

ESTROGEN INCREASES NITRIC OXIDE PRODUCTION IN HUMAN BRONCHIAL EPITHELIUM

**Elizabeth A. Townsend, Lucas W. Meuchel, Michael A. Thompson, Christina
M. Pabelick, and Y S Prakash**

**Department of Physiology and Biomedical Engineering (EAT, LWM, CMP, YSP),
and Department of Anesthesiology (MAT, CMP, YSP)**

Mayo Clinic, Rochester, MN 55905 USA

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Address for Correspondence: Y. S. Prakash, MD, PhD, Professor of Anesthesiology and Physiology, Department of Anesthesiology, 4-184 W Joseph SMH, Mayo Clinic, 200 1st ST SW, Rochester MN 55905. Phone: 507-255-0165; Fax: 507-255-7300; Email: prakash.ys@mayo.edu

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ABSTRACT

While sex differences in asthma severity are recognized, the mechanisms by which sex steroids such as estrogen influence the airway are still under investigation. Airway tone, a key aspect of asthma, represents a balance between bronchoconstriction and dilation. Nitric oxide (NO) from the bronchial epithelium is an endogenous bronchodilator. We hypothesized that estrogens facilitate bronchodilation by generating NO in bronchial epithelium. In acutely dissociated, human bronchial epithelial cells from female patients, exposure to 17 β -estradiol (E₂; 10 pM-100 nM) resulted in rapid increase of diaminofluorescein (DAF-2) fluorescence (NO indicator) within minutes, comparable to that induced by ATP (20 μ M). Estrogen receptor (ER) isoform-specific agonists THC (ER α) and DPN (ER β) stimulated NO production to comparable levels and at comparable rates, while the ER antagonist ICI 182,780 (1 μ M) was inhibitory. Estrogen effects on NO were mediated via caveolin-1 (blocked using the caveolin-1 scaffolding domain peptide) and by increased intracellular calcium ([Ca²⁺]_i; prevented by 20 μ M BAPTA but not by blocking Ca²⁺ influx using LaCl₃). Estrogen increased endothelial NOS activation (inhibited by 100 μ M L-NAME) and phosphorylated Akt. In epithelium-intact human bronchial rings contracted with ACh (1 μ M), E₂, THC and DPN all produced acute bronchodilation in a dose-dependent fashion. Such bronchodilatory effects were substantially reduced by epithelial denudation. Overall, these data indicate that estrogens, acting via ER α or ER β , can acutely produce NO in airway epithelium (akin to vascular endothelium). Estrogen-induced NO and its impairment may contribute to altered bronchodilation in women with asthma.

INTRODUCTION

Asthma is more common in women than men (Schatz and Camargo, 2003; Melgert et al., 2007), with symptoms exacerbated during the menstrual cycle and with pregnancy (Vrieze et al., 2003; Farha et al., 2009). These data suggest that sex steroids (e.g. estrogens) play a role in asthma. However, the mechanisms by which sex steroids influence the airway in a beneficial or detrimental fashion are still under investigation. An important aspect of asthma pathophysiology is enhanced airway tone, which represents a balance between bronchodilation and bronchoconstriction, both of which could potentially be modulated by estrogens on a rapid timescale, thus dynamically altering airway tone. In this regard, we have previously shown that clinically-relevant concentrations of estrogens can acutely (i.e. within minutes) reduce intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in airway smooth muscle (ASM) (Townsend et al., 2010), thus rapidly reducing bronchoconstriction. However, the mechanisms by which estrogens may influence bronchodilation have not been well-studied.

Nitric oxide (NO) is a major endogenous as well as exogenous bronchodilator (Nijkamp and Folkerts, 1995; Feletou et al., 2001; Folkerts and Nijkamp, 2006). In airways, NO may be derived from airway epithelium, (Di Maria et al., 2000; Shaul, 2002; Bove and van der Vliet, 2006) non-adrenergic/non-cholinergic innervation, (Lammers et al., 1992; Belvisi et al., 1995) and ASM (Nijkamp and Folkerts, 1995). All three NOS isoforms (nNOS, eNOS and iNOS) are known to be expressed within the respiratory tract (Shaul, 2002; Ricciardolo et al., 2004; Bove and van der Vliet, 2006), with airway epithelium being a major site for eNOS and iNOS, the latter especially in the asthmatic airway (Ortiz and Garvin, 2003; Jiang et al., 2009). While NO has several mechanisms of

action, the bronchodilatory properties of NO on ASM are of particular interest in terms of regulation of airway tone and its dysfunction in airway hyperreactivity and inflammation.

The effects of sex steroids such as estrogen or progesterone on the airway epithelium have not been well-studied (Ricciardolo et al., 2004; Bove and van der Vliet, 2006). In vascular endothelium, estrogens, acting via estrogen receptors (ERs) induce rapid NO production (and vasodilation) by facilitating caveolar dissociation from eNOS (Hisamoto and Bender, 2005). Whether a similar set of mechanisms is involved or whether estrogens actually produce NO in airway epithelium is not known. Estrogen receptors (ERs) are known to be present in airway epithelium (Ivanova et al., 2009), with both cytoplasmic and nuclear localization. A previous study found levels of exhaled NO production, a marker of inflammation in asthma, to be higher during the late-follicular phase (Kharitonov et al., 1994), but this finding likely reflect iNOS activity (the major source of exhaled NO). A single study using NCI-H441 lung adenocarcinoma epithelial cells showed that acute exposure to estradiol increases the conversion of [³H]-L-arginine to [³H]-L-citrulline (Kirsch et al., 1999), but did not demonstrate NO production. In the present study, using acutely dissociated, normal human bronchial epithelial cells (BECs), and epithelium-intact vs. -denuded bronchial rings, from females, we tested the hypothesis that estrogen enhances rapid (likely non-genomic) NO production via eNOS activation thereby potentiating bronchodilation.

MATERIALS AND METHODS

Isolation of Human BECs

Human bronchial epithelium was isolated from bronchial tissue obtained from lung samples incidental to thoracic surgery (lobectomy, pneumonectomy) in female patients at Mayo Clinic, Rochester. Following completion of analysis by the pathologist for surgical purposes, the discarded lung sample was dissected to identify normal-appearing airways (confirmed via review of clear margins by the pathologist). Patient ages ranged between 45 and 75 years. Patient histories were noted, but samples were de-identified, thus considered exempt by Mayo Institutional Review Board and approved for research use. Airway samples were restricted to patients with focal disease not involving the airway (e.g. focal non-airway cancers or granuloma) and without pre-existing airway disease (asthma, emphysema, COPD) at the time of surgery and thus considered normal. No attempts were made to determine hormonal status (pre- or post-menopausal) of the patients at the time of surgery; however ER expression was confirmed in all samples prior to further experimentation.

Airway samples were rapidly transferred to the laboratory in ice-cold Hanks Balanced Salt Solution (HBSS). The bronchial epithelium was dissected from the smooth muscle and cartilage on ice, minced, and transferred T25 flasks or 8-well Lab-Teks (Nalgene Nunc International, Rochester, NY). Explants were grown in a 95% air/ 5% CO₂ humidified incubator using Basal Epithelial Growth Medium supplemented with BPE, 2 ml; Hydrocortisone, 0.5 ml; hEGF, 0.5 ml; Epinephrine, 0.5 ml; Transferrin, 0.5 ml; Insulin, 0.5 ml; Retinoic Acid, 0.5 ml; Triiodothyronine, 0.5 ml; GA-1000, 0.5 ml

(Lonza, Portsmouth, NH). Explants were removed from culture at confluence. The BECs were then trypsinized and re-plated for experiments. All experiments were performed in cells from primary explants and passages 1 and 2 of subculture. Cell purity was verified by periodic Western analyses for expression of E-cadherin, but absence of fibroblast surface protein or smooth muscle myosin.

Isolation of Cellular Fractions

Human BECs were harvested and cellular fractions were prepared by separation into heavy (nuclear/Golgi), cytosolic, and membrane fractions using the FractionPREP Cell Fractionation System (BioVision Inc. Mountain View CA; manufacturer-provided protocol) as described previously for ASM (Townsend et al., 2010).

Western Blot Analysis

Standard SDS-PAGE (Criterion Gel System; Bio-Rad, Hercules, CA; 10% gradient gels) and PVDF membrane (Bio-Rad) transfer techniques were used. Membranes were blotted for ER α (1 μ g/ml (1:200 dilution) rabbit anti-ER α ; Santa Cruz Biotechnology Inc. SC-542), ER β (1 μ g/ml (1:200 dilution) mouse anti-ER β ; Santa Cruz SC-53494), eNOS (1 μ g/ml (1:250 dilution); mouse anti-eNOS; BD Transduction Laboratories, Franklin Lakes, NJ), p-eNOS (1 μ g/ml (1:250 dilution); mouse anti-p-eNOS (pSer1177); BD Transduction Laboratories), iNOS (1.5mg/ml (1:650 dilution); rabbit anti-iNOS; Cell Signaling Technology #2977), AKT (1 μ g/ml (1:1000 dilution); rabbit anti-AKT; Cell Signaling Technology #9272), and p-AKT (1 μ g/ml (1:1000 dilution); rabbit anti-p-AKT; Cell Signaling Technology #9271). GAPDH (1 μ g/ml (1:1000 dilution); rabbit anti-GAPDH; Cell Signaling Technology #2118) was used for

normalization. Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and signals developed by Supersignal West Pico or West Femto (p-eNOS, p-AKT) Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL). Blots were imaged on a Kodak ImageStation 4000mm (Carestream Health, New Haven, CT) and quantified using densitometry.

Immunofluorescence Microscopy

ER expression in BECs was verified using immunofluorescence as described for other proteins (Townsend et al., 2010). Briefly, BECs grown on 8-well Lab-Teks were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton-X, blocked in 4% normal donkey serum, and incubated overnight with an antibody against eNOS along with an antibody against either ER α or ER β . Immunopositivity was detected with appropriate AlexaFluor488- and Cy3-conjugated secondary antibodies (donkey anti-rabbit or anti-mouse IgG, Jackson Immunoresearch, 1:200 dilution). Samples were visualized using an Olympus FluoView laser scanning confocal microscope equipped with Ar and Kr lasers and appropriate filters (1024x1024 pixel images, 0.4 μ m optical section thickness; 40X/1.3NA oil-immersion lens).

Real-time NO imaging

BECs grown in 8-well Labtek chambers were loaded with the NO-sensitive fluorescent dye 4,5-diaminofluorescein diacetate (Calbiochem; DAF-2 DA: 5 μ M, 60 min at room temperature). The NO-insensitive dye DAF-4 (5 μ M) was used as a negative control for NO detection. Cells were imaged as previously described (Meuchel et al., 2011) using a real-time fluorescence imaging system (MetaFluor; Universal Imaging,

Downingtown, PA) mounted on a Nikon Diaphot inverted microscope, with a 40X/1.3NA oil immersion lens (Fryer Instruments, Edina, MN). Cells were initially perfused with 2.5 mM Ca^{2+} HBSS and baseline fluorescence established. The responses of ~10 cells per chamber (average number of cells in field of view) were obtained from software-defined regions of interest (0.2 Hz acquisition of 510 nm emission following 488 nm excitation using a Photometric Cascade digital camera system (Roper Scientific, Tucson, AZ)).

Real-time simultaneous NO and Ca^{2+} imaging

BECs grown in 8-well Labtek chambers were loaded with DAF-2 DA as well as the fluorescent calcium indicator X-Rhod-1 AM (Invitrogen: 1 μ M, 45 minutes at room temperature). Each dye was sequentially visualized using appropriate excitation/emission filter sets in the imaging system as above (quality control experiments done to verify absence of bleed through between the fluorescence channels). Calcium calibration was performed using X-Rhod-1 tripotassium salt (#84049 Anaspec, Fremont, CA) according to calcium calibration kit protocol (Invitrogen)

Force Measurements

Bronchial samples for force studies were collected from 3rd to 6th generation bronchi of female patients (as above). The connective tissue was removed by microdissection, and the bronchial epithelium was either retained (epithelium-intact) or mechanically removed (epithelium-denuded) by abrasion within the bronchial lumen via a fine wire brush. The bronchial rings (epithelium -intact or -denuded) were then suspended in a 5 ml organ bath (Radnotti Systems) and force measurements performed using calibrated force transducers (Grass Instruments FT03). Rings were maintained at 37°C in physiological saline solution. Each ring was stretched to optimal length using

repeated contractions with 1 μ M ACh. The rings were then contracted with 1 μ M ACh until a stable contraction was reached. Samples were then exposed to vehicle (time control) or to increasing concentrations of E₂, THC, DPN and the extent of bronchodilation measured. Force responses were measured using custom-built software based on LabView (National Instruments).

