NO upregulation of a cyclic nucleotide-gated channel contributes to calcium elevation in endothelial cells

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Abstract

We investigated whether NO upregulates a cyclic nucleotide-gated (CNG) channel and whether this contributes to sustained elevation of intracellular calcium levels ([Ca\(^{++}\)]_i) in porcine pulmonary artery endothelial cells (PAEC). Exposure of PAEC to a NO donor, NOC 18 (1 mM), for 18 hr increased the protein and mRNA levels of CNGA2 40 % and 50 %, respectively (p < 0.05). [Ca\(^{++}\)]_i in NO-treated cells was increased 50%, and this increase was maintained for up to 12 hr after removal of NOC-18 from medium. Extracellular calcium is required for the increase in [Ca\(^{++}\)]_i in NO-treated cells. Thapsigargin induced a rapid cytosolic calcium rise, whereas both a CNG and a non-selective cation channel blocker caused a faster decline in [Ca\(^{++}\)]_i, suggesting that capacitative calcium entry contributes to the elevated calcium levels. Antisense inhibition of CNGA2 expression attenuated the NO-induced increases in CNGA2 expression and [Ca\(^{++}\)]_i, and in capacitative calcium entry. Our results demonstrate that exogenous NO upregulates CNGA2 expression and that this is associated with elevated [Ca\(^{++}\)]_i and capacitative calcium entry in porcine PAEC.
INTRODUCTION

Pathological conditions associated with infections or injury lead to increased levels of nitric oxide (NO) and consequent cell injury and apoptosis in a variety of cell types including endothelial cells (35, 39, 48, 59). Excessive NO has been reported to elevate intracellular calcium levels (5, 10). The sustained high levels of intracellular calcium are cytotoxic to cells and are essential for NO-induced endothelial apoptosis (9, 23, 30, 32, 34). NO is known to elevate intracellular calcium levels through calcium release from intracellular stores, through the influx and extrusion of calcium across the plasma membrane, and through intracellular and intercellular diffusion of molecules, such as inositol-1,4,5-trisphosphate which binds to its receptor on the endoplasmic reticulum and subsequently enhances the release of calcium from intracellular stores.

It is not known how NO-elevated calcium levels are sustained in a cell after the NO stimulus is removed, but NO upregulation of calcium permeable channels, e.g. nonselective cation channels and voltage-dependent channels, may contribute to the sustained elevation of intracellular calcium levels (21, 27, 28, 57). Calcium-permeable, nonselective cation channels are believed to be a critical calcium entry pathway in the vascular endothelium (1, 26). Resting membrane potential is the electromotive force for moving ions, including calcium, through these channels. The calcium-permeable, nonselective cation channels in vascular endothelium have not been cloned; however, cyclic nucleotide-gated (CNG) cation channels, originally thought to be unique to sensory signal transduction in retinal and olfactory cells (22, 54), have been cloned and shown to be abundantly expressed in vascular endothelial cells in vivo and in vitro (18, 20, 52, 53). For instance, the mRNA for a rod-type CNG channel has been detected in tissues such as eye, lung, spleen, thymus, and smooth muscle (18, 20) and in various endothelia including those from aorta, medium-sized mesenteric arteries, and small mesenteric arteries (52, 53). The identified CNG channel subunits belong to two physiologically distinct subfamilies, CNGA and CNGB (11). There are four members, CNGA1, CNGA2, CNGA3 and CNGA4, in the CNGA
subfamily and two members, CNGB1 and CNGB3, in the CNGB subfamily (12, 40, 50, 58). CNGA2 and CNGA4 can be activated by both cAMP and cGMP (13). CNGA2 can form a functional channel when expressed alone in heterologous expression systems. In contrast, CNGA4 does not form a functional channel alone but can modulate the channel properties of CNGA2. Native functional CNG channels may exist as heteromultimers containing some combination of both subunits. CNG cation channels are permeable to both calcium and monovalent cations, and channel activity is regulated by cyclic nucleotides (cAMP or cGMP).

The physiological role of CNG channels in endothelial cells is not fully understood, but it has been recently shown that CNG channels are involved in store-operated calcium entry in rat pulmonary artery endothelial cells (52). Thus, it is possible that exogenous NO can upregulate the expression and/or activity of CNG channels in lung endothelial cells. An NO-induced increase in CNG channel expression or activity could contribute to elevation of intracellular calcium. Since an increase in CNG channels would not be degraded immediately after removal of exogenous NO, it may also contribute to a sustained elevation of intracellular calcium. NO cytotoxicity is associated with sustained elevation of intracellular calcium (9, 23, 30, 32, 34). However, there is very little information about mechanisms to explain how exogenous NO-induced elevation of intracellular calcium is maintained after removal of NO. In the present studies, we investigated whether NO upregulates CNG channels and if so, whether this contributes to sustained elevation of intracellular calcium levels via store-operated calcium entry in porcine PAEC.

