subtype channels were also shown to be involved in the *E*-CAOx vasorelaxant effect. The present study showed that *E*-CAOx acts like an NO donor, activating NO/sGC/PKG pathway and thus $K^+$ channels.

**Key Words:** endothelium, mesenteric artery, NO donor, oxime, rat, vasorelaxation

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From the *Departamento de Ciências Farmacêuticas, Universidade Federal da Paraíba, João Pessoa, Paraíba, Brazil; †Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Pampulha, Belo Horizonte, Minas Gerais, Brazil; ‡Departamento de Patologia Básica, Universidade Federal do Paraná, Jd. Americas, Curitiba, Paraná, Brazil; §Centro de Biotecnologia, Universidade Federal da Paraíba, João Pessoa, Paraíba, Brazil; and ¶Departamento de Biorregulação, Universidade Federal da Bahia, Salvador, Bahia, Brazil.

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Reprints: Darizy F. Silva, PhD, Departamento de Biorregulação, Universidade Federal da Bahia, Vale do Canela, Salvador, Bahia, Brazil 40110-902 (e-mail: darizy@gmail.com).



## INTRODUCTION

Since the discovery of nitric oxide (NO), its biological activities have been a matter of extensive research. An important feature of the vascular endothelium is the adequate output of this messenger, whose production occurs via *N*-hydroxylation and oxidative catalysis of the guanidine group on L-arginine in the presence of molecular oxygen and various cofactors, resulting in the stoichiometric production of NO and L-citrulline.[1–3] The NO radical, acting as a paracrine or an autocrine messenger, permeates biological membranes and can react with other molecules that contain unpaired electrons, as such thiol groups and heme groups binding to an iron ion.[4] The effects of NO differ depending upon its concentration: protector and vasorelaxation effects are obtained at lower concentrations (picomolar or nanomolar), whereas higher concentrations are associated with cytotoxic effects.[5,6] In the vascular bed, NO acts directly on soluble guanylyl cyclase (sGC), leading to an increase in guanosine 3,5-cyclic monophosphate (cGMP) concentration and subsequent activation of cGMP-dependent protein kinase (PKG), which ultimately facilitates the phosphorylation of various proteins that promote vasorelaxation.[7–9] The relaxation induced by this cyclic nucleotide is associated with several molecular modifications, including a decrease in cytosolic $Ca^{2+}$ concentration ($c[Ca^{2+}]$),[10] the activation of the sarcoplasmic reticulum $Ca^{2+}$-ATPase,[11] the activation of different potassium channels,[12] decreased inositol 1,4,5-trisphophate ($IP_3$) production,[9,13] and closure of voltage-dependent L-type $Ca^{2+}$ channels (L-type $Ca_v$).[14] In addition, $Ca^{2+}$-independent mechanisms involving a direct reduction of $Ca^{2+}$sensitivity of the contractile apparatus have also been reported to mediate cGMP-induced relaxation.[15,16] Another possibility is that NO directly activates $Ca^{2+}$-dependent $K^+$ channel ($K_{Ca}$) or voltage-dependent $K^+$ channel ($K_v$) currents.[1,3,17,18] A decrease in the production of NO or NO synthase (NOS) activity leads to endothelial dysfunction, which is characterized by diminished endothelium-dependent vasodilatation and a reduced capacity of endothelial cells to suppress inflammation, thrombosis, and oxidative stress. Endothelial dysfunction is a central component of the pathophysiological process during the initiation and progression of atherosclerotic lesions,[19–21] which can be detected in hypercholesterolemia, hypertension, and heart failure.[21–23]

Alternatively, restoring NO deficiency has lead to the development of NO donor molecules, such as oximes or

nitroglycerin, which do not require endothelium participation to release NO. Studies have demonstrated that oxidative cleavage of the C=N bond of C=NOH functions are steps catalyzed not only by NOS but also by other enzymes, such as hemeproteins, peroxidases, and catalases.[24–26] These actions result in the generation of stable nitrogen oxides, with the possible intermediate formation of NO.

The existence of an NOS-independent pathway capable of nitrogen oxide production has been suggested in cultured smooth muscle cells from the rat aorta[27] and from trachea.[28] In both cases, nitrite formation was blunted by a cytochrome P450 (CYP450) inhibitor, suggesting the involvement of CYP450.

Previous studies report that non-amino acid compounds containing a C=NOH function, which are present in oxime molecules, can result in the relaxation of rat endothelium-denuded aortic rings, with NO/sGC/PKG pathway participation.[29–34]

In the present study, 3 aromatic substituted oxime derivatives, containing C=NOH function group, were evaluated as possible vasorelaxant drugs acting through NO donor mechanisms.

## MATERIALS AND METHODS

### Drugs

*E*-cinnamaldehyde oxime (*E*-CAOx, molecular mass [MM] = 147),[35] benzophenone oxime (BF, MM = 197.23),[35] and 4-Cl-benzophenone oxime (4-Cl-BF, MM = 231.68) were obtained according to the reported methods.[36]

