Nitric oxide: inhibitory effects on endothelial cell calcium signaling, prostaglandin I$_2$ production and nitric oxide synthase expression

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Abstract

Objective: Nitric oxide (NO) produced in large amounts by inducible nitric oxide synthase exerts many harmful effects such as stimulation of inflammation and induction of apoptosis. The effects of excessive NO production on functions of endothelial cells, the major physiological source of NO, are not completely known. The aim of this study was to investigate the role of NO on the regulation of endothelial cell Ca$^{2+}$ signaling and endothelial cell function.

Methods: Primary porcine aortic endothelial cells (PAECs) were used for all these studies. Intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]) were measured using fura-2/AM. Production of prostaglandin I$_2$ (PGI$_2$) and cyclic GMP were assessed using enzyme immunoassays, and endothelial NO synthase protein expression was evaluated by Western blotting.

Results: Bradykinin (BK, 10 nM) and thapsigargin (TG, 1 $\mu$M) provoked large increases in [Ca$^{2+}$]. The NO donor NOC12 reduced these responses, respectively, by 21% and 31% at 100 $\mu$M, 60% and 55% at 300 $\mu$M, and 74% and 78% at 500 $\mu$M. These effects were also observed with other NO donors including spermine NONOate and NOC18, and were completely prevented by carboxy-PTIO (200 $\mu$M), an NO scavenger. 8-Bromo-cGMP, however, had no effects on BK- and TG-stimulated Ca$^{2+}$ responses. A 30-fold increase in PGI$_2$ production was observed in cells stimulated with BK. NOC12 again reduced this response by 12%, 54%, 83% and 95% at 10, 100, 300 and 500 $\mu$M, respectively. Endothelial NO synthase protein level was reduced by 2%, 15%, 36 and 47% after 2, 6, 12 and 24 h, respectively, of incubation with NOC18, a NO donor with long half-life.

Conclusions: NO, when produced in large amounts, can inhibit agonist-induced Ca$^{2+}$ responses independently of cyclic GMP, reduce the production of endothelium-derived relaxing factors (EDRFs) and interfere with endothelial NO synthase protein expression.

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1. Introduction

Maintenance of vascular homeostasis is dependent to a great extent on the activities of endothelium-derived relaxing factors (EDRFs), including nitric oxide (NO) and prostaglandin I$_2$ (PGI$_2$), and endothelium-derived hyperpolarizing factor (EDHF). Production of EDRFs requires Ca$^{2+}$ entry, which represents the major portion of the increase in free intracellular Ca$^{2+}$ in response to physiological stimuli such as shear stress and receptor agonists. Endothelial NOS (eNOS), the enzyme responsible for the physiological production of NO, also requires Ca$^{2+}$ entry for sustained activation.

NO produced by eNOS has multiple beneficial effects including modulation of platelet aggregation, inhibition of leukocyte adhesion and control of vascular smooth muscle cell proliferation [1–3]. However, when it is produced or administrated in large quantities, NO could have such harmful effects as causing inflammation and inducing apoptosis [4–6].
The effects of excessive NO on endothelial cell signaling and functions are nevertheless not completely known. Studies examining the effects of NO donors on Ca\textsuperscript{2+} entry have described controversial findings \cite{7,8}, and little is known as to whether NO can affect the production of other EDRFs. In this study, we examined the effects of NO donors on Ca\textsuperscript{2+} signaling and PGI\textsubscript{2} production, and eNOS protein expression in primary cultured endothelial cells. The results indicate that large amounts of NO inhibit agonist-stimulated Ca\textsuperscript{2+} entry, substantially block the production of PGI\textsubscript{2} and interfere with eNOS protein expression.

2. Methods

2.1. Cell culture

Porcine aortic endothelial cells (PAECs) were isolated, as previously described \cite{9}, by gently scraping the intima of descending aortas from pigs from slaughter house. After centrifugation at 250  \times  g for 10 min in M199 solution (Boehringer, Mannheim, Germany), the fraction of endothelial cells was purified from this suspension, resuspended in M199 solution with Earle’s salts, supplemented with 100 IU/ml penicillin G, 100 \mu g/ml streptomycin and 20% newborn calf serum (NCS), then seeded onto polybiphenyl dishes fixed on 10\times10 mm glass cover slips, and cultured at 37 °C under 5% CO\textsubscript{2} for 2 days. The medium was renewed everyday.

