Exogenous, but not endogenous nitric oxide inhibits
adhesion molecule expression in human endothelial cells

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INTRODUCTION

Nitric oxide (NO) has many beneficial actions on the vascular wall including suppression of inflammation. The mechanism(s) by which NO antagonizes cytokine signaling are poorly understood, but are thought to involve inhibition of the pro-inflammatory transcription factor, NF-κB. NO represses nuclear translocation of NF-κB via the S-nitrosylation of its subunits which decreases the expression of target genes including adhesion molecules. In previous studies, we have shown that the intracellular location of endothelial nitric oxide synthase (eNOS) can influence the amount of NO produced and that NO levels are paramount in regulating the S-nitrosylation of target proteins. The purpose of the current study was to investigate the significance of subcellular eNOS to NF-κB signaling induced by pro-inflammatory cytokines in human aortic endothelial cells (HAECs). We found that in HAECs stimulated with TNF-α, L-NAME did not influence the expression of intercellular adhesion molecule 1 (ICAM-1) or vascular cell adhesion molecular 1 (VCAM-1). In eNOS “knock down” HAECs reconstituted with either plasma membrane or Golgi restricted forms of eNOS, there was no significant effect on the activation of the NF-κB pathway over different times and concentrations of TNF-α. Similarly, the endogenous production of NO did not influence the phosphorylation of IkBα. In contrast, higher concentrations of NO derived from the use of the exogenous NO donor, DETA NONOate, effectively suppressed the expression of ICAM-1/VCAM-1 in response to TNF-α and induced greater S-nitrosylation of IKKβ and p65. Collectively these results suggest that neither endogenous eNOS nor eNOS location is an important influence on inflammatory signaling via the NF-κB pathway and that higher NO concentrations are required to suppress NF-κB in HAECs.

Keywords: nitric oxide, NF-κB, inflammation, endothelial, S-nitrosylation
cell adhesion molecule 1 (VCAM-1; Janssen-Heininger et al., 2000).

NF-κB is a protein complex that is maintained in an inactive form by sequestration in the cytosol via interaction with inhibitory subunits (IkB). Activation of cytokine receptors and elevated ROS promotes the phosphorylation of IkB, which leads to its ubiquitination and degradation and translocation of the active complex to the nucleus (Baud and Karin, 2001; Mohan et al., 2003). Multiple mechanisms have been proposed to account for the ability of NO to inhibit NF-κB. These include the stabilization of IkB, induction of IkBa mRNA, and reduced nuclear translocation of NF-κB. Given the multiple mechanisms of inhibition, it is not surprising that NO has been shown to S-nitrosylate multiple targets within the NF-κB pathway including Cys62 of p50 which compromises DNA binding (Matthews et al., 1996), Cys38 of p65 which prevents heterodimer formation (Kelleher et al., 2007) and Cys-179 of IKKβ which prevents IkB phosphorylation (Reynaert et al., 2004).

Previously we have shown that the location of eNOS with the endothelial cell is important for enzyme activity as well as the ability to influence the S-nitrosylation of target proteins such as NSF (Fulton et al., 2002; Zhang et al., 2006; Qian et al., 2010). When targeted to the plasma membrane, eNOS produces more NO than eNOS residing on endomembranes of the Golgi and also greater S-nitrosylation of target proteins such as NSF. However, this difference in S-nitrosylation was negated when the amount of NO produced was normalized through the use of calcium-insensitive forms of eNOS and suggests that the amount of NO has a greater influence on the S-nitrosylation of target proteins versus the proximity of NO production. The majority of studies investigating the role of NO in NF-κB activation have used NO donors or induced the high capacity NOS2 isoform. Comparatively little is known about whether endogenously produced NO from eNOS can influence endothelial NF-κB activation and whether the location of eNOS is important for NF-κB activation. In the current study we addressed both of these questions by investigating whether endogenously produced NO, the location of NO synthesis and the amount of NO produced was normalized through the use of eNOS or iNOS from cells transfected with eNOS or iNOS was calculated after subtracting NO2 levels from cells lacking NOS activity (RFP transfected group).