Stock solutions of aromatic oximes were prepared with dimethyl sulfoxide (DMSO) (50%) on the day of the experiment. Tyrode solution was used in serial dilution. The final concentration of DMSO in the bath never exceeded 0.1% and had no effect when tested in control preparations. DMSO, phenylephrine (Phe), acetylcholine, $N^W$-nitro-L-arginine methyl ester (L-NAME), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), *N*-acetyl-L-cysteine (NAC), proadifen, 7-ethoxyresorufin (7-ER, 7-ethoxy-3*H*-phenoxazin-3-one, and 7-ethoxy-3*H*-phenoxazin-3-one), 1*H*[1,2,4,]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ), (Rp)-8-(*para*-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate [(Rp)-8pCPT-cGMPS], tetraethylammonium (TEA), iberiotoxin, 4-aminopyridine (4-AP), glibenclamide, *S*(−)1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl]-3-pyridinecarboxylic acid, methyl ester [*S*(−)-BAY K 8644], 5-(methylamino)-2-({(2*R*,3*R*,6*S*,8*S*,9*R*, 11*R*)-3,9,11-trimethyl-8-[(1*S*)-1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl}methyl)-1,3-benzoxazole-4-carboxylic acid (A23187), 9,11-dideoxy-9a, 11a-methanoepoxy prostaglandin $F_{2a}$ (U46619), diaminofluorescein 4,5-diacetate (DAF-DA, NO-specific probe), trypsin, hyaluronidase, bovine serum albumin, fetal bovine serum, penicillin plus streptomycin, and Dulbecco modified Eagle medium (DMEM) were all purchased from Sigma Aldrich Brazil Ltd (São Paulo, Brazil). All drugs were dissolved in distilled water, except ODQ, glibenclamide, 7-ER, proadifen, DAF-DA, and the tested oximes, which were dissolved in DMSO. Nifedipine and PTIO were dissolved in absolute ethanol. The solutions were kept at 0°C to −4°C.

### Animal and Superior Mesenteric Artery Ring Preparation

Experiments were conducted in accordance with Brazilian federal law no. 11794/08, which establishes procedures for scientific use of laboratorial animals, and Animal Care and Use Committee of the Federal University of Paraiba (CEPA#1003/07). Superior mesenteric artery (first branch) was removed from male Wistar rats (250–300 g), cleaned of connective and fat tissues in Tyrode solution (composition in milliMolar: NaCl, 158.3; KCl, 4.0; $MgSO_4$, 1.05; $CaCl_2$, 2.0; $NaH_2PO_4 \cdot H_2O$, 0.42; $NaHCO_3$, 10; and glucose, 5.6) and cut into rings (1–2 mm). In some experiments, the endothelium was removed by gently rubbing the intimal surface of the rings with thin wire.

### Organ Bath Studies

For isomeric tension recording, superior mesenteric artery rings were suspended in an organ bath containing 10 mL of Tyrode solution, maintained at 37°C, and gassed with a 95% $O_2$ + 5% $CO_2$ mixture (pH 7.4), as previously reported.[37] Rings were stabilized with an optimal resting tension of 0.75*g*, which had been determined previously by length–tension relationship experiments and studies using the optimal contraction to 10 μM Phe against passive tension. The tissues were then allowed to equilibrate for 60 minutes while resting tension was readjusted to 0.75*g* when necessary. The isometric contraction was recorded by a force transducer (FORT-10; WPI, Sarasota, FL) coupled to an amplifier–recorder (Miobath-4, WPI) and to a personal computer equipped with an analog-to-digital converter board. Endothelial removal was qualitatively assessed by the failure to relax to acetylcholine (10 μM) after contractile tone was induced by Phe (10 μM). Rings that relaxed less than 10% were considered endothelium denuded, and relaxation more than 90% was considered endothelium intact. Rings with relaxation between 10% and 90% were again submitted to endothelium removal process. After the washout, the rings were precontracted again with Phe (10 μM) to produce a similar level of precontraction. When the Phe-induced contraction plateued, oxime derivatives or vehicles were then added in cumulative manner. In some experiments, 30 minutes before Phe contraction, inhibitors were added individually or in combination. To investigate NO production, the following inhibitors were used: L-NAME, an inhibitor of NOS; PTIO, a cell-permeable scavenger of NO[38,39]; NAC, an $NO^-$ scavenger[40] proadifen, inhibitor of CYP450[30]; and 7-ER, a selective inhibitor of CYP450 from the 1A family and of NAD(P)H-dependent reductases. To evaluate the NO/sGC/PKG pathway participation, the following antagonists were used: ODQ, an inhibitor of the sGC,[41,42] and (Rp)-8pCPT-cGMPS, an inhibitor of PKG.[43] Participation of $K^+$ channels was addressed by incubation with TEA or iberiotoxin, a $K^+$ channel blocker[39]; 4-AP, a $K^+$ channel voltage-sensitive, $K_v$, blocker; and glibenclamide, a $K^+$ channel ATP-sensitive ($K_{ATP}$) blocker[40,41] or in combination with ODQ. Furthermore, the participation of these channels was also investigated by increasing the $K^+$ concentration from 4 to 20 mM, which results in $K^+$ efflux reduction (30 minutes before Phe contraction, which was

maintained until the end of the experiment). In another set of experiments, the endothelium-denuded rings were contracted with an alternative agonist, U46619 (1 μM), with a thromboxane $A_2$ analog[42] or A23187 (10 μM), or with KCl (60 mM). Once the plateau contraction was obtained, *E*-CAOx was added cumulatively. The modified $K^+$ solution (20 or 60 mM $K^+$) was prepared by replacing $Na^+$ with an equimolar concentration of $K^+$ to maintain constant ion strength in the bath solution. In a third set of experiments, the endothelium-denuded rings were contracted with 60 mM KCl in Tyrode solution, washed, incubated with 20 mM $K^+$ in Tyrode solution, and after 30 minutes, *S*(−)-BAY K 8644 (200 nM, a direct L-type $Ca_v$ activator) was added into the bath.[43] After the plateau was attained, *E*-CAOx was added cumulatively. This final process produced a partial depolarization required to obtain the appropriate response to *S*(−)-BAY K 8644.