MATERIALS AND METHODS

Chemicals: The CNG channel antibodies were purchased from Alpha Diagnostic International (San Antonio, TX). PolyATtract mRNA isolation system was purchased from Promega Corporation (Madison, WI). Digoxigenin-labeled dideoxyuridinetriphosphates and Genius Labeling and Detection kits were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). NOC-18 [2,2′-(hydroxynitrosohydrzino)bis-ethanamine] and PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) were purchased from Calbiochem (San
Diego, CA), and L-cis-Diltiazem HCl from Biomol Research Laboratories (Plymouth Meeting, PA). All other chemicals were obtained from Fisher Scientific (Orlando, FL).

**Cell culture and exposure to NO donor:** PAEC were obtained from the main pulmonary artery of 6-month-old pigs and were propagated in monolayer cultures as described by Zhang et al (55). Fifth- to seventh passage cells in postconfluent monolayers maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) with 4% fetal bovine serum (HyClone Laboratories, Logan, UT) and antibiotics were used for all experiments.

In the present study, NOC-18 and PTIO were used as NO donor and NO scavenger, respectively. To examine effects of prolonged exposure to NOC-18 and PTIO on cell viability, PAEC in wells of a 96-well plate were incubated in RPMI 1640 medium containing 0, 0.01, 0.02, 0.039, 0.078, 0.16, 0.31, 0.625, 1.25, 2.5, 5 and 10 mM NOC-18, and 0, 0.01, 0.02, 0.039, 0.078, 0.16, 0.31, 0.625, 1.25, 2.5, 5 and 10 mM PTIO for 18 hr, respectively. Rapid Cell Viability kit (Oncogene) was used to determine cell viability by following the manufacturer’s instructions (data not shown). Viabilities of cells treated with concentrations of NOC-18 >5 mM or of PTIO >5 mM were lower than those of control cells, i.e. incubated in 0 mM NOC-18/PTIO. Therefore, 1 mM NOC-18, 2 mM PTIO or 1 mM NOC-18+2 mM PTIO were used in the present studies, since cell viabilities under these conditions were comparable to their controls.

It has been reported that NO released from 1 mM NOC-18 results in steady state levels of 1-3 µM NO in medium without any cofactors (3) (product technique data of Calbiochem). This is comparable to concentrations (1-30 µM) produced by endogenous inducible NOS in culture media and in plasma after cytokine stimulation or lung injury (43, 44). NO concentrations used in the clinical arena for inhalation treatment of pulmonary hypertension, acute lung injury, and cardiopulmonary failure are 15-30 ppm (5-10µM) (4, 6, 24, 47). Given that the NO concentrations to which the lung endothelium is exposed are slightly lower than that in the inhaled NO gas, exposure of PAEC to 1-3 µM NO constitutes a physiologically-relevant cellular
model with which to study NO-mediated changes in CNG channel expression, intracellular calcium levels, and CNG channel-mediated capacitative calcium entry in vascular endothelial cells.

**Immuno-blot analysis of protein levels of CNG channels in PAEC:** Cell monolayers in 100-mm dishes were incubated in serum-free RPMI medium containing 0 (control) or 1 mM NOC-18 (NO) at 37°C for 18 hr. Cell lysates (40 µg protein) were loaded on 7.5 % SDS-PAGE gels, electrophoresed, and blotted on PVDF membranes as previously described (56). Anti-rat CNGB1 and anti-rat CNGA2 & 4 antibodies (10 µg/ml) were used to detect the CNG channel proteins in porcine PAEC. Band densities were determined on a densitometer scanner (MultiImager, Bio-Rad) to measure relative protein levels of the CNG channels in the endothelial cells.

To verify effects of NO released from NOC-18 on protein levels of CNG channels, a NO scavenger, PTIO, was used. PAEC were pre-incubated in medium containing PTIO (2 mM) for 2 hr, after which 0 mM (control) or 1 mM NOC-18 were added to the medium. Cell lysates (40 µM) were subjected to immuno-blot analysis of CNGA2.

To determine whether NO-modulated CNGA2 expression in PAEC is sustained after NO is removed from the medium, some control and NO-treated cells were subsequently incubated in medium without NOC-18 for 12 hr. Then, cells were collected for immuno-blot analysis of CNGA2.

To determine whether the by-product of NOC-18, H₂N-CH₂-NH-CH₂-NH₂, affects protein levels of CNG channels, 10 mM NOC-18 was dissolved in a HBSS buffer and kept as a stock solution at room temperature for 10 days (about 5X half life of NOC-18) to deplete NO. Then, PAEC were incubated in the NO-depleted solution (1 mM) and analyzed for protein levels of CNGA2. Relative levels of CNGA2 protein in by-product-treated PAEC were comparable to
those in control cells (data not shown), indicating that elevated levels of CNGA2 protein in cells exposed to 1 mM NOC-18 were due to effects of NO and not NOC-18 by-products.