2.2. Measurement of intracellular Ca\textsuperscript{2+} concentration

After 2 days of culture, PAECs adhering to glass cover slips were incubated for 45 min in a modified Tyrode’s solution (composition in mM: 150.0 NaCl, 2.7 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, 1.0 CaCl\textsubscript{2} and 10.0 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, with pH 7.4 at 25 °C) containing 10% NCS and 2 \mu M fura-2/AM, a fluorescent Ca\textsuperscript{2+} indicator. The cells were subsequently washed three times with modified Tyrode’s solution to remove the fura-2/AM and the serum from the extracellular fluid, then left to equilibrate in the cell buffer for 20 min before measurements were started. All experiments were performed at 25 °C. The absorption shift of fura-2 that occurs upon binding can be determined by scanning the excitation spectrum between 340 and 380 nm while monitoring the emission at 510 nm. Fluorescent images were analyzed every 30 s from individual cells with an [Ca\textsuperscript{2+}]\textsubscript{i} analyzer (Argus 50, Hamamatsu Photonics) using an ultrahigh sensitivity television camera (CCD). After background subtraction, the fluorescence ratio (F340/F380) was obtained by dividing, pixel by pixel, the 340 nm image by the 380 nm image. Changes in this ratio were used to express changes in intracellular Ca\textsuperscript{2+} concentration to eliminate potential artifacts caused by variations in cell thickness, intracellular dye distribution or photobleaching. Bradykinin (BK), thapsigargin (TG), NOC12, NOC18, spermine NONOate, carboxy-PTIO, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and 8-bromo-cyclic guanosine-3’,5’-monophosphate (cGMP) had no effect on fura-2 fluorescence itself, or on autofluorescence of unloaded cells when examined at concentrations employed in this study.

2.3. Measurement of cGMP concentration

Determinations of cGMP level were performed as previously described \cite{10}. PAECs were plated onto 96-well culture dishes at a density of 10\textsuperscript{4} cells/well and incubated with M199 medium for 2 days at 37 °C under 5% CO\textsubscript{2} – air and saturated humidity. When PAECs reached confluence, the medium was removed and the cells were washed with cell buffer. PAECs were then incubated for 45 min at room temperature in the cell buffer containing NOC12, an NO donor, and/or ODQ, a guanylyl cyclase inhibitor. The cells were then resolved and cyclic GMP levels were determined using an enzyme-linked immunoassay according to the manufacturer’s instructions (EIA kit, Amersham, USA).

2.4. Measurement of prostaglandin I\textsubscript{2}

The amount of PGI\textsubscript{2} released from PAECs was measured as the concentration of its stable metabolite 6-keto-PGF\textsubscript{1\alpha}. PAECs were plated in 35-mm culture dishes and incubated with M199 medium for 2 days at 37 °C under 5% CO\textsubscript{2} – air and saturated humidity. When PAECs reached confluence, the medium was removed and the cells were washed with cell buffer. PAECs were then incubated for 45 min at room temperature in the cell buffer containing various concentrations of NOC12. Bradykinin (10 nM) was then added, and the incubation was continued for 10 min at room temperature. The concentrations of 6-keto-PGF\textsubscript{1\alpha} in the cell medium were then determined using an enzyme-linked immunoassay according to the manufacturer’s protocol (EIA, Assay Designs, Amersham).