## NO Measurement

### Explant Culture

Rat aortic smooth muscle cells were isolated in accordance with a method adapted from Kirschenlohr et al.[44] DMEM containing 20% fetal calf serum and antibiotics (penicillin and streptomycin) was used. Initially, 5–10 small muscle cubes, with less than 1 $mm^2$, were transferred onto tissue culture plates (2 $cm^2$, BD Biocoat Matrigel Matrix) and covered with 50 μL of DMEM. The preparation was incubated at 37°C in a humidified atmosphere (5% $CO_2$–21% $O_2$). After 12 hours, DMEM (500 microliters per well) was added. The medium was changed every 2 days. Confluent monolayers typically formed within 7 days. After formation, the fragments were removed and the cells were washed 3 times with phosphate-buffered saline (composition in milliMolar: NaCl, 137; KCl, 2.7; $KH_2PO_4$, 4.17; and $Na_2HPO_4$, 4.3) to remove nonadherent cells and debris. Trypsin (0.2% per 5 minutes in 5% $CO_2$ atmosphere) was then added to suspend the adherent cells. The dispersed cell suspension was washed out, resuspended in DMEM, and aliquoted into 24-well tissue culture plates (12,000 cells per square centimeter). Thenceforth, the medium was changed every 2 days, and confluent monolayers were obtained within 5 days. Cells from the third passage were used in these studies.

### Determination of $[NO]_i$ and $c[Ca^{2+}]$ From Aortic Myocytes

NO donation by *E*-CAOx was determined by, DAF-2DA, a cell-permeable, sensitive, fluorescent indicator commonly used for the detection of NO.[45,46] Initially, the cells were preincubated with 10 μM DAF-2DA plus Phe (10 μM) for 30 minutes at 37°C. The purpose of the presence of Phe was to maintain consistency with the experiments performed on mesenteric artery rings. NO bioavailability in aortic myocytes was quantified after exposure to *E*-CAOx (10 μM) or vehicle for 30 minutes. The fluorescent signal was also verified in cells pretreated with 7-ER (10 μM) for 30 minutes before the addition of *E*-CAOx (10 μM). The cells were then washed twice with phosphate-buffered saline containing bovine serum albumin and analyzed with 10,000 cells per sample by flow cytometry using the FACSCalibur (Becton Dickinson, San Jose, CA).

Cytofluorographic analysis was performed using a Becton–Dickinson FACScan with an argon ion laser tuned to 488 nm at 15 mW output. The cells were analyzed at the flow cytometer in the absence of the fluorescent dyes or drugs (Blank). After this, the cells were incubated with a selective fluorescent dye for $Ca^{2+}$ (Fluo-4AM, 10 μmol/L) and Phe (10 μM) for 30 minutes. Afterward, the vehicle or *E*-CAOx (100 μM) was added to the cells loaded with selective dyes.[47,48] After the addition of *E*-CAOx, the samples were analyzed for 15 minutes in intervals of 5 minutes. Acquisition was set at 10,000 cells, and the mean fluorescence intensity (obtained in fluorescein isothiocyanate channel—interval of 515–545 nm with peak emission at 525 nm) was measured in all the samples by using WinMDI software version 2.9. Changes in fluorescence were normalized to the fluorescence obtained in the presence of FLUO-4 alone (F0) and were expressed as (F/F0). Values were expressed as mean ± SEM (normalized to percentage of control) from 3 independent experiments in duplicate.

### Data Analysis

Results were expressed as mean ± SEM. In each experiment, "n" indicates the number of rings from different rats. Relaxation responses are represented as percentage of the contraction induced by the vasoconstrictor. Statistical analysis was performed using analysis of variance for repeated measures followed by Bonferroni posttest or Student *t* test for unpaired data. $P < 0.05$ was considered statistically significant.

# RESULTS

## Influence of the Endothelium on the Vasorelaxation Induced by Aromatic Oximes in Superior Mesenteric Artery Rings

After stable contraction elicited by Phe, aromatic oximes (BF and 4-Cl-BF) caused a concentration-dependent vasorelaxation in superior mesenteric artery rings during a 10-minute time course. The presence of a functional endothelium influenced the relaxation induced by 4-Cl-BF, as shown in Figure 1. Endothelium removal shifted the curve to right and reduced the relaxant effect at the highest administered concentrations (endothelium intact 66.6 ± 12.5 vs. endothelium removed 28.7 ± 4.7; n = 6). BF produced identical concentration-dependent relaxations in superior mesenteric rings with or without a functional endothelium. In the experiments illustrated in Figure 1, relaxation reached 38.0 ± 1.5 (n = 6). As 4-Cl-BF, BF did not demonstrate any promising effect.

Contrary to what was observed with BF and 4-Cl-BF, the *E*-CAOx demonstrated a significant and sustainable concentration-dependent vasorelaxation effect in superior mesenteric artery rings. Figure 1 demonstrates that *E*-CAOx–induced relaxation of endothelium-intact rings was only significantly altered at the highest administered concentrations (endothelium intact, 70.7 ± 7.0; endothelium denuded, 91.0 ± 4.5; $P < 0.05$; n = 6). After successive washes, rings demonstrated the same contractile magnitude to Phe (10 μM) when compared with first Phe contraction (data not show).