**RNA isolation and reverse transcriptase (RT) PCR:** To assess the mRNA levels of CNGA2, total polyA RNA was extracted directly from control and NOC-18 (1 mM, 18 hr) exposed PAEC with the PolyATract mRNA isolation system (Promega, Madison, WI) following the manufacturer's instructions (56). Based on the 3’ end region of the isolated porcine CNGA2 cDNA (unpublished data), two gene-specific primers were designed: a sense primer (5’- CCC TCG AAA GCA ATA AAG ATG AGA AGA –3’) and an antisense primer (5’- TTC CTG ATG GAA AGG TTT ACG GGA ACA –3’). One step RT-PCR was performed to amplify a 274 bp fragment out of the mRNA target using Titan One Tube TR-PCR Kit (Boehringer-Mannheim, Indianapolis, IN). Cycling parameters were as follows: one cycle of 94°C for 2 min; 10 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 50 s; and 25 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 55 s cycle elongation of 5 s for each additional cycle. Primers for β-actin were used under identical conditions as an internal control. The RT-PCR products were analyzed on a 2% agarose gel. Band intensities were quantified on the densitometer scanner.

**Determination of intracellular calcium levels:** Changes in intracellular free ionized calcium concentration were measured using the fluorescent calcium indicator Fluo-3 acetoxyethyl ester (Fluo-3/AM, Molecular Probes, Eugene, OR) and confocal microscopy. This cell-permeable dye can be hydrolyzed by intracellular esterases and trapped in cells as Fluo-3. PAEC were grown in RPMI 1640 medium in 35 mm dishes with coverslips until cells reached 80-90% of confluence. The pre-confluent cell monolayers were then incubated in HEPES-buffered HBSS (Hanks’ Balanced Salt Solution) alone and in HBSS+NOC-18 (1 mM) at 37°C for 6 hr. To assess effects of calcium influx on NO-regulation of intracellular calcium levels, some cells were incubated in the calcium-free HBSS containing 100 µM EGTA with or without exposure to 1 mM NOC-18 for 6 hr, since cells incubated in the calcium-free HBSS for 6 hr did
not exhibit changes in morphology or viability. Thereafter, the cells were washed three times with ice-cold HBSS and loaded with 4 µM Fluo-3/ AM in HBSS for 30 min at room temperature in the dark. The dye-loaded cells were washed three times with cold HBSS and measured for fluorescent intensity using a Zeiss LSM 510 laser scanning confocal microscope with Exλ 488 nm and Emλ 526. Fluorescence intensities (a.u.) were measured in 50 selected cells and 5 empty spots (backgrounds). Average relative fluorescence intensities (a.u.) of control and NO-treated cells were calculated after being normalized to backgrounds.

To determine whether capacitative calcium entry into NO-treated endothelial cells contributes to the rise in intracellular calcium levels, an inhibitor of endoplasmic reticulum calcium ATPase, thapsigargin (Tg), was used to liberate calcium from intracellular stores. Cell monolayers on 35 mm dishes with coverslips were incubated in medium with NOC-18 (1 mM) for 18 hr. Intracellular calcium levels were measured using Fluo-3/ AM and confocal microscopy. Fluorescence intensities of 50 cells and 5 empty spots (backgrounds) were monitored. After stable baseline were obtained, Tg (final concentration: 10 µM) was added to the medium. Tracings of fluorescence intensity (a.u.) changes of cells induced by Tg were recorded. Means of background fluorescence intensities (a.u.) were subtracted from means of cell fluorescence intensities (a.u.). The ratios of F (a.u.) and F₀ (means of a.u. for the first 220 seconds) were calculated. To verify that calcium influx into cells contributes to the rise in intracellular calcium levels, some Fluo-3/AM-loaded cells were incubated in calcium-free HBSS. Tracings of fluorescence intensities (a.u.) were assessed, and the F/F₀ ratios were calculated.