2.5. Western blotting

After incubation of subconfluent PAECs with NOC18 for 2, 6, 12 and 24 h, the cells were lysed in a lysis buffer containing (in mM) 150.0 NaCl, 1.0 EDTA, 1.0 EGTA, 2.5 sodium pyrophosphate, β-glycerophosphate, 1.0 Na\textsubscript{3}VO\textsubscript{4}, 20.0 Tris–HCl, 1.0 PMSF, with 1 \mu g/ml leupeptin and 1% (vol/vol) Triton X-100. Total cell protein concentration was determined using the Dc protein assay kit (Bio-Rad, CA, USA). Samples containing equal amounts (20 \mu g) of total cellular protein were loaded and separated on 7.5% SDS-polyacrylamide gel electrophoresis. The gel was then transferred onto a PVDF membrane, which was then incubated at 4 °C for 24 h with a primary anti-eNOS monoclonal antibody (1 \mu g/ml). After washes in transfer buffer, the membrane was incubated with goat anti-mouse IgG-horse-radish peroxidase. The washes were repeated before the membrane was developed by a light-emitting nonradioactive
method using ECL reagent (Amersham). The membrane was then subjected to autoradiography for 1–5 min. Band intensities were analyzed densitometrically using the software IMAGE (National Institutes of Health, MD, USA).

2.6. Statistical analysis

Unless otherwise indicated, data are means±S.D. Statistical analysis was performed using Student’s t-test for unpaired data or one-way ANOVA followed by post-hoc tests in the Systat MGLH program, where appropriate. Differences with \( p<0.05 \) are considered significant.

2.7. Materials

Medium 199 was purchased from Boehringer (Manheim, FRG). Newborn calf serum and penicillin-streptomycin were from GIBCO (New York, USA). Fura-2/AM, NOC12 and NOC18 were purchased from Dojindo (Kumamoto, Japan). Bradykinin, thapsigargin, spermine NONOate and ODQ were from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grades.

3. Results

3.1. \( \text{Ca}^{2+} \) signals produced by bradykinin and thapsigargin in PAECs

To trigger increases intracellular \( \text{Ca}^{2+} \) concentration in PAECs, we used BK, a receptor agonist, and TG, an irreversible inhibitor of the endoplasmic reticulum \( \text{Ca}^{2+} \)-ATPase. In the presence of extracellular \( \text{Ca}^{2+} \), BK (10 nM) rapidly increased F340/F380 from 0.6±0.1 (basal) to 5.1±0.4 (maximal), which was slowly attenuated (open circles, Fig. 1A). The rapid increase in \( [\text{Ca}^{2+}]_i \) was dependent on both intracellular and extracellular \( \text{Ca}^{2+} \), while the sustained increase was dependent mainly on the presence of extracellular \( \text{Ca}^{2+} \), because BK induced only a small and transient increase in F340/F380 from 0.5±0.1 (basal) to 1.6±0.5 (maximal) in the absence of extracellular \( \text{Ca}^{2+} \).

TG (1 \( \mu \)M) provoked a slightly delayed but long lasting increase in F340/F380 from 0.7±0.1 (basal) to 4.7±0.7 (maximal) (open circles, Fig. 1B) in the presence of extracellular \( \text{Ca}^{2+} \). Under \( \text{Ca}^{2+} \)-free conditions with the cell buffer containing 1 mM EGTA, TG (1 \( \mu \)M) provoked a small transient rise in F340/F380 from 0.5±0.1 (basal) to 1.2±0.2 (maximal) \( (n=21) \). Thus, the sustained \( \text{Ca}^{2+} \) increases in response to TG depend on \( \text{Ca}^{2+} \) entry.

3.2. Effects of different NO donors, NOC12, NOC18 and spermine NONOate, on the BK- and TG-induced \( \text{Ca}^{2+} \) responses in PAECs

Pretreatment of PAECs with NOC12 (500 \( \mu \)M) for 45 min almost completely prevented the \( \text{Ca}^{2+} \) responses to BK (10 nM) and TG (1 \( \mu \)M). Basal and maximal F340/F380 ratios were, respectively, 0.7±0.1 and 1.3±0.4 for BK (closed squares, Fig. 1A), and 0.8±0.1 and 1.0±0.1 for TG (closed squares, Fig. 1B) in the presence of 1 mM extracellular \( \text{Ca}^{2+} \). NOC12 dose-dependently (100–500 \( \mu \)M) inhibited the BK- and TG-stimulated \( \text{Ca}^{2+} \) responses, as shown in Fig. 1A and B. The reversibility of the effect of NOC12 was confirmed in the experiments in Fig. 2A, in which BK (10 nM, A) or TG (1 \( \mu \)M, B) was added. Values are means±S.D.; \( n=21 \) cells from three separate experiments.
3.3. Testing the involvement of cGMP in the inhibition of the Ca\(^{2+}\) signals by NO