Materials

Compounds were obtained from sources as indicated. Chemical structure and formal name are indicated by the company. (R,R)-5,11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol ((R,R)-THC: Tocris #1990), Diaryl-propionitrile (DPN: Tocris #1494), and G-1 (Tocris #3577) were obtained from Tocris Biosciences (Ellisville MO). MAHMA-NONOate (Cayman Chemical #82130), DEA-NONOate (Cayman Chemical #82100), and Sulpho-NONOate (Cayman Chemical #83300) were obtained from Cayman Chemical, Ann Arbor, MI. N^G-Nitro-L-arginine Methyl Ester (L-NAME: Calbiochem #483125), 1,2-*bis*(*o*-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA: Calbiochem #196419), 4,5-Diaminofluorescein diacetate (DAF-2 DA: Calbiochem #251505), 4-Aminofluorescein diacetate (DAF-4: Calbiochem #121745) and Caveolin-1 scaffolding domain peptide (CSD: Calbiochem #219482) were purchased from Calbiochem (San Diego, CA). 17 β -estradiol (E₂: Sigma #E8875), ICI 182, 780 (ICI: Sigma #I4409), 1400W (Sigma #W4262) and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless mentioned otherwise.

Statistical analysis

Bronchial samples were obtained from 6 female patients and used for BEC isolation. NO experiments were performed in at least 15 cells each from 4 different patient samples, although not all protocols were performed in each sample obtained. Force responses were measured from 4 patient samples. Results were compared using unpaired *t*-test or one-way ANOVA with repeated measures as appropriate. Bonferroni correction was applied for multiple comparisons. Statistical significance was established at $p < 0.05$. Values are expressed as means \pm SE.

RESULTS

ER Expression in Human BECs

Western analysis of cell fractions from unstimulated BECs revealed that both ER α and ER β were expressed in the cytosol as well as plasma membrane (Figure 1), with a relatively higher membrane expression of ER α compared to ER β . Nuclear expression of ER α or ER β was small. Two-color fluorescent immunostaining confirmed the co-localization of both ERs with eNOS within the plasma membrane (Figure 1C). However, it should be noted that ER β was also observed separate from eNOS, suggesting a non-caveolar expression of this isoform.

Sensitivity and Specificity of DAF-2 in BECs

The NO sensitive dye DAF-2 DA is intracellularly metabolized and converted to DAF-2, which fluoresces in proportion to the total NO that the dye binds over time (i.e. a cumulative response). To establish sensitivity and specificity of DAF-2 in isolated BECs for NO, a variety of NO and nitrous oxide (N₂O) donors were used.

The fast-acting NO donor MAHMA-NONOate was perfused and exhibited a rapid increase in intracellular fluorescence that plateaued in approximately 3 min (Figure 2A). Sensitivity of DAF-2 for NO within BECs *per se* was determined as described recently for pulmonary artery endothelial cells (Meuchel et al., 2011) by constructing an empirical curve of amplitudes of fluorescence changes (difference from baseline at 15 min) using a range of MAHMA-NONOate concentrations (1nM to 10 μ M). Concentrations of 1 nM MAHMA-NONOate showed minimal changes over baseline fluorescent levels and were comparable to vehicle controls. Maximal fluorescence as

well as rate increased in a dose dependent manner with saturation occurring at 10 μM MAHMA-NONOate. The data were fitted with a 4 parameter sigmoid curve with R^2 value equal to 0.996 (Figure 2B). The empirical calibration was used to determine the rate of NO production in subsequent protocols.

We verified that the rate of change in DAF-2 fluorescence reflected NO release or production. At similar concentrations, the NO donor DEA-NONOate (DEANO) showed a slower rate of increase (Figure 2D) and reached a smaller amplitude (Figure 2C) compared to MAHMA-NONOate, and did not saturate at 15 min. The N_2O donor Sulpho-NONOate had no effect on DAF-2 fluorescence in BECs (thereby establishing selectivity of DAF-2 for NO). Additionally, lack of change in fluorescence of the NO insensitive dye DAF-4 in BECs with exposure to DEANO was verified.

DAF-2 fluorescence was low at baseline in untreated BECs, indicating a low level of NO. Both acetylcholine (ACh: 1 μM) and ATP (20 μM), well-known inducers of NO in airway epithelium (Ricciardolo et al., 2004; Bove and van der Vliet, 2006) increased NO levels (Figure 3; $p < 0.05$ compared to vehicle) further demonstrating the applicability of DAF2. The amount of NO produced by ACh and ATP was comparable to the NO donor DEANO (50 μM).

ER activation induces NO production in BECs

Exposure of BECs to E_2 (10 nM; $\text{ER}\alpha$ and $\text{ER}\beta$ activation), THC (10 nM; $\text{ER}\alpha$ activation) or DPN (10 nM; $\text{ER}\beta$ activation) all increased DAF-2 fluorescence (Figure 4A) such that by 15 min, these ER agonists had produced significant increases in NO, comparable to that achieved by 20 μM ATP (Figure 4B; $p < 0.05$). No differences in maximal

amplitude measured after 15 min or the rate of NO production were observed between ER α vs. ER β stimulation (Figures 4B and 4C).

There is recent evidence in non-airway tissues for involvement of a G-protein coupled receptor (GPCR30 or GPER) in non-genomic estrogen signaling (Langer et al.). Whether GPCR30 is present and functional in BECs is not known. However, the GPCR30 specific agonist G-1 (50 μ M) had no effect on NO levels (Figure 4). Additionally, Western analysis of BECs for GPCR30 showed no detectable expression of this receptor (data not shown). Accordingly, this avenue of estrogen signaling was not explored further.

A concentration-response for E₂ induced-NO production (10 pM to 100 nM E₂) revealed minimal NO production at 10 pM E₂, and maximal response at 10 nM (Figure 5). Qualitatively similar results were obtained for ER-specific agonists THC (ER α) and DPN (ER β) (Figure 6). Both of these ER isoform-specific agonists showed minimal NO production at 10 pM. DPN-induced NO response saturated at 10 nM, while THC-induced response was maximal at 100 nM. Maximal amplitudes were taken at 15 min and the curve was fitted with a 4-parameter sigmoid ($R^2 = 0.993$; E₂, $R^2=0.992$; THC, $R^2=0.996$; DPN).

Mechanisms of E₂-induced NO production in BECs

Pre-treatment of BECs with the non-specific ER antagonist ICI 182,780 (ICI: 1 μ M, 30 min) completely abrogated subsequent E₂-induced increase in NO (Figure 7; $p<0.05$). Additionally, chelation of [Ca²⁺]_i using the cell-permeant BAPTA-AM (20 μ M, 30 min) resulted in substantially blunted NO response to 10 nM E₂ (Figure 7; $p<0.05$). Finally, inhibition of NOS with L-NAME (100 μ M, 45 min) severely blunted E₂-induced increase in NO (Figure 7; $p<0.05$).

In a separate set of experiments, BECs were pretreated with the iNOS specific antagonist 1400W (10 μ M) for 30 min prior to NO imaging. There was no difference in DAF-2 fluorescence achieved with ATP, E₂, THC, or DPN in 1400W-treated cells compared to values obtained in the absence of this iNOS inhibitor (Figure 7). Furthermore, Western analyses showed minimal iNOS expression in BECs grown in growth media (Supplemental Figure 1). Fifteen minute pre-treatment with E₂ (to match functional NO imaging experiments) did not change iNOS expression compared to vehicle control. Overnight treatment with inflammatory cytokine TNF α (20 ng/ml: used as a positive control) significantly upregulated iNOS expression in these cells (Supplemental Figure 1; $p < 0.05$).

In cells dual-labeled with Ca²⁺ indicator XRhod-1 AM as well as DAF-2 DA, exposure to either 20 μ M ATP or 10 nM E₂ resulted in an increase in [Ca²⁺]_i that preceded NO production (Figure 8). Following the peak of the [Ca²⁺]_i response, a plateau level was reached that was higher than basal levels. NO production continued to increase while [Ca²⁺]_i levels remained elevated (Figure 8).

In separate sets of experiments to determine the mechanism of E₂-induced increase in [Ca²⁺]_i, 1 mM LaCl₃ was added for 20 min prior to exposure to either ATP or E₂ to non-specifically inhibit Ca²⁺ influx across the plasma membrane. In such cells, treatment with ATP resulted in minimal changes in [Ca²⁺]_i (suggesting a predominant role for influx) and no increase in DAF-2 fluorescence was detected (Figure 8). In contrast, pre-treatment with LaCl₃ did not inhibit increases in [Ca²⁺]_i caused by 10 nM E₂ nor did it abolish the observed increase in NO production (Figure 8). To further determine the source of E₂-induced increases in Ca²⁺, BECs were pre-treated with LaCl₃ as well as the

IP₃ receptor antagonist Xestospongin C (XeC, 1 μM). The presence of both inhibitors resulted in substantial blunting of the Ca²⁺ response and complete abolishment of E₂-induced NO production (Figure 8; p<0.05).

Previous studies have already established the association between caveolae and eNOS (Garcia-Cardena et al., 1997; Ju et al., 1997), and the importance of Ca²⁺-induced dissociation of eNOS in subsequent NO production (Kone, 2000). Human bronchial epithelium is known to express the constitutive caveolar protein caveolin-1 (Krasteva et al., 2006). Pre-treatment of BECs with the caveolin-1 scaffolding domain peptide (CSD: 5 μM, 4 hour pre-treatment), which inhibits caveolin-1 function, significantly attenuated both THC- and DPN-induced NO production by ~50% (p<0.05; Figure 9).

Effect of E₂ on Akt and eNOS phosphorylation

Phosphorylation of Akt and eNOS are known to be key steps in the production of NO (Hurt et al., 2002). In airway epithelium this pathway is initiated by many agonists including ACh. Exposure of bronchial epithelial tissue to ACh (1 μM; 2 min) or to E₂ (10 nM; 2 min) resulted in increased phosphorylation of Akt compared to vehicle controls (Figure 10A; p<0.05). Phosphorylation induced by E₂ was comparable to that induced by 1 μM ACh. The combination of E₂ and ACh resulted in greater phosphorylation of Akt as compared to either treatment individually (Figure 10A, 10B; p<0.05). A similar trend in ACh vs. E₂ effects was observed for phosphorylation of eNOS at Ser1177 when exposed to ACh (1μM; 10 min) or E₂ (10 nM; 20 min) (Figure 10C; p<0.05). Phosphorylation induced by E₂ was comparable to that induced by 1μM ACh. The combination of E₂ and ACh resulted in greater phosphorylation of eNOS as compared to either treatment individually (Figure 10C, 10D; p<0.05).

E₂ effects on bronchodilation

In epithelium-intact human bronchial rings from female patients, exposure to 1 μ M ACh produced a typical, sustained force response (Figure 11). Exposure to 10 nM E₂ THC or DPN each produced rapid relaxation within a 15 min time frame that was comparable between treatments (Figure 11). It should be noted that a 15 min time period was selected to match the NO imaging data. However, longer exposure to ER agonists resulted in >50% relaxation (not shown). Denudation of epithelium blunted E₂ effects on bronchodilation (Figure 11 shows E₂, but a similar lack of effect was observed for THC and DPN).