To determine whether CNG channels contribute to the capacitative calcium influx, a known blocker of native CNG channels, L-cis-Diltiazem HCl (Dil) (15, 25, 41, 46) (final concentration: 500 µM), and a non-selective cation channel blocker, LaCl₃ (La³⁺) (final concentration: 100 µM) (16, 49), were used after cells were depleted of calcium stores by Tg. Then, tracings of F/F₀ ratio changes were assessed.
Antisense inhibition of porcine CNGA2 gene expression: PAEC were transfected with an inducible vector, pIND/V5-His-Topo (Invitrogen, San Diego, CA), containing antisense CNGA2 gene or GFP (a green fluorescent protein, Clontech) gene (transfection control). In brief, the cDNA for CNGA2 was rescued from plasmid pNO18 (obtained from our differential screening experiment, unpublished data), and the ends were treated with Taq DNA polymerase and cloned into a mammalian expression vector to form pIND-CNGA2. The antisense orientation of the insert was verified by restriction enzyme digestion and sequencing [DNA Sequencing Laboratory, Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida]. As a control for the transfection study, cDNA for GFP was cloned downstream of the inducible promoter to form pIND-GFP. PAEC were stably transfected with the inducible expression system encoding the antisense porcine CNGA2. To generate inducible clones, cell monolayers (passage 3) were first transfected with pVgRXR (a gene required for induction of antisense CNGA2 gene expression), and then with the pIND-CNGA2 construct. Transfection was carried out as described previously (55). Stable transfects were selected by culturing the cells in RPMI medium containing an antibiotic (neomycin) to eliminate the untransfected cells. For controls, clones containing GFP gene were generated as above with pIND-GFP instead of pIND-CNGA2 vectors.

The transfects were cultured in RPMI medium with or without 5 µM Ponasterone A for 24 hr. Immuno-blot analysis of CNGA2 in the pIND-GFP or pIND-CNGA2-transfected cells was carried out to confirm the inhibition of CNGA2 expression. To determine the effects of inhibition of CNGA2 expression on NO-increased intracellular calcium levels and calcium influx, the antisense CNGA2- and the GFP-transfected PAEC were incubated in medium with or without NOC-18 (1 mM) for 18 hr. Intracellular calcium levels were measured in these cells using Fluo-3/AM calcium indicator and confocal microscopy. Tg, Dil, and La^{3+} were used to examine calcium store-operated calcium influx in antisense CNGA2- and GFP-transfected cells as described above.

Statistical analysis: Within each experiment, control and treated cells were matched for cell line, number of passages, and state of cell confluence. Statistical significance for the effects of NO on
CNGA2 protein/ mRNA and fluorescence intensities was determined by analysis of variance and Student’s t-test (51).

RESULTS

Immu-no-blot analysis of CNG channel proteins in porcine PAEC

Family members of CNG cation channels have been reported to be present in vascular endothelium and associated with calcium influx (18, 20, 52, 53). To determine whether NO exposure alters expression of these channels in endothelial cells, immuno-blot analysis was carried out to assess the steady-state levels of CNGA2, CNGA4 and CNGB1 protein. Panel A of Figure 1 shows that relative protein levels of CNGA2 in NO-treated cells were increased 40% compared to control cells (p<0.05 vs control; n=4), whereas relative levels of CNGA4 in NO-treated and control cells were comparable. Since the CNGB1 has been reported to be present in vascular endothelium (18, 53), the weak reaction between CNGB1 antibodies and CNGB1 protein in porcine PAEC may be due to lack of antibody cross reactivity, i.e. anti-rat CNGB1 antibody vs porcine CNGB1 protein. Alternatively, the level of CNGB1 may be much lower than that of the CNGA2 or CNGA4 protein in PAEC. As such, we focused subsequent experiments on CNGA2.

To verify that upregulation of CNGA2 is caused by NO released from NOC-18, PTIO, a NO scavenger, was used pre-NO-exposure. Panel B of Figure 1 shows that PTIO attenuated the NO-increased CNGA2 protein level, indicating that the upregulation of CNGA2 in NOC-18-treated cells is mediated through generation of NO from NOC-18.

To determine whether NO-upregulated CNGA2 expression is sustained after NO-treatment, control and NO-treated PAEC were washed and then returned to control medium for 12 hr, after which protein levels of CNGA2 were detected using immunoblot analysis. Panel C of Figure 1 shows that the levels of CNGA2 protein remain elevated for up to 12 hr after being removed from a source of exogenous NO (NOC-18) in the medium.
To assess whether NO exposure also increased transcription of CNGA2, PAEC were exposed to control medium or medium containing 1 mM NOC-18 for 18 hr and mRNA levels of CNGA2 were determined using RT-PCR analysis (Figure 2, panel A). Densitometric analysis demonstrated that levels of CNGA2 transcripts in NO-exposed cells were increased 50 % compared to the controls (Figure 2, panel B). These observations suggest that 1) CNGA2 exists in porcine PAEC as detected at protein and mRNA levels and 2) gene expression of CNGA2 is upregulated by NO exposure.