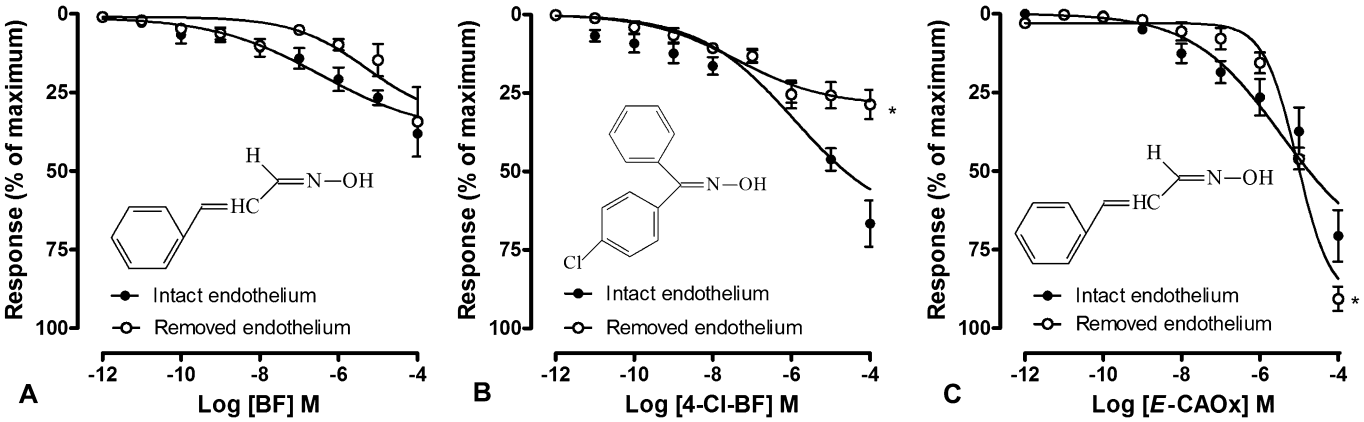


**FIGURE 1.** Relaxing effects of aromatic oximes in rat mesenteric rings precontracted with Phe in the presence (●) and in the absence (○) of the functional endothelium. Concentration–response curves for relaxant effects of (A) BF, (B) 4-Cl-BF, and (C) *E*-CAOx in rings precontracted with Phe. *$P < 0.05$ versus rings with endothelium.

## Effect of *E*-CAOx on Endothelium-Denuded Rings Contracted With Multiple Contractile Agents and on Cytosolic Calcium From Myocytes

In a separate set of experiments designed to study the influence of the vasoconstrictor agonists, relaxations produced by *E*-CAOx were significantly different when precontraction was induced by 60 mM KCl, U46619, or A23187. The relaxation induced by 100 μM *E*-CAOx was similar in rings precontracted by calcium ionophore (A23187) or Phe. However, the effect of *E*-CAOx was lesser in rings precontracted with U46619 (73.0% ± 5.0%; $P < 0.05$) or 60 mM KCl (62.0% ± 10.0%, $P < 0.05$) than in contractions by Phe (91.0% ± 4.5%).

In endothelium-denuded rings precontracted with *S*(−)-BAY K 8644 (200 nM) in the presence of 20 mM KCl, *E*-CAOx induced a significantly greater concentration-dependent relaxation than was observed in Phe-precontracted rings under the same conditions. Furthermore, the relaxation at 100 μM *E*-CAOx was significantly more pronounced in *S*(−)-BAY K 8644 contraction (98% ± 1.8%, $P < 0.05$) compared with Phe in 20 mM KCl (79.0% ± 5.0%, Fig. 2B); however, it was not possible to confirm whether *E*-CAOx acts directly on L-type $Ca_v$ or downstream of $Ca^{2+}$ channel activity.

The fluorescence emitted by FLUO-4, a $Ca^{2+}$ ion–sensitive dye, was detected in myocytes stimulated by Phe (10 μM); this signal was significantly reduced ($P < 0.05$) in the presence of *E*-CAOx (100 μM). A significant reduction in fluorescence was observed after 5, 10, and 15 minutes of incubation with *E*-CAOx (~17.5%, ~33%, and ~19%, respectively).

## NO Donation From *E*-CAOx: Pharmacological and Chemiluminescence Characterization

In rings without endothelium, the relaxation induced by *E*-CAOx was not affected by the presence of either the NOS inhibitor, L-NAME (100 μM), or the CYP450 inhibitor, proadifen (30 μM); however, 7-ER (10 μM), an inhibitor of cyt450 1A1 and NAD(P)H-dependent reductases (10 μM), partially inhibited the relaxation effect induced by *E*-CAOx, shifting the concentration–response curve to the right and reducing the relaxant effect (100 μM *E*-CAOx = 33.0% ± 4.0%, $P < 0.05$), as shown in Figure 3A. Data from the literature demonstrate the ability of various enzymes to catalyze, by oxidative cleavage, the C=N bond of the C=NOH functional group in NO.[29,34] This result strengthens the hypothesis that NO donation by *E*-CAOx is mediated through enzymatic NAD(P)H-dependent reductase activity.

