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NITRIC OXIDE DECREASES IN ENDOTHELIN-1 SECRETION THROUGH THE ACTIVATION OF SOLUBLE GUANYLATE CYCLASE

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ABSTRACT

The use of exogenous nitric oxide (NO) has been shown to alter the regulation of other endothelially- derived mediators of vascular tone, such as endothelin-1 (ET-1). However, the interaction between NO and ET-1 appears to be complex and remains incompletely understood. One of the major actions of NO is the activation of soluble guanylate cyclase (sGC) with the subsequent generation of cyclic GMP (cGMP). Therefore, we undertook this study to test the hypothesis that NO regulates ET-1 production via the activation of the sGC/cGMP pathway. The results obtained indicated that the exposure of primary cultures of four-week-old ovine pulmonary arterial endothelial cells (4wkPAECs) to the long acting nitric oxide donor DETA NONOate induced both a dose- and time-dependent decrease in secreted ET-1. This decrease in ET-1 secretion occurred in the absence of changes in endothelin converting enzyme-1 or sGC expression but in conjunction with a decrease in preproendothelin-1 mRNA. The changes in ET-1 release were inversely proportional to the cellular cGMP content. Further, the NO-independent activator of sGC, YC-1, or treatment with a cGMP analogue, also produced significant decreases in ET-1 secretion. Conversely, pretreatment with the sGC inhibitor, ODQ blocked the NO induced decrease in ET-1. Therefore, we conclude that exposure of 4wkPAECs to exogenous NO decreases secreted endothelin-1 due to the activation of soluble guanylate cyclase and increased cGMP generation.

Key Words: Endothelin-1, nitric oxide, soluble guanylate cyclase, cyclic GMP

INTRODUCTION

Persistent Pulmonary hypertension of the newborn (PPHN) is a disease characterized by a failure of the normal pulmonary vascular transition at birth leading to increased pulmonary vascular resistance, decreased pulmonary blood flow and severe hypoxemia (23). PPHN affects between 2-6/1000 live born term infants and continues to have significant morbidity and mortality despite recent advances in therapy (23). Important among these advances is the use of inhaled nitric oxide which has been shown in several multi-center randomized controlled trials to improve oxygenation and decrease the need for Extracorporeal Membrane Oxygenation (ECMO) in term and near term infants (16, 24).

Endogenous nitric oxide is an endothelially-derived regulator of vascular tone which is formed by nitric oxide synthase (NOS) (13). NO can then freely diffuse to the smooth muscle cell and activate soluble guanylate cyclase (sGC) increasing the conversion of guanine triphosphate (GTP) to the intracellular signaling molecule cyclic guanine monophosphate (cGMP) (39). Cyclic GMP works, at least in part, by activating cyclic GMP-dependent protein kinase (PKG) another important intracellular signaling enzyme (1). It is presumed that when NO is given by inhalation it freely diffuses through the pulmonary epithelium to the vascular smooth muscle where it exerts its vascular dilating effect in a similar fashion (13).

In approximately 40% of the infants with PPHN treated with inhaled NO have an incomplete or unsustained response (16, 24). Further, upon the acute withdrawal of inhaled NO there can be a significant increase in the pulmonary vascular resistance which may occur independent of the initial response to inhaled NO therapy, this phenomenon has been termed rebound pulmonary hypertension (3, 35). In sheep models, we have shown that inhaled NO exposure increases plasma endothelin-1 (ET-1) levels and decrease NOS activity (5, 34). ET-1 is a potent endothelially-derived vasoconstricting peptide, which exists in its nascent form as preproET-1 a non-secreted, physiologically inactive 203 amino acid protein (21). Preproendothelin-1 is cleaved intracellularly in an incompletely understood step to Proendothelin-1 or Big ET-1, a 39 amino acid peptide (21). Big ET-1 has been shown to have weak vasodilating effects when infused intravenously and is converted to the active 21 amino acid peptide ET-1 by the tightly regulated enzyme endothelin converting enzyme-1 (ECE-1) (33, 38). There are at least 2 isoforms of ECE-1 in the pulmonary vasculature, with ECE-1α the predominant form (11). It has been shown by multiple investigators that ET-1 and NO exert a paracrine regulation on each other (8, 33, 42). There have been several *in vitro* studies utilizing cultures of systemic vascular endothelial cells that demonstrated that increasing endogenous NO generation, through activation of NOS by various means, will lead to decreased ET-1 (8, 28). However, *in vivo* studies in sheep and pig models demonstrated that inhaled NO at concentration of 30-40 ppm produces increased circulating ET-1 levels (15, 34). These conflicting data prompted us to evaluate the effect of exogenous NO on cells cultured from the pulmonary arteries of sheep in order to examine the mechanism by which NO regulates the release on ET-1. Here we report that in

pulmonary vascular endothelial cells, NO decreases ET-1 secretion through activation of soluble guanylate cyclase and increased cellular generation of cGMP.

MATERIALS AND METHODS

Cell Culture and treatment regimens: Primary cultures of pulmonary arterial endothelial cells were isolated from sheep by the explant technique as we have described (41). Briefly, a segment of the main pulmonary artery from a 4-week old lamb was excised and placed in a sterile 10 cm dish containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 1g/L glucose, 10% Fetal Bovine Serum, fibroblast growth factor (10ng/ml), antibiotics (penicillin and streptomycin) and antimycotics (fungizone). The segment was stripped of adventitia with sterile forceps. The main pulmonary artery segment was then opened longitudinally, and the endothelial layer was removed by gentle rubbing with a cell scraper, and the cells were grown in growth media at 37°C in 21% O_2-5% CO_2. After a few days in culture, moderate-sized aggregates of endothelial cells (4wkPAECs) were transferred using a micropipette, grown to confluence and maintained in culture.

For experimental treatments, 4wk PAECs were transferred to serum-free DMEM-H16 media supplemented with antibiotics and antimycotics 12 hours prior to the experiments to achieve cell cycle synchronization. All experiments were carried out using 500,000 cells of passage 6-10, unless otherwise specified. Cells were then treated (in a 3 ml volume) for 0-24 hours with the long acting nitric oxide donor DETA-NONOate (0-1mM, Alexis Biochemical), unless otherwise specified, or with the nitric oxide independent activator of soluble guanylate cyclase (sGC), YC-1 (30 μM, Calbiochem) (4). Further cells were treated with the sGC inhibitor, ODQ (10 μM,

Biomol) (12), or the inhibitory cGMP stereoisomer of protein kinase G (PKG), Rp-8-pCPT-cGMP (RcGMP, Biomol) at 2.5 μM and 5 μM, corresponding to 5 and 10 times the inhibitory constant (14) alone and prior to the addition of NO. In additional experiments, cells were treated with the long acting cGMP analogue, Sp-8-pCPT-cGMP (Biomol), at concentrations ranging from 1.5- 9 μM.

Western Blot Analysis: Cells were harvested using MPER, mammalian protein extraction reagent (Pierce), then sonicated. Protein concentrations were estimated using Bradford reagent. Total protein (20 μg) was separated on a 4-20% SDS-Polyacrylamide gel (BioRad) and then electrotransferred to polyvinylidene difluoride membranes (Amersham, Arlington Heights, IL) The gels were stained with 0.1% Coomassie blue stain and evaluated by densitometry to determine equal protein loading. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20. After blocking, the membranes were incubated at room temperature with the appropriate dilution of the antiserum of interest (1:1000 for ECE-1α, 1:500 for ECE-1β, 1:100 for sGC-α and 1:1000 for sGC-β). The ECE-1α antiserum was generated as previously described (34). The sGC antisera were a generous gift from Peter Yuen (UT Memphis). After washing membranes hybridized with anti-rabbit horseradish peroxidase antibody and the bands visualized with chemiluminescence using a Kodak Digital Science™ Image Station (NEN) and analyzed using the same software. Specificity was demonstrated using a mammalian expression vector containing full-length rat ECE-1 α transiently transfected into COS-7 cells. This construct was generously provided by Dr. Yanagisawa (UT Southwestern).