**NO elevates intracellular calcium levels in PAEC**

To determine whether intracellular calcium levels are elevated after exposure of endothelial cells to NO and whether calcium influx into cells contributes to the elevation of intracellular calcium levels, calcium levels of PAEC incubated in the calcium-replete or calcium-free HBSS after exposure to NO or control medium were measured using a fluorescent dye, Fluo-3/ AM, for calcium and confocal microscopy. In the presence of extracellular calcium, exposure of PAEC to 1 mM NOC-18 for 6 hr increased intracellular calcium levels by 50 % compared to controls (1\textsuperscript{st} and 2\textsuperscript{nd} bars in Panel B, Figure 3; p<0.05, n=4). In the absence of extracellular calcium, the NO elevation of intracellular calcium was attenuated, since intracellular calcium levels in cells exposed to NOC-18 or control medium were comparable (3\textsuperscript{rd} and 4\textsuperscript{th} bars in Panel B, Figure 3). These data suggest that NO elevates intracellular calcium levels and calcium influx contributes to the NO-elevated calcium levels.

**CNG channels contribute to store-operated calcium influx in PAEC**

To determine whether calcium influx contributes to intracellular calcium levels in PAEC after NO exposure, tracings of fluorescence intensity (a.u.) changes in Fluo-3/AM-loaded cells were determined before and after intracellular calcium stores were emptied in the presence or absence of extracellular calcium. Intracellular calcium levels in cells with emptied intracellular calcium stores were elevated and the rise in calcium levels was sustained for up to 1500 seconds as determined by fluorescence intensities (a.u.) (panel A of Figure 4) and F/F\textsubscript{0} ratios (panel B). In
the absence of extracellular calcium, Tg transiently elevated fluorescence intensities (a.u.) (panel C) and F/F₀ ratios (panel D), but the sustained calcium signal was eliminated. These data suggest that calcium influx into endothelial cells contributes to NO-increased intracellular calcium levels in PAEC.

To examine whether CNG channels contributed to the capacitative calcium influx, a CNG channel blocker (Dil) and a non-selective cation channel blocker (La³⁺) were used in cells with depleted calcium stores. As shown in Figure 5, Dil decreased F/F₀ ratios of cells without (panel A) or with (panel B) NO-exposure, suggesting that CNG channels contributed to calcium store-operated calcium entry in PAEC. La³⁺ caused a fast drop in F/F₀ ratios in NO-treated and control cells. In addition, rates of decline in F/F₀ ratios induced by La³⁺ were higher than those induced by Dil. These results indicate that CNG channels contribute to capacitative calcium influx.

**Antisense inhibition of CNGA2 gene expression prevents NO-mediated elevations in intracellular calcium levels and CNG channel-mediated calcium entry in PAEC**

To examine whether suppression of the expression of the functional subunit of the CNGA gene attenuates the NO-induced increase in intracellular calcium levels and the CNG channel-mediated capacitative calcium influx, PAEC were transfected with antisense CNGA2 or a GFP gene (transfection control). After the antisense gene was induced to express for 24 hr, the CNGA2 protein level in the antisense CNGA2-transfected cells was decreased to 20 % of that observed in the GFP-transfected cells as measured by immunoblot analysis (Figure 6). Inhibition of CNGA2 expression also attenuated the NO-induced upregulation of CNGA2 expression in PAEC incubated with or without NOC-18 for 18 hr (Figure 6).

To determine whether inhibition of CNGA2 expression in endothelial cells prevents the NO-induced increase in intracellular calcium levels and the CNG channel-mediated capacitative calcium entry, antisense CNGA2- and GFP-transfected PAEC were incubated in medium containing 5 µM Ponasterone A for induction of gene expression and then with medium
containing 1 mM NOC-18 for 18 hr. Thereafter, intracellular calcium levels (Figure 7) and store-operated calcium influx (Figure 8) in the NO-treated transfects were measured. As shown in Figure 7 the intracellular calcium levels in the antisense transfects after exposure to NO and control medium were comparable; in contrast, the calcium levels in GFP transfected control cells after NO exposure were elevated 50% (p< 0.05, n=4). These results suggest that inhibition of CNGA2 channel expression prevents elevation of intracellular calcium levels caused by NO. As shown in Figure 8 Dil did not alter capacitative calcium entry in antisense CNGA2-transfected cells with or without NO-exposure, suggesting that inhibition of CNGA2 gene expression prevents CNG channel-mediated capacitative calcium entry in PAEC.

DISCUSSION

In vascular endothelium NO has been associated with activation of a calcium permeable non-specific cation channel (28). Our data demonstrate that NO upregulates steady state protein and mRNA levels of CNGA2 but not CNGA4 in porcine PAEC. The increased expression of CNGA2 is retained for up to 12 hr after cessation of NO exposure. Since CNGA2 can form a functional channel, increased CNGA2 can lead to enhanced channel activity in NO-exposed endothelial cells. Increased protein levels were correlated with elevated mRNA, suggesting that NO may regulate CNGA2 gene expression by enhanced synthesis and/or stability of the mRNA for this CNG channel.