DISCUSSION

In this study, biologically relevant concentrations of estrogen as well as ER-specific agonists induced physiologically significant levels of NO in human BECs and produced bronchodilation via an epithelium-dependent mechanism. Estrogen acts on the bronchial epithelium via both major ER isoforms which are expressed in the membrane and cytosol, and induce NO within a matter of minutes. These data point to non-genomic mechanisms (involving Akt and eNOS phosphorylation), providing novel evidence for a functional role of non-nuclear ERs in the airway epithelium. Combined with our previous study in human ASM cells (Townsend et al., 2010), the novel findings of E₂-induced NO production suggest an important bronchodilatory role for sex steroids. The relevance of such effects lies in the normal effects of estrogens on the airway, and the dysregulation of estrogen signaling in the setting of diseases such as asthma which involves impaired NO-mediated bronchodilation and enhanced ASM contractility.

Estrogen signaling involves the potent ER agonist 17 β -estradiol (E₂). While airway ER expression, especially in humans, has not been well-studied, based on perimenstrual fluctuations in asthma symptoms, ER expression is likely. One previous study reported ER α and ER β expression in human BECs (Ivanova et al., 2009), but examined only genomic actions of ER activation in these cells. Genomic responses to estrogens are known to be complex, cell-specific, estrogen concentration and duration dependent, and involve a host of signaling proteins and pathways (Heldring et al., 2007). Given these complexities, and the relative lack of knowledge on estrogen signaling within the airway epithelium, we did not address the issue of prolonged estrogen exposure in BECs in this study, while recognizing its importance *in vivo*. In this regard, the results of our present

study are novel, highlighting the localization of both plasma membrane receptors as well as cytosolic in human BECs and focusing on rapid, non-genomic mechanisms of ER action.

While rapid and likely non-genomic effects of E₂ occurring within seconds to minutes have been observed in other tissues (Levin, 2002; Simoncini and Genazzani, 2003), the underlying mechanisms are still under investigation. Previous work, but not in the lung, has focused on non-genomic actions of ER α and ER β (Levin, 2002; Heldring et al., 2007). The presence of both ER isoforms in the BEC plasma membrane, and the current results on NO production and bronchodilation using ER isoform specific agonists (THC vs. DPN) suggest that both ER α and ER β are functional in terms of non-genomic regulation of NO in airway epithelium. Blocking ER activation with the ER antagonist ICI 182,780 attenuated NO production in BECs further confirming that rapid, non-genomic ER α or ER β activation was responsible for the observed NO effects. In other tissues, there is recent evidence for a G-protein coupled receptor (GPCR30 or GPER) in non-genomic estrogen signaling (Langer et al., 2010). This receptor is not blocked by ICI 182,780. To date, we have not detected GPCR30 in human airway, and the GPCR30 agonist G-1 had no effect on BEC-induced NO production, suggesting that this receptor is not a key player in estrogen-induced NO effects in the airway.

While the present study has demonstrated that BECs have ERs, there is currently limited data on estrogen signaling *per se* within the airway epithelium. A previous study using H441 epithelial cells found that acute exposure to E₂ increases conversion of [³H]L-arginine to [³H]L-citrulline (Kirsch et al., 1999), although NO production was not measured. We now show that estradiol rapidly increases NO production in acutely

dissociated BECs from female patients, and increases phosphorylation of Akt and eNOS (Ser1177). In the previous study, the authors noted the necessity of determining which ERs (ER α and/or ER β) are responsible for eNOS activation and subsequent NO production. Using ER specific agonists THC (ER α) and DPN (ER β), we addressed this question, finding that both receptor types can increase NO levels in a concentration-dependent manner.

Only a single report in airway epithelium (Kirsch et al., 1999) has reported that estradiol-effects on NOS are Ca²⁺ dependent. Our results showing the blunting effects of the Ca²⁺ chelator BAPTA on E₂-induced NO are consistent with this idea. The novel aspect of our study here is exploration of the mechanisms by which estrogens alter [Ca²⁺]_i. Dual imaging of [Ca²⁺]_i and NO allowed for time resolution of the Ca²⁺ events leading to NOS activation. Inhibition of influx with LaCl₃ blunted ATP-induced Ca²⁺ elevation, and prevented changes in NO levels. In contrast, LaCl₃ did not substantially affect E₂-induced elevation in Ca²⁺ or NO, suggesting an intracellular source for Ca²⁺. This is in contrast to previous work (Kirsch et al., 1999) showing a role for influx. However, inhibition of IP₃ receptor channels with XeC abolished E₂-induced Ca²⁺ and NO changes. Previous studies have established a role for IP₃ receptor channels in BEC [Ca²⁺]_i regulation (Boitano et al., 1992). However, this is the first evidence for estrogen effects on BEC NO via mobilization of intracellular Ca²⁺ stores.

Caveolin-1 is an important regulator of membrane localization of eNOS with dissociation of eNOS from caveolae being an important step in NO production (Kone, 2000). Accordingly, interactions of caveolin-1 scaffolding domain with eNOS inhibits NO production (Ju et al., 1997) In this regard, our results with CSD are consistent. What

is novel is the caveolar association of both ER α and ER β in human BEC. Studies in vascular endothelium have established the importance of ER α -caveolin-1 association which influences eNOS activity (Klinge et al., 2008). However, little is known about the interaction of ER β with caveolin-1 and eNOS, especially in the airway. Our studies showed that inhibition of caveolin-1 with CSD reduced eNOS activation by 50% in BECs, presumably by dissociating caveolin-1 from eNOS. However, significant amounts of NO were still produced by ER α or ER β activation in the presence of CSD, suggesting a novel role for ER-induced eNOS signaling independent of caveolin-1.

The functional relevance of E₂-induced epithelial NO lies in modulation of airway tone: a balance between bronchoconstriction and bronchodilation. Our novel findings in epithelium-intact bronchial rings show that ER activation produces rapid and sufficient epithelial NO to induce bronchodilation even in the presence of ACh, a well-known bronchoconstrictor. It should, however, be noted that ACh can also induce epithelial NO generation (as shown in this study), and thus estrogens could enhance this epithelial effect. On the other hand, by reducing ASM [Ca²⁺]_i (Townsend et al., 2010), estrogens may counteract the bronchoconstricting action of ACh on ASM itself, albeit less effectively than if the epithelium was present. This latter aspect may be particularly important in the role of estrogens in determining the balance between bronchoconstriction and bronchodilation in the setting of dysfunctional epithelium in airway diseases.

The clinical relevance of our study lies in diseases such as asthma which are more prevalent in women (Melgert et al., 2007; Postma, 2007), with increased severity and frequency of exacerbations (Becklake and Kauffmann, 1999), and catamenial variations

in airway reactivity in ~40% of women with pre-existing disease (Chhabra, 2005; Murphy and Gibson, 2008). Clinical data suggest that estrogens are detrimental in asthma; however, asthma exacerbations are greater during late luteal phase when estrogen levels are lowest (Hanley, 1981; Gibbs et al., 1984), and can be alleviated in postmenopausal women receiving hormone replacement therapy (Bellia and Augugliaro, 2007). We and others have shown that estrogen can exhibit relaxant effects directly on the ASM, in part by decreasing $[Ca^{2+}]_i$ via L-type (Townsend et al., 2010) and BK_{Ca} channels (Dimitropoulou et al., 2005) (the latter being responsive to cGMP, a downstream effector of NO). Indeed, it is likely that estrogen-induced epithelial NO stimulates guanylyl cyclase in the underlying ASM, producing bronchodilation in humans similar to murine models (Dimitropoulou et al., 2005). Our study suggests that the epithelial-derived NO may stimulate guanylyl cyclase in ASM. Thus, estrogens may have a two-pronged effect in inducing bronchodilation.

A potential confounding factor in examining the role of estrogens is the role of airway inflammation, and how estrogens interact with inflammatory mediators in modulating airway tone. Here, inflammation-induced alterations in NO (e.g. due to enhanced epithelial iNOS (Ortiz and Garvin, 2003; Jiang et al., 2009)) may also be relevant. Asthma severity correlates with increasing levels of exhaled NO, a marker of airway inflammation. Menstrual variations in exhaled NO have been reported (Mandhane et al., 2009) which suggest regulation of NO by sex steroids; however the underlying mechanisms remain unknown. It must be noted that in our study, we examined only normal BECs not exposed to inflammatory mediators. Accordingly, we found very low levels of iNOS, and as expected, iNOS inhibition did not alter NO production by E_2 ,

THC, or DPN. Whether estrogens influence epithelial NO production via iNOS in the presence of inflammation remains to be determined. Furthermore, other sex steroids (and their metabolites) may play roles in modulating airway reactivity. Here, the interaction between progesterone and estrogen may be important in women, while testosterone-induced changes in airway tone may be relevant in men. These avenues are largely unexplored.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Townsend, Meuchel, Thompson, Pabelick, Prakash

Conducted experiments: Townsend, Meuchel, Pabelick, Prakash

Contributed new reagents or analytic tools: Prakash, Pabelick

Performed data analysis: Townsend, Meuchel

Wrote or contributed to the writing of the manuscript: Townsend, Meuchel, Thompson, Pabelick, Prakash

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Human bronchial epithelial cells (BECs) express estrogen receptors

(ERs). (A) Western analysis of fractions of unstimulated BECs shows that ER α and ER β both localize to the plasma membrane and cytosol, with a small degree of expression in the nucleus. (B) Bar graph summarizes results from 4 patients. (C) Fluorescent immunostaining demonstrated ER α and ER β expression in BECs, with substantial co-localization of eNOS with either isoform. An AlexaFluor488 dye was used to visualize ERs while Cy3 was used for eNOS. Rb: rabbit; Mo: mouse. Scale bar is 10 μ m.

Figure 2. Validation and calibration of the nitric oxide (NO) sensitive

fluorescent dye 4,5 diaminofluorescein diacetate (DAF-2 DA) in BECs. (A) Exposure of DAF-2 DA loaded cells to NO donors such as MAHMA-NONOate (1 μ M) or DEA-NONOate (DEANO; 50 μ M) resulted in rapid increase in fluorescence, consistent with the NO release properties of each agent. The nitrous oxide donor, Sulpho-NONOate had no effect on DAF-2 fluorescence, compared to vehicle controls. The NO-insensitive indicator, DAF-4, showed no change in fluorescence with 50 μ M DEANO. Overall, these results were used to validate the use of DAF-2 in human BECs. (B) A concentration-response curve of the NO donor MAHMA-NONOate was constructed (1 nM – 10 μ M). Data points were maximal amplitudes taken after 10 minutes and corrected for baseline. The empirical response curve was fitted with a 4-parameter sigmoid ($R^2 = 0.996$). (C) Amplitude of changes in gray level (GL) of DAF-2 fluorescence with exposure to different agents. (D) Rate of change in DAF-2 fluorescence reflecting rate of NO

production with exposure to different agents. In bar graphs, values are means \pm SE (n=4 patients). * indicates significant difference from vehicle control (p<0.05). N.D.: not determinable (rate of change was negligible).

Figure 3. NO induction in BECs. Exposure of DAF-2 DA loaded cells to the sustained NO donor DEANO (50 μ M), or the known endogenous inducers of epithelial NO such as acetylcholine (ACh; 1 μ M) and ATP (20 μ M) resulted in substantial increases in fluorescence. Amplitude of changes in gray level (GL) of DAF-2 fluorescence was calculated as the maximal fluorescence achieved after 15 min of exposure corrected for baseline fluorescence. ACh-induced NO was comparable to that by DEANO in rate (not shown) and amplitude, while ATP produced even greater NO. Values are means \pm SE (n=4 patients). * indicates significant difference from vehicle control (p<0.05),

Figure 4. ER α and ER β activation results in NO production in human BECs.