DETECTION OF S-NITROSYLATION

Human aortic endothelial cells were treated with L-NAME (1 mM) or DETA NONOate (1 mM) for 6 h and cells were lysed and biotin labeled using biotin switch assay with some modification (Jaffrey and Snyder, 2001; Forrester et al., 2009). In brief, 48 h after transfection, cells were washed two times with cold PBS, and lysates prepared by incubation with HEN buffer containing 250 mM HEPES, 1 mM EDTA, 0.1 mM neocuproine (pH 7.7), SDS (2.5% final concentration), and methyl methanethiosulfonate (Sigma-Aldrich) at 50°C for 20 min and vortex every 4 min. Proteins were precipitated with acetone, washed three times with 70% acetone, and mixed with 0.2 mM biotin-HPDP (Pierce) with or without 50 mM ascorbate at ambient temperature for 1 h. Biotinylated proteins were pulled down by using streptavidin–agarose beads (Sigma-Aldrich), separated by SDS-PAGE, and detected by chemiluminescence with anti-p65 (Abcam) or IKKβ (Cell Signaling) antibody.

CELL VIABILITY

Human aortic endothelial cells were plated into 96-well plate and treated with DETA NONOate (1 mM), TNFα (1 ng/ml), and thapsigargin (10 μM) was used as a positive control. Cell viability was determined after 24 h treatment by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT reagent was prepared by dissolving 5 mg/ml in PBS and then diluted with complete media (1:10) and allowed to incubate for 3 h, after that the MTT was removed and DMSO (100 μl) was added to each well and shaken for 15 min. Viability was determined by recording absorbance at λ = 570 nm. The effect was assessed as percent cell viability where vehicle-treated cells (i.e., control) were taken as 100% viable.

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. Comparisons were made using analysis of variance (ANOVA) with a post hoc test. Differences were considered as significant at p < 0.05.
RESULTS

Our first experimental goal was to determine whether endogenous NO is capable of influencing the ability of TNFα to upregulate the expression of ICAM-1 in HAECs. HAECs were treated with TNFα (1 ng/ml) for 4, 8, 24 h, with or without pre-treatment with the NOS inhibitor L-NAME (1 mM). ICAM-1 expression was robustly upregulated at all time points in a time-dependent manner. As shown in Figure 1A, L-NAME did not influence the level of induction of ICAM-1. We next investigated where NO influences the induction of ICAM-1 to lower concentrations of TNFα and similar effects of L-NAME were observed (Figure 1B). We next increased the concentration of L-NAME to 2 mM, but again found no significant change in the expression of ICAM-1 in response to TNFα (Figure 1C). To exclude the possibility that more stable S-nitrosylation is not reversed with short term inhibition of eNOS, we increased the L-NAME pre-treatment time from 30 min to 24 h. Despite the longer inhibition of NO synthesis, we did not observe any effect on ICAM-1 upregulation or VCAM-1 induction (Figure 1D).

We next determined whether the location of eNOS was important in regulating the TNFα-dependent induction of adhesion molecules. To do so, we utilized a strategy of knocking down endogenous eNOS using a stably delivered shRNA and then re-expressing novel forms of eNOS that contain silent mutations to bypass the siRNA knockdown and are specifically targeted to the Golgi (S17) or plasma membrane (CAAX; Zhang et al., 2006). As shown in Figure 2 and consistent with our previous findings, we did not detect any significant difference in ICAM-1 expression between HAECs expressing the Golgi (S17) or PM (CAAX) eNOS versus a control adenovirus (RFP).