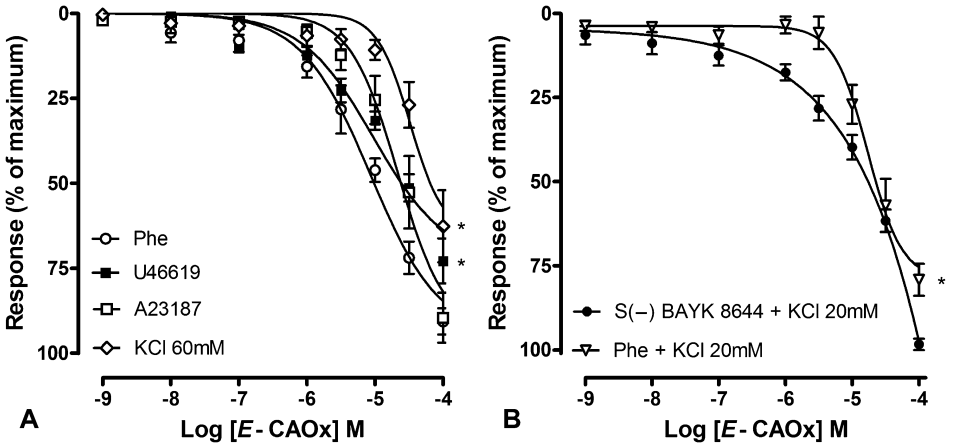


**FIGURE 2.** Relaxant effect induced by *E*-CAOx in endothelium-denuded rings precontracted with different contractile agents. A, Concentration–response curves for relaxant effect of *E*-CAOx in rings precontracted with Phe (○), U46619 (■), A23187 (□), or 60 mM KCl (◊). B, Concentration–response curves for relaxant effect of *E*-CAOx in rings precontracted with *S*(−)-BAY K 8644 plus 20 mM KCl (●) or Phe plus 20 mM KCl (▽).*$P < 0.05$ versus Phe.

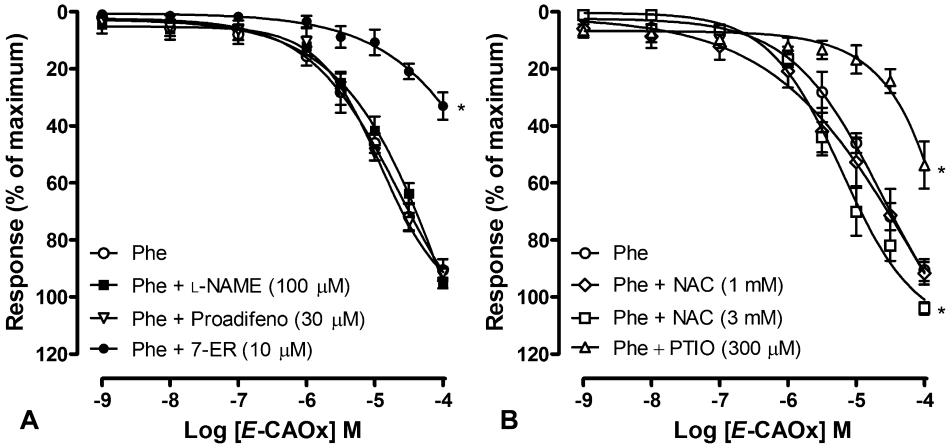


**FIGURE 3.** Effect relaxant of *E*-CAOX involves the NO production dependent on 7-ER–sensitive enzymes. Concentration–response curves for relaxant effect of *E*-CAOx (A) in the absence Phe (○) and in the presence of L-NAME, 100 μM (■); proadifen, 30 μM (▽); or 7-ER, 10 μM (●) and (B) in the absence of Phe (○) and in the presence of NAC (1 mM, [□]) or PTIO (300 μM, Δ) in endothelium-denuded rings precontracted with Phe. *$P < 0.05$ versus Phe.

As NO can be generated in radical ($NO^{\bullet}$) or ionic ($NO^{-}$) form, concentration–response curves to *E*-CAOx were tested in the presence of NAC (1 or 3 mM), an intracellular scavenger of free radicals, such as $NO^{-}$. In these conditions, the inhibitor did not reduce the *E*-CAOx relaxation response, but a significant reduction in relaxation (to 54.0% ± 6.0%, $P < 0.05$) was observed when challenged with PTIO (300 μM), an $NO^{\bullet}$ scavenger, as shown in Figure 3B.

The present data suggest a potential new hypothesis for the mechanism(s) underlying endothelium-independent relaxation elicited by compounds with a C=NOH function: enzymatic conversion and NO production in radical form.

In a vascular bed, the major target to NO is sGC, a heme enzyme, closely related to relaxant mechanisms. In this sense, the guanylyl cyclase inhibitor ODQ was used to further investigate the involvement of the sGC/cGMP pathway in relaxation.

As shown in Figure 4A, incubation with ODQ (1 and 10 μM) partially inhibited relaxations caused by *E*-CAOx. When the inhibitor concentration was increased 10-fold (1–10 μM), the observed inhibition was augmented approximately 1.7-fold (72.0% ± 7.0%, $P < 0.05$ and 49.0% ± 4.0%, $P < 0.05$, respectively). The relaxant effect of *E*-CAOx was also reduced after incubation with the PKG inhibitor, (Rp)-8pCPT-cGMPS (10 μM) (72.0% ± 5.0%, $P < 0.05$). Taken together, these results indicate the involvement of the sGC/cGMP/PKG pathway in the *E*-CAOx–induced relaxation response.

Using flow cytometry (Fig. 5), the NO donation hypothesis was further supported when fluorescence from cells treated with *E*-CAOx (10 μM) increased by approximately 54% (4,5-Diaminoluorescein triazolo [DAF-2T] fluorescence: control = 100%; Phe + *E*-CAOx = 154% ± 23%, $P < 0.05$) when compared with basal fluorescence from control cells (10 μM of DAF-DA plus vehicle). This increase in fluorescence disappeared in cells pretreated with 7-ER (10 μM) (DAF-2T fluorescence: 74% ± 25%, $P > 0.05$ vs. control).

