

## Title Page

# Endothelin mediates superoxide production and vasoconstriction through activation of NADPH oxidase and uncoupled NOS in the rat aorta

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## Running Title Page

Running title: ET-1-mediated superoxide production

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Abbreviations: Endothelin (ET-1), Nitric oxide (NO), Nitric oxide synthase (NOS), Superoxide ( $O_2^{\bullet-}$ ), Tetrahydrobiopterin ( $BH_4$ ), Reactive oxygen species (ROS), physiological saline solution (PSS).

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## Abstract

Experiments were designed to test the hypothesis that elevated levels of ET-1 in the vasculature activate NADPH oxidase and/or uncoupled NOS resulting in  $O_2^{\bullet-}$  production and mediate increased constriction. Rat aortic rings were incubated with ET-1 or vehicle in the presence and absence of SOD, ebselen (glutathione peroxidase mimetic), apocynin (NADPH oxidase inhibitor), L-NAME (NOS inhibitor),  $BH_4$  (NOS cofactor), or selective  $ET_A$  and  $ET_B$  receptor antagonists (BQ-123 and A-192621, respectively).  $O_2^{\bullet-}$  production was monitored by oxidized dihydroethidine staining and/or lucigenin chemiluminescence. ET-1 significantly increased  $O_2^{\bullet-}$  production compared to vehicle. SOD, ebselen, and apocynin inhibited the ET-1 induced increase in  $O_2^{\bullet-}$  in intact and endothelium-denuded aorta. L-NAME and  $BH_4$  inhibited the ET-1 induced increase in  $O_2^{\bullet-}$  in intact tissue, while these two compounds had no effect on ET-1 induced  $O_2^{\bullet-}$  in endothelium-denuded aorta. Pre-incubation with BQ-123 or A-192621, individually, had no effect on ET-1 induced  $O_2^{\bullet-}$ , however combining both antagonists inhibited the ET-1 stimulated increase in  $O_2^{\bullet-}$ . Rat aortic rings were incubated with ET-1 or vehicle in the presence or absence of sepiapterin ( $BH_4$  synthesis substrate) or apocynin and mounted on wire myographs to determine isometric force generation in response to increasing KCl concentrations. ET-1 increased the contractile response to KCl compared to vehicle. Treatment with either sepiapterin or apocynin attenuated the ET-1 mediated increase with no effect of sepiapterin or apocynin alone. These data support the hypothesis that ET-1 increases vascular tone, in part, through  $ET_A/ET_B$  receptor activation of  $O_2^{\bullet-}$  production from NADPH oxidase and NOS uncoupling.

## Introduction

Not long ago, superoxide ( $O_2^{\bullet-}$ ) was considered simply a destructive by-product of cellular reactions. However, it is becoming clear that  $O_2^{\bullet-}$  and other reactive oxygen species (ROS) play a role in maintaining cellular homeostasis and cell signaling (Wolin, 2000).  $O_2^{\bullet-}$  is generated by virtually every cell type within the vascular wall (Madamanchi et al., 2005). Furthermore, experimental animal models and clinical studies of atherosclerosis, hypertension, and diabetes have demonstrated increased vascular  $O_2^{\bullet-}$  and/or ROS production (see reviews Madamanchi et al., 2005; Munzel et al., 2005; Griendling and FitzGerald, 2003).

Endothelial dysfunction and the bioavailability of nitric oxide (NO) have been documented as one of the many risk factors for cardiovascular disease. Several possible abnormalities may account for the dysfunction including 1) reduced NO synthesis, 2) uncoupling of NO synthase (NOS) to produce  $O_2^{\bullet-}$ , and 3) degradation of NO via its interaction with  $O_2^{\bullet-}$  from other enzymatic sources. NOS uncoupling has been shown to occur when the NOS cofactor, tetrahydrobiopterin ( $BH_4$ ), is oxidized or in limited quantities (Vasquez-Vivar et al., 1998; Laursen et al., 2001; Milstein and Katusic, 1999). It has recently been reported that  $BH_4$  oxidation and subsequent uncoupling of NOS contributes to blood pressure elevation in a model of low renin hypertension (Landmesser et al, 2003).

The most widely studied enzymatic source of  $O_2^{\bullet-}$  in the vascular wall is NADPH oxidase, but uncoupled NOS, xanthine oxidase, cytochrome P450, and enzymes of the mitochondrial electron transport chain are also possible sources of  $O_2^{\bullet-}$ .  $O_2^{\bullet-}$  and NO react to form another oxidant, peroxynitrite ( $ONOO^-$ ; Beckman et al., 1996). Recently, Kuzkaya et al. (2003) have reported that  $BH_4$  is more sensitive to oxidation by  $ONOO^-$  than  $O_2^{\bullet-}$ , suggesting that there is increased NOS uncoupling in the presence of  $ONOO^-$ . Landmesser et al (2003) suggested

that activated NADPH oxidase is critical for producing ROS that lead to the oxidation of BH<sub>4</sub>. Recently, it has been postulated that activated NADPH oxidase provides the priming event (“kindling” radicals) which may lead to NOS uncoupling and production of O<sub>2</sub><sup>•-</sup> or “bonfire” radicals within the endothelium and vascular wall (Munzel et al., 2005).