To clarify if cGMP is involved in the observed inhibitory effect of NOC12, we tested the effect of the cGMP analog 8-bromo-cGMP on BK- and TG-induced Ca\(^{2+}\) responses in PAECs. As shown in Fig. 3A and B, pretreatment of 2 mM 8-bromo-cGMP had absolutely no effect on the BK- and TG-induced Ca\(^{2+}\) increases; the peak F340/F380 ratios were 5.1±0.4 and 4.3±0.2, respectively, for BK and TG. These data indicate that NOC12 does not inhibit BK- and TG-stimulated Ca\(^{2+}\) responses via the accumulation of cGMP.

To further confirm that the observed inhibitory effect of NOC12 does not involve the cGMP pathway, we tested if the guanylate cyclase inhibitor ODQ could prevent the observed effect of NOC12. The effect of ODQ to inhibit NOC12-stimulated cGMP production was first confirmed. As shown in Fig. 3C, NOC12-induced cGMP accumulation was inhibited by ODQ (375.7±75.9 and 248.1±59.9 pg/10\(^6\) cells at 0 and 10 \(\mu\)M ODQ, respectively), and was almost completely inhibited at 20 \(\mu\)M ODQ (46.2±8.8 pg/10\(^6\) cells in control vs. 65.8±24.3 pg/10\(^6\) cells with 20 \(\mu\)M ODQ, N.S., \(n=6\)). However, even at this dose ODQ could not prevent the inhibitory effect of NOC12 on the Ca\(^{2+}\) responses (Fig. 3A and B). These results suggest that the inhibition by NOC12 of the BK- and TG-stimulated Ca\(^{2+}\) responses in PAECs does not involve cGMP accumulation.

3.4. Effect of excessive NO on PGI\(_2\) production induced by BK in PAECs

Besides NO, PGI\(_2\) plays important role in endothelium-dependent vasorelaxation and its production also depends on increases in cytosolic Ca\(^{2+}\) concentration. Phospholipase A\(_2\), the rate-limiting enzyme for the liberation of arachidonic acid from phospholipids to produce PGI\(_2\), is strictly Ca\(^{2+}\)-dependent [11–14]. We therefore tested the effects of various doses (10–500 \(\mu\)M) of NOC12 on PGI\(_2\) production in PAECs. PGI\(_2\) production was assessed by measuring its stable metabolite 6-keto-PGF\(_{1\alpha}\). As shown in Fig. 4, BK (10 nM) greatly increased 6-keto-PGF\(_{1\alpha}\) from 16.0±6.2 ng/10\(^6\) cells to 481.4±68.0 ng/10\(^6\) cells. This effect was inhibited by NOC12 in a dose-dependent manner (420.3±27.3, 220.6±55.6, 76.4±8.6 and 21.3±1.8 ng/10\(^6\) cells at 10, 100, 300 and 500 \(\mu\)M NOC12, respectively). There is a good correlation between the inhibitory effects of NOC12 on BK-stimulated peak [Ca\(^{2+}\)]\(_i\) and PGI\(_2\) production (\(R=0.96\), \(p<0.05\)).

**Table 1**

<table>
<thead>
<tr>
<th>NO donors</th>
<th>Peak F340/F380</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bradykinin (10 nM)</strong></td>
<td><strong>Thapsigargin (1 (\mu)M)</strong></td>
</tr>
<tr>
<td>Buffer</td>
<td>5.1±0.4</td>
</tr>
<tr>
<td>NOC12, 500 (\mu)M</td>
<td>1.3±0.4*</td>
</tr>
<tr>
<td>NOC18, 1 (\mu)M</td>
<td>2.5±0.5*</td>
</tr>
<tr>
<td>NONOate, 500 (\mu)M</td>
<td>1.4±0.5*</td>
</tr>
</tbody>
</table>

Data are shown as means±S.D.; \(n=21\). Cells were incubated with each of these compounds for 45 min before BK (10 nM) or TG (1 \(\mu\)M) was added. Data are mean (\(n=21\) cells) peak F340/F380 ratios from three separate experiments.