## Participation of $K^+$ Channels in the Vasorelaxation Induced by *E*-CAOx

In this experimental condition, all inhibitors did not alter the precontraction levels (not shown); however, when $K^+$ concentration in Tyrode solution was changed from 4 to 20 mM KCl or in the presence of TEA (1 mM), the rings developed a small and transient contraction (less than 20%) that spontaneously reverted within 5–10 minutes during incubation. KCl (20 mM), TEA (1 mM), and iberiotoxin (100 nM) have similar vasorelaxation inhibitory effects in *E*-CAOx–induced relaxation, shifting the curve to right (Fig. 6A). In contrast, preincubation of 4-AP and glibenclamide did not significantly impact *E*-CAOx–induced relaxation (Fig. 6B).

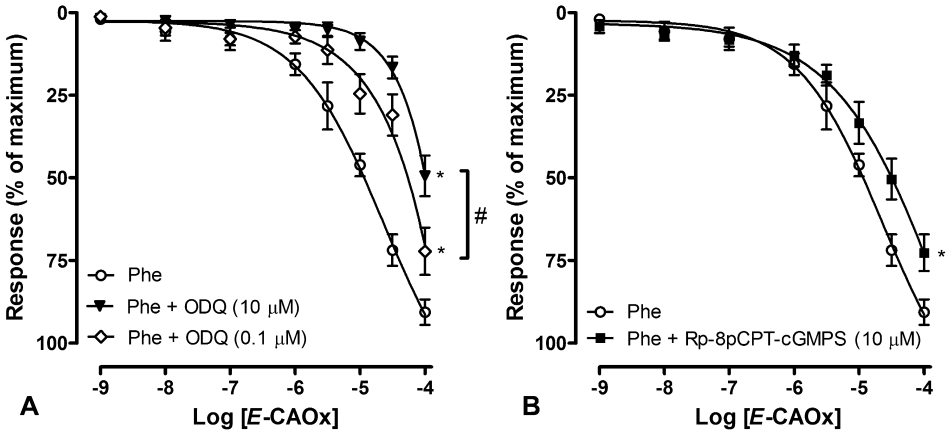


**FIGURE 4.** The involvement of sGC and PKG in the vasorelaxant effect of *E*-CAOx. Concentration–response curves for relaxant effect of *E*-CAOx in the absence of Phe (○) and in the presence of (A) ODQ (10 μM, ▼, and 0.1 μM, ◊) or (B) (Rp)-8pCPT-cGMPS (10 μM, ■) in endothelium-denuded rings precontracted with Phe. *$P < 0.05$ versus Phe.

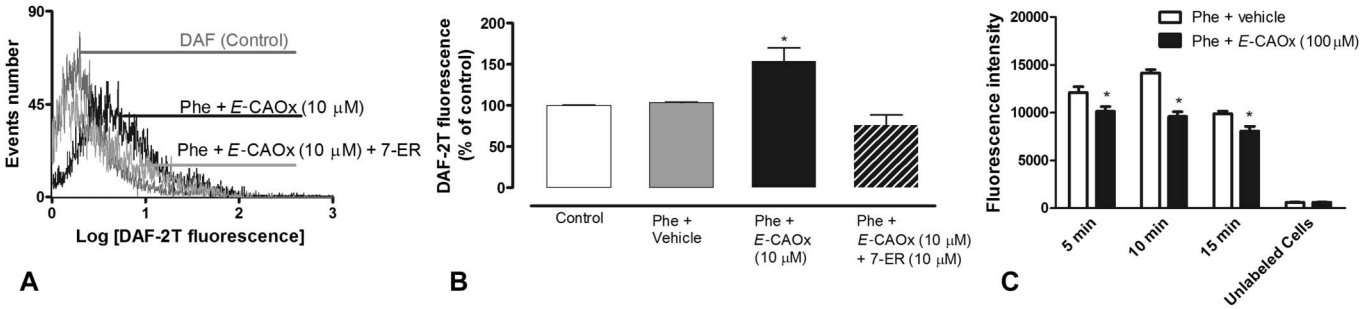


**FIGURE 5.** Effect of *E*-CAOx on the intracellular fluorescence of NO-sensitive probe DAF-2DA in rat myocytes. A, Histograms from indicated condition of a representative experiment. B, Representative of intracellular fluorescence of DAF-2T in different experimental situations. C, Effect of *E*-CAOx on the intracellular calcium concentration in rat myocytes. The cells loaded with FLUO-4 were treated with vehicle or *E*-CAOx (100 μM) in the presence of Phe (10 μM). $*P < 0.05$ versus control.

Several reports have noted that NO can directly activate $K^+$ channels, without participation of sGC/cGMP/PKG pathway. To identify this possibility, we carried out a similar investigation to that presented above in the presence of ODQ (10 μM). The original relaxation mediated by *E*-CAOx was similarly depressed in the presence of ODQ and OQD plus $K^+$ channel blockers, such as 4-AP, glibenclamide, and TEA. However, *E*-CAOx response was significantly reduced by ODQ in the presence 20 mM KCl solution (27% ± 3%, $P < 0.05$, Figs. 6C, D).