Endothelin (ET-1) is a potent endothelial-derived vasoconstrictor peptide. ET-1 is generally known to stimulate ET<sub>A</sub> receptors in vascular smooth muscle cells to produce vasoconstriction and ET<sub>B</sub> receptors on endothelial cells to produce vasodilation. The balance between ET<sub>A</sub> and ET<sub>B</sub> receptor activity may contribute to the pathogenesis of vascular disease (Ram, 2003; Kirchengast and Luz, 2005). Some, but not all, experimental models of hypertension, atherosclerosis, and diabetes display high levels of circulating ET-1 and are associated with endothelial dysfunction (see reviews, Muller et al., 2003; Cernacek et al., 2003; Boussette and Giaid, 2003; Schiffrin, 2005). Both ET-1 and O<sub>2</sub><sup>•-</sup> have been the subjects of investigation within the cardiovascular field over the past decade, yet little is known about the precise relationship between these important modulators of vascular function. Wedgwood et al. (2001a) demonstrated that ET-1 stimulated O<sub>2</sub><sup>•-</sup> production in isolated pulmonary arterial smooth muscle cells is blocked by the actions of an ET<sub>A</sub> receptor antagonist. Li et al. (2003) have shown that an ET<sub>A</sub> receptor antagonist blocks the O<sub>2</sub><sup>•-</sup> production in rat carotid arteries. The ET<sub>B</sub> receptor has been shown to stimulate the production of ROS in human umbilical endothelial cells and sympathetic neurons (Dong et al, 2005; Dai et al, 2004).

While ET-1 has been linked to the production of O<sub>2</sub><sup>•-</sup> in the vasculature, thus far, no studies have examined the role of NOS in ET-1 mediated O<sub>2</sub><sup>•-</sup> production and its functional consequences on vasoconstriction. Therefore, we hypothesized that elevated levels of ET-1 in the vasculature activate NADPH oxidase and/or uncoupled NOS resulting in O<sub>2</sub><sup>•-</sup> production and

increased vasoconstriction. Both  $ET_A$  and  $ET_B$  receptors have been reported to mediate  $O_2^{\bullet-}$  production, thus we also sought to determine whether ET-1 stimulated  $O_2^{\bullet-}$  production is receptor subtype dependent.

## Methods

*Isolation and incubation of rat aortic rings.* Experiments were conducted in male 200-250 g Sprague-Dawley rats (Harlan, Indianapolis, IN). The institutional animal care and use committee approved all procedures. Rats were anesthetized with sodium pentobarbital (50mg/kg intraperitoneally, Abbott Laboratories, North Chicago, IL), thoracic aorta was removed, cleaned of adhering tissue in ice-cold physiological saline solution (PSS), and cut into 3-4 mm segments. Composition of PSS was as follows (mM): NaCl 130, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.8, MgSO<sub>4</sub> 1.17, NaHCO<sub>3</sub> 14.9, dextrose 5.5, EDTA 0.26, CaCl<sub>2</sub> 1.6.

*Measurement of ET-1 mediated O<sub>2</sub><sup>•-</sup> production with lucigenin-enhanced chemiluminescence.* Vessels were left intact or denuded of endothelium, incubated for 4 hours at 37°C in either PSS (basal) or ET-1 (0.001 μM to 1 μM) in siliconized polypropylene microcentrifuge tubes. Denudation of the aorta was accomplished by rubbing the tissue with blunt forceps. The denudation technique was verified in a subset of aortic tissue by testing the vascular reactivity of the aortic ring to constrict to phenylephrine (10 μM) followed by testing the relaxation of the ring to acetylcholine (100 μM). The denudation technique utilized demonstrated constriction to phenylephrine followed by a total absence of relaxation to acetylcholine, while the endothelium-intact tissue fully relaxed. A subset of rings were pre-incubated for 1 hour with one of the following inhibitors: superoxide dismutase (SOD; 300 U/mL), Nω-nitro-L-arginine methyl ester (L-NAME; 100 μM), ebselen (50 μM), BH<sub>4</sub> (3 μM), BQ-123 (cyclo [D-Trp-D-Asp-Pro-D-Val-Leu]; selective ET<sub>A</sub> antagonist, 100 nM), A-192621 ([2-(4-propoxyphenyl)-4-(1,3-benzodioxol-5-yl)-1-(2,5-ethylphenyl)aminocarbonylmethyl]-pyrrolidine-3-carboxylic acid]; selective ET<sub>B</sub> antagonist, 100 nM), or vehicle. Aortic rings were placed in duplicate wells containing 200 μL lucigenin (5 μM; Li et al., 1998) in a 96-well

microplate (OptiPlate-96; Perkin-Elmer; Wellesley, MA) and sealed with Topseal-A (Perkin-Elmer; Wellesley, MA). Background wells contained lucigenin solution without tissue. Lucigenin solutions were maintained in the dark. Plates were dark adapted for 30 minutes and counted on a TopCount<sup>TM</sup> scintillation & luminescence counter (Perkin-Elmer; Wellesley, MA) set to single photon counting mode for 3 seconds/well. The counts were repeated 8 times to reduce variability, averaged, and normalized to per minute (cpm). Background luminescence was subtracted and the specific cpms were normalized to the dry weight of the tissue. The lucigenin method utilized in this study is a modified high throughput method, which accommodates comparisons of multiple aortic segments simultaneously. Validation of the high throughput method utilized a purified substrate/enzyme source of  $O_2^{\cdot-}$ , xanthine/xanthine oxidase, in the presence and absence of SOD.