As endogenous NO failed to inhibit TNFα-stimulated expression of adhesion molecule expression, we next looked at whether exogenous NO could prove more effective. HAECs were treated with vehicle or 1 mM DETA NONOate for 24 h and then challenged with TNFα in the presence and absence of L-NAME. As shown in Figure 3A, DETA NONOate robustly inhibited TNFα stimulated expression of VCAM-1 and also ICAM-1. Consistent with previous results, L-NAME did not have any effect. To exclude the possibility that reduced viability of cells may also lead to the decrease in ICAM-1 or VCAM-1 expression, we performed the MTT assay after 24 hr exposure to TNFα and DETA NONOate (Figure 3B). There was no significant difference in viability in cells exposed to DETA NONOate or the combination of TNFα and DETA NONOate. Thapsigargin (10 μM) was used as a positive control and showed a robust decrease in cell viability. Therefore the decreased expression of adhesion molecules we observed with DETA NONOate was not due to reduced cell viability. To compare the amount of NO produced from endogenous NO synthases versus that obtained from an exogenous source of NO, we measured NO levels in the media using NO-specific chemiluminescence. As shown in Figure 3C, we found that 100 μM DETA NONOate produced about threefold more NO than that produced by iNOS, fivefold higher than a PM-restricted eNOS and 100-fold higher than Golgi restricted eNOS. The 10-fold higher concentrations of NO donor which are necessary to inhibit NF-κB signaling in HAECs are thus much greater than that obtained from enzymatic sources.
Human aortic endothelial cells were treated with L-NAME (1 mM) or DETA NONOate (1 mM) for 30 min, and then TNFα (1 ng/ml) was added for 24 h. Activation of NF-κB was detected by anti-ICAM-1 or anti-VCAM-1 antibodies. GAPDH was used as a loading control.

(B) HAECs were plated into 96-well plate and cells were treated with the same condition as above, cell viability was detected by MTT assay 24 h after treatment.

(C) Nitric oxide release from cells expressing RFP, S17-eNOS, CAAX–eNOS, or iNOS or from different concentrations (10, 30, 50, 100 μM) of DETA NONOate over 24 h. Results are presented as mean ± SEM (n = 5). *p < 0.05 versus control (RFP).

To further determine the underlying mechanism of NO inhibition of adhesion molecules, we next monitored the activation of NF-κB by multiple approaches. NO has been reported to inhibit NF-κB activation via the S-nitrosylation and inhibition of IKKβ activity and a corresponding reduction in the phosphorylation of its substrate IκBα. In HAECs, we found that inhibition of endogenous NO with L-NAME did not prevent the increase in IκBα phosphorylation in response to TNFα (Figure 4A). Activation of NF-κB by TNFα was confirmed by an increase in phosphorylated IκBα and the degradation of total IκBα (Figure 4A). To further determine if higher amounts of NO, as generated by DETA NONOate, can inhibit ICAM-1/VCAM-1 expression through the S-nitrosylation of IKKβ (Reynaert et al., 2004) or p65 (Kelleher et al., 2007) as previously reported, we treated HAEC cells with L-NAME (1 mM) or DETA NONOate (1 mM) for 6 h and the S-nitrosylation of both proteins was detected using the biotin switch assay. Endogenous NO, inhibited with L-NAME, did not alter the S-nitrosylation level of IKKβ or p65, while DETA NONOate significantly increased the nitrosylation of both proteins (Figures 4B,C).