NO is known to increase cGMP levels via activation of the soluble guanylate cyclase (13, 38), which can activate CNGA2 (2). Levels of cGMP, but not cAMP, were elevated in cultured porcine PAEC exposed to 1 mM NOC-18 for 18 hr (data not shown). The NO-increased cGMP may keep CNGA2 active, leading to a sustained rise in calcium. It is also possible, though less likely, that NO directly activates CNGA through modification of sulfhydryl groups (14).

NO-upregulated CNGA2 expression was associated with elevation of intracellular calcium levels and capacitative calcium entry in porcine PAEC in the current studies. Both the
release of calcium from intracellular stores, most likely the endoplasmic reticulum, and the influx of calcium from the extracellular space, i.e. capacitative calcium entry, contribute to the NO-induced elevation of cytosolic calcium. NO and other oxidant stresses are known to mediate calcium influx in endothelial cells (5, 28, 33). Exogenous NO increased intracellular calcium levels in cultured porcine aortic endothelial cells (5). As such, our results are in agreement with previous observations in endothelial cells (5, 28, 33).

The present study also demonstrates that the CNG channel blocker, Dil, inhibited capacitative calcium influx induced by calcium store depletion both in control and NO-treated PAEC, suggesting that CNG channels contribute to the calcium influx. Inhibition of calcium entry by La\(^{3+}\) indicates that non-selective cation channels including CNG channels may play a role in maintaining intracellular calcium levels. Although CNGB1 has been shown to be present in vascular endothelial cells derived from systemic arteries (53) and sensitive to Dil (25), our results showed that CNGA2, but not CNGB1, was abundant, and CNGA2 expression was upregulated by NO exposure in porcine PAEC. Differences in CNGB1 abundance among vascular endothelial cells may be due to different animal species (guinea pig/ rat vs porcine) and/or origin of vascular bed (aorta/ mesenteric arteries vs pulmonary arteries). Differences in CNGA2 sensitivity to Dil may be due to a non-specific chemical modulation and/or a selective response to Dil in specific cell types (rod cells vs endothelial cells). However, irrespective of the effects of chemical modulation, the association between increased CNGA2 expression and elevated intracellular calcium levels has been verified using antisense methodology to specifically suppress CNGA2 gene expression. Antisense inhibition of CNGA2 attenuated NO elevation of intracellular calcium levels and CNG channel-associated capacitative calcium entry in porcine PAEC, suggesting a role for NO modulation of CNG channels.

NO upregulation of CNGA2 expression may increase the number of the channels in a cell, consequently enhancing calcium entry and leading to elevation of intracellular calcium levels. CNG channels have been shown to play an important role in the regulation of calcium
influx in endothelial cells (42, 52). Recently, it has been shown that in rat PAEC a non-selective cation conductance attributed to channels regulated by cyclic nucleotides and endogenously expressed CNG channels mediated the non-selective cation current (52). Furthermore, a portion of the CNGA2 gene has been cloned from the rat PAEC and linked to store-operated calcium entry (52). Our results are in agreement with these reports and demonstrate the presence of functional CNG channels which contribute to capacitative calcium entry in PAEC.

Intracellular calcium has been shown to act as a signal transducer that modulates multiple cellular process including apoptosis (19, 31). Enhanced calcium influx has been observed in NO-induced apoptosis of mouse oligodendrocytes (9) and in glucocorticoid-stimulated thymocytes (29, 36). The initial calcium increase in apoptotic cells occurs via a controlled, physiological mechanism such as surface antigen receptor engagement on B cells leading to calcium increases that promote cell death (8). Therefore, NO-upregulated CNGA2 channels and consequent increase in capacitative calcium influx and intracellular calcium levels may contribute to the triggering of apoptotic cascades in endothelial cells. In this study we did not examine the downstream effects of NO-increased intracellular calcium levels and its link with selective pathways in induction of apoptosis. However, accumulating evidence indicates that a calcium signal is associated with apoptosis (37). For example, elevated intracellular calcium can activate calcium-dependent protein kinases and phosphatases in B cell lines that have been reported to control apoptosis (7). Intracellular calcium can activate calpain, a calcium-dependent neutral proteinase, to induce apoptosis since calpain is rapidly activated in apoptotic cells and the apoptosis is prevented by blocking calpain activation using specific inhibitors (45). Increase in intracellular calcium can also activate Ca\(^{2+}/Mg\(^{2+}\)-dependent endonuclease, leading to DNA fragmentation, the hallmark of apoptosis (17).

In conclusion, exogenous NO upregulates CNGA2 expression which contributes to enhanced capacitative calcium influx and increased intracellular calcium levels in pulmonary vascular endothelial cells. The increased cytosolic calcium levels may be involved in cytotoxicity
of NO, e.g. apoptosis, in endothelial cells. Our observations support the notion that the CNG channel, a type of non-selective cation channel, may play physiological and pathological roles in lung endothelium via calcium influx. Since the lung endothelium is a potential target for NO generated by the action of inducible NO synthase in alveolar macrophages and lung inflammatory cells and for NO administered as inhalation therapy, a better understanding of NO upregulation of CNG channel expression and calcium influx will help us to prevent potential pulmonary vascular dysfunction associated with NO-inhalation therapy, lung inflammation, and acute lung injury that are associated with increased endogenous NO production.