*In situ detection of ET-1 mediated  $O_2^{\cdot-}$  production.* Vessels were incubated for 4 hours at 37°C in either PSS (basal) or ET-1 (1  $\mu$ M) in siliconized polypropylene microcentrifuge tubes. A subset of rings were pre-incubated for 1 hour with one of the following inhibitors: polyethylene glycol-SOD (PEG-SOD; 250 U/mL) or apocynin (300  $\mu$ M). Aortic rings were frozen in Tissue-Tek OCT media, cut on a cryostat (Leica Cyrostat CM3000) into 30  $\mu$ m thick sections and mounted on glass slides. Slides were protected from light and incubated in dihydroethidine (DHE; 1  $\mu$ M) for 30 minutes at 37°C. Images were obtained with a Zeiss LSM 510 META confocal microscope (Thornwood, NY), with an excitation of 488 nm and emission of 574-595 nm or a 560 nm long pass filter.

*Vascular reactivity.* Aortic rings were incubated as previously outlined. Rings were mounted between pins in a multichamber myograph for the measurement of isometric force generation. The chamber was filled with warmed (37°C) gassed (95%  $O_2$ , 5%  $CO_2$ ) PSS. Tension



was set to 25 mN and the buffer was replaced with calcium-free PSS to determine the passive tension. ET-1 treatment resulted in the development of active tension and in calcium-free buffer passive tension was determined to be  $10 \pm 1$  mN. Once passive tension was established, cumulative doses of KCl (8-100 mM) were accomplished in calcium-replete PSS in the presence or absence of inhibitors. Force generation (mN) was monitored using a computer analysis program (Myodaq; Denmark).

*Statistics.* For  $O_2^{\cdot-}$  production using lucigenin-enhanced chemiluminescence, statistical analysis was performed using an ANOVA with Newman-Kuels post-hoc test (Prism; GraphPad Software, San Diego, CA). Maximum responses in vasoconstriction were compared by ANOVA with Fisher's PLSD post-hoc test (Statistica, StatSoft, Tulsa, OK).

*Materials.* All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. OCT was purchased from VWR International (So. Plainsfield, NJ). NaCl, KCl, EDTA, and apocynin were purchased from Fisher Scientific (Pittsburgh, PA). Endothelin-1 was purchased from American Peptide (Sunnyvale, CA). Tetrahydro-L-biopterin was purchased from Cayman Chemical Company (Ann Arbor, MI). ET-1 receptor antagonists were kindly provided by Abbott Laboratories (Abbott Park, IL).

## Results

ET-1 increased  $O_2^{\bullet-}$  production in both endothelium-intact and endothelium-denuded aortic tissue using lucigenin-enhanced chemiluminescent  $O_2^{\bullet-}$  detection (figure 1A, 1B). Specificity of the assay was demonstrated by a significant reduction of the signal in the presence of SOD (figure 1A, 1B). ET-1 significantly increased  $O_2^{\bullet-}$  at 0.1 and 1  $\mu$ M in endothelium-intact and denuded tissue. The endothelium-denuded segments appear to have a higher  $O_2^{\bullet-}$  production at 0.01  $\mu$ M ET-1, however this failed to reach statistical significance (figure 1B). Aortic tissue dissected from the same animal was used to directly compare the response to ET-1 in endothelium-intact and denuded rings. No significant difference was detected in the ET-1 stimulated  $O_2^{\bullet-}$  production between endothelium-intact and endothelium-denuded aortic tissue (figure 1C).

ET-1 mediated  $O_2^{\bullet-}$  production was verified with the oxidative fluorescent dye, DHE. Increased nuclear staining, indicating enhanced  $O_2^{\bullet-}$  production, is evident in the tissues incubated with ET-1 when compared to those incubated in PSS (figure 2A, 2B). The NADPH oxidase inhibitor, apocynin, and the antioxidant, PEG-SOD, prevented the increase in DHE staining (figure 2C, 2D).

ET-1 increased  $O_2^{\bullet-}$  production in endothelium-intact aortic tissue was significantly inhibited in the presence of the NOS inhibitor, L-NAME, the NOS cofactor,  $BH_4$ , and an antioxidant, ebselen (figure 3A, 3C, 3E). To determine whether the endothelium was the source for the L-NAME-inhibitable  $O_2^{\bullet-}$ , the aortic tissue was endothelium-denuded prior to incubation with ET-1. ET-1 mediated  $O_2^{\bullet-}$  production in the endothelium-denuded aortic tissue was inhibited only in the presence of ebselen (figure 3B, 3D, 3F). ET-1 stimulated  $O_2^{\bullet-}$  production in endothelium-intact tissue was significantly inhibited in the presence of both specific ET-1

receptor antagonists (figure 4B), while individually the antagonists did not inhibit  $O_2^{\bullet-}$  production (figure 4A). L-NAME,  $BH_4$ , ebselen, BQ-123, or A-192621 had no effect on basal aortic tissue  $O_2^{\bullet-}$  detection (data not shown).