Generation of ECE-1β antisera: A peptide was designed that was specific for the ECE-1β. This peptide (CLGKKGPGLTVSLPL) corresponds to the N terminal domain of the ECE-1β, which can immunologically distinguish between the ECE-1 isoforms (8). The protein was purified and synthesized at >90% purity. The peptide was then conjugated, via addition of an N- terminal cysteine. Two female New Zealand White Rabbits (12 wk of age and 2kg in weight) were then injected with 200 μg of conjugated peptide and 200 μg Fruend's complete adjuvant. This injection was repeated after 14, 28, 42, and 56 days with the exception that Fruend's incomplete adjuvant was used. Bleeds (15 ml) were collected at 42, 56 and 70 days. Aliquots of antisera were then stored at -20°C until used. Antibody generation was carried out commercially by Biosynthesis (Lewisville, TX). Specificity was demonstrated using a mammalian expression vector containing full-length rat ECE-1 βtransiently transfected into COS-7 cells. This construct was generously provided by Dr. Yanagisawa (UT Southwestern).

Endothelin-1 Determinations: Conditioned media was collected at multiple timepoints and stored at -80°C until assayed. The media was acidified with 0.1% trifluoroacetic acid and loaded in 3x18 C18 SepPak columns (Peninsula Laboratories) pre-activated with 60% acetonitrile, 0.01% trifluoroacetic acid and equilibrated with 0.01% trifluouroacetic acid. The adsorbed material was eluted with 2 ml of 0.01% trifluoroacetic acid- 60% acetonitrile. The eluant was dried in a Savant speed vacuum centrifuge then dissolved in assay buffer and stored at -20°C or assayed immediately for immunoreactive endothelin-1 (ET-1). The ET-1 standard, ^{125}I-labelled ET-1, anti-ET-1 antibody, and secondary

antibody were purchased from Peninsula Laboratories. Cross-reactivity for measured human and bovine ET-1 antiserum is 100%, 7% for human ET-2, and ET-3, and <1% for bovine ET-2 and ET-3. Inter-assay and intra-assay variabilities were 8 and 4% respectively. Each sample was run in duplicate. This assay was modified from a previously published procedure (43).

Cell proliferation assays: 4wkPAECs were seeded onto 96-well plates (Costar) at approximately 5,000 cells per well (approximately 75% confluence) and allowed to adhere for at least 18 hours. The initial number of viable cells was then determined to correct for differences in starting cell number between experiments, and to monitor changes in cell number over time. The cells were transferred to serum free media and treated with the long acting NO donor, DETA NONOate at various concentrations (6 μM-1mM). After 24 hours of treatment, the cell number was determined using the Cell Titer 96 AQ_{ueous} One Solution kit (Promega) according to the manufacturers instructions.

*Determination of mitochondrial membrane potential integrity***:** Mitochondrial membrane potential has been analyzed previously using the lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (17). This dye fluoresces red in its multimeric form in healthy mitochondria, and is the active reagent in the DePsipher Mitochondrial Potential Assay kit (Trevigen) PAECs were seeded onto 24-well plates and incubated with or without 500 μM DETA NONOate. The DePsipher reagent (25 μg/ml) was then added 24h later, and incubated for a further 20 min. Fluorescent images

were captured using a CoolSnap digital camera and the average fluorescent quantified using Metamorph imaging software (Fryer).

cGMP quantification: PAECs were treated as described and were washed with cold PBS, then scraped into PBS and lysed by sonication. An aliquot was removed for protein quantification. Cold ethanol was added to a final concentration of 66% ethanol to the desiccate the cells and elute cGMP. The cell lysate/ ethanol solution was then centrifuged at 5000g for 15 minutes and the pellet discarded. The cell extract was then dried in a Savant speed vacuum at room temperature, resuspended in assay buffer, acetylated using acidified sodium acetate, then used immediately for cGMP quantification by commercially available ELISA kit (Cayman Chemical, Ann Arbor, MI). The antiserum has a specificity for acetylated cGMP of 100%, cGMP 9%, acetylated cAMP 0.05% and cAMP <0.01%. The results were determined by colorimetry in a Labsystems Multiskan EX automated plate reader (Biotech) at 490nM wavelength and compared to an acetylated cGMP standard curve. All samples were run in duplicate with two concentrations. The interassay and intraassay variability was <10%.

Semiquantitative RT-PCR: Total RNA was collected using the Trizol reagent as previously described. After extraction with chloroform and purification with isopropanol and ethanol, total RNA was quantified by spectrophotometry. Then total RNA (0.1 μg) was reverse transcribed and amplified using a single-step RT-PCR kit (Invitrogen). The following cycling conditions were used for the amplifications: 1 cycle at 50ºC for 45 min (RT step), followed by a denaturation step at 94 °C for 2 min, and a set of 15-25 cycles

of: 94 °C for 30 s, 55 °C for 60 sec and 68 °C for 90 sec. Cycle curves were carried out for ET-1 and 18S (as a normalizer) to determine the linear amplification range. Based on these studies the following cycle numbers were chosen: 15 cycles for 18S (internal control) and 25 cycles for ET-1. The following set of primers were used: ET-1 sense primer: 5'- GCCCTGAGTTCTTTTCCTGCTTGG-3'; ET-1 antisense primer: 3'-CCAAGGAGCTCCAGAAACAGC-3'; 18S sense primer: 5'-AGGGTTCGATTCCGGAGAGGG-3'; 18S antisense primer: 5'-CATTCCAATTACAGGGCCTCG-3'.

Statistical analysis: ET-1 data over time was analyzed by ANOVA for repeated measures. Other data was analyzed by two-way ANOVA with Student Neuman-Kiehl post hoc testing. A $P < 0.05$ was considered statistically significant.

RESULTS

To determine the effect of exogenous NO on endothelin-1 release, 4wk PAECs were treated with DETA NONOate (500 μM) a long acting NO donor. The results obtained indicated both a time and dose-dependent increase in secreted ET-1 (Fig. 1) Treatment with the NO donor, DETA NONOate (500μM), significantly reduced the amount of ET-1 secreted into the media after 24 hours (2743 ± 92 pg/ml vs 561± 112 pg/ml, $P < 0.05$, Fig. 1 A). Similarly, the addition of DETA NONOate at concentrations ≥ 250μM produced as significant decrease in secreted ET-1 ($P<0.05$ vs. untreated, Fig. 1 B). The decrease in secreted ET-1 was not related to a change in the expression of ECE-1 or sGC as determined by Western blot analysis (Fig. 2). To verify that this decrease in ET-1 release was not related to a change in cell viability, cells were treated with the NO donor DETA NONOate, at concentrations ranging from 6 μM to 1mM and cell number was determined. No change in viable cell number was observed (Fig. 3 A). Further, treatment of the cells with 500 μM DETA NONOate did not disrupt the mitochondrial membrane potential as detected by the DePsipher fluorophore, which fluoresces red in the presence of a polarized membrane (Fig. 3 B, C). In addition, TUNEL analysis indicated that these cells were not undergoing apoptosis under these conditions (data not shown). These data demonstrate that treatment of 4wkPAECs with the long acting NO donor, DETA NONOate, significantly decreases ET-1 release but does not alter cell viability at 24 hours.

To investigate whether the decrease in ET-1 was related to NO-induced activation of sGC, 4wkPAECs were treated with YC-1 (30 μM), a NO-independent activator of sGC. This produced a significant decrease in secreted immunoreactive ET-1 at 24 hours (515 ± 312 pg/ml, $P<0.05$ vs untreated, Fig. 4). This decrease was similar to that seen in cells treated with DETA NONOate (500 μM). Treatment with doses higher than 30μM had no greater effect on ET-1 release (data not shown). Treatment of 4wkPAECs with ODQ (10μM), a heme site inhibitor of sGC, had no effect on the amount of ET-1 secreted (2037±454 pg/ml vs 2743 ± 92 pg/ml for control cells). However, pretreatment of 4wkPAECs with ODQ prior to adding DETA NONOate prevented the NO-induced decrease in secreted ET-1, (2365± 230 pg/ml) compared to control values of 2743 ±92 pg/ml ($P<0.05$ vs untreated, Fig. 4).