(A) In DAF-2 loaded human BECs, 20 μ M ATP (shown) and 1 μ M ACh (not shown) both induced an increase in NO production (15 min). Treatment with the non-selective physiologically-relevant 17- β -estradiol (E₂; 10 nM), or with ER α vs. ER β selective agonists (THC and DPN, respectively; 10 nM each) caused an increase in fluorescence comparable to that of ATP (20 μ M). In comparison, the GPCR30 agonist G-1 had no effect on fluorescence. (B) Amplitude of agonist or estrogen induced DAF-2 response. Based on the DAF-2 calibration in Figure 2, NO levels were in the 30-85 nM range. Vehicle control as well as G1 showed minimal change in fluorescence. (C) Rate of NO production. Estrogen induced NO production was only slightly slower than that by ATP. In bar graphs, values

are means \pm SE (n=5 patients). * indicates significant difference from vehicle control (p<0.05). N.D.: not determinable (rate of change was negligible).

Figure 5. E₂ effects on NO production are concentration-dependent. (A) In DAF-2 loaded BECs, increasing physiologic concentrations of E₂ (10 pM-100 nM) increased NO production in a concentration-dependent manner. No difference was observed between 10 and 100 nM concentrations. (B) Summary of E₂-induced NO amplitudes taken after 15 minutes. The curve was fitted with a 4-parameter sigmoid ($R^2 = 0.993$). (C) Rate of NO production. Increasing E₂ concentrations until 10 nM resulted in greater rates of NO production. In bar graphs, values are means \pm SE (n=5 patients). * indicates significant difference from vehicle control (p<0.05). N.D.: not determinable (rate of change was negligible).

Figure 6. ER isoform-specific agonist effects on NO production are concentration-dependent. In DAF-2 loaded BECs, increasing concentrations of ER α -specific agonist, THC and ER β -specific agonist, DPN (10 pM-100 nM) resulted in progressively increased NO production (measured at 15 min). DPN-induced NO response saturated at 10 nM, while THC-induced response was maximal at 100 nM. The effects of DPN and THC were comparable overall. The curves were fitted with a 4-parameter sigmoid ($R^2=0.992$; THC, $R^2=0.996$; DPN).

Figure 7. Mechanisms of E₂-induced NO production in BECs. (A) In DAF-2 loaded BECs pretreatment with the ER antagonist ICI 182,780 (ICI; 1 μ M; 30 min), the calcium chelator BAPTA (20 μ M; 30 min), or the eNOS inhibitor L-NAME (100 μ M; 45 min) substantially attenuated the subsequent effect of 10 nM

E₂ on NO production. In contrast, the iNOS inhibitor 1400W (10μM; 30 min), did not alter the amount of NO produced by E₂ (or that by THC or DPN; not shown). In bar graphs (B), values are means ± SE (n=5 patients). * indicates significant difference from vehicle control, # significant inhibitor effect (p<0.05).

Figure 8. Estrogen increases [Ca²⁺]_i via intracellular store depletion. In cells dual-labeled with the Ca²⁺ indicator XRhod-1 AM as well as with DAF-2 DA, exposure to either 20 μM ATP or 10 nM E₂ resulted in an increase in Ca²⁺ that preceded NO production (A). Following peak [Ca²⁺]_i response, a plateau level was reached that was higher than basal calcium levels. NO production continued to increase while [Ca²⁺]_i levels remained elevated. To determine the role of intracellular stores in E₂-induced elevation in [Ca²⁺]_i, 1 mM LaCl₃ was added for 20 min prior to introduction of either ATP or E₂, thus non-specifically inhibiting plasma membrane Ca²⁺ influx. Subsequent treatment with ATP resulted in no change in DAF-2 fluorescence (B) and only a small increase in [Ca²⁺]_i (C). In contrast, pre-treatment with LaCl₃ did not inhibit increases in [Ca²⁺]_i caused by 10 nM E₂ nor did it abolish the increase in NO. However, the additional presence of the IP₃ receptor antagonist Xestospongine C (XeC; 1 μM) completely abolished E₂-induced NO response as well as elevation of [Ca²⁺]_i, indicating an intracellular source for Ca²⁺. Values are means ± SE (n=4 patients). * indicates significant difference from vehicle control, # significant inhibitor effect (p<0.05).

Figure 9. Inhibition of caveolin-1 attenuates ER activation-induced NO production. In DAF-2 loaded BECs, pretreatment with the inhibitory caveolin-1 scaffolding domain peptide (CSD: 5 μM 4 h) significantly attenuated the

subsequent effect of 10 nM THC or DPN on NO production, but did not abolish it.

* indicates significant difference from vehicle control, # significant inhibitor effect ($p < 0.05$).

Figure 10. Estrogen increases Akt and endothelial nitric oxide synthase (eNOS)

phosphorylation. To determine E₂ effects on Akt phosphorylation, BECs were treated with ACh (1 μM; 2 min), E₂ (10 nM; 2 min), or a combination of E₂ and ACh (2 min). Western analyses indicated that ACh or E₂ alone or in combination increased phosphorylated Akt (p-Akt) levels (A). In summary bar graph (B), values are means ± SE (n=4 patients) relative to total Akt expression. To determine eNOS phosphorylation, BECs were treated with ACh (1 μM; 10 min), E₂ (10 nM; 20 min), or a combination of E₂ and ACh (E₂ alone 10 min, E₂+ACh additional 10 min). Western analyses indicated increased phosphorylation of eNOS at Ser 1177 (C). In summary bar graph (D), values are means ± SE (n=4 patients). * indicates significant difference from unstimulated controls ($p < 0.05$).

Figure 11. Estrogen acutely induces bronchodilation.

In epithelium-intact human bronchial rings (from female patients), 1 μM ACh produced a typical, sustained force response. Exposure to 10 nM E₂, THC or DPN each produced rapid relaxation within a 15 min time frame (selected to match the NO imaging data). Longer exposure to ER agonists resulted in >50% relaxation (not shown). Denudation of epithelium blunted E₂ effects on bronchodilation.