Aortic vasoconstriction was assessed by monitoring the increase in force generation by increasing KCl concentrations. Incubation of endothelium-intact aortic tissue with ET-1 increased the vasoconstriction in comparison to incubation with vehicle alone. The maximal increase in force to KCl in the vehicle-treated vessels was  $90 \pm 5\%$ , while in the ET-1-treated vessels, the increase in force was  $118 \pm 11\%$  (figure 5). The NADPH oxidase inhibitor, apocynin, significantly decreased the maximal ET-1 induced vasoconstriction ( $68 \pm 16\%$  increase in force). Since we observed that uncoupled NOS via oxidized  $BH_4$  was a source of the  $O_2^{\bullet-}$  (figure 3) we determined contractile responses to KCl in vessels pretreated with sepiapterin, a precursor in the salvage pathway for the synthesis of  $BH_4$ . Sepiapterin also significantly decreased the maximal ET-1 induced vasoconstriction ( $83 \pm 18\%$  increase in force). Apocynin and sepiapterin had no effect on vehicle-treated vessels ( $75 \pm 8\%$  and  $87 \pm 9\%$  increase in force, respectively).

## Discussion

The new findings of this study are: 1) ET-1 mediates  $O_2^{\bullet-}$  production through uncoupled NOS in an endothelium-dependent manner, 2) endothelium-independent  $O_2^{\bullet-}$  production stimulated by ET-1 does not appear to be via activation of uncoupled NOS, 3) ET-1 activation of  $O_2^{\bullet-}$  production involves both  $ET_A$  and  $ET_B$  receptor subtypes in aortic tissue, and 4) two sources of  $O_2^{\bullet-}$ , NADPH oxidase and uncoupled NOS, contribute to increased vasoconstriction mediated by ET-1. Additionally, we have confirmed the previous findings that ET-1 stimulated  $O_2^{\bullet-}$  production is dependent on NADPH oxidase. The present study supports the hypothesis that ET-1 mediates  $O_2^{\bullet-}$  production from activated NADPH oxidase providing the “kindling” radicals to uncouple NOS and facilitate endothelial dysfunction in a mechanism similar to that proposed by Munzel et al (2005).

### *ET-1 mediated $O_2^{\bullet-}$ production and NOS uncoupling*

We utilized two methods of  $O_2^{\bullet-}$  detection in this study, lucigenin-enhanced chemiluminescence and oxidized dihydroethidine staining. Specificity of both  $O_2^{\bullet-}$  detection methods was verified by the inclusion of SOD, PEG-SOD, or ebselen. Ebselen is an antioxidant that has been reported to have thioredoxin reductase and/or glutathione peroxidase-like activity (Zhao et al., 2002). The lucigenin method for  $O_2^{\bullet-}$  detection has been reported to reflect levels of  $O_2^{\bullet-}$  extracellularly and intracellularly because lucigenin is cell permeable (Munzel et al., 2002). Interestingly, we found that both the endothelium-intact and endothelium-denuded aortic tissue was stimulated by ET-1 to generate  $O_2^{\bullet-}$ . No quantitative difference was detected between the  $O_2^{\bullet-}$  production in endothelium-intact and denuded tissue from the same animal. This led us to speculate that regardless of whether the endothelium is present, ET-1 maximally stimulated  $O_2^{\bullet-}$  production.

The present study extended the findings of Li et al. (2003) that ET-1 increases  $O_2^{\bullet-}$  via NADPH oxidase activation by showing that ET-1 also activates uncoupled NOS in an endothelium-dependent manner. Experimental evidence to determine whether NOS is uncoupled requires measurements of  $O_2^{\bullet-}$  that is inhibited by a non-specific NOS inhibitor, L-NNA or L-NAME (Munzel et al., 2002). These inhibitors can block the transfer of electrons to either L-arginine or oxygen (Andrew and Mayer, 1999). In our experiments, L-NAME inhibited the ET-1 mediated  $O_2^{\bullet-}$  production in the endothelium-intact but not denuded tissue. These results indicate that endothelial NOS is a source of ET-1 stimulated  $O_2^{\bullet-}$ . Landmesser et al (2003) have suggested that uncoupled endothelial NOS, via oxidized  $BH_4$ , is a source of  $O_2^{\bullet-}$  production in aortic tissue in the DOCA-salt treated rat. Therefore, exogenous  $BH_4$  was added to the tissue to provide a source of reduced  $BH_4$  for NOS (Cosentino and Luscher, 1999). These data revealed that exogenous  $BH_4$  prevented the ET-1 stimulated  $O_2^{\bullet-}$  production in the endothelium-intact tissue.  $BH_4$  did not inhibit the ET-1 mediated  $O_2^{\bullet-}$  production in the endothelium-denuded aorta, thus confirming that  $BH_4$  is not acting as a non-specific antioxidant.