We next verified that the pharmacologic regulation of sGC activity produced the expected changes in cellular cGMP levels. Cyclic GMP levels were significantly increased with activation of sGC by DETA NONOate (11.8±.13 pmol/ μg protein, $P<0.05$ v control) or YC-1 (12.9 ±1.1 pmol/μg protein, $P<0.05$ vs control) compared to 4±1.3 pmol/μg protein for control cells (Fig. 5 A). Treatment with doses higher than 30μM had no greater effect on cGMP levels (data not shown). Blocking sGC activity with ODQ (10 μM) did not alter basal cGMP levels. However, pretreatment of 4wkPAECs with ODQ significantly reduced the NO induced increase in intracellular cGMP ($P<0.05$ vs untreated, Fig. 5).

In order to determine if cGMP alone could alter ET-1 secretion, cells were treated with the long-acting cell-permeable cGMP analogue, Sp-8-pCPT-cGMP. The results obtained (Fig. 6) demonstrated a significant decrease in secreted ET-1 at concentrations of 3 μM (898 ±316 pg/ml, $P<0.05$ vs untreated), 4.5μM (1040 ± 198pg/ml, $P<0.05$ v untreated), and 9 μM (790± 170 pg/ml, $P<0.05$ v untreated) but not at 1.5 μM (2445±64 pg/ml), compared to untreated cells (2743±92 pg/ml). To determine if the mechanism for the regulation of ET-1 release occurred through cGMP-dependent protein kinase (PKG), cells were treated with the antagonistic stereoisomer of cGMP, Rp-CPT-8cGMP (RcGMP), prior to the addition of the NO donor. Inhibition of PKG significantly, but only partially, blocked the effect of NO on ET-1 release (1192 ± 166 pg/ml at a concentration 2.5μM and 1416± 156 pg/ml at concentration of 5μM RcGMP, both $P<0.05$ vs NO alone, Fig. 6). Treatment with RcGMP alone had no effect on ET-1 release (data not shown).

To begin to investigate if NO decreases ET-1 release through changes in RNA levels, cells were again treated with DETA NONOate (500 μM) and total RNA isolated and quantified by RT-PCR. The results obtained indicated that exposure to the NO donor produced a significant decrease in preproET-1 mRNA to nearly undetectable levels after NO treatment ($P<0.05$ vs untreated, Fig. 7).

DISCUSSION

Our results demonstrate that in primary cultures of PAECs isolated from juvenile lambs exposed to exogenous NO there is a decrease in secreted ET-1. This decrease occurs in the absence of alterations in ECE-1 expression but is associated with a decrease in ET-1 gene expression. The decrease in ET-1 secretion appears to be related to activation of sGC, as YC-1-mediated, NO-independent, activation of sGC, can also decrease ET-1 secretion. Conversely, the inhibition of sGC activity with ODQ, a heme-site inhibitor of sGC, can block the NO-mediated decrease in ET-1 secretion. Moreover we found that the decreases in ET-1 secretion were inversely proportional to changes in intracellular cGMP. In addition, decreases in ET-1 secretion could be replicated using a cell permeable analogue of cGMP. The NO-mediated decrease in ET-1 secretion could also be significantly attenuated by an antagonist of PKG. However, this attenuation was only partial suggesting that there may be other important effects of increased intracellular cGMP besides the activation of PKG that may be involved in the reduction of ET-1 secretion. In addition, our data indicate that the addition of NO-donors to 4-week old PAECs does not appear to alter either sGC-α1 or sGC-β1 protein after 24h of exposure. This is in contrast to the data from Fillipov et al who found that, in vascular smooth muscle cells, NO donors decreased sGC subunit expression (22). The reason for this apparent differential regulation is unclear but may represent divergent regulatory mechanisms in the two cell types. However, further studies will be needed to explore this possibility.

The endothelium is important in regulating vascular tone under a wide variety of physiologic conditions and stresses. There are several important endothelial-derived regulators of vascular tone with NO and ET-1 among them. NO exerts its action in the vasculature through several distinct pathways. Important amongst them is the activation of sGC to increase intracellular cGMP, direct nitration and nitrosylation of proteins and induction of reactive oxygen species (1, 39). Exposure of the endothelium to pharmacologic levels NO undoubtedly alters the coordination of vasoactive agents and the results are complex and may vary according to the vascular bed and developmental stage. Although the effect of NO on ET-1 release has been studied in a variety of systems, the mechanism by which NO regulates ET-1 production and release remain incompletely understood.

Previous studies utilizing primary cultures of systemic vascular endothelial cells isolated from adult animals have shown that stimulating endogenous NO production with agonists will decrease secreted ET-1 (8). However, as it is known that NO and ET-1 release are developmentally regulated and the response of the vasculature to these agents may change with developmental stage (26, 37). Therefore, in the context of pulmonary hypertension of the newborn, we felt it was important to investigate the response of pulmonary vascular endothelial cells of newborn and juvenile animals to begin to understand the changes seen in the pulmonary vasculature after treatment with exogenous NO. It has also been previously demonstrated, using human umbilical venous endothelial cells, that blocking NO production can increase ET-1 release (36). This increased ET-1 release was associated with an increase in ET-1 transcription (36). Conversely,

treatment with the NO donor molsidomine, reduced the hypoxia induced increase in ET-1 release in the lungs of rats (7). Although the exact mechanism of the interaction was not resolved, these data indicate that NO and ET-1 have an inverse relationship, and provide important information in the context of the regulation of the two vascular mediators. Similarly, the addition of an NO donor has been shown to reduce the hypoxia dependent increase in ET-1 release in human umbilical vein endothelial cells (28). The response of the umbilical venous endothelial cells, which line vessels that constrict in the presence of normal oxygen tension and pH, may not be the same as that of the pulmonary vascular endothelial cells. However, the fact that the results obtained are similar to those obtained in our studies suggests that this may be a common regulatory pathway for the effect of NO on ET-1.

We have previously demonstrated that NO donors can increase ROS levels in pulmonary endothelial cells (9, 10) and other investigators have sought to evaluate the role of ROS on ET-1 release. The data obtained have indicated that increased superoxide production, but not increased NO or hydrogen peroxide, decrease ET-1 release via the inhibition of ECE-1(32). The mechanism for this inhibitory effect was found to be through the displacement of the heavy metal zinc from the ECE-1 enzyme (32). Thus, we investigated the effect of NO on ECE-1 expression. Our data indicated that exogenous NO had no effect on ECE-1 α or ECE-1 β expression and although we cannot rule out an affect on ECE-1 activity this suggests that the mechanism by which NO exerts its effect on ET-1 is not via alterations in ECE-1 expression. In addition, studies utilizing primary

cultures of coronary artery endothelial cells have shown that increased superoxide levels increased transcription of preproendothelin-1 mRNA and increased ET-1 release into the media (27). However, our results indicate that preproET-1 mRNA levels and ET-1 release are both decreased by NO. The conflicting data obtained in these studies, although evaluated in different cell types, highlight the potential but complex role that ROS may play in the regulation of ET-1 gene expression and secretion and underscore the importance of understanding vascular specific and cell specific effects.

The data we present in these studies are in contrast to our previously published results where we found that 4wk old lambs exposed to 40ppm inhaled NO for 24 hours had a 2-fold increase in the circulating immunoreactive ET-1 levels (34). Thus, it still remains unclear if this increase in ET-1 secretion is directly related to the use of inhaled NO rather than NO donors or may be due to changes in cell-cell communication or to alterations in biomechanical forces that exist in the whole animal situation. Important among biomechanical forces playing an active role in the whole animal is shear stress. It has previously been shown that exposure of human umbilical venous cells to shear stress increases ET-1 release and increases transcription of preproendothelin-1 mRNA after 24 hours (36). Therefore, the effects of the increased blood flow in the pulmonary system we have observed (5) may increase shear stress in the animal exposed to inhaled NO may account in part for the differences between our *in vivo* and *in vitro* data. In addition, the vessel is made up of more than one cell type and it is possible that more "organotypic" cultures such as endothelial and smooth muscle co-cultures may be required to more

adequately evaluate the effect of NO on ET-1 expression and secretion. However, further studies will be required to examine these possibilities.