* \(p<0.05\) vs. buffer.
3.5. Effect of NOC18 on eNOS protein expression in PAECs

Endothelial NOS is the major physiological source of NO production. We thus examined the effect of excessive...
NO on eNOS protein levels in PAECs using NOC18, an NO donor with a half-life of 21 h at 37 °C. Subconfluent PAECs were pretreated with NOC18 for various periods of time before the cells were lysed and eNOS protein were blotted. As shown in Fig. 5, NOC18 time-dependently reduced eNOS protein levels by 2%, 15%, 36% and 47% after 2, 6, 12 and 24 h, respectively.

4. Discussion

In this study, we have found that large amounts of NO can substantially inhibit BK- and TG-stimulated Ca\(^{2+}\) responses independently of the cGMP pathway, decrease PGI\(_2\) production and suppress eNOS protein expression in vascular endothelial cells.

Changes in Ca\(^{2+}\) concentration, in particular capacitative Ca\(^{2+}\) entry, in response to agonists and shear stress are important for many endothelial cell functions [15]. The literature has been controversial as regards the effects of NO on Ca\(^{2+}\) entry. Several groups have reported that NO donors can inhibit capacitative Ca\(^{2+}\) entry (CCE) through a cGMP-linked pathway [7,16–19]. Dedkova et al. reported that sodium nitroprusside (SNP, 100 μM) and 8-bromo-cGMP (300 μM) inhibited ATP-induced CCE in bovine vascular endothelial cells and concluded that NO could exert an autoregulatory negative feedback on its own Ca\(^{2+}\)-dependent synthesis. On the contrary, Gilon et al. [8] reported that neither NO nor cGMP had any effect on CCE in rat pancreas acinar cells. In the present study, we found that excessive NO could inhibit BK- and TG-induced Ca\(^{2+}\) responses independently of cGMP pathway, as evidenced by the observations that NOC12 significantly prevented these responses while 8-bromo-cGMP did not, and that inhibition of guanylyl cyclase with ODQ could not reverse the inhibitory effect of NOC12. While it is possible that differences among cell types could attribute to some extent to the apparently opposing findings in these studies, further studies are needed to address this issue.

Several studies have demonstrated that exogenous NO can inhibit the vascular response to NO. Dinerman et al. [20] demonstrated that endothelium desensitized the relaxation of vascular smooth muscle in response to nitroglycerin and postulated a competitive interaction between endothelial NO and nitroglycerin. Our studies suggest that desensitization might occur at the level of the endothelial cell through attenuation of the Ca\(^{2+}\) response to vasorelaxing agonists.

The mechanism underlying the inhibitory effect of excessive NO on the Ca\(^{2+}\) responses remains unclear. However, it is possible that excessive NO does this by inhibiting cytochrome P450 via NO-heme interactions. Previous studies have suggested that the cytochrome P450 product 5,6-epoxy eicosatrienoic acid (5,6-EET) can regulate Ca\(^{2+}\) entry. Thus, CYP inhibitors were shown to inhibit agonist-induced 5,6-EET production and Ca\(^{2+}\) entry in endothelial cells. Also in these studies, induction of CYP potentiated agonist-induced Ca\(^{2+}\) entry, and nano- to picomolar 5,6-EET induced Ca\(^{2+}\) elevation consistent with Ca\(^{2+}\) entry [21]. The activity of cytochrome P450 isoymes depends on a heme-protein in their structures. NO has been demonstrated to form a tight complex with the heme [22] and inhibit CYP-related reactions [23–25]. Therefore, large quantities of NO may inhibit agonist-induced Ca\(^{2+}\) signals in endothelial cells by suppressing 5,6-EET production. Additionally, excessive NO could inhibit Ca\(^{2+}\) entry via the formation of peroxynitrite. Elliott [26] reported that peroxynitrite decreased BK-induced Ca\(^{2+}\) entry in vascular endothelial cells. Large amounts of NO will diffuse easily through the mitochondrial membrane and reversibly inhibit cytochrome oxidase. This inhibition suppresses the mitochondrial respiratory chain and as a consequence increases mitochondrial O\(_2^–\) radical release, leading to peroxynitrite formation [27,28]. The effects of NO on CYP450 and cytochrome oxidase could converge to bring about its inhibition of the Ca\(^{2+}\) responses in endothelial cells.