### ***ET-1 receptor mediated $O_2^{\bullet-}$ production***

$ET_A$  receptors have been demonstrated to mediate  $O_2^{\bullet-}$  production in vascular smooth muscle cell cultures as well as pulmonary vascular preparations (Laplante et al., 2005; Sedeek et al., 2003; Wedgwood et al., 2001a; Galle et al., 2000; Wedgwood et al., 2001b).  $ET_B$  receptor mediated ROS production in cultured human umbilical vein endothelial cells has also recently been reported (Dong et al., 2005). These authors demonstrated that apocynin inhibited the ET-1 mediated ROS production and endothelial cell proliferation, although the authors did not determine whether ET-1 stimulation also produced NOS uncoupling. The present study demonstrated that both  $ET_A$  and  $ET_B$  receptor signaling is critical for stimulation of  $O_2^{\bullet-}$

production in aortic tissue, which was a puzzling finding. However, it is plausible that ET-1 may activate NADPH oxidase through both ET<sub>A</sub> and ET<sub>B</sub> receptors since both receptor subtypes have been reported to increase O<sub>2</sub><sup>•-</sup> (Laplante et al., 2005; Sedeek et al., 2003; Wedgwood et al., 2001a; Galle et al., 2000; Dong et al., 2005; Dai et al., 2004). NADPH oxidase is expressed in all cell types in aortic tissue. Thus, we would speculate that ET-1 mediates stimulation of NADPH oxidase via both ET<sub>A</sub> and ET<sub>B</sub> receptors on smooth muscle and endothelial cells which produce the so-called “kindling” radicals to further cause oxidation of BH<sub>4</sub> resulting in NOS uncoupling. We have hypothesized that this may result from some type of “cross-talk” between the ET receptor signaling pathways. Recently, Just et al. (2004) have suggested that both ET receptors function synergistically in renal vasoconstriction studies.

#### ***ET-1 mediated O<sub>2</sub><sup>•-</sup> production and vasoconstriction***

ET-1 is a known vasoconstrictor, thus we hypothesized that O<sub>2</sub><sup>•-</sup> from NADPH oxidase and/or uncoupled NOS will mediate an increase in constriction. We found an increase in contraction with ET-1 treatment and our results implicate at least two sources of O<sub>2</sub><sup>•-</sup> that mediate the increased vasoconstriction, NADPH oxidase and uncoupled NOS. As previously discussed, BH<sub>4</sub> is a critical factor in mediating O<sub>2</sub><sup>•-</sup> production from uncoupled NOS in the present study as well as others (see review, Channon 2004). For vascular reactivity studies, we utilized sepiapterin to provide a source for intracellular production of BH<sub>4</sub> in the aortic tissue. Sepiapterin is a substrate in the salvage pathway for the synthesis of BH<sub>4</sub> (Alp and Channon, 2004). Neither apocynin nor sepiapterin affected the vasoconstriction in the absence of ET-1. Both apocynin and sepiapterin lowered maximal constriction when compared to ET-1 alone. Thus, functionally, these data indicate that ET-1 mediated increased constriction is facilitated through products of both NADPH oxidase and uncoupled NOS.

### *Perspectives*

In the present study, aortic tissue was incubated with exogenous ET-1 to model pathological states in which there are high levels of ET-1 such as salt-sensitive hypertension, atherosclerosis, and diabetes (Lariviere et al., 1993; White et al., 1994; Muller et al., 2003; Cernacek et al., 2003; Bousette and Giaid, 2003). ET-1 is known to be a paracrine factor and to act locally within a given tissue or cell type, thus this ex vivo method models the paracrine effects of ET-1 within the vascular tissue. It is difficult to assess the local concentrations of ET-1 within any given tissue at different points in a pathological state. Elmarakby et al. (2004) reported that plasma ET-1 levels are increased approximately 10-fold in ET<sub>B</sub> receptor deficient rats on a high salt diet compared to wild-type rats on a high salt diet. When the same rats are chronically treated with an ET<sub>A</sub> receptor antagonist, plasma ET-1 levels increased about 100-fold compared to wild-type rats on a high salt diet. Under conditions of reduced receptor availability, we reasoned that the tissue ET-1 would be “displaced” into the circulation. Thus, the plasma levels of ET-1 would be a reasonable approximation of the tissue levels, suggesting that tissue levels can become extremely high.

There are many pathological states in which the vasculature is exposed to high levels of ET-1 and endothelial dysfunction is present. These conditions may or may not be associated with chronic hypertension. Indeed, we recently reported that chronic ET-1 infusion in whole animals on a high salt diet increased aortic O<sub>2</sub><sup>•-</sup> production as well as producing a hypertensive state (Elmarakby et al, 2005). The NADPH oxidase inhibitor, apocynin, reduced vascular O<sub>2</sub><sup>•-</sup> production without affecting the hypertension. Although the mechanisms of endothelial dysfunction may be unknown, it appears that O<sub>2</sub><sup>•-</sup> and/or ROS production from diverse enzymatic and cellular sources may be involved in many vascular pathologies (Munzel et al.,

2005). Our data are consistent with the hypothesis that ET-1 stimulates NADPH oxidase in all cell types of the vasculature as well as the uncoupling of NOS in the endothelium via the oxidation of BH<sub>4</sub>. These events translate to enhanced vasoconstriction and may contribute to vascular disorders. Future studies will focus on additional ET-1 mediated dysfunction of NOS regulatory pathways in the vasculature.