ET-1 is constitutively released by endothelial cells at a rate determined by the cellular or vascular milieu. Previous studies in capillary endothelial cells have shown that there is no intracellular store of the active 21 amino acid peptide ET-1, although there is a small store of the 39 amino acid precursor Big ET-1 (25). This suggests that ET-1 secretion may be regulated predominantly at the level of preproET-1 transcription. In addition, it has previously been shown that preproET-1 mRNA has a short half-life in the cultured endothelial cells (< 30 min) (20). However, the transcriptional regulation of preproET-1 mRNA and the role of NO in this regulation remain incompletely understood. Our data indicate that NO decreases ET-1 mRNA levels in 4wkPAECs. Even allowing for the semi-quantitative nature of our RT-PCR analysis, our data suggest that the decrease in secreted ET-1 is related to decrease in transcription of the precursor, preproET-1. The PreproET-1 promoter has putative binding sites for activator protein-1 (AP-1), c-myc, NFκB, and GATA-2 binding protein (6, 19, 30). Previous experiments in fibroblast cell lines have shown that c-myc at low concentrations will promote preproET-1 transcription, while at higher concentrations it inhibits transcription to nearly undetectable levels (40). Further, activation of the cis-elements that comprise the AP-1 sequence, c-jun and c-fos, by phosphorylation and increased nuclear translocation will promote preproET-1 transcription (20, 29, 31). In our current study we have shown that activating sGC and increasing intracellular cGMP decreases ET-1 release and may decrease preproET-1 mRNA. Previous studies in a neuroblastoma cell line demonstrated

that increased intracellular cGMP through activating PKG significantly increases c-Myc expression and phosphorylation and increases cytosolic c-Jun. However, the increased c-Jun is not associated with an increase in phosphorylated c-Jun nor nuclear translocation (2). While these experiments were not carried out in endothelial cells, they provide evidence for a possible mechanism to explain the decrease ET-1 release seen in our system. Further studies will be needed to determine if in the pulmonary vascular endothelium increased cGMP regulates transcription of preproET-1 through either an increase in phosphorylated c-myc or alteration in the activation of c-Jun.

In conclusion, our studies provide further mechanistic insight into the interaction between NO and ET-1. Moreover, our cell culture system indicates that the interactions between ET-1 and NO involve complex signaling pathways and further studies will be required to determine how cGMP regulates ET-1 release in the pulmonary vasculature. It is our hope that as we can gain further insight into the complex interactions between NO and ET-1 in the endothelium, that improved therapies for pulmonary hypertensive disorders can be developed.

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FIGURE LEGENDS

Figure 1. Exogenous NO decreases the secretion of ET-1 from pulmonary arterial endothelial cells isolated from 4-week old lambs. 4wkPAECs were treated or not with DETA NONOate (0-1mM) and the level of secreted ET-1 determined over a 24h time period.

Panel A. There is a time-dependent increase in secreted ET-1 that is abrogated in the presence of DETA NONOate. * $P<0.05$ vs. T=0h. † $P<0.05$ vs. untreated at the same time point (A).

Panel B. There is a decrease in secreted ET-1 in the presence of increasing concentrations of DETA NONOate. * $P<0.05$ vs. Untreated.

Figure 2. Western blot analysis for ECE-1 α and ECE-1 β in pulmonary arterial endothelial cells isolated from 4-week old lambs. Protein extracts (20 μg) from 4wkPAECs, exposed or not to DETA NONOate (500μM) for 24h, were separated on a 7.5% SDS-polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed using specific antisera raised against either ECE-1 α, ECE-1 β, sGC-α1, or sGC-β1.

Panel A. A representative Western blot for ECE-1 α and ECE-1 β expression is shown. Positive control for each Western is whole cell extract (20 μg) prepared from COS cells transiently transfected a mammalian expression vector containing either full-length ECE-1 α or ECE-1 β.

Panel B. The densitometric values for ECE-1 α and ECE-1 β protein was determined in the presence and absence of DETA NONOate (500μM) for 24h. ECE-1 α and ECE-1 β protein levels are unchanged by DETA NONOate exposure. Values are mean ± sd. N=8 for ECE-1 α and N=4 for ECE-1β.

Panel C. A representative Western blot for sGC-α1 and sGC-β1 expression is shown.

Panel D. The densitometric values for sGC-α1 and sGC-β1 protein was determined in the presence and absence of DETA NONOate (500μM) for 24h. sGC-α1 and sGC-β1 protein levels are unchanged by DETA NONOate exposure. Values are mean ± sd. N=6 for sGC-α1 and N=4 for sGC-β1.

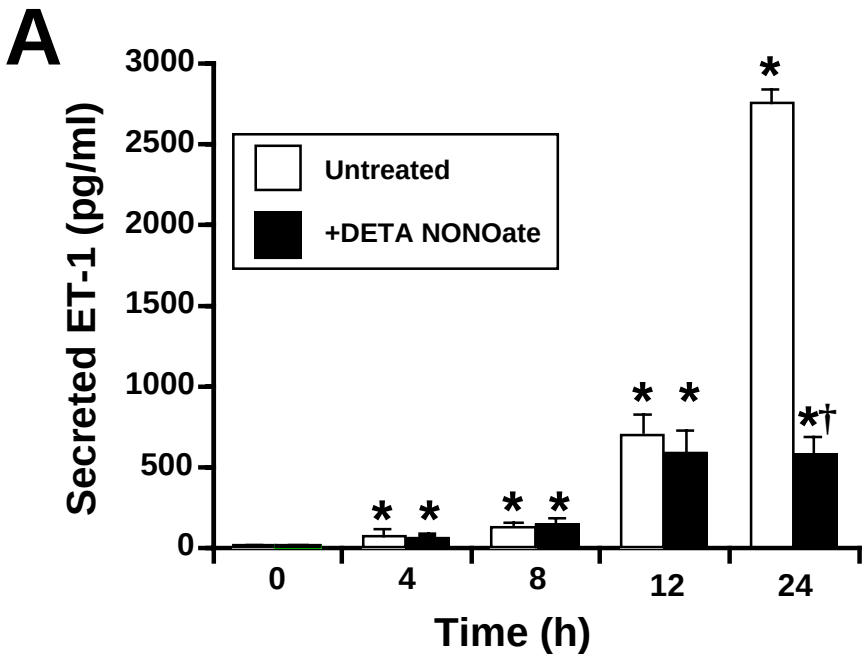
Figure 3. Exogenous NO does not alter the viability of pulmonary arterial endothelial cells isolated from 4-week old lambs. 4wkPAECs were exposed for 24h to increasing concentrations (6μM-1mM) of DETA NONOate. Even at the highest concentration (1mM) there was no effect on the level of biochemically active cells (A). Values are normalized to the level seen in normal growth serum (100%). N=8. In addition, treatment of the cells with 500μM DETA NONOate has no effect on mitochondrial membrane integrity as analyzed by DePsipher, a fluorophore which fluoresces red in the presence of a polarized membrane. Panel B is a representative micrograph captured under identical imaging conditions of 4wkPAECs in the presence and absence of DETA NONOate (500 μM), while panel C is average pixel data as obtained using metamorph software. Also, included is an image obtained from cells exposed to vitamin C (18h, 500μM), as a positive control, that results in a decrease in mitochondrial membrane integrity. N=4, * $P < 0.05$ vs. untreated.

Figure 4. Modulation of soluble guanylate cyclase activity alters the secretion of ET-1 from of pulmonary arterial endothelial cells isolated from 4-week old lambs. 4wkPAECs were exposed for 24h to the NO independent activator of sGC, YC-1 (30 μM), DETA NONOate (500μM), the heme site inhibitor of sGC, ODQ (10μM), or DETA NONOate and ODQ in combination. YC-1 produces a significant decrease in the secretion of ET-1 compared to untreated cells (A). * $P<0.05$ compared to control, N=6. Pretreatment of 4wkPAECs with the heme site inhibitor of sGC blocks the NO-mediated reduction in secreted ET-1, while ODQ alone has no effect on the secreted ET-1 levels (B). † $P<0.05$ compared to DETA NONOate alone, N=3.

