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Exogenous nitric oxide activates the endothelial glucocorticoid receptor

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Abstract

This study investigated the effect of exogenous nitric oxide (NO) on endothelial glucocorticoid receptor (GR) function. The NO donor diethylenetriamine NONOate (DETA, 50–500 μM) caused concentration dependent nuclear localization of transfected chimeric green fluorescent protein GFP-GR and elevated expression of secreted alkaline phosphatase (SEAP) from a glucocorticoid response element (GRE) promoter construct in bovine aortic endothelial cells. Other weaker NO donors (*S*-nitroso-*N*-acetylpenicillamine and spermine NONOate) failed to induce GFP-GR nuclear localization, but all the NO donors activated GRE-SEAP expression, a response unaffected by the antioxidant *N*-acetyl-L-cysteine. Overall, exogenous NO from high concentration donors can directly activate GR, suggesting a potential feedback mechanism for NO to regulate endothelial inducible nitric oxide synthase (iNOS) expression.

Keywords: Glucocorticoid receptor; Nitric oxide; Endothelium; Inflammation

The endothelium plays a central role in regulating inflammatory responses to stimuli that include biochemical triggers (e.g., TNFα and interleukins) and disturbed hemodynamics. Atherosclerosis, a chronic inflammatory disease, preferentially develops at bifurcation sidewalls where reversing or vortexing flows generate regions of low and oscillating wall shear stress [1]. Sustained unidirectional shearing of endothelial cells elevates the expression of atheroprotective genes such as endothelial nitric oxide synthase (eNOS), superoxide dismutase, and C-type natriuretic peptide [2–4]. Fluid shear stress on cultured endothelial cells induces nitric oxide (NO) and prostacyclin (PGI_2) production and can lead to generation of reactive oxygen species (ROS) [5–7].

Distinct from its vasodilatory action, NO has atheroprotective properties such as reducing platelet aggregation, inhibiting smooth muscle cell proliferation, and reducing leukocyte adhesion. In human endothelial cells, NO donors such as *S*-nitroso-glutathione (GSNO) decrease TNFα or IL-1α-stimulated induction of interleukins (IL-6 and IL-8) and adhesion molecules (E-selectin) [8]. NO from GSNO also suppresses vascular cell adhesion molecule-1 (VCAM-1) gene transcription after TNFα stimulation by interfering with NFκB binding activity. TNFα induced VCAM-1 expression is inhibited by antioxidants [9], suggesting that reactive oxygen species and oxidative stress are also involved during inflammatory cytokine activation.

Anti-inflammatory glucocorticoid drugs such as dexamethasone inhibit the expression of a number of cytokines, including TNFα, IL-6, IL-8, as well as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and inducible nitric oxide synthase (iNOS) in various cell types [10,11]. NFκB is an inducible transcription factor that mediates the expression of these inflammatory and immune response genes upon activation by exposure to proinflammatory cytokines such as TNFα, oxidative stress, and various bacterial or viral molecules. In most cases, the active form of NFκB is a heterodimer of RelA (p65) and NFκB1 (p50) released from its inhibitor IκB. Steroid-liganded glucocorticoid receptor (GR) can

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directly interact with the p65 subunit of NFκB [12] or interfere with transcriptional cofactors such as CREB binding protein (CBP) and steroid receptor coactivator-1 (SRC-1) [13]. Other studies have demonstrated the repression of NFκB responsive targets such as IL-6 by steroid activated GR in endothelial cells [14]. Pretreatment of endothelium with dexamethasone inhibits cytokine induction of the iNOS [15]. While short term (10–60 min) treatment with glucocorticoid causes a rapid non-transcriptional activation of eNOS and enhanced NO production [16], prolonged administration of dexamethasone (7 days) leads to downregulation of eNOS expression. This downregulation is the likely cause of hypertension, a major cardiovascular side effect of systematically administered glucocorticoids [17,18].

Steady arterial shear stress causes endothelial GR activation and glucocorticoid response element (GRE) regulated gene transcription through mechanisms that are sensitive to inhibitors of shear-activated MEK1/2 and PI-3 kinases [19]. This finding suggests crosstalk between the atheroprotective role of unidirectional shear stress and anti-inflammatory actions of the GR. We now investigate the role of exogenous NO on the endothelial GR pathway in the context of inflammatory cytokines since NO donors suppress endothelial VCAM-1 induction by TNFα [8]. We hypothesize that exogenous nitric oxide can directly activate the GR pathway in the endothelium.