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## References

- Alp NJ and Channon KM (2004) Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. *Arterioscler Thromb Vasc Biol.* **24**: 413-420.
- Andrew PJ and Mayer B (1999) Enzymatic function of nitric oxide synthases. *Cardiovasc Res.* **43**: 521-531.
- Beckman JS and Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol.* **271**: C1424-C1437.
- Bousette N and Giaid A (2003) Endothelin-1 in atherosclerosis and other vasculopathies. *Can J Physiol Pharmacol.* **81**: 578-587.
- Cernacek P, Stewart DJ, Monge JC, Rouleau JL (2003) The endothelin system and its role in acute myocardial infarction. *Can J Physiol Pharmacol.* **81**:598-606.
- Channon KM (2004) Tetrahydrobiopterin: regulator of endothelial nitric oxide synthase in vascular disease. *Trends Cardiovasc Med.* **14**: 323-327.
- Cosentino F and Luscher TF (1999) Tetrahydrobiopterin and endothelial nitric oxide synthase activity. *Cardiovasc. Res.* **43**:274-278.
- Dai X, Galligan JJ, Watts SW, Fink GD, Kreulen DL (2004) Increased O<sub>2</sub><sup>•-</sup> production and upregulation of ET<sub>B</sub> receptors by sympathetic neurons in DOCA-salt hypertensive rats.

*Hypertension* **43**: 1048-1054.

Dong F, Zhang X, Wold LE, Ren Q, Zhang Z, Ren J (2005) Endothelin-1 enhances oxidative stress, cell proliferation and reduces apoptosis in human umbilical vein endothelial cells: role of ET<sub>B</sub> receptor, NADPH oxidase and caveolin-1. *Br J Pharmacol* **145**: 323-333.

Elmarakby A, Loomis ED, Pollock JS, Pollock DM (2004) ET<sub>A</sub> receptor blockade attenuates hypertension and decreases reactive oxygen species in ET<sub>B</sub> receptor-deficient rats. *J Cardiovasc Pharmacol* **44**: S7-S10.

Elmarakby A, Loomis ED, Pollock JS, Pollock DM (2005) NADPH oxidase inhibition attenuates oxidative stress but not hypertension produced by chronic ET-1. *Hypertension* **45**: 283-287.

Galle J, Lehmann-Bodem C, Hubner U, Hinloth A, and Wanner C (2000) CyA and OxLDL cause endothelial dysfunction in isolated arteries through endothelin-mediated stimulation of O<sub>2</sub>(-)<sup>•</sup> formation. *Nephrol. Dial. Transplant* **15**:339-346.

Griendling KK, and FitzGerald GA (2003) Oxidative stress and cardiovascular Injury Part II: Animal and human studies. *Circulation* **108**:2034-2040.

Just A, Olson AJ, Arendhorst WJ (2004) Dual constrictor and dilator actions of ET(B) receptors in the rat renal microcirculation: interactions with ET(A) receptors. *Am J Physiol: Renal Physiol* **286**: F660-F668.

Kirchengast M and Luz M (2005) Endothelin receptor antagonists: clinical realities and future directions. *J Cardiovasc Pharm* **45**: 182-191.

Kuzkaya N, Weissmann N, Harrison DG, Dikalov S (2003) Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric oxide synthase. *J Biol Chem*. **278**:22546-22554.

Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, and Harrison DG (2003) Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J. Clin. Invest.* **111**:1201-1209.

Lapante M, Wu R, Moreau P, deChamplain J (2005) Endothelin mediates superoxide production in angiotensin II-induced hypertension in rats. *Free Rad Biol & Med* **38**: 589-596.

Lariviere R, Thibault G, and Schiffrin EL (1993) Increased endothelin-1 content in blood vessels of deoxycorticosterone acetate-salt hypertensive but not in spontaneously hypertensive rats. *Hypertension*. **21**:294-300.

Laursen, JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukai T, and Harrison DG (2001) Endothelial regulation of vasomotion in ApoE-deficient mice. Implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation* **103**: 1282-1288.

Li L, Fink GD, Watts SW, Northcott CA, Galligan JJ, Pagano PJ, and Chen AF (2003) Endothelin-1 increases vascular superoxide via endothelin(A)-NADPH oxidase pathway in low-

renin hypertension. *Circulation* **107**: 1053-1058.

Madamanchi, NR, Vendrov, A, Runge, MS (2005) Oxidative stress and vascular disease. *Arterioscler Thromb Vasc Biol* **25**: 29-38.

Milstein S and Katusic Z (1999) Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun* **263**: 681-684.

Muller DN, Fiebeler A, Park JK, Dechend R, Luft FC (2003) Angiotensin II and endothelin induce inflammation and thereby promote hypertension-induced end-organ damage. *Clin Nephrol.*

**Suppl 1**: S2-12.

Munzel T, Afanas'ev IB, Kleschyov AL, Harrison DG (2002) Detection of superoxide in vascular tissue. *Arterioscler Thromb Vasc Biol.* **22**:1761-1768.

Munzel T, Daiber, A, Ullrich, V, Mulsch, A (2005) Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP dependent protein kinase. *Arterioscler Throm Vasc Biol* **25**: 1-7.

Ram CV (2003) Possible therapeutic role of endothelin antagonists in cardiovascular disease. *Am J Ther.* **10**:396-400.

Schiffrin, EL (2005) Vascular endothelin in hypertension. *Vascul Pharmacol* **43**: 19-29.

Sedeek, MH, Llinas, MT, Drummond, H, Fortepiani, L, Abram, SR, Alexander, BT, Reckelhoff, JF, Granger, JP (2003) Role of reactive oxygen species in endothelin-induced hypertension. *Hypertension* **42**: 806-810.

Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, and Pritchard Jr. KA (1998) Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc. Natl. Acad. Sci. U. S. A.* **95**:9220-9225.