Figure 5. Modulation of soluble guanylate cyclase activity alters cGMP levels in pulmonary arterial endothelial cells isolated from 4-week old lambs. 4wkPAECs were exposed for 24h to the NO independent activator of sGC, YC-1 (30 μM), DETA NONOate (500μM), the heme site inhibitor of sGC, ODQ (10μM), or DETA NONOate and ODQ in combination. Stimulating sGC with either the NO donor, DETA NONOate (500μM), or YC-1 (30μM) increases cellular cGMP by 3-fold. Pretreatment with the inhibitor of sGC, 10 μM ODQ, prior to treating with the NO donor prevented the increase in cGMP levels. Treatment with ODQ alone had no effect on cellular cGMP levels. * $P<0.05$ compared to control; † $P<0.05$ compared to DETA NONOate alone. N=4.

Figure 6. Modulation of protein kinase G activity alters ET-1 secretion from pulmonary arterial endothelial cells isolated from 4-week old lambs. 4wk PAECs were exposed for 24h to the cell permeable cGMP analogue, Sp-CPT-8-cGMP (1.5 μM to 9μM) or DETA NONOate (500μM) in the presence and absence of the PKG inhibitor, Rp-8-pCPT-cGMP (RcGMP, 2.5 μM and 5 μM). Sp-CPT-8-cGMP significantly inhibited ET-1 secretion at 3μM and 9 μM compared to control while Rp-8-pCPT-cGMP blocked the NO-mediated decrease in ET-1 secretion. * $P<0.05$ compared to control; † $P<0.05$ compared to DETA NONOate alone. N=4.

Figure 7. Exogenous NO decreases the expression of prepro ET-1 mRNA in pulmonary arterial endothelial cells isolated from 4-week old lambs. 4wkPAECs were treated with DETA NONOate (500 μM) for 24h and total RNA extracted to determine preproET-1 mRNA levels via semi-quantitative RT-PCR. DNA samples were then run on a 1.5% agarose gel A and visualized by ethidium bromide staining. A representative ethidium bromide gel is shown (A). Densitometric analysis of preproET-1 mRNA levels was then carried out and the data normalized to the level of 18S. PreproET-1 levels are decreased in the presence of DETA NONOate (B). * $P < 0.05$ vs untreated. N=3.



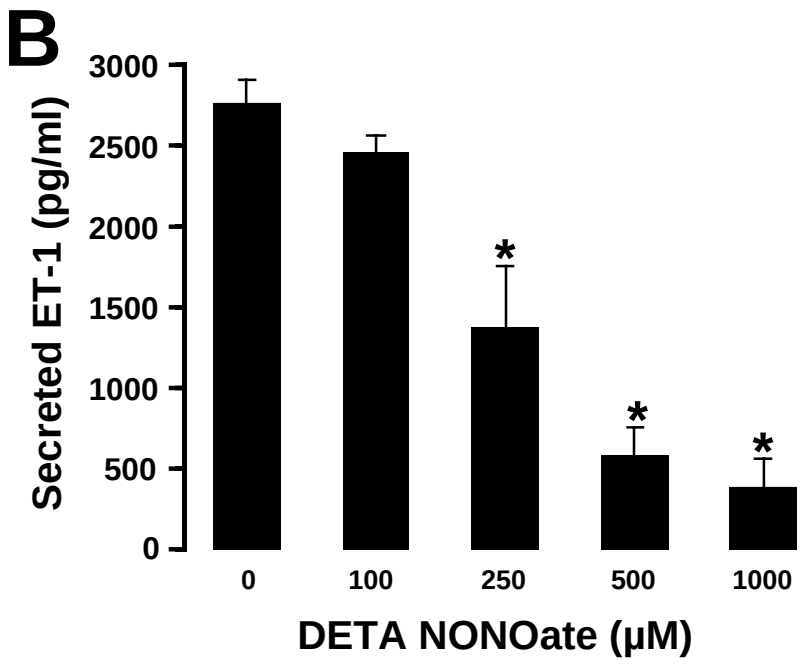


Fig. 1

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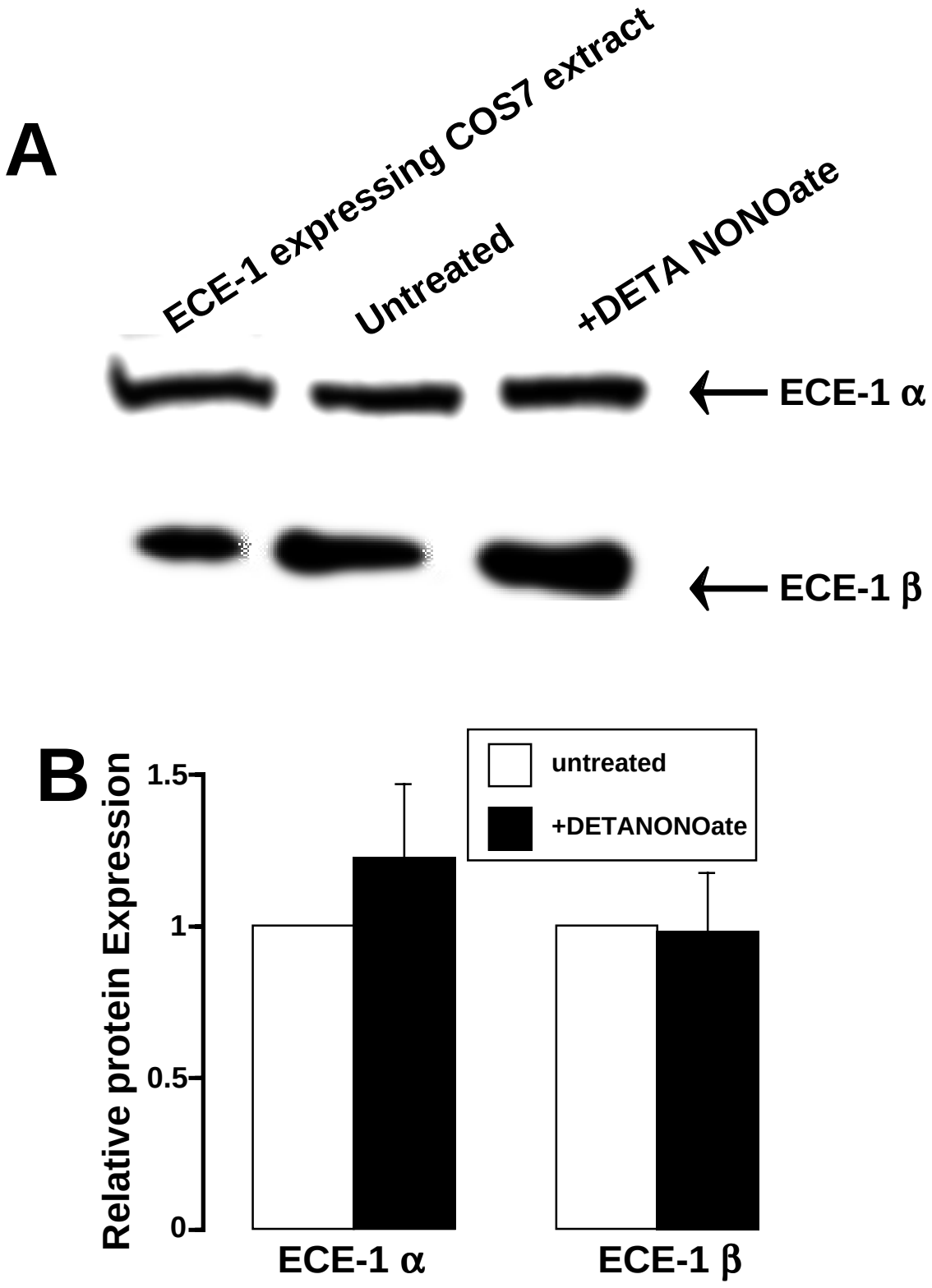