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REFERENCE


FIGURE LEGENDS

**Figure 1.** Immuno-blot analysis of protein levels of CNG channels in PAEC. **Panel A:** proteins of CNGB1, CNGA2 and CNGA4 in cells exposed to 0 mM (control) and 1 mM NOC-18 were immuno-detected as described in Materials and Methods (inserts show representative bands). Band densities of CNGA2 and CNGA4, but not CNGB1 due to a weak reaction between anti-rat CNGB1 antibody and porcine CNGB1 protein, were analyzed. Relative levels of CNGA2 and CNGA4 in NO-treated and control cells were plotted (bar graphs). Levels of CNGA2 protein in NO-treated cells were increased 40% compared to control. Levels of CNGA4 in NO-treated and control cells were comparable. Lane 1: cells exposed to control medium; and lane 2: cells exposed to NOC-18. **Panel B:** verification of effects of NO released from NOC-18 on increased CNGA2 protein levels in PAEC. Lane 1: CNGA2 protein in cells exposed to 0 mM NOC-18; lane 2: CNGA2 in cells exposed to 1 mM NOC-18; lane 3: CNGA2 in cells in the presence of 2 mM PTIO; and lane 4: CNGA2 in cells exposed to 1 mM NOC-18 in the presence of 2 mM PTIO. **Panel C:** upregulated CNGA2 expression is sustained after removal of NO from the medium for up to 12 hr. Lane 1: cells exposed to 0 mM NOC18; lane 2: cells exposed to 1 mM NOC-18; lane 3: cells exposed to control medium for 18 hr, and then washed and transferred to fresh medium for 12 hr; and lane 4: cells exposed to NOC-18 for 18 hr, and then washed and transferred to fresh medium for 12 hr. Data represent mean ± S. E. (n=4) for each sample. * p<0.05 vs controls for all panels.

**Figure 2.** Exogenous NO increased mRNA levels of CNGA2 in PAEC. One step RT-PCR was performed to amplify a 274 bp fragment out of the CNGA2 mRNA target as described in “Materials and Methods” (Panel A). The amplified products were analyzed, quantified, and normalized to levels of actin mRNA (Panel B, relative mRNA levels). The relative steady state levels of CNGA2 transcripts in NO-exposed PAEC were increased 50% compared to controls.
C=cells exposed to control medium; and NO=cells exposed to NOC-18 (1 mM). Data represent mean ± S. E. (n=4) for each sample. *: p<0.05 vs control.

**Figure 3.** Calcium influx into PAEC contributed to the NO-induced elevation of intracellular calcium levels. PAEC were incubated in calcium-replete HBSS (+Calcium) with (NO) or without (C) NOC-18 (1 mM) for 6 hr, after which relative intracellular calcium levels were measured using a calcium indicator (Fluo-3/ AM) and confocal microscopy as described in “Materials and Methods”. To examine whether calcium entry plays a role in NO-elevated levels of the intracellular calcium, cells were incubated in calcium-free HBSS (-Calcium) with (NO) or without (C) NOC-18 (1 mM) for 6 hr. Panel A shows representative photomicrographs of cells loaded with Fluo-3/ AM in calcium-replete (1st and 2nd photomicrographs) or calcium-free HBSS (3rd and 4th photomicrographs) and with (NO) or without (C) exposure to NO for 6 hr. Means of fluorescence intensities of 50 cells with or without incubation in calcium-free medium after exposure to NO or control conditions are plotted in panel B. Data represent mean ± S. E. (n=4) for each sample. * p<0.05 vs control.

**Figure 4.** Calcium influx contributes to intracellular calcium levels. Changes of intracellular calcium levels in NO-treated PAEC with depleted calcium stores were assessed using Fluo-3/ AM and confocal microscopy as described in “Materials and Methods”. Panels A and C show representative tracings of fluorescence intensity (a.u.) changes of 6 cells induced by Tg in the presence and absence of extracellular calcium, respectively. The F/F₀ ratios were calculated and plotted in panels B and D, respectively.

**Figure 5.** CNG channels contribute to capacitative calcium entry in PAEC. Changes in calcium levels in control and NO-treated PAEC after emptying calcium stores and blocking CNG or non-
selective channels were examined as described in “Materials and Methods”. F/F₀ ratios had a fast drop in both control (panel A) and NO-treated (panel B) cells with depleted calcium stores, after Dil was added to the medium. La³⁺ induced fast drops in calcium store-depleted cells with (panel C) or without (panel D) NO exposure. Panels A and B: changes of F/F₀ ratios in control (A) and NO-treated (B) cells after Tg and Dil were added to the medium. Panels C and D: changes of F/F₀ ratios of cells without (C) or with (D) NO exposure to which Tg and La³⁺ were added to the medium.