Wedgwood S, Dettman RW, Black SM (2001a) ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. *Am J Physiol Lung Cell Mol Physiol* **281**: L1058-L1067.

Wedgwood S, McMullan DM, Bekker JM, Fineman JR, and Black SM (2001b) Role for endothelin-1-induced superoxide and peroxynitrite production in rebound pulmonary hypertension associated with inhaled nitric oxide therapy. *Circ. Res.* **89**:357-364.

White CR, Brock TA, Chang LY, Crapo J, Briscoe P, Ku D, Bradley WA, Gianturco SH, Gore J, Freeman BA, and Tarpey MM (1994) Superoxide and peroxynitrite in atherosclerosis. *Proc Natl Acad Sci U S A.* **91**:1044-1048.

Wolin MS (2000) Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol* **20**: 1430-1442.

Zhao R, Masayasu H, Holmgren A (2002) Ebselen: a substrate for human thioredoxin reductase

strongly stimulating its hydroperoxide reductase activity and a superfast thioredoxin oxidant.

*Proc Natl Acad Sci U S A.* **99**: 8579-8584.

## Footnotes

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## Figure Legends

**Figure 1.** Lucigenin-enhanced chemiluminescence demonstrates ET-1 mediated  $O_2^{\bullet-}$  production in (A) endothelium-intact and (B) endothelium-denuded aortic tissue. SOD (300 U/mL) prevents the increase in  $O_2^{\bullet-}$  production. (C) ET-1 (1000 nM) stimulates  $O_2^{\bullet-}$  production similarly in both endothelium-intact and endothelium-denuded tissue. Values are means  $\pm$  SE. (A) n=6-12; (B) n=5-13; (C) n=9-10. \*p<0.05 vs. basal; \*\*p<0.01 vs. basal; \*\*\*p<0.001 vs. basal; †p<0.05 vs. ET-1; ††p<0.01 vs. ET-1

**Figure 2.** Dihydroethidine (DHE) staining confirms ET-1 mediated  $O_2^{\bullet-}$  production. Aortic tissue incubated in (A) PSS, (B) ET-1 (1000 nM), (C) ET-1 + PEG-SOD (250 U/ml), (D) ET-1 + apocynin (300  $\mu$ M). (n=4-7) Lumen indicated by arrow.

**Figure 3.** ET-1 (1000 nM) mediated  $O_2^{\bullet-}$  production was assessed from (A, C, E) endothelium-intact aortic tissue or (B, D, F) endothelium-denuded aortic tissue in the presence of (A, B) L-NAME (100  $\mu$ M), (C, D)  $BH_4$  (3  $\mu$ M), or (E, F) ebselen (50  $\mu$ M). Values are means  $\pm$  SE. (A) n=5, (B) n=9-18; (C) n=6-12; (D) n=10-11; (E) n=7-8; (F) n=11-12. \*p<0.05 vs. basal; \*\*p<0.01 vs. basal; \*\*\*p<0.001 vs. basal; †p<0.05 vs. ET-1; †††p<0.001 vs. ET-1

**Figure 4.** ET-1 (1000 nM) mediated  $O_2^{\cdot-}$  production in endothelium-intact aortic tissue in the presence of specific ET-1 receptor antagonists. (A)  $ET_A$  receptor antagonist (BQ-123; 100 nM) and  $ET_B$ -receptor antagonist (A-192621; 100 nM) individually (n=11) or (B) simultaneously (n=7). Values are means  $\pm$  SE. \*p<0.05 vs. basal; \*\*p<0.01 vs. basal; \*\*\*p<0.001 vs. basal; †p<0.05 vs. ET-1

**Figure 5.** ET-1 induced vasoconstriction of aortic rings in the absence or presence of (upper panel) sepiapterin,  $BH_4$  synthesis substrate (100  $\mu$ M, n=7) or (lower panel) the NADPH oxidase inhibitor, apocynin (300  $\mu$ M; n=8). Values are means  $\pm$  SE. Maximal responses of ET-1 treated aortic segments (n=8) are significantly increased compared to PSS treated aortic segments (n=8; p<0.05), ET-1 + apocynin (p<0.001), and ET-1 + sepiapterin (p<0.05).