**DISCUSSION**

In the current study, we investigated the ability of NO to regulate NF-κB signaling and adhesion molecule expression in HAECs. We found that endogenous NO was unable to influence NF-κB activation or the induction of ICAM-1 or VCAM-1 expression either under basal conditions or in response to TNFα in both a time and dose-dependent manner. Selective expression of eNOS at the plasma membrane which results in greater NO release also failed to suppress the induction of ICAM-1. Significantly higher amounts of NO, greater than that produced endogenously, were necessary to observe suppression of adhesion molecule expression in human endothelium.
We found no change in the expression of NF-κB by NO can vary depending on the cell type studied. In A540 cells, NO operates at the nuclear level and inhibits the binding of NF-κB to DNA through a reversible, redox-based modification of the p50–p65 heterodimer (Kelleher et al., 2007). In Jurkat T cells, NO functions in the cytoplasm by inhibiting IκBα degradation, thus preventing the nuclear translocation of NF-κB. In intact blood vessels and cultured endothelium, eNOS can be found localized to either the perinuclear Golgi or the plasma membrane (Zhang et al., 2006) and NO production and the S-nitrosylation of target molecules can be found concentrated at sites of eNOS localization (Iwakiri et al., 2006). We found that the restricted expression of eNOS at the Golgi or plasma membrane of HAECS had no significant effect on TNFα induced activation of NF-κB. Even at the higher concentrations of NO afforded by the PM-localization of eNOS, which could compensate for the reduced basal NO production secondary to the absence of hemodynamic stress in culture conditions, there was no meaningful change in adhesion molecule expression.

When targeted to the plasma membrane, eNOS releases more NO, generates more cGMP in adjacent reporter cells and produces more pronounced endothelium-dependent relaxations when expressed in intact blood vessels from eNOS−/− mice. Expression of eNOS at the plasma membrane also results in greater S-nitrosylation of the redox sensitive fusogen N-ethylmaleimide-sensitive factor (NSF) which functionally translates into a more significant suppression of vWF release from endothelial cells. In contrast to WT-eNOS, expression of calcium-insensitive forms of eNOS at different intracellular locations results in equal production of NO. The equalization of the amount of NO produced by eNOS targeted to the Golgi and plasma membrane resulted in similar increases in cGMP, S-nitrosylation of NSF, and equal suppression of vWF exocytosis (Qian et al., 2010). These results suggest that the amount of NO released is the most influential factor in controlling cellular function via S-nitrosylation. This concept is supported by the data in the current study showing that higher concentrations of NO are necessary to effectively constrain the NF-κB-dependent expression of cellular adhesion molecules. While it is clear that there is significant variability in the sensitivity of substrate proteins to S-nitrosylation and also the sites of S-nitrosylation within those proteins (Hess et al., 2005; Greco et al., 2006), the variables involved are not well defined (Doulias et al., 2010). Our results suggest that, at least in the context of HAECS, the S-nitrosylation of NF-κB requires higher NO concentrations than other more susceptible proteins such as GAPDH and NSF. This is supported by a number of studies mapping the S-nitrosylation proteome which have failed to identify constituents of the NF-κB pathway (Greco et al., 2006; Hao et al., 2006). The reversal of protein S-nitrosylation is a poorly understood process and the rate of reversal is known to vary significantly with the substrate (Paige et al., 2008). Two enzymes have been discovered that can accelerate the loss of S-NO from substrate proteins, S-nitrosoglutathione reductase (GSNOR) or alcohol dehydrogenase 5 (ADH5) and Thioredoxin-1 (Trx1; Jensen, 1998; Benhar et al., 2010). To exclude the possibility that in HAECS, NF-κB is a low turnover or stable S-NO protein we preincubated cells with the competition with other molecules such as superoxide or free thiols.

Previous studies have found that NF-κB is sensitive to both oxidative and nitrosative stress (Marshall et al., 2000; Marshall and Stamler, 2002). NO can suppress NF-κB signaling via allosteric modification of its subunits including p50, p65, and also IKKβ (Matthews et al., 1996; Reynaert et al., 2004; Kelleher et al., 2007). We found no change in the expression of NF-κB-dependent genes and no change in the phosphorylation of IκBα suggesting that the levels of endogenous NO produced from eNOS are insufficient to influence these pathways. Indeed, the majority of studies that have shown inhibition of NF-κB have relied on higher concentrations of NO, either from an NO donor or from immune cells that expresses high levels of iNOS (Reynaert et al., 2004; Kelleher et al., 2007).