Fig. 2 A & B

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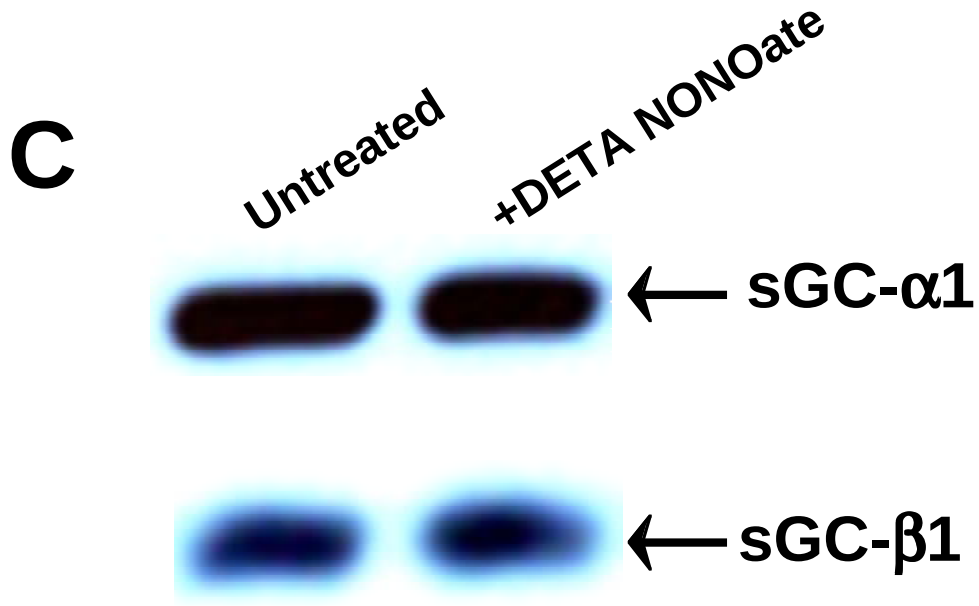

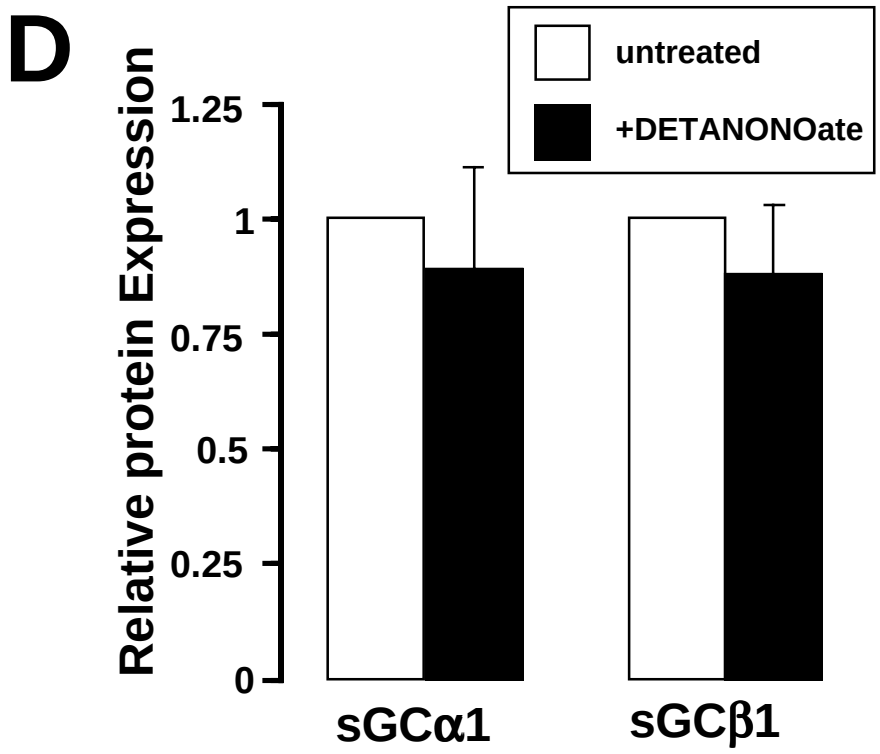

Fig. 2 C & D

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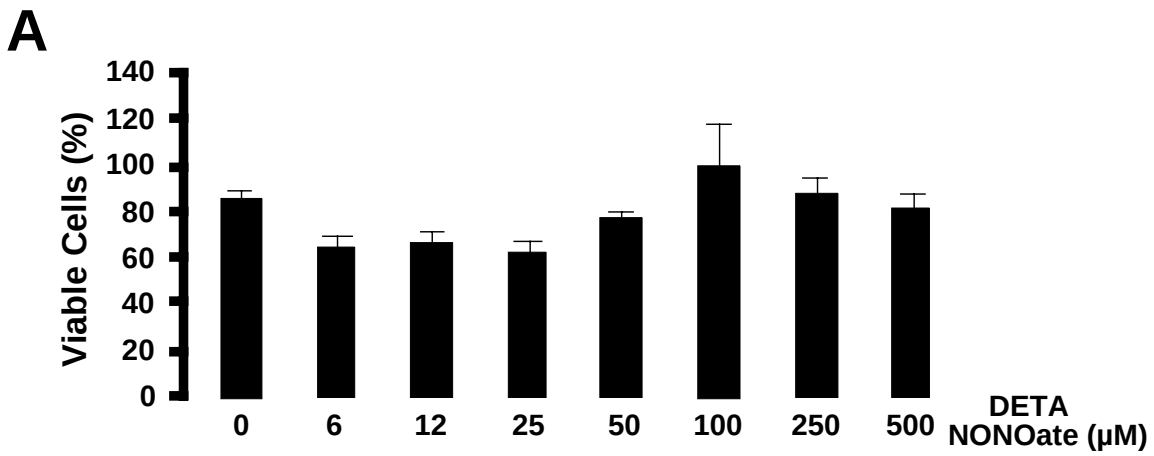

Fig. 3 A

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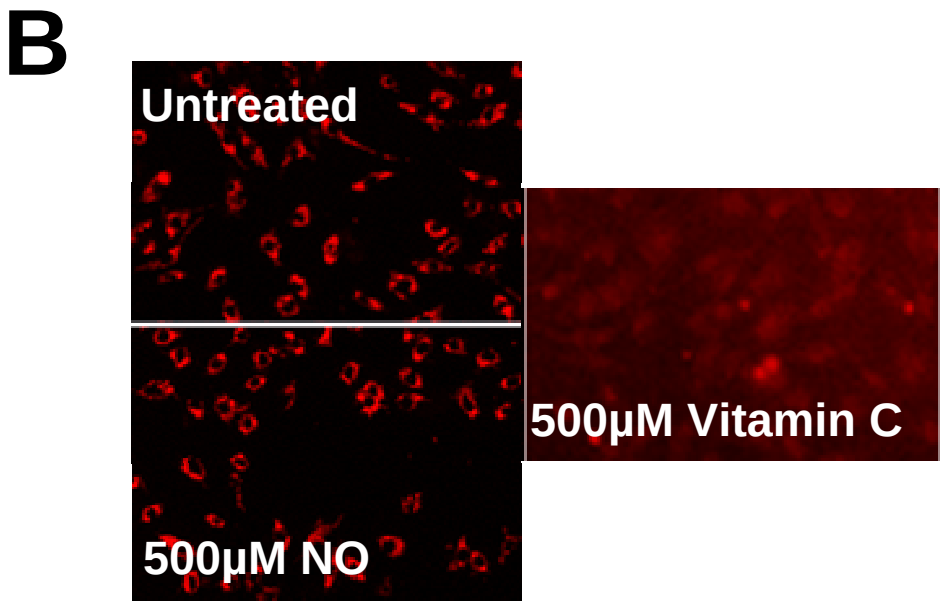