Endothelial nitric oxide synthase is activated by increases in cytosolic Ca\(^{2+}\) [29–32] and is dependent on Ca\(^{2+}\) entry for its sustained activation [33]. We now show that NO can inhibit Ca\(^{2+}\) entry independently of cGMP production. However, Parkinson et al. [34] have shown that Ca\(^{2+}\) can directly inhibit NO-stimulated soluble guanylyl cyclase. From these studies, it is possible that cGMP production is balanced in part by Ca\(^{2+}\)-dependent eNOS activity and Ca\(^{2+}\)-inhibitable soluble guanylyl cyclase activity, with possible superiority of eNOS in this regards [29–32].

The present study also demonstrates that excessive NO can reduce eNOS protein expression. Chen and Mehta [35] previously showed that NO, authentic or derived from nitroglycerin, decreased NOS activity without affecting NOS protein expression in human platelets after incubation for 3 h. These findings suggest that the negative feedback mechanism can be formed in short term. In the present study, a NO donor with a long half-life reduced eNOS protein expression by ~50% after 24-h incubation. This clearly confirms that exogenous NO can interfere with eNOS protein expression and influence intrinsic NO production. The underlying mechanism is still not clear. However, excessive NO has been shown to induce apoptosis [4–6]. It is likely that apoptosis induced by such large amounts of NO over a long period of time could lead to cleavage of eNOS and possibly other proteins in the endothelial cell.

EDRFs other than NO, including PGI\(_2\) and EDHF, play important roles in endothelium-dependent vasorelaxation in various physiological and pathological settings [36–38] and the production of these also depends on increases in cytosolic Ca\(^{2+}\) concentration [11–14,38]. The activity of phospholipase A\(_2\), and thus PGI\(_2\) production, has been shown to strictly require Ca\(^{2+}\) [11–14]. The finding that excessive NO inhibits Ca\(^{2+}\) entry suggests that it could thereby inhibit also the production of PGI\(_2\). Indeed, NOC12 dose-dependently inhibited both the BK-stimulated increase in cytosolic Ca\(^{2+}\) concentration and the production of 6-
keto-PGF$_{1\alpha}$, a stable metabolite of PGI$_2$ in PAECs. The inhibitory effects of NOC12 on Ca$^{2+}$ entry and PGI$_2$ production appear to be well correlated. We previously demonstrated that Ca$^{2+}$ entry also plays a predominant role in the effects of EDHF stimulated by agonists. Thus, BK produces very little or no hyperpolarization of porcine coronary artery under Ca$^{2+}$-free conditions or in the presence of extracellular Ca$^{2+}$ with Ca$^{2+}$ entry inhibited by the non-specific Ca$^{2+}$ channel inhibitor, SKF96365 [38]. This is in line with the observations by Bauersachs et al. [39] that NO attenuates the release of EDHF in cultured human endothelial cells. NO thus probably modifies EDHF production by regulating cytosolic Ca$^{2+}$ concentration.

In conclusion, we have shown that excessive NO can inhibit Ca$^{2+}$ entry, PGI$_2$ production and eNOS expression in endothelial cells. The functions of many protein kinases in the cell are dependent on Ca$^{2+}$ signals and the vasculature has its tone maintained by the production of EDRFs. These effects of excessive NO clearly will disturb many endothelial functions and promote endothelial injury.

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References