S-nitrosylation, the formation of an S-nitrosothiol (S-NO) by the covalent addition of a NO moiety to the SH-group of accessible cysteine residues requires higher concentrations of NO than does activation of sGC (Gow and Ischiropoulos, 2001). There are several reasons for this including the requirement that NO must first be converted to a nitrosating species such as N₂O₃ which may not occur uniformly throughout the cell (Espey et al., 2001). Also as NO is a rapidly diffusing gas, the proteins in closest proximity to the source of NO have the best chance of S-nitrosylation versus
NOS inhibitor for 0.5–24 h prior to stimulation with TNFα and saw no difference in ICAM-1 induction. While the possibility exists that this was not sufficient time to observe an effect, other reports have shown effects of NO on NF-κB over a more acute time scale. For example, the activity of IKKβ was markedly enhanced 4 h after treatment with the NOS inhibitor, L-NMMA, and occurred contemporaneously with a decrease in IKKβ S-nitrosylation (Reynaert et al., 2004).

It is difficult to extrapolate the quantity of NO made by eNOS in cultured endothelial cells to that made in vivo. The anti-inflammatory effect of eNOS-derived NO has been shown in vivo following the genetic deletion of eNOS (Mashimo and Goyal, 1999). Thus the possibility exists that eNOS in cultured endothelial cells may be less active than in the in vivo setting, although the expression of an active form of eNOS did not have additional effects. Alternatively it might be that endogenous sources of NO (i.e., eNOS) are important in modulating inflammatory signaling but that this occurs under select pathophysiological conditions that have a multitude of mediators such as tissue/cell injury (e.g., I/R injury) versus the single inflammatory cytokine used in the current study. The regulation of inflammatory signaling cascades are complex and exhibit multiple redundant pathways so that TNFα alone may not be sufficiently sensitive to endogenous NO versus the multitude of signaling pathways and cell types involved in vivo.

NF-κB signaling, leading to increased ICAM-1 expression, is also sensitive to oxidative stress. Indeed, compounds such as resveratrol, which have anti-oxidant properties and can also blunt the effects of peroxynitrite, have been shown to inhibit TNFα-mediated activation of NF-κB and ICAM-1 expression (Holthoff et al., 2010). It is therefore possible that peroxynitrite may be a more efficient regulator of NF-κB activation than NO. However, the pursuit of this hypothesis is beyond the scope of the current study. The regulation of inflammatory signaling (e.g., I/R injury) versus the single inflammatory cytokine used in vivo is, at least in part, NF-κB-dependent; however, we would expect that if Sp1 and API were involved in this response, then they too would be sensitive to NO inhibition. Recently, it was discovered that TNFα stimulated ICAM-1 expression can also occur downstream of p38 MAPK signaling (Zerr et al., 2011). However, NO has been shown to activate p38 MAPK signaling (Wang et al., 2006; Kaddai et al., 2008) which is the opposite of its actions on NF-κB. Although increased p38 MAPK activity could account for some of the reduced NO-sensitivity of adhesion molecular upregulation, it does not explain the lack of sensitivity of 1kBα, IKKβ, and p65 to endogenous NO. The mechanism by which NO regulates p38 MAPK signaling and whether S-nitrosylation is involved remains unclear.

Collectively, we have shown that the endogenous production of NO in HAEC is not sufficient to inhibit the upregulation of adhesion molecules in response to cytokine stimulation. Higher concentrations of NO, in the pharmacological range, are required to suppress the induction of ICAM-1 and VCAM-1. Our results reinforce the concepts that NO concentration, susceptibility of target proteins and the cell type are critical factors regulating the control of cellular function by S-nitrosylation.

ACKNOWLEDGMENTS

This work was supported by NIH grants R01 HL085827, R01HL092446, and P01 HL101902-01A1 and an established investigator award from the AHA.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 August 2011; paper pending published: 30 November 2011; accepted: 04 January 2012; published online: 18 January 2012.