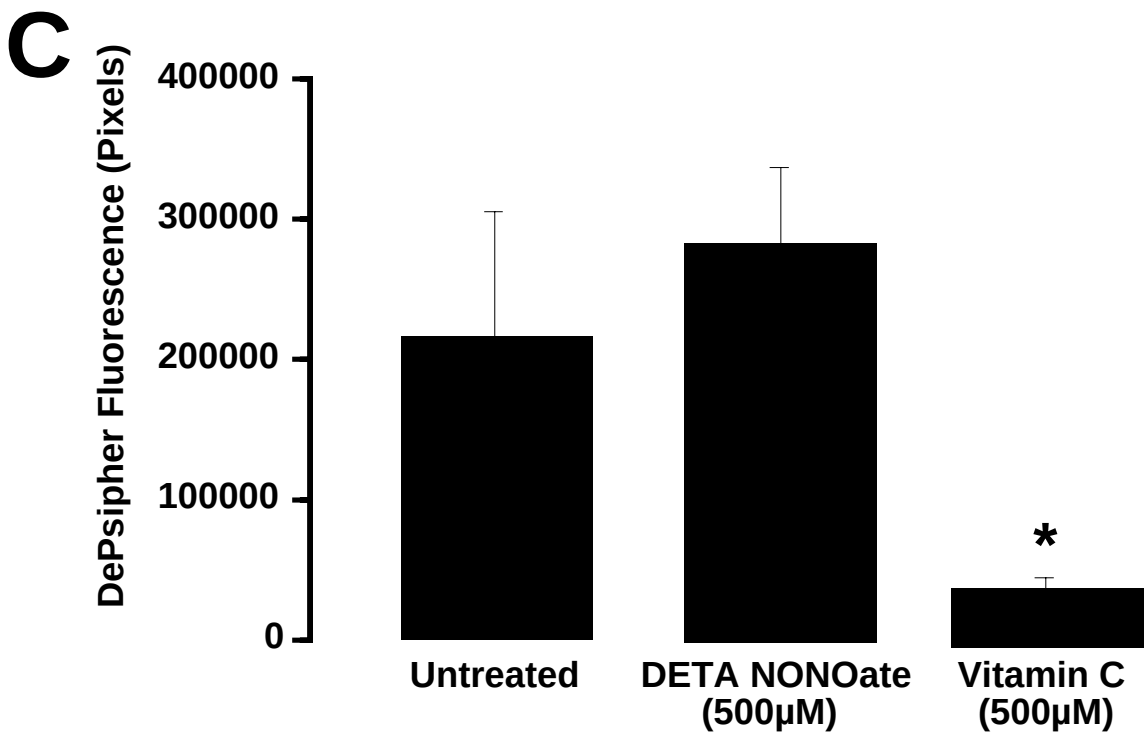

Fig. 3 B & C

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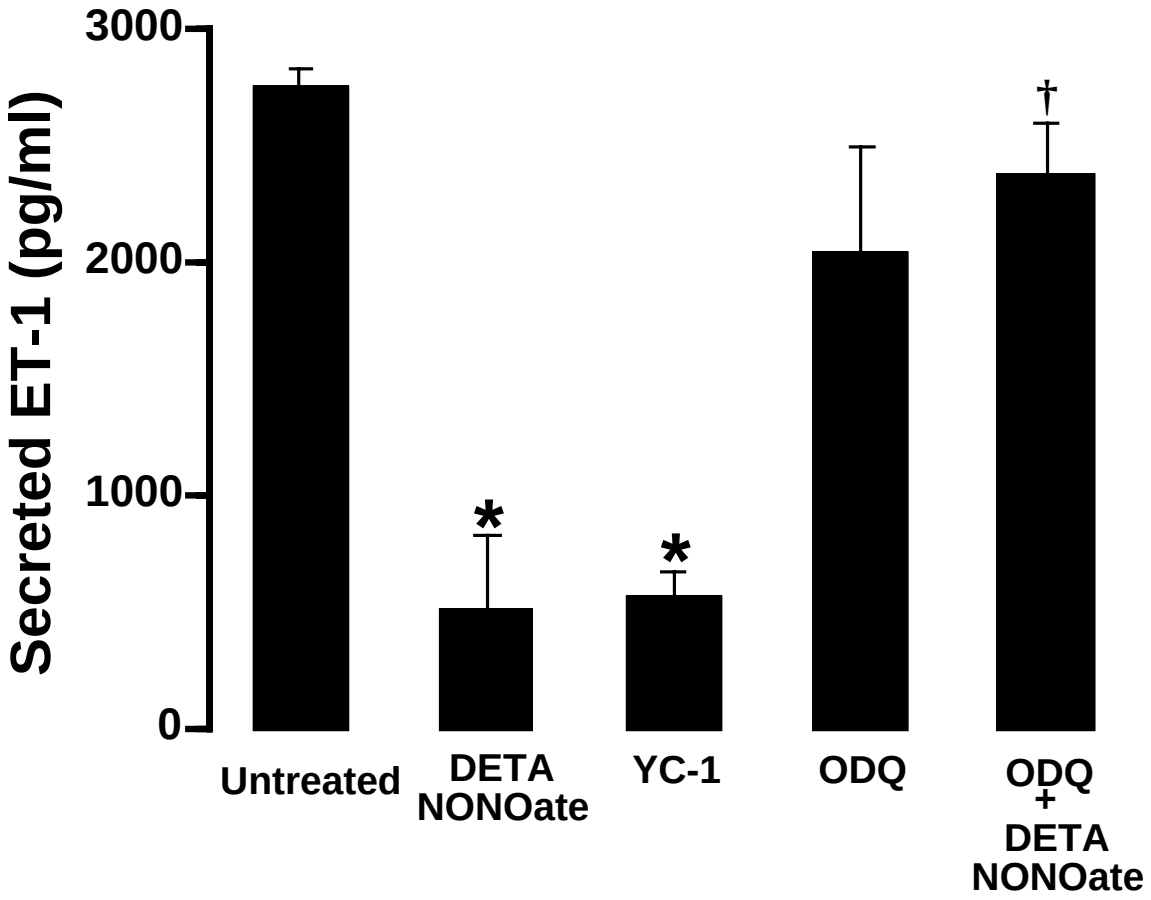

Fig. 4

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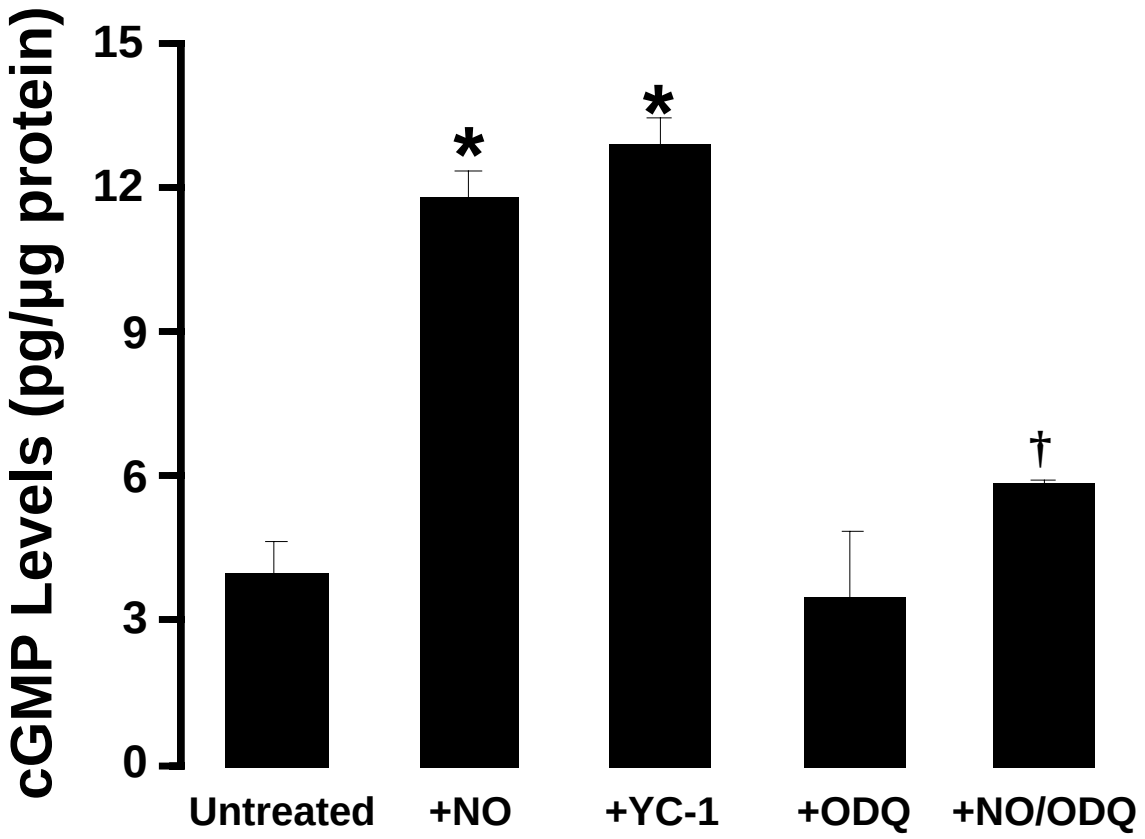