Materials and methods

Materials. Dexamethasone, 1,4-pregnadien-9α-fluoro-16α-methyl-11β, 17,21-triol-3, 20-dione (Steraloids) was dissolved in DMSO and stored at −20 °C. Nitric oxide donors *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 2,2′-(hydroxynitrosohydrazino) bis-ethanamine (DETA NONOate or "DETA") were from Sigma, and the NO donor *N*-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine (Spermine NONOate or "SPER") was obtained from Cayman Chemicals. *N*-acetyl-L-cysteine (NAC) was also obtained from Sigma. The pGFP-GR plasmid was provided by Dr. Mario Galigniana (Univ. Michigan Med. School) [20]. In this vector, the mouse GR cDNA N-terminus was fused in-frame to the C-terminus of green fluorescence protein (GFP) on the pEGFP-C3 plasmid (Clontech). The pGRE-SEAP plasmid (Clontech) utilizes a secretable heat-stable form of human placental alkaline phosphatase (SEAP) driven by three tandem copies of the GRE sequence, $GGTACA(N)_3TGTTCT$, fused to a weak TATA-like (P_{TAL}) region of the Herpes simplex virus thymidine kinase (HSV-TK) promoter.

Cell culture. Bovine aortic endothelial cells (BAECs) were cultured (passage <12) in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker) supplemented with 10% heat-inactivated newborn calf serum, 1% L-glutamine (200 mM), and 2% penicillin streptomycin (Invitrogen) [21]. BAEC were transfected for 2 h with pGFP-GR or pGRE-SEAP using Lipofectamine (Invitrogen) as previously described [19] and allowed to incubate overnight. Transfected BAEC were then maintained in phenol-free DMEM with 2.5% charcoal/dextran-treated fetal bovine serum (HyClone) for minimal background growth factor and steroid concentrations.

Fluorescence assays. For GFP-GR localization studies, cells were digitally imaged at 40× using a Zeiss Axiophot2 fluorescence microscope (FITC cube) and a cooled CCD camera (MTI, CCD-300T-RC) with Scion Image software (Scion). SEAP was quantified using a Fluorescence Detection Kit (Clontech). Heating samples at 65 °C and incubating them in assay buffer containing L-homoarginine, a SEAP inhibitor, eliminated endogenous alkaline phosphatase activity. Total SEAP activity was measured using the fluorescent substrate 4-methylumbelliferyl phosphate (MUP) in black 96-well plates read by the Fluoroskan Ascent FL microplate fluorometer (EX 360 nm; EM 449 nm).

Results

Exogenous NO causes GR nuclear localization in a donor-dependent manner

To investigate the role of NO in endothelial GR regulation, we tested the effect of exogenous NO donors on GFP-GR nuclear localization. We chose these different NO donor drugs based on their use in the literature. DETA and SPER are members of the diazeniumdiolates (NONOates) class of NO donors with nucleophilic cores. They are stable in solid form and decompose in solution to generate up to two NO molecule per donor following a first-order decomposition. SNAP belongs to the *S*-nitrosothiol class (general form: RS—N=O) that has variable stability in solution, with a half-life depending on the R-group. In GFP-GR expressing cells, adding DETA to culture media induced nuclear localization in a dose-dependent manner, with clearly detectable nuclear localization at supraphysiologic NO achieved at 250 or 1000 μM DETA (Fig. 1). However, adding NO donors SNAP or SPER failed to induce GFP-GR nuclear localization over the same concentration range. GFP-GR remained cytoplasmic even at high (1 mM) concentration of SNAP or SPER, and no nuclear localization was observed even after extended incubation for 4 h (not shown). The data in Fig. 1 are consistent with the finding that DETA is a superior NO donor to SNAP and SPER on endothelial cells [22]. All imaging in Fig. 1 was conducted after 2 h incubation in media containing NO donors. This experiment indicated that only very high dose NO could serve as an activator of GR nuclear localization. Non-specific biochemical effects from high dose SNAP or SPER (e.g., ROS) were not sufficient to induce GFP-GR nuclear localization. The NO effect was donor-specific based on the GFP-GR localization effect, likely due to variability in donor structure and consequent mechanism and half-life of NO release.