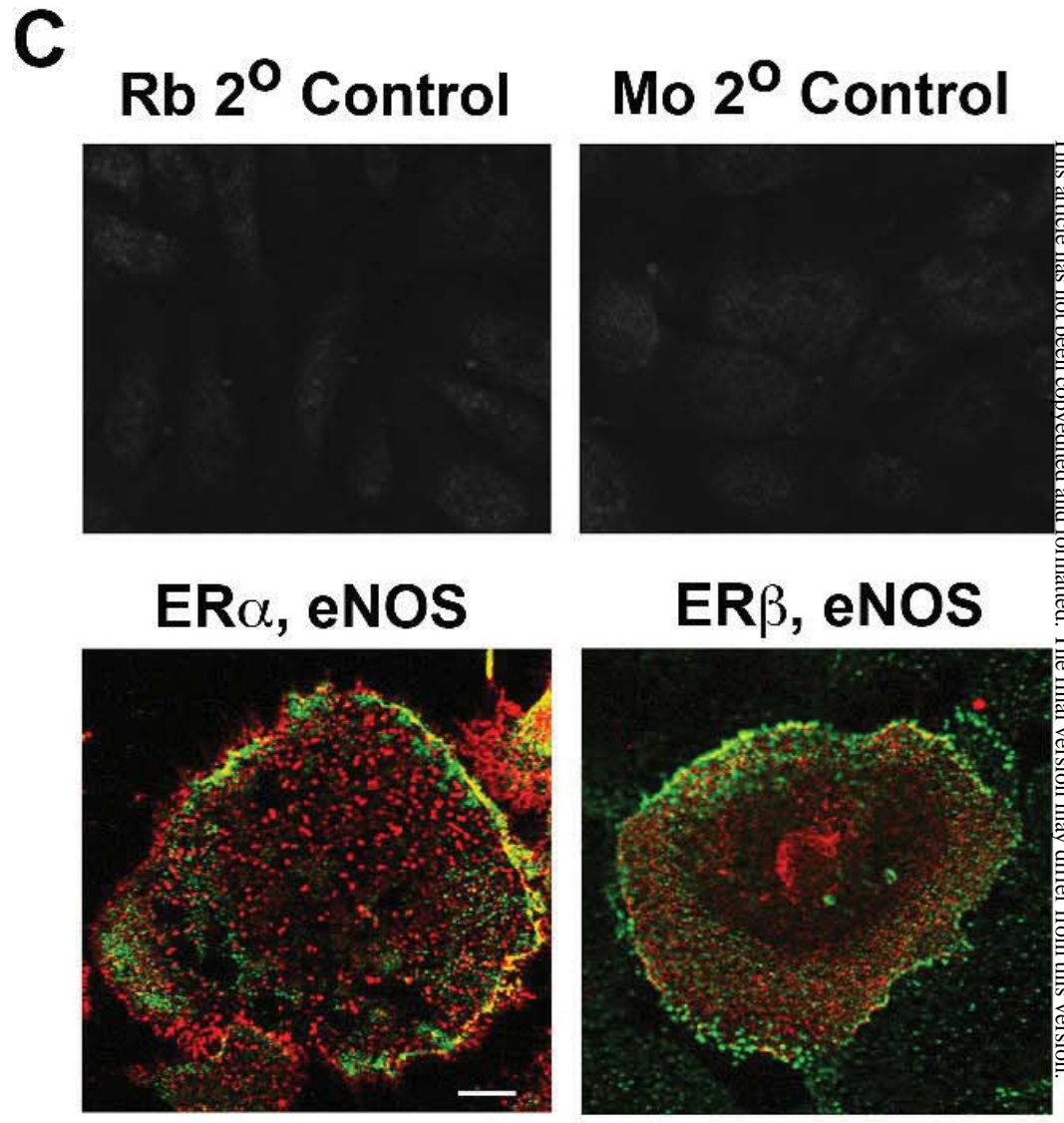
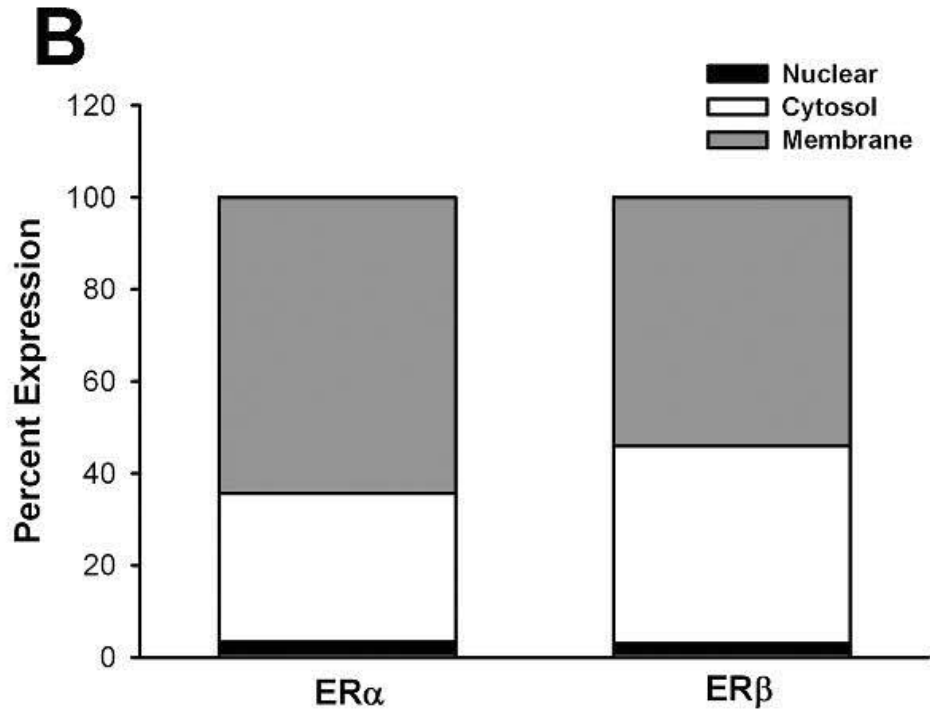
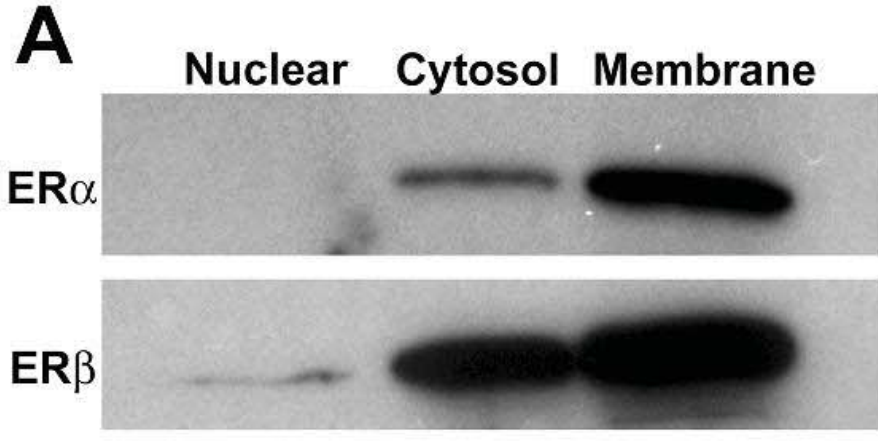
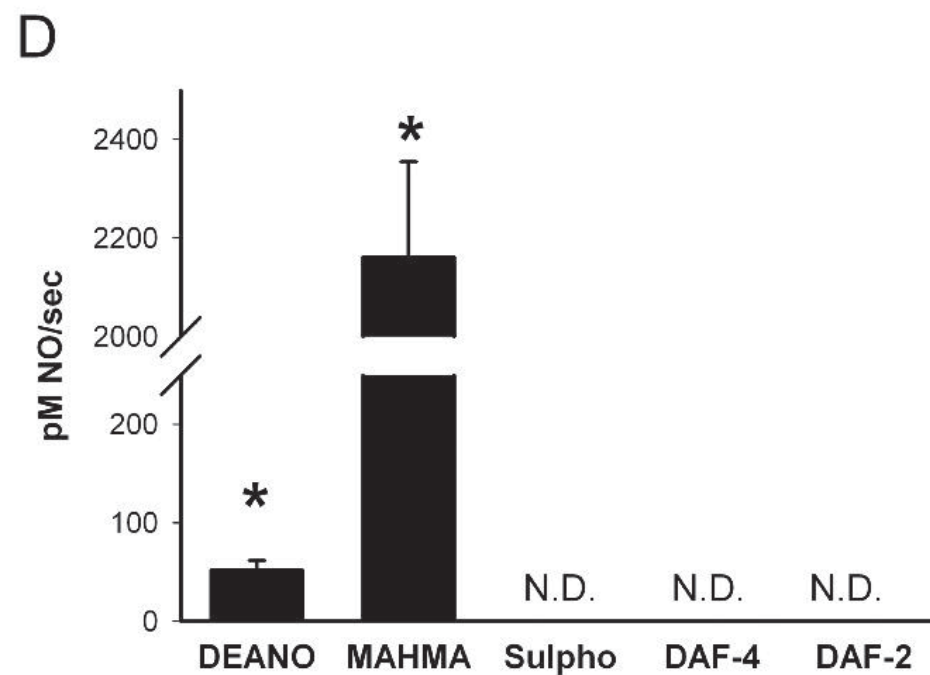
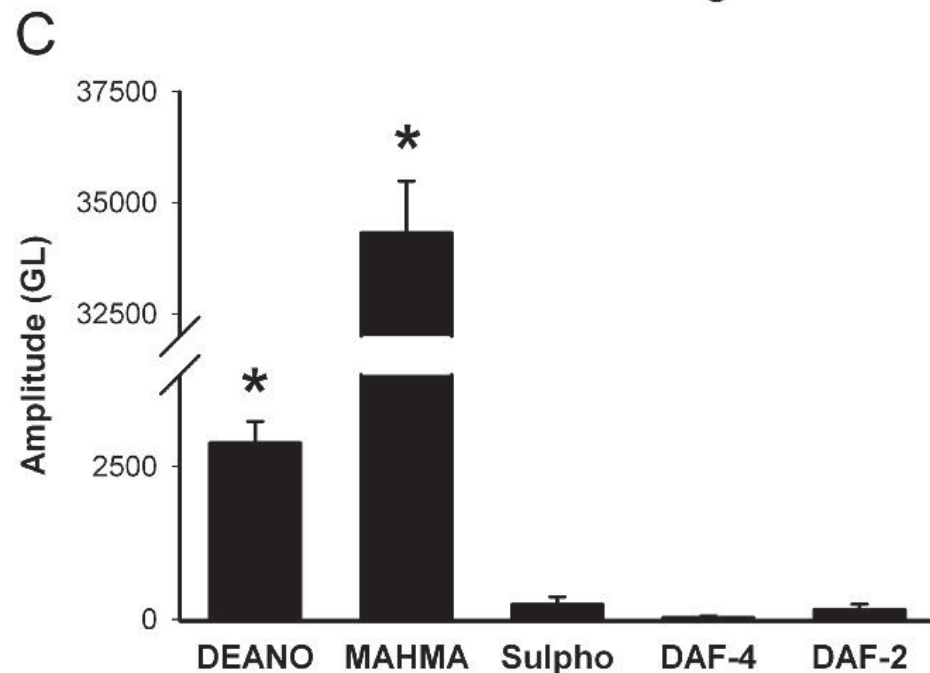
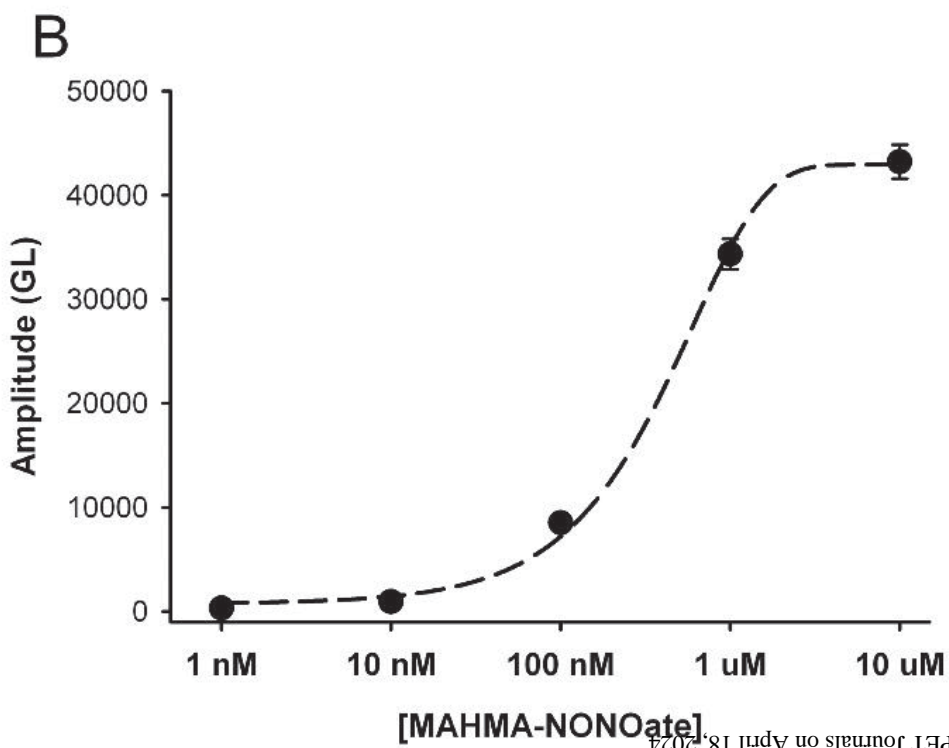
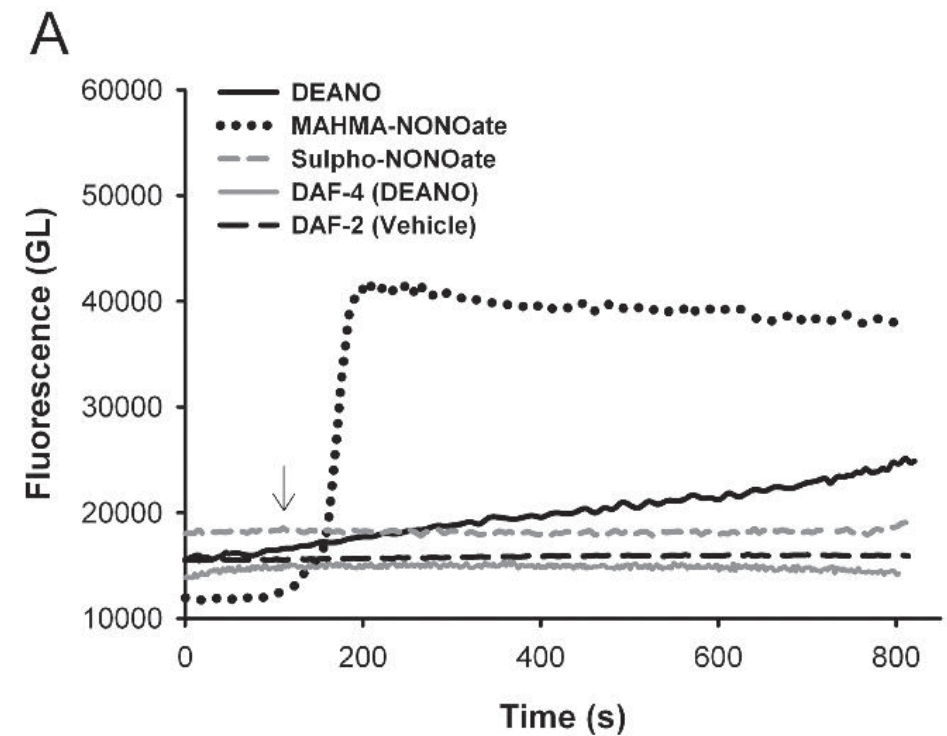