Figure 6. Antisense inhibition of CNGA2 gene expression. Cell monolayers were transfected with constructs containing GFP (a transfection control) or antisense CNGA2 gene. The transfected cells expressing GFP or antisense CNGA2 were exposed to 1 mM NOC-18 for 18 hr. Then, relative levels of CNGA2 protein in the transfects were assessed using immunoblot analysis (insert), while band densities were determined on a densitometer scanner (bar graphs in each panel). Inhibition of CNGA2 expression attenuated NO-induced upregulation of CNGA2 gene expression. Lane 1: cells transfected with GFP and exposed to control medium; lane 2: cells transfected with GFP and exposed to 1 mM NOC-18 for 18 hr; lane 3: cells transfected with antisense CNGA2 gene and exposed to control medium; and lane 4: cells transfected with antisense CNGA2 and exposed to 1 mM NOC-18 for 18 hr. C: cells exposed to control medium; NO: cells exposed to 1 mM NOC-18 for 18 hr; GFP: cells transfected with GFP gene; and antisense CNGA2: cells transfected with antisense CNGA2 constructs. Data represent mean ± S. E. (n=3) for each sample. ∗: p<0.05 vs control and **: p<0.05 vs cells transfected with GFP gene and exposed to control medium.

Figure 7. Inhibition of CNGA2 expression abolished NO-induced elevation of intracellular calcium levels. PAEC were transfected with constructs containing antisense CNGA2 or GFP
gene. The transfects were incubated in medium without (control conditions) or with NOC-18 (1 mM) for 18 hr, after which relative intracellular calcium levels were measured using Fluo-3/AM and confocal microscopy as described in “Materials and Methods”. Panel A shows representative photomicrographs of cells loaded with Fluo-3/AM after exposure to NO and control conditions, while panel B shows means of fluorescence intensities (a.u.) of 50-100 cells. NO caused 50% elevation of intracellular calcium levels in GFP-transfected cells. Fluorescence intensities of antisense CNGA2-transfected cells exposed to NO were comparable to those measured in cells not exposed to NO. C: cells incubated in control medium without NOC-18; NO: cells incubated in medium containing 1 mM NOC-18 for 18 hr; GFP: GFP-transfected cells; and antisense CNGA2: cells transfected with antisense CNGA2 constructs. Data represent mean ± S. E. (n=4) for each sample. * p<0.05 vs control.

**Figure 8.** Antisense inhibition of CNGA2 expression abolished CNG channel-mediated capacitative calcium entry in PAEC. Antisense CNGA2-transfected cells in 35 mm dishes with coverslips were incubated in medium with or without NOC-18 (1 mM) for 18 hr. Relative levels of intracellular calcium were assessed using Fluo-3/AM and confocal microscopy as described in “Materials and Methods”. In antisense CNGA2-transfected cells without (panel A) or with (panel B) NO-exposure, Dil did not alter F/F₀ ratios in cells with depleted calcium stores, suggesting that inhibition of CNGA2 expression abolished CNG channel-associated capacitative calcium entry. **Panels A and B:** fluorescence intensities (F/F₀) of antisense CNGA2-transfected cells without (A) or with (B) NO exposure to which Tg and Dil were added to the medium.
Figure 1

A

[Images of CNGB1, CNGA2, and CNGA4 proteins with bar graphs showing relative levels of CNG channel proteins.]

B

[Images of relative levels of CNGA2 protein with bar graphs showing control (C) and NO treatment (NO) with PTIO.]

C

[Images of relative levels of CNGA2 protein with bar graphs showing control (C) and NO treatment (NO) 12 hr post NO-treatment.]
Figure 2

A

CNGA2

Actin

B

Relative levels of CNGA2 mRNA

C

NO

*
Figure 3
Figure 4

A

B

C

D

Tg

fluo-3 intensity (a.u.)

Time (sec)

fluo-3 intensity (F/F₀)

Time (sec)

Tg

fluo-3 intensity (a.u.)

Time (sec)

fluo-3 intensity (F/F₀)

Time (sec)
Figure 5
Relative levels of CNGA2

CNGA2 levels were measured under different conditions: GFP and Antisense CNGA2. The graph shows significant increases in CNGA2 levels under the NO condition compared to the C condition. The statistical significance is indicated by asterisks: * denotes a significant increase, ** denotes a highly significant increase.

Figure 6
Figure 7
Figure 8

A

B

Dil
t

Tg

fluo-3 intensity (F/F_0)

Time (sec)

fluo-3 intensity (F/F_0)

Time (sec)