## DISCUSSION

In this study, *E*-CAOx presented the most pronounced vasorelaxant effect when compared with other aromatic oximes tested (BF and 4-Cl-BF). These results indicate that *E*-CAOx donates $NO^{\bullet}$ through NAD(P)H-dependent reductase actions, promoting the sGC/cGMP/PKG pathway and subsequently large conductance calcium-activated potassium channels ($BK_{Ca}$) activation.

Previous studies have demonstrated that cinnamaldehyde, a natural compound, induces endothelium-dependent

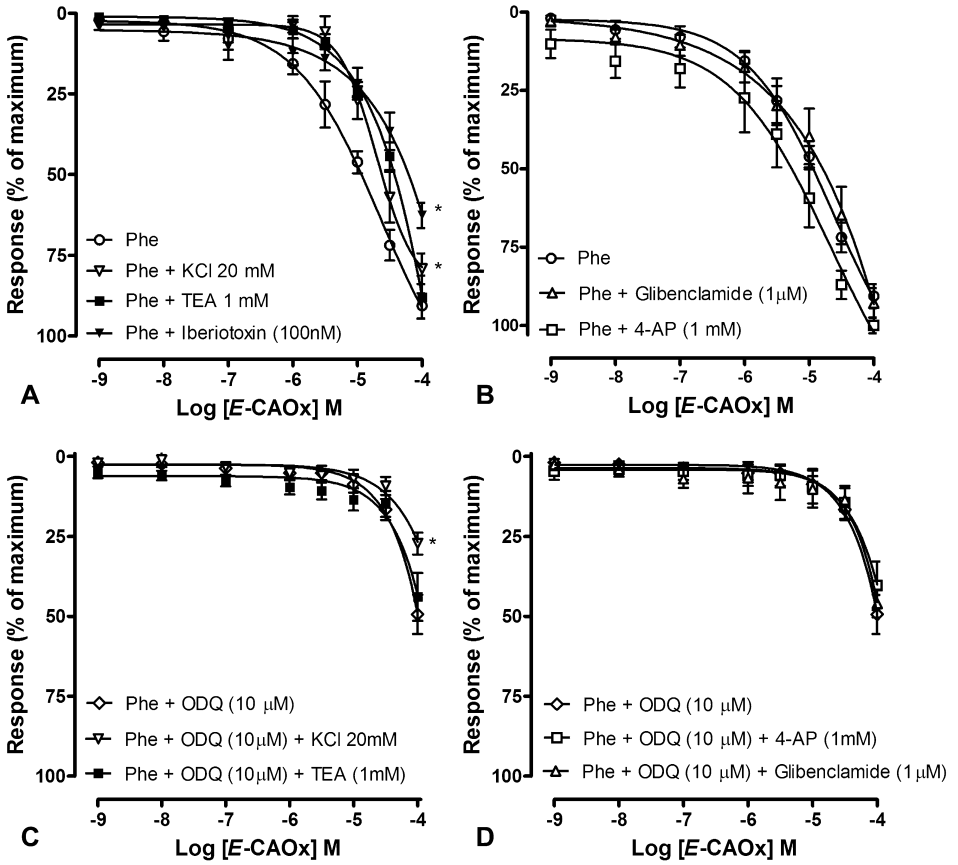


**FIGURE 6.** Participation of TEA-sensitive $K^+$ channels on relaxant response to *E*-CAOx. Concentration–response curves for relaxant effect of *E*-CAOx in the absence of Phe (○) and in the presence of (A) KCl, 20 mM (), TEA, 1 mM (■), or Iberiotoxin, 100 nM (▼); (B) 4-AP, 1 mM (■), or glibenclamide, 1 μM (Δ); (C) ODQ, 10 μM, plus KCl, 20 mM (), or ODQ, 10 μM, plus TEA, 1 mM (■); and (D) ODQ, 10 μM, plus 4-AP, 1 mM (□), or ODQ, 10 μM, plus glibenclamide, 1 μM (Δ). $*P < 0.05$ versus Phe.

and independent vasorelaxant actions in isolated aorta from rats.[49] In this study, we demonstrated that *E*-CAOx induces potent vasorelaxation response in the rat superior mesenteric artery. Compared with other aromatic oximes in endothelium-intact and endothelium-denuded mesenteric artery rings pre-contracted with Phe, *E*-CAOx caused statistically greater relaxation. When rings were contracted with U46619, *E*-CAOx also induced a concentration-dependent relaxation response, demonstrating that the *E*-CAOx action is not directly related to the adrenergic receptor antagonist.

NO in the cardiovascular system is mainly produced in the endothelium by NOS, also known as endothelial NOS (eNOS).[3] The vascular smooth muscle is another source of NO, produced by NOS 1, which was first described in smooth muscle from bovine coronary,[50] rat aorta,[51] and mouse mesenteric arterial bed.[52]

This investigation demonstrated that neither the endothelium nor the NOS was involved in the relaxation induced by *E*-CAOx, as indicated by the observation that endothelium removal or treatment with L-NAME (100 μM) resulted in unaltered vasorelaxant responses mediated by *E*-CAOx. This result differed from other studies investigating different tissues, in which eNOS has an important role in the relaxation induced by compounds bearing the C=NOH functional group, which is also present in this oxime,[53] and corroborates with other reports, demonstrating that the metabolization by endothelial or non-endothelial NOS was of minor importance and had no influence on oxime relaxation effects.[29,30,34] This discrepancy seems to be justified by tissue and/or species differences.[29]

Previous studies demonstrated that oximes can be metabolized by different enzymes, for example, CYP450, and are capable of NO donation.[24,25,27,29] Our results initially suggest the involvement of NO and subsequently sGC/cGMP/PKG pathway activation in the *E*-CAOx–mediated relaxation response.