Figure 1

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Figure 2



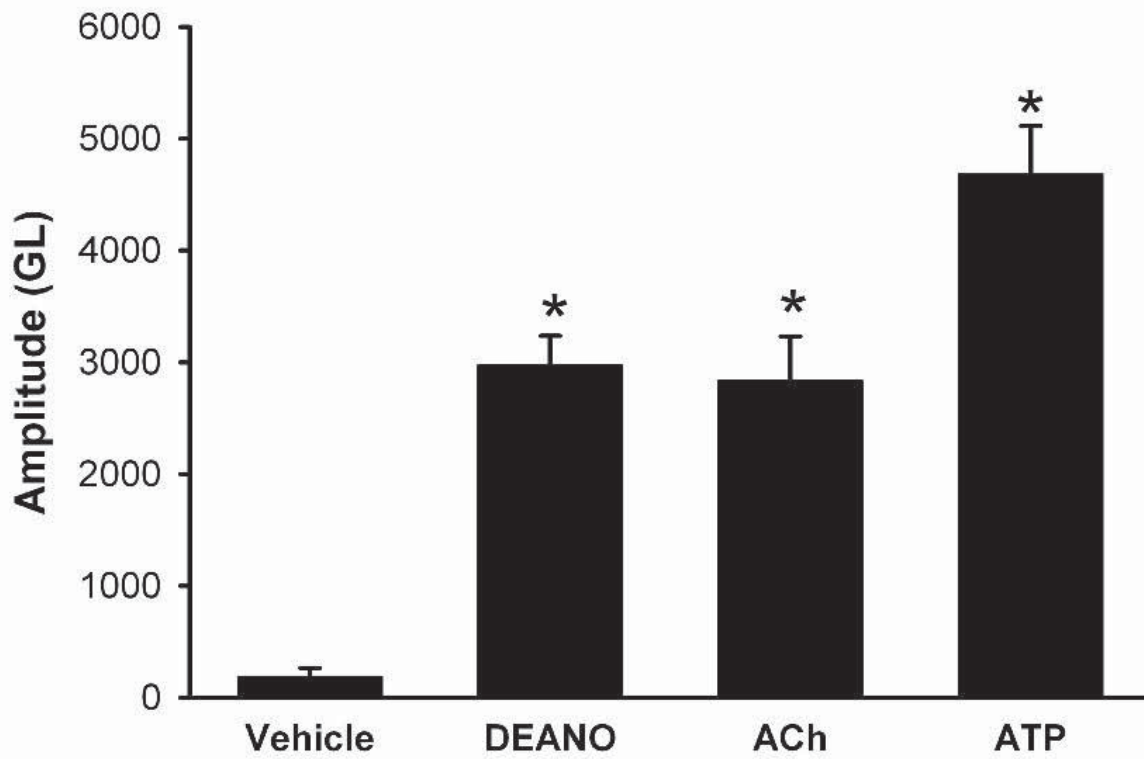


Figure 3

Figure 4

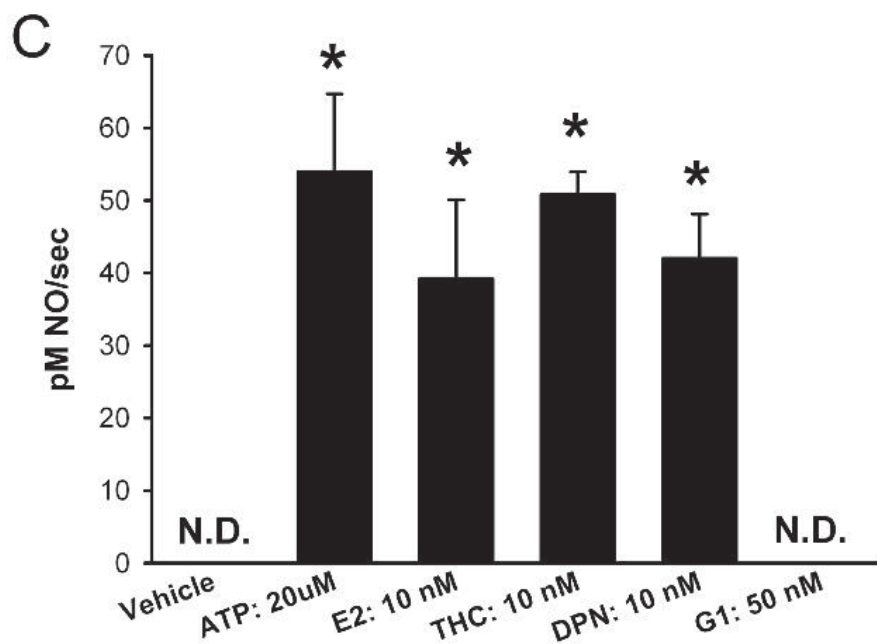
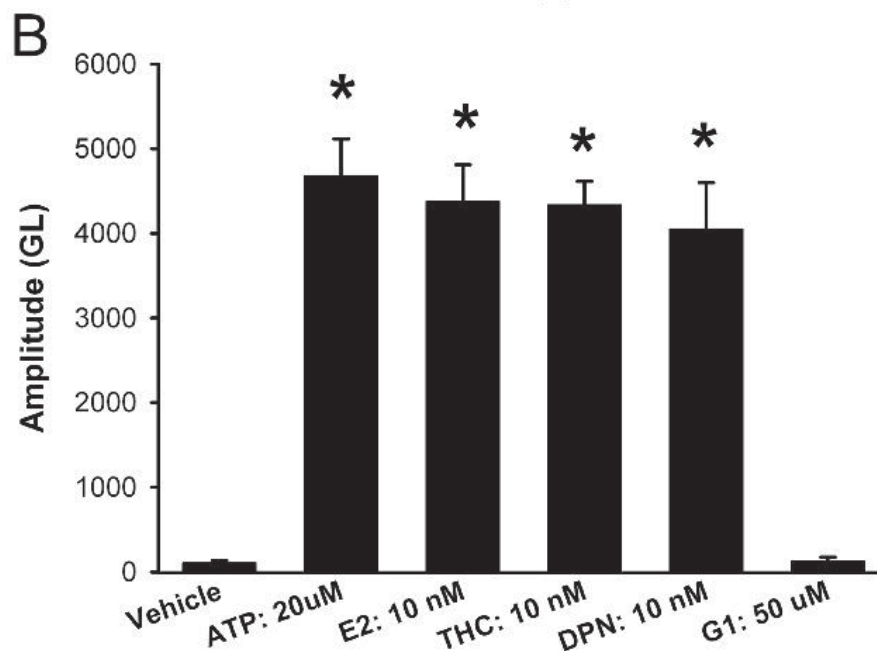
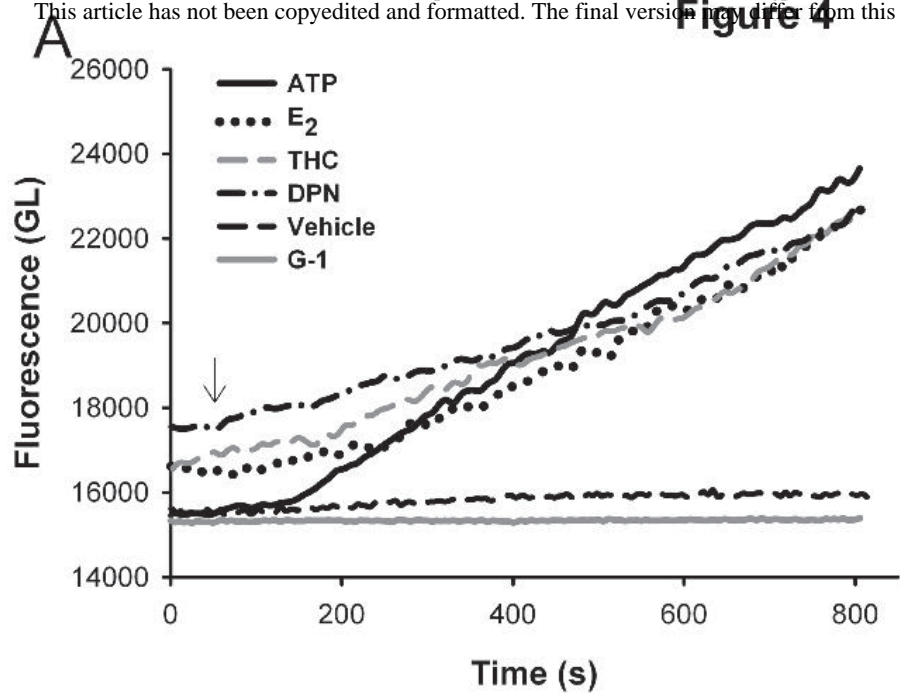
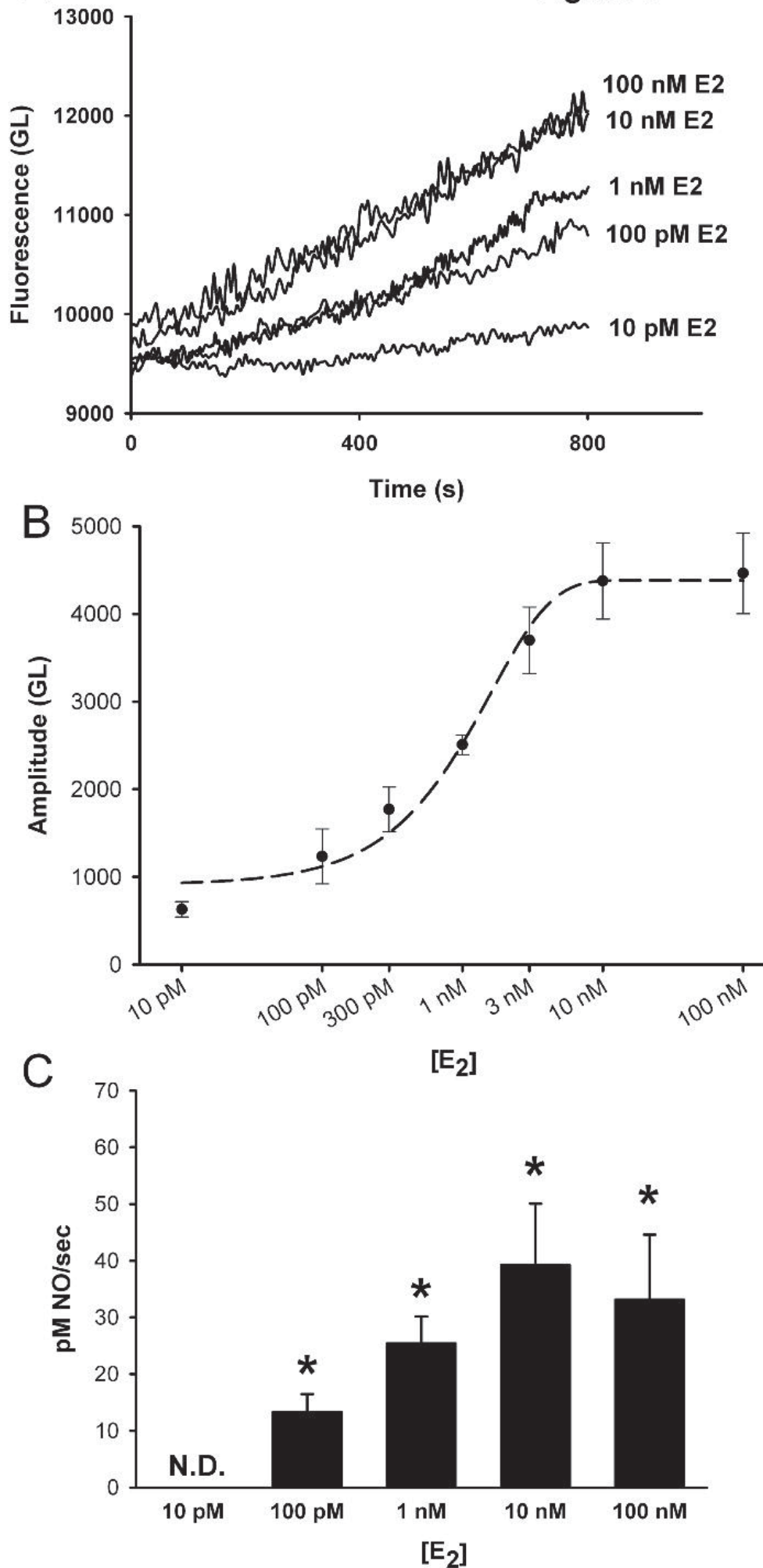


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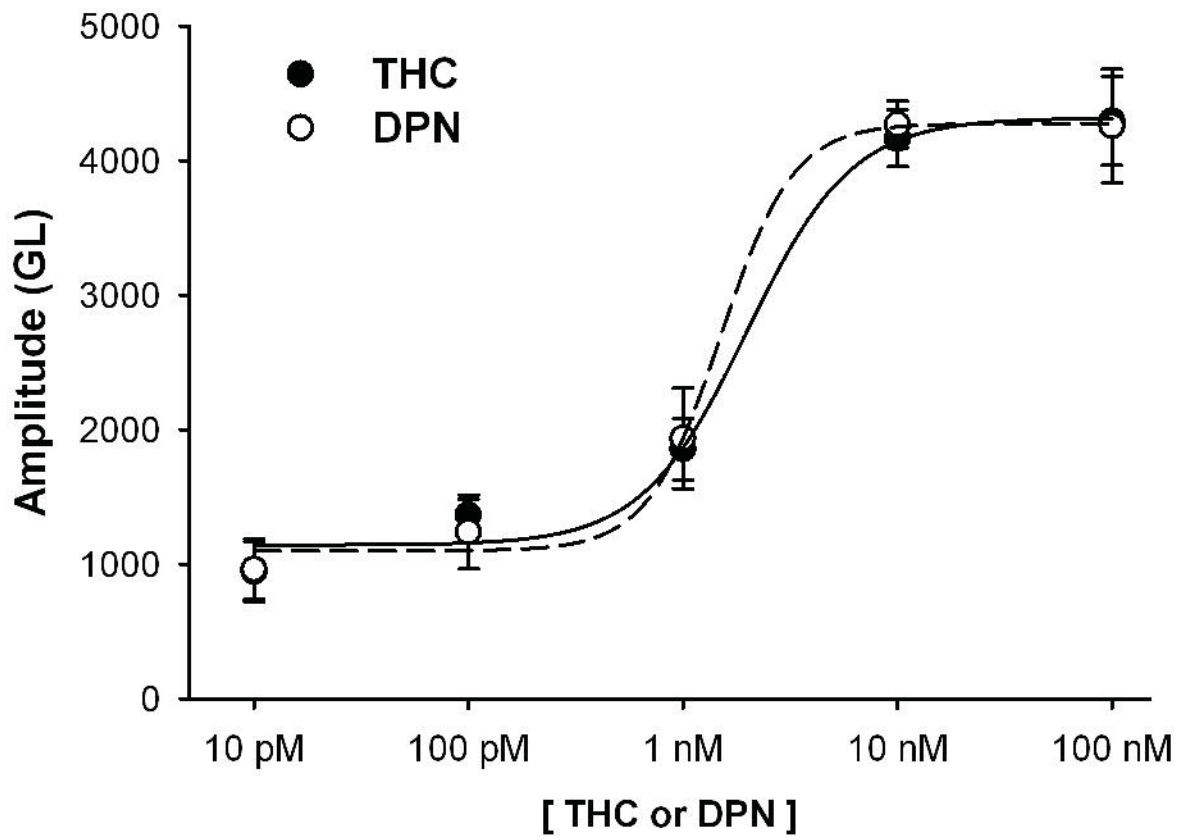