FIGURE 1

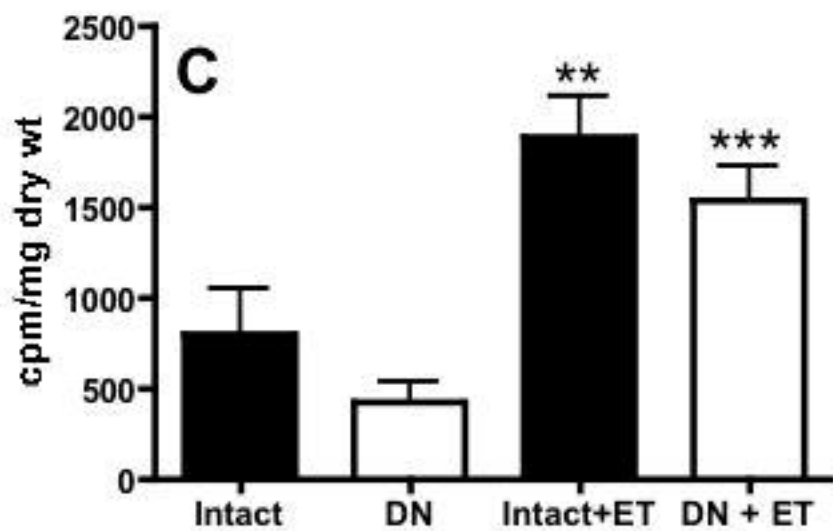
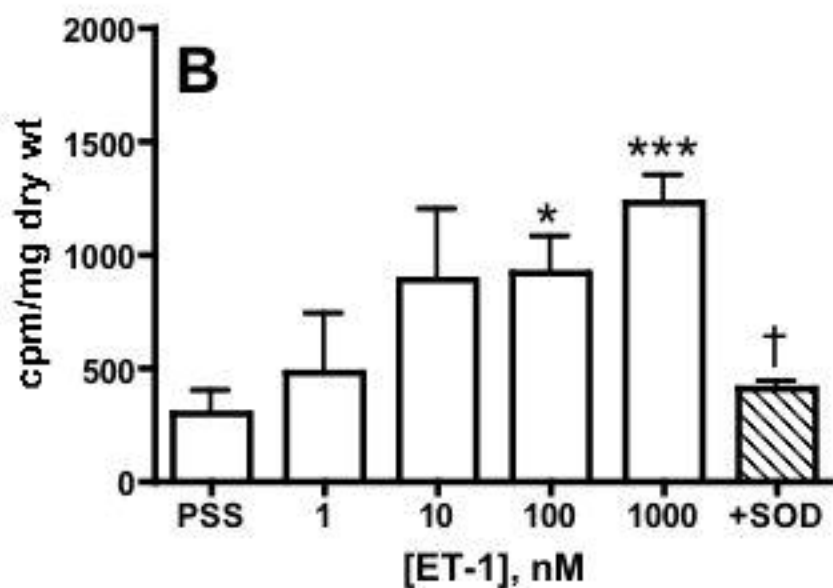
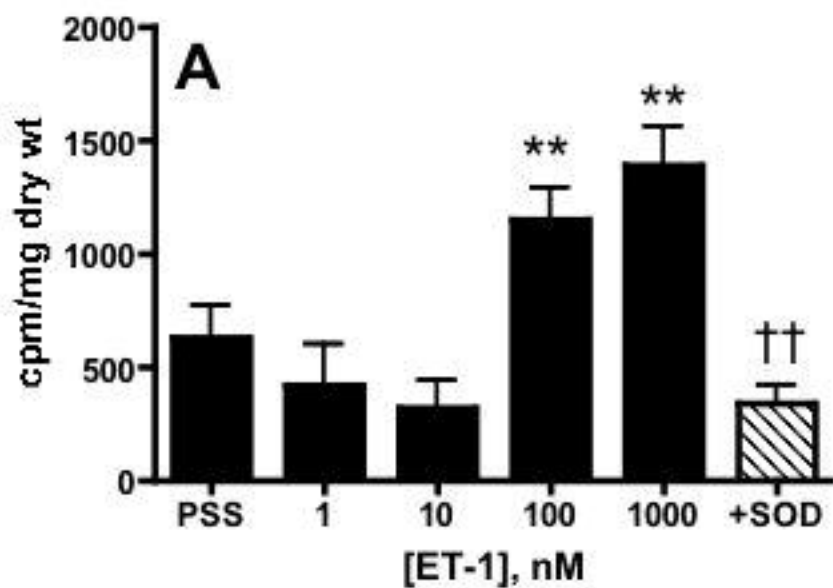
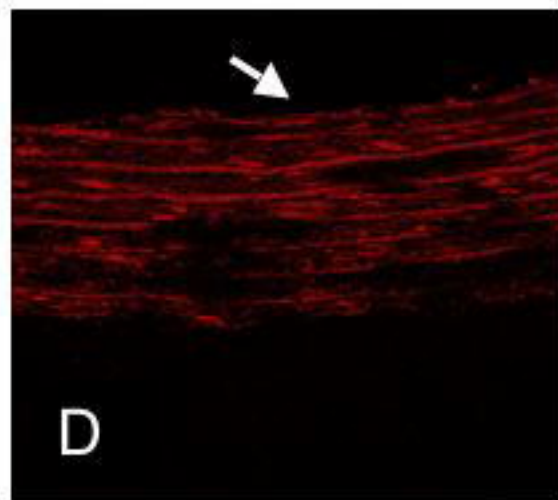
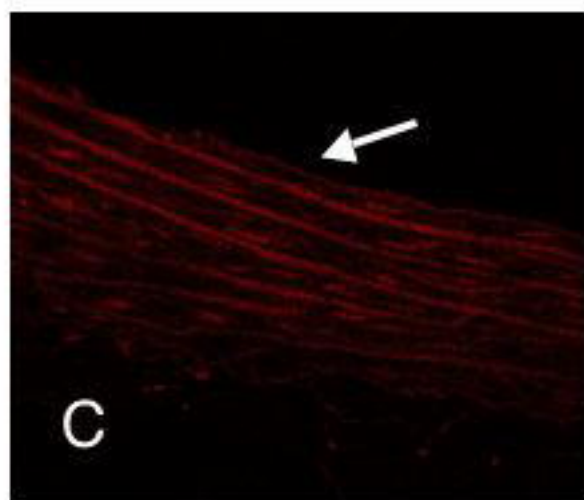