Enzymatic oxidation of the C=N bond of the C=NOH oxime group and further cyt450-mediated NO production do not seem to occur with *E*-CAOx, which is different from other oximes, such as $N^{w}$-hydroxy-L-arginine (L-NOHA) and 4-chlorobenzamidoxime (CIBZA).[29] Our results demonstrate that proadifen, a nonspecific inhibitor of cyt450, was unable to alter the relaxation response mediated by *E*-CAOx. *E*-CAOx relaxation response was significantly reduced only in the presence of 7-ER, an inhibitor of CYP450 1A1[54] and NAD(P)H-dependent reductase enzymes.[29,55] This finding suggests that the reductase domain of an NAD(P)H-dependent enzyme might be necessary to cleave the C=NOH functional group of *E*-CAOx. The sGC/cGMP/PKG pathway activity can be investigated by using several pharmacological tools: PTIO, an NO scavenger; ODQ, a sGC inhibitor; and through (Rp)-8pCPT-cGMP, a PKG inhibitor used as a pharmacological tool in intact tissue studies.[56]

The presence of NAC, a nitroxyl anion scavenger, at different concentrations (1 or 3 mM), did not affect the vasorelaxant response induced by *E*-CAOx, suggesting that this form of NO is not involved. This form of NO is released by a different group of NO donors, such as sodium trioxodinitrate (Angeli salt), which also can induce sGC activation.[57] Moreover, strong evidence exists demonstrating NO participation in *E*-CAOx response. Using flow cytometry, *E*-CAOx was shown to induce a significant increase in DAF-2T fluorescence when compared with control (in the absence of *E*-CAOx and in the presence of vehicle).[59] Assays using flow cytometry demonstrated that *E*-CAOx significantly increased NO levels in myocytes, which was prevented by preincubation with 7-ER, thereby reinforcing the argument that the NO donation involves NAD(P)H-dependent reductases.

Taken together, our results corroborate the current literature, where activation of sGC by NO culminates in the intense conversion of GTP into cGMP, which leads to the activation of PKG and several effector molecules that can mediate cGMP-dependent relaxing effects in vascular smooth muscle cells (VSMCs).[60]

It is well established that NO and NO donors can induce hyperpolarization of arterial VSMC[58] and PKG can alter the functionality of the $K^+$ channels resulting in vasorelaxation.[12] The opening of these channels induces a change in the resting membrane potential to more negative values (repolarization or hyperpolarization) because of efflux of $K^+$ ions, thus leading to vasodilatation.[41,59] A common property of drugs that promotes vasorelaxation through $K^+$ channel activation is the effective reduction in vascular smooth muscle contraction, in an environment composed of a moderate increase in the extracellular $K^+$ concentration (<40 mM).[41] The increase in extracellular $K^+$ from 4 to 20 mM (with isosmotic correction) significantly reduces the *E*-CAOx–mediated vasorelaxation, suggesting that hyperpolarization/repolarization mechanisms contribute to the effectiveness of this compound. Considering that in smooth muscle there are several different types of $K^+$ channels, among them $BK_{Ca}$,[60] we investigated the possible role of different $K^+$ channels involved in the vasorelaxing response elicited by *E*-CAOx.

Therefore, in the presence of 4-AP ($K_v$ blocker) or glibenclamide (a $K_{ATP}$ blocker), no curve shift in either condition was observed, confirming that these channels are not involved in the *E*-CAOx–mediated vasorelaxant response. On the other hand, treatment with TEA (1 mM) or iberiotoxin[39] reduced the *E*-CAOx–mediated response by shifting the concentration–response curve to the right. This result suggests the involvement of $BK_{Ca}$ channels but does not confirm if it occurs by direct interaction with the channel. To answer this question, we evaluated the participation of $K^+$ channel in ODQ-insensitive pathways. The results showed that only in the ODQ plus 20 mM KCl combination, *E*-CAOx response had a significant reduction.

*E*-CAOx also produces a significant reduction of $c[Ca^{2+}]$ in vascular myocytes stimulated with Phe (10 μM); this action can include the participation of calcium channels and intracellular calcium stores.[15] L-type $Ca_v$ are the major $Ca^{2+}$ influx pathway in smooth muscle cells resulting in muscle contraction.[61] When contractions are induced by a high-$K^+$ or an L-type $Ca_v$ agonist, such as *S*(−)-Bay K 8644, $Ca^{2+}$ currents ($I_{Ca}$) are generated and the $c[Ca^{2+}]$ increases, leading to contraction. *E*-CAOx, similar to other NO donors, also produces relaxant responses in arterial rings contracted in both situations, suggesting a possible inhibition of $Ca^{2+}$ influx as a candidate for the additional mechanism involved in pharmacological effect of *E*-CAOx.

## CONCLUSION

In conclusion, our results indicate that *E*-CAOx, an oxime bearing the C=NOH functional group, represents a new NO donor that can induce vasorelaxation in an endothelium- and NOS-independent manner in the rat superior mesenteric artery. NO donation is dependent upon NAD(P)H-dependent reductases, and the relaxation response occurs via sGC/cGMP/PKG pathway activation, associated with $BK_{Ca}$ activation and a reduction in $Ca^{2+}$ influx. In addition, *E*-CAOx has the potential to be a very important tool for future prevention or treatment of conditions where NO production is deficient, such as atherosclerosis, hypertension, cardiac ischemia, and thrombus formation.

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