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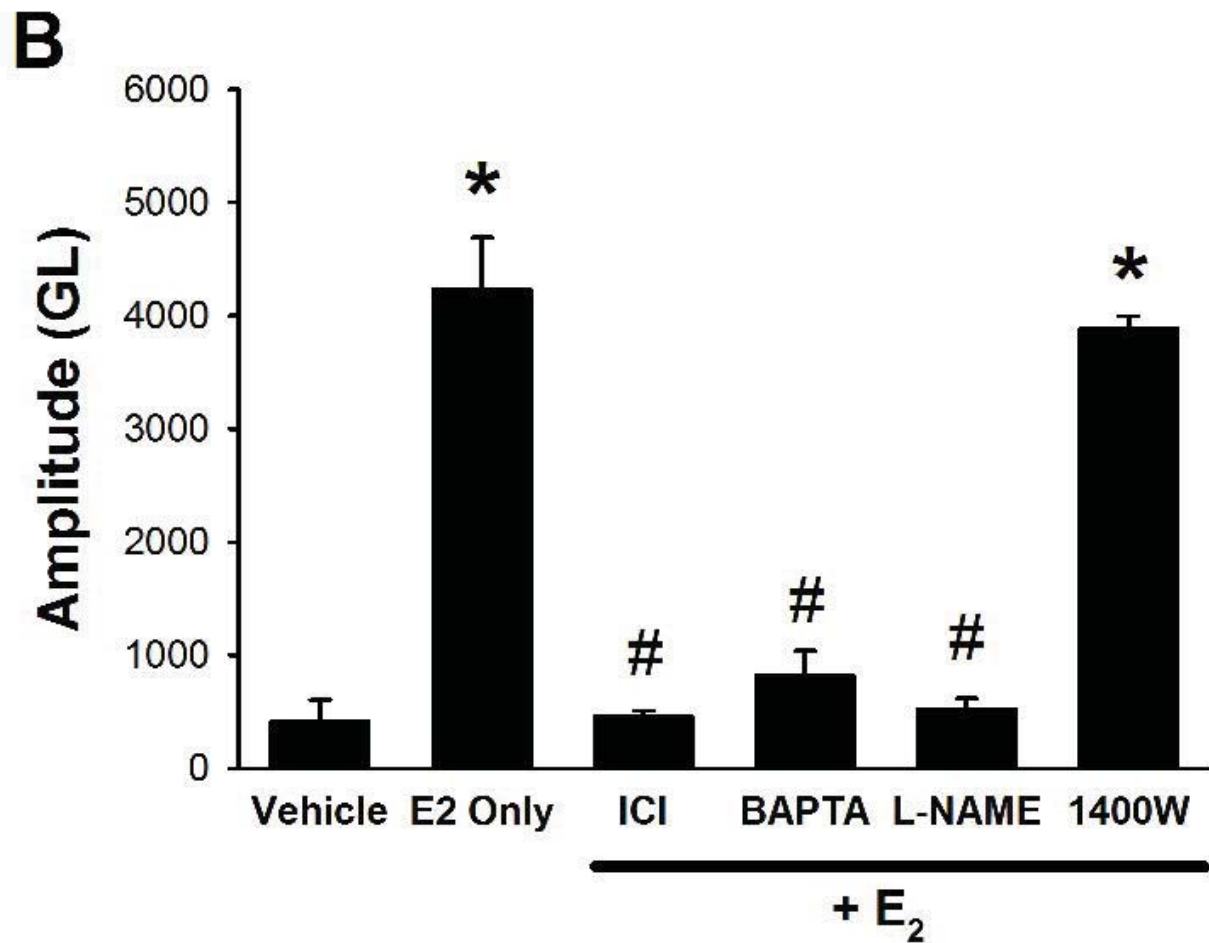
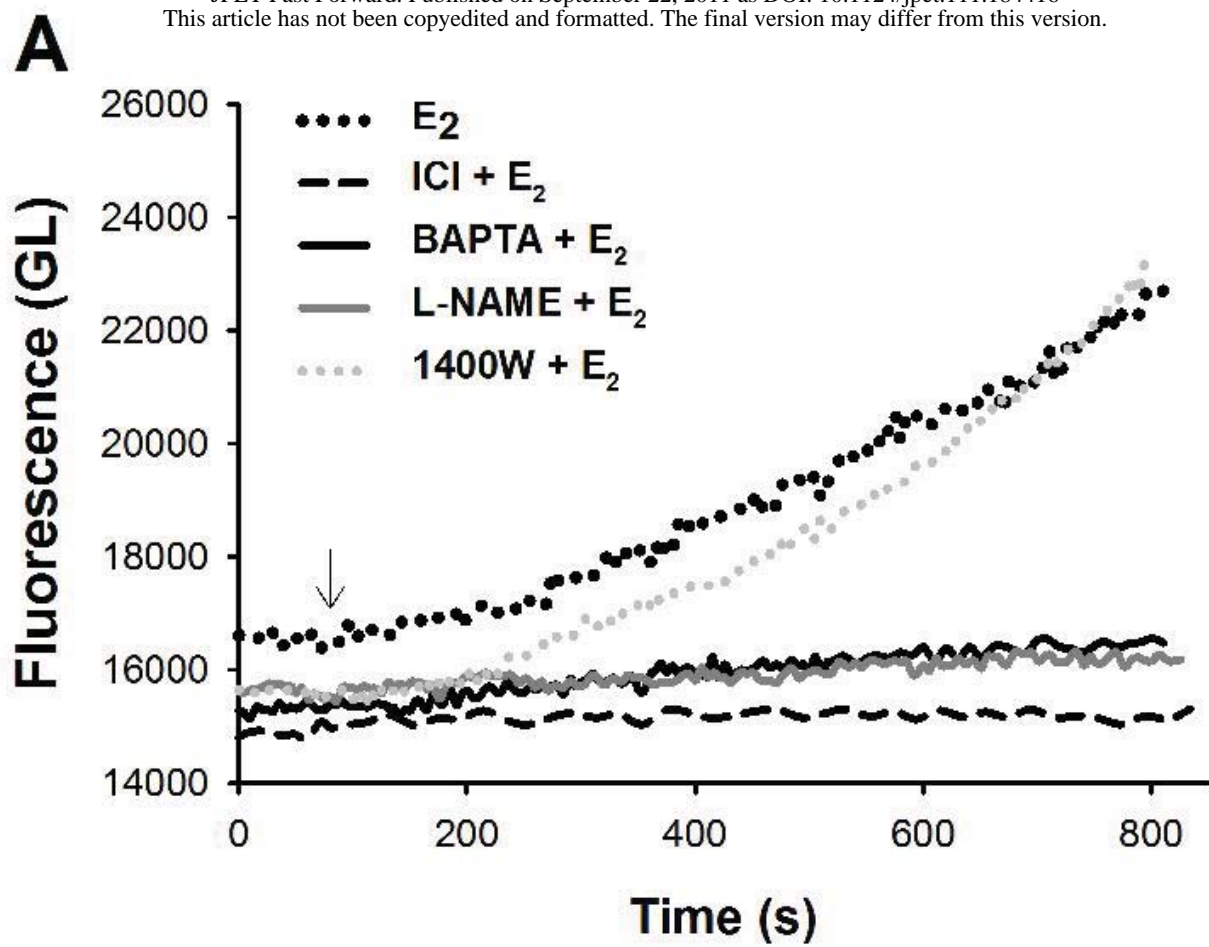
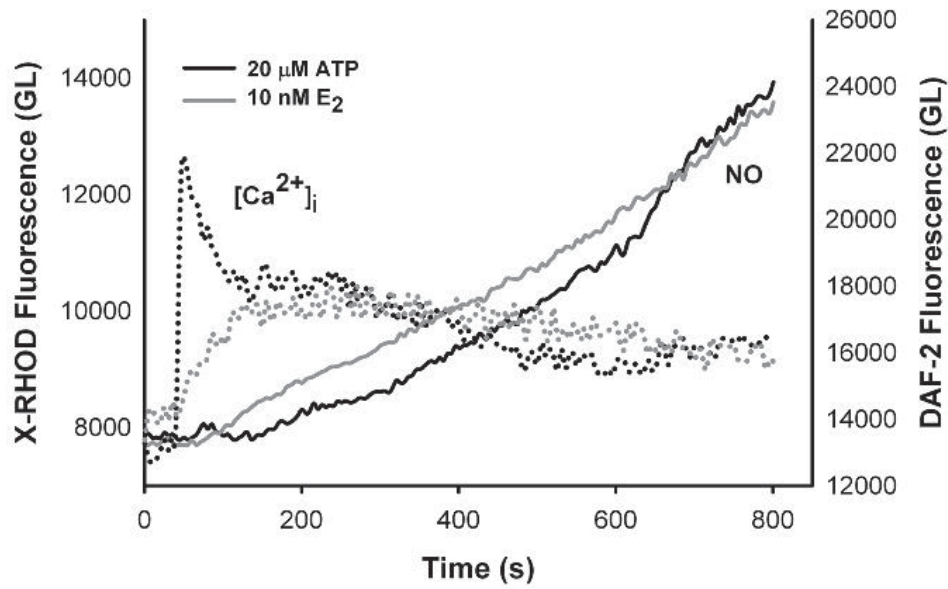
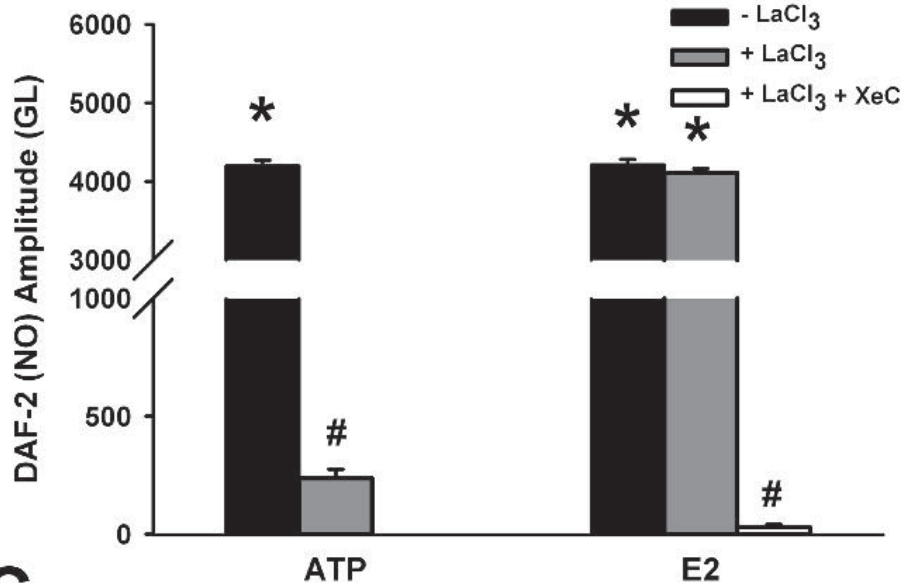