GRE activation by NO donors is dose-dependent and ROS-independent

To test whether exogenous NO also activates endogenous GR to regulate transcription from a GRE promoter, a GRE-SEAP promoter construct was used. BAEC transfected with GRE-SEAP plasmid were

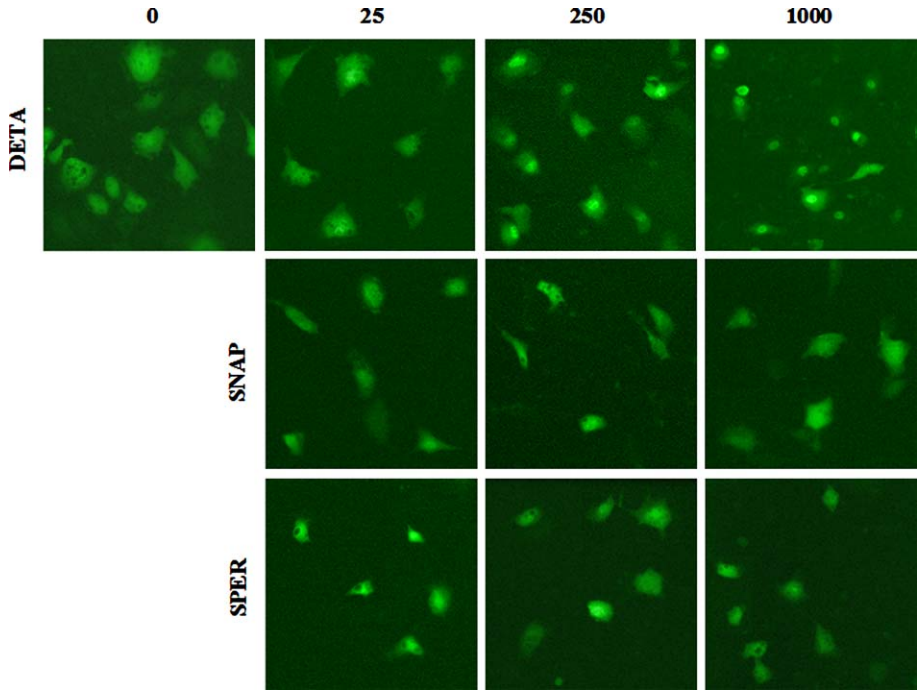

Fig. 1. Nuclear translocation in BAEC of GFP-GR with NO donors: DETA, SNAP, or SPER. Concentrations are in μM. For control, images of GFP-GR transfected BAEC (no DETA) demonstrated that fluorescence was uniform throughout cell cytoplasm. NO donors were added to culture media, and all fluorescent imaging was done after 2 h incubation at 37 °C. GFP-GR nuclear localization can be observed at high concentrations of DETA, but not SNAP or SPER.

incubated in media containing various NO donors. In all cases, production of SEAP was calculated as fold induction over the static control cells in media without NO donors. Adding DETA at concentrations of 5, 125, 250, and 500 μM increased SEAP production in a dose-dependent manner. Fig. 2A shows the dose–response curve of SEAP production from a GRE promoter after 6 h induction with DETA at various concentrations. SEAP production over control increased from 3.2-fold to 17.5-fold as DETA concentration increased from 50 to 500 μM. This result of GRE transcriptional activation correlated with the previous GFP-GR nuclear localization data for DETA.

BAEC transfected with GRE-SEAP plasmids were also treated with one of three NO donors (DETA, SPER, and SNAP) at 250 or 500 μM concentrations as well as dexamethasone (DEX) at 25 μM for up to 8 h (Fig. 2B). NO donor-induced SEAP production began by 2 h and continued to increase for the next several hours. Again, adding DETA to media yielded the most noticeable SEAP induction during the 8 h time course, and fold induction over control was consistent with dose–response curve of Fig. 2A, with maximum increase of about 25-fold after 6 h induction. Other NO donors at either concentration yielded GRE induction curves that peaked at different times although most led to an increase of 10–20-fold induction between 4 and 8 h. Finally, induction with dexamethasone caused a large increase in SEAP production, reaching maximum after 8 h induction of 25-fold over control. High levels of DETA were as potent as high dose dexamethasone in inducing expression from the GRE promoter construct.

One common concern with high dose nitric oxide donors is the excess oxidative stress placed on cells due to formation of reactive oxygen species (ROS) [23]. To help confirm that the results observed were not due to an increase in ROS, an antioxidant *N*-acetyl-L-cysteine (NAC), a glutathione precursor, and free radical scavenger were used. Cells were pretreated with NAC (10 mM) for 2 h before adding NO donors in culture media. Expression of the transfected GRE-SEAP promoter was measured after 6 h induction with NO donors (Fig. 2C). The antioxidant NAC in culture media did not cause a significant reduction in NO-induced SEAP production in the presence of DETA, SPER, and SNAP (500 μM), or dexamethasone (25 μM). These data indicated that the GRE activation and increased SEAP production observed in the presence of NO donor were due to exogenous NO and not from ROS.

Discussion

Adding NO donors as a source of excess NO production induced GR nuclear localization in a donor-specific manner at 2 h, although all the donors up-regulated GRE

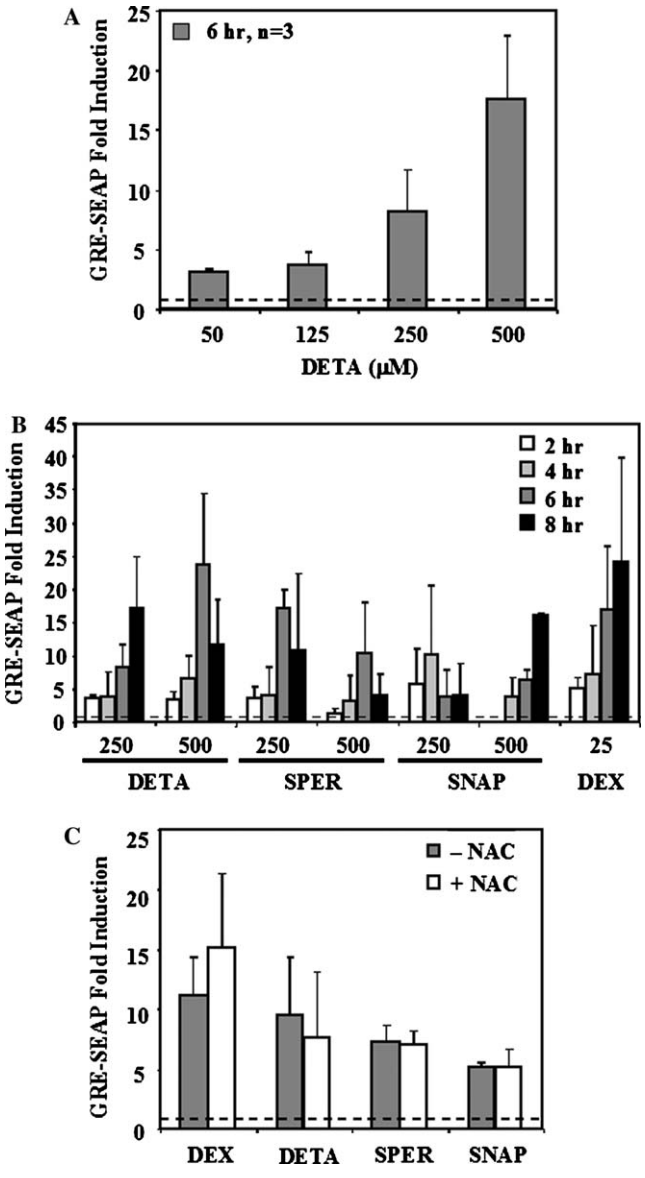

Fig. 2. Fold-induction of GRE-SEAP promoter construct expression with NO donors. (A) Dose–response curve for DETA induction of SEAP production. (B) BAEC were treated with dexamethasone (DEX, 25 μM) or different NO donors (DETA, SNAP or SPER) at 250 or 500 μM for up to 8 h, and media were collected every 2 h and measured for SEAP activity. (C) BAEC transfected with pGRE-SEAP were pretreated with or without the antioxidant NAC (10 mM) for 2 h before dexamethasone (DEX, 25 μM) or NO donor (DETA, SPER, or SNAP at 500 μM) was added to media for 6 h incubation. Results are compared to cells without pretreating with NAC. All data are presented as means ± SE ($n = 3$ for each condition).

promoter, to varying levels, by 8 h. The promoter construct study, however, is a measure of endogenous GR response to elevated NO, while GFP-GR is a highly expressed transgenic construct. The antioxidant NAC had no effect on GRE activation by NO donors; therefore, the ability of high levels of NO to activate GR and its transcription pathway was demonstrated in this study. Although concentrations of NO from NO donors used in the experiments were notably higher than that expected physiologically [24], several types of cells at site of inflammation, such as macrophages and vascular smooth muscle cells, are able to release NO at significantly higher quantities, affecting endothelial cells in cytokine-activated neighborhoods. This may also be the case when iNOS becomes expressed at a site of vascular inflammation. Overall, our data are consistent with observations in the literature on anti-inflammatory roles of glucocorticoids [10] and NO donors in suppressing cytokine-activated endothelial function [8]; and suggest a cooperative relationship between exogenous NO and the atheroprotective properties of shear-activated GR under flow [19].

While previous studies have shown long-term glucocorticoids as an upstream repressor of eNOS and NO production by reducing the binding activity of transcription factor GATA [25], an acute treatment of dexamethasone can activate eNOS [16]. Also, exogenous NO induction of GR nuclear localization and GRE promoter activity suggests that the presence of excess NO, as in acute inflammatory conditions with elevated iNOS, can lead to strong induction of GR, GRE regulated gene expression, and other anti-inflammatory actions, including potential feed-back mechanisms to regulate iNOS expression. Furthermore, the ability of exogenous NO to down-regulate TNFα activated pro-inflammatory cytokine expression (IL-6 and IL-8) has been shown in previous studies [8,26,27] which have linked the NO inhibitory effect on NFκB activation to scavenging oxygen species, and stabilization of NFκB inhibitor IκBα [28]. Figs. 1 and 2 suggest that NO-activated GR may help downregulate NFκB in cytokine-activated endothelium.

While applied shear induces a rapid release of NO by endothelial cells, the concentration of generated NO is still significantly less than that achieved by NO donors at 250 or 500 μM [29]. Shear stress may exert its inhibitory effects against TNFα through the activation of GR and GRE pathway. Given that TNFα initiates the NFκB pathway, a process that is sensitive to both NO and glucocorticoids, it would be important to further investigate the actions of shear activated GR on NFκB, especially the antagonism expected between GR and NFκB at their corresponding promoter sites. It should also be noted that shear induces *fos* expression [30] and activates *fos*/*jun* binding to AP-1 which can also antagonize NFκB function [31].

In summary, we have shown that exogenous supply of NO activated GR and GRE transcription pathways. This result adds to previous findings that shear stress alone can induce NO production, activate GR, and that both glucocorticoids and NO can inhibit NFκB activation, largely independent of shear-induced NO (Fig. 3). Also, this is the first report that exogenous NO from high concentration donors can directly activate GR, a

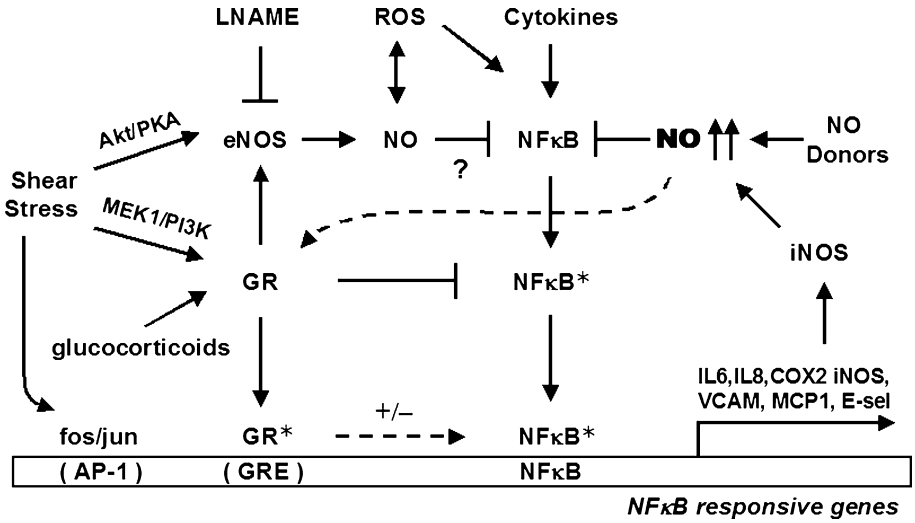

Fig. 3. Potential interactions between shear stress, NO, and transcriptional factors (GR, NFκB, and AP-1). Arterial shear stress activates eNOS [5] and GR [19]. In endothelium, the GR activated by shear stress, dexamethasone, or high dose NO donors (this work, dotted line) may antagonize TNFα activation of NFκB responsive genes (e.g., shear attenuates TNFα induction of IL-6 and IL-8 expression; data not shown). Shear stress prevention of inflammation (i.e., NFκB) does not necessarily require shear-induced NO, and possibly involves additional modulators of NFκB beyond GR. The activation of GR by high NO may be a feed-back mechanism to restrict iNOS expression.

potential feed-back mechanism to regulate iNOS expression. Our finding that exogenous NO is an upstream regulator of GR transcriptional pathway suggests that the GR pathway could be an additional mechanism by which NO exerts its anti-inflammatory functions.

Acknowledgments

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