Fig. 5

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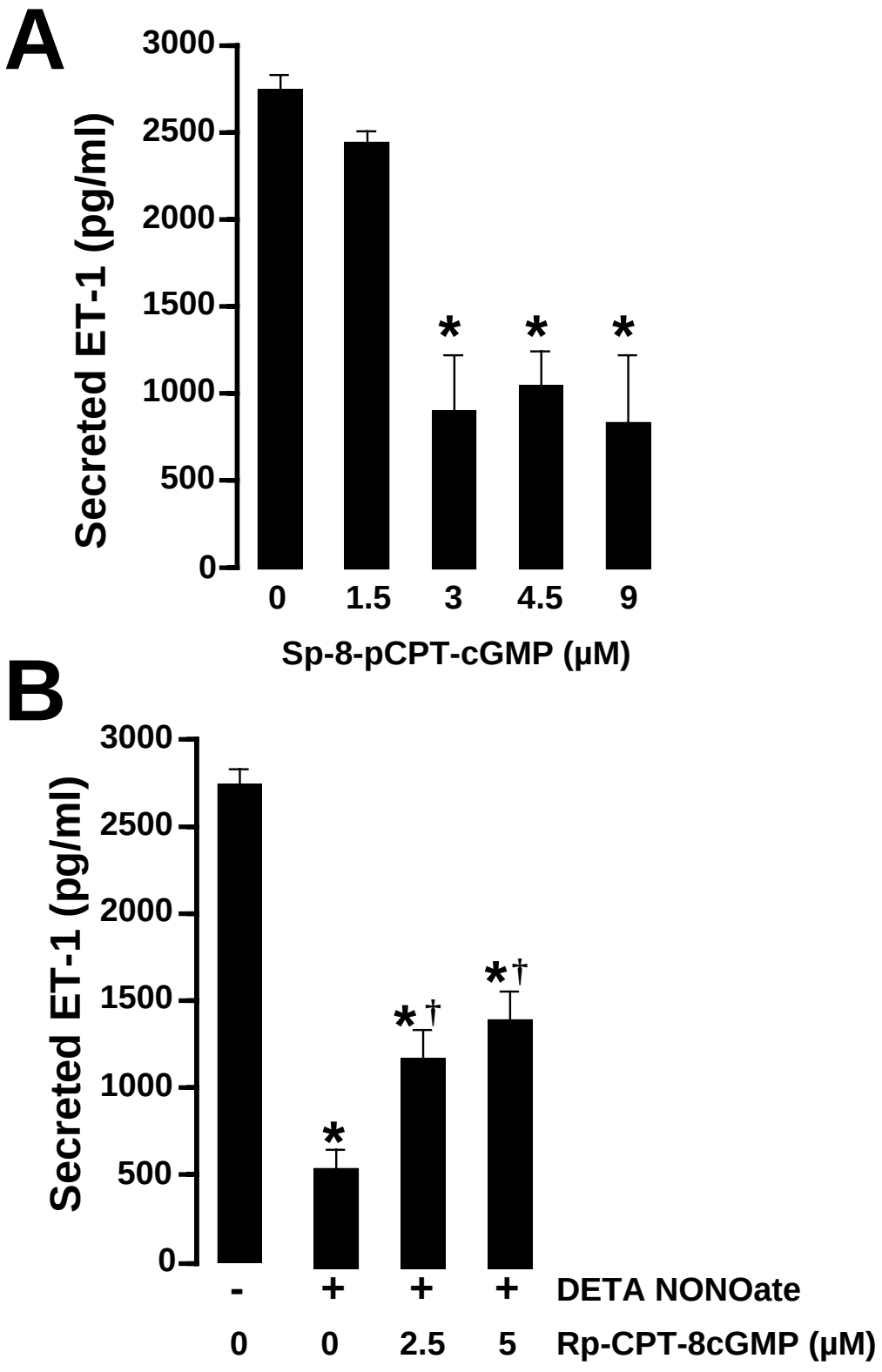

Fig. 6

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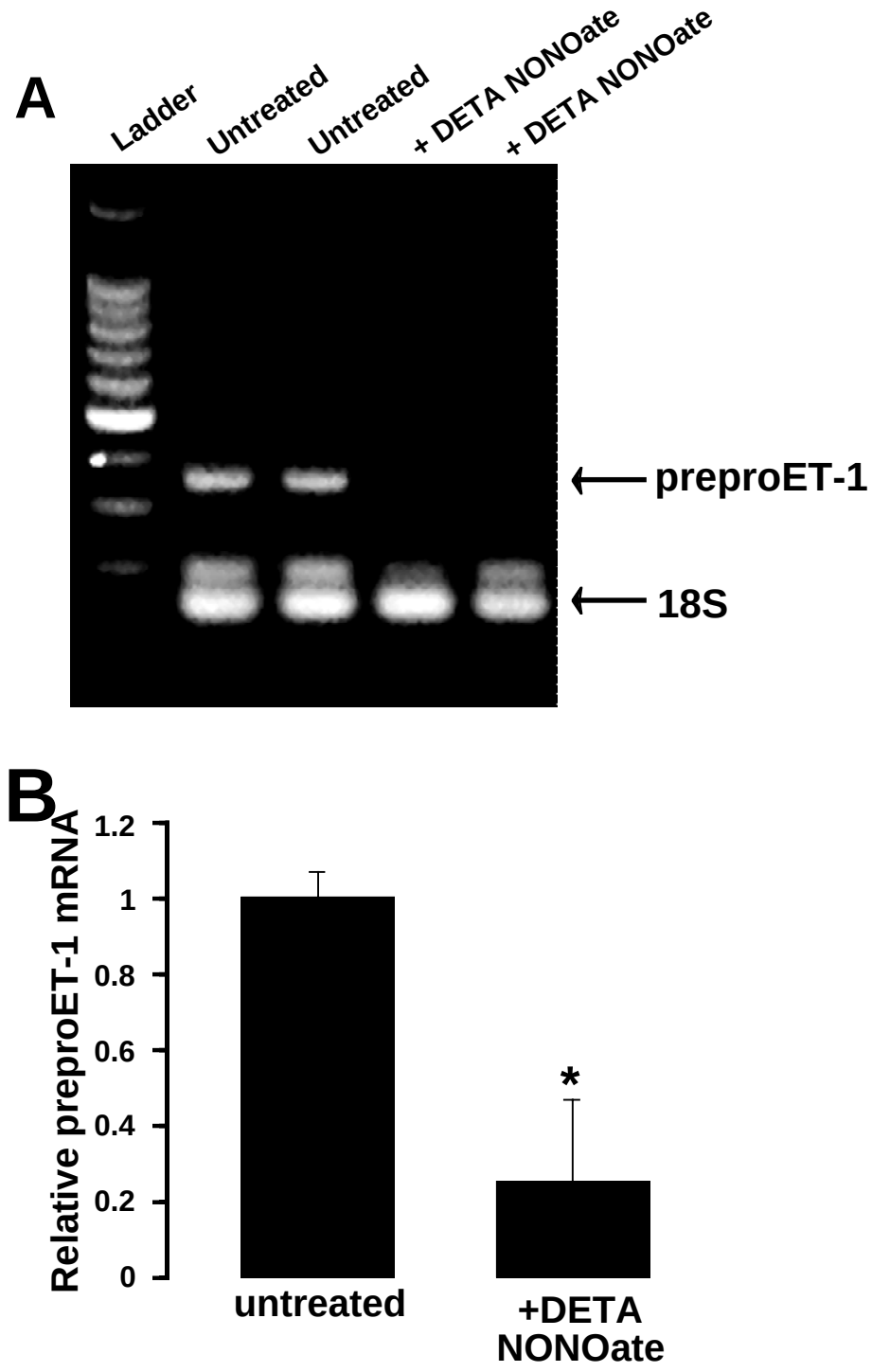

Fig. 7

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