Figure 7

A



B



C

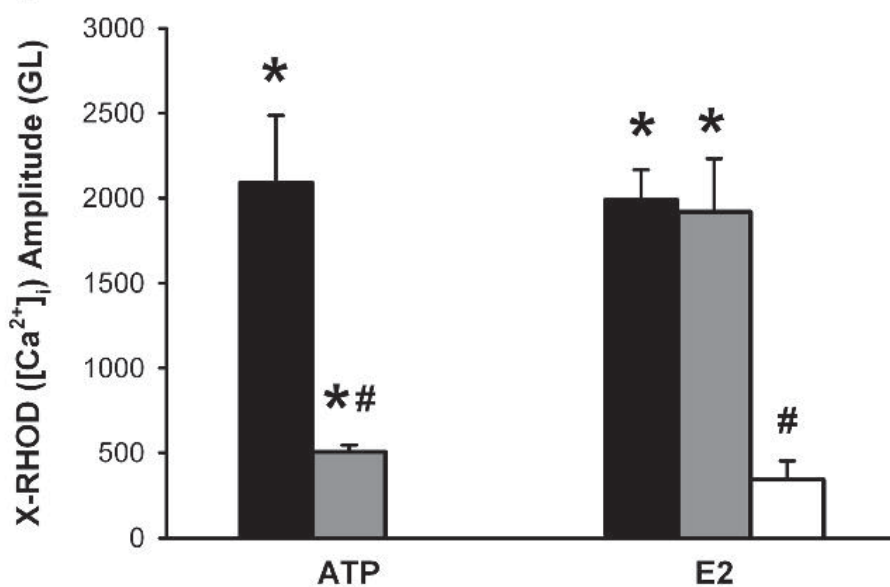


Figure 8

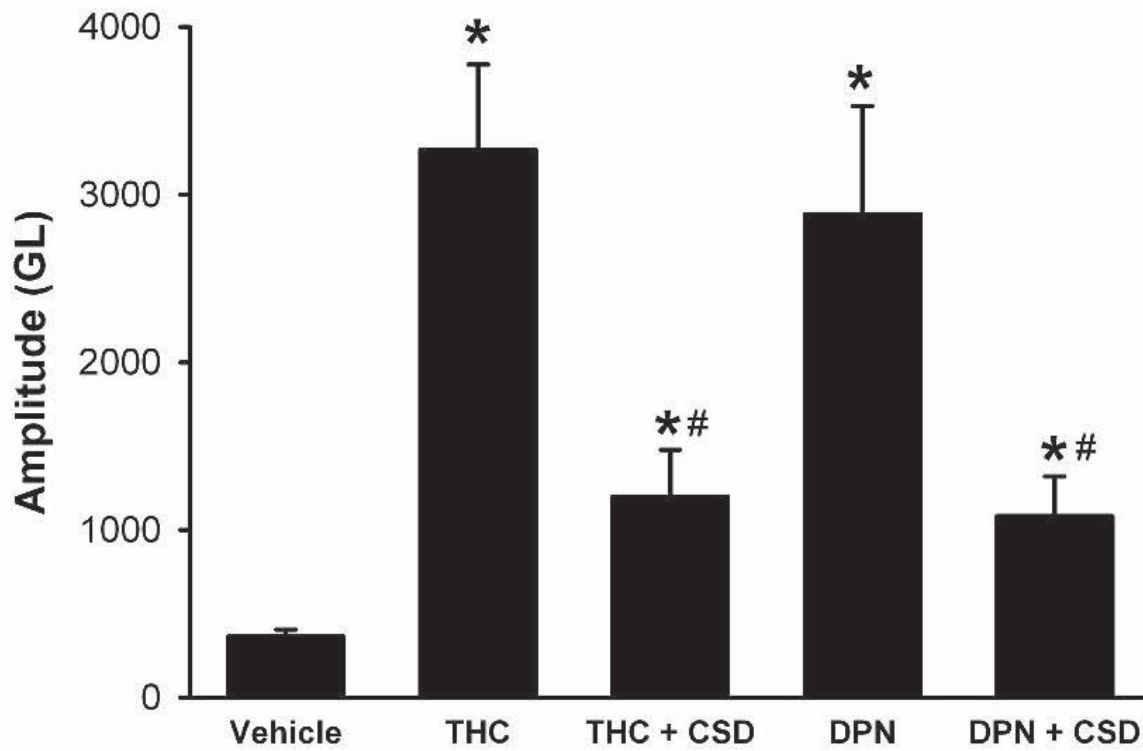


Figure 9

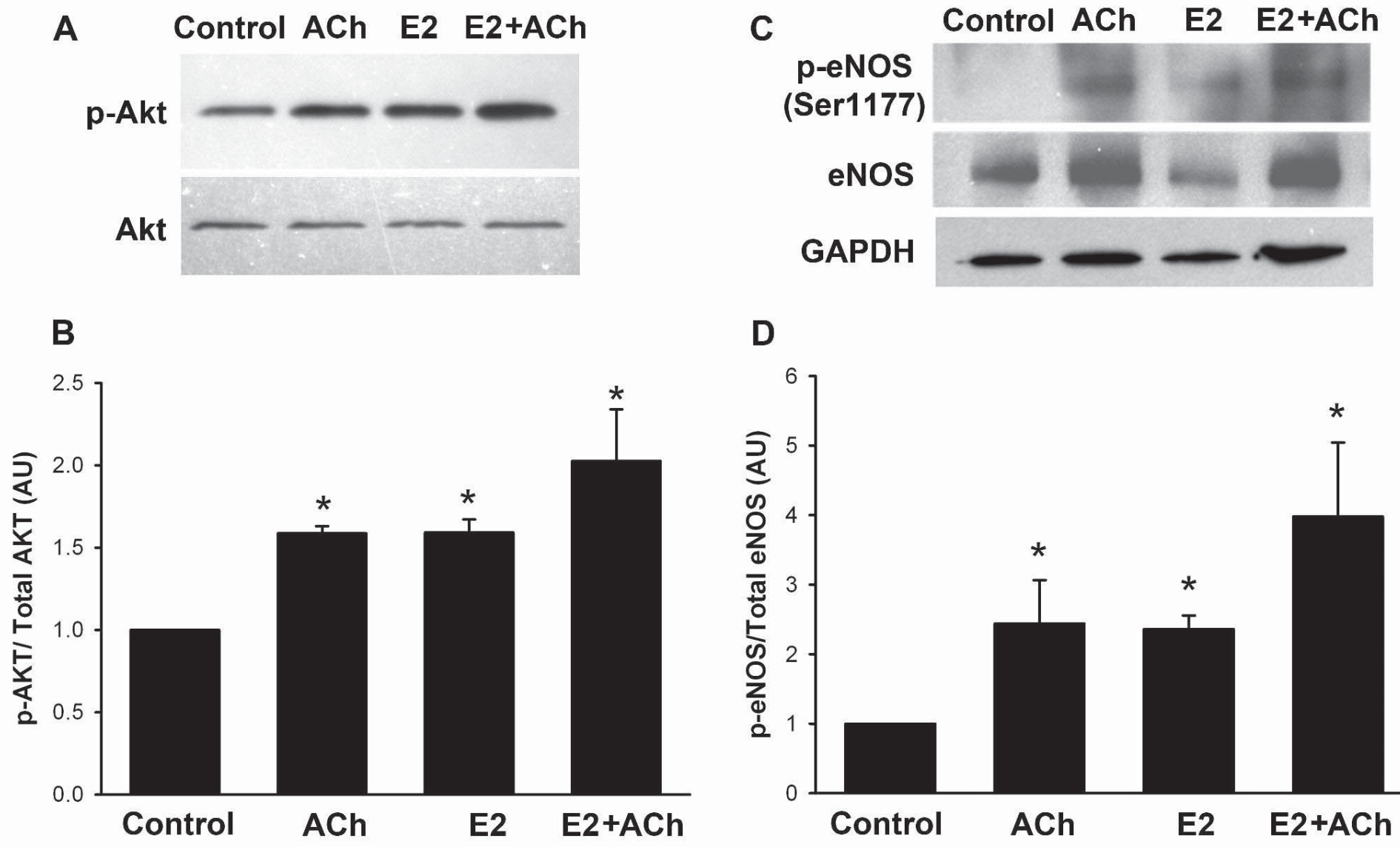


Figure 10

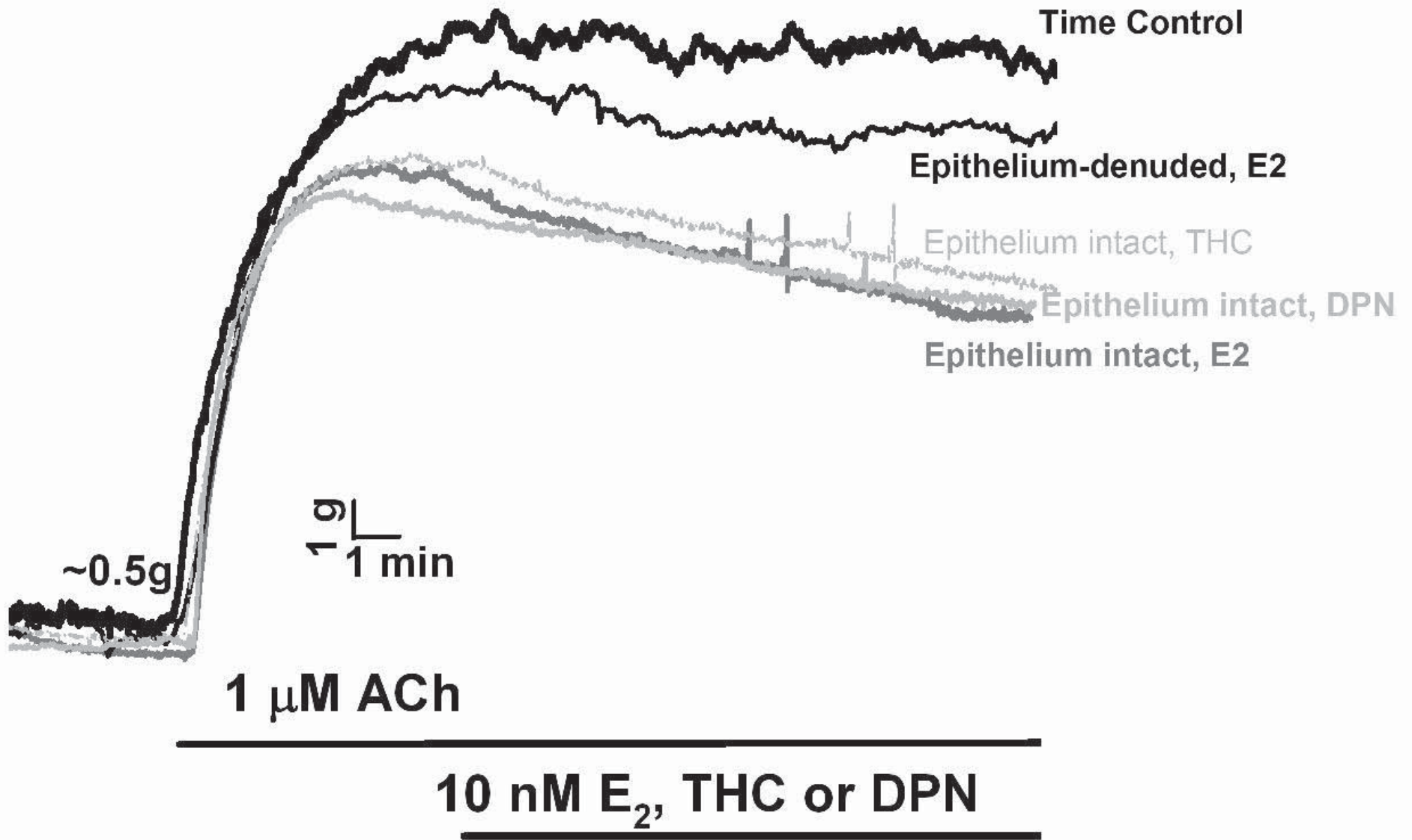


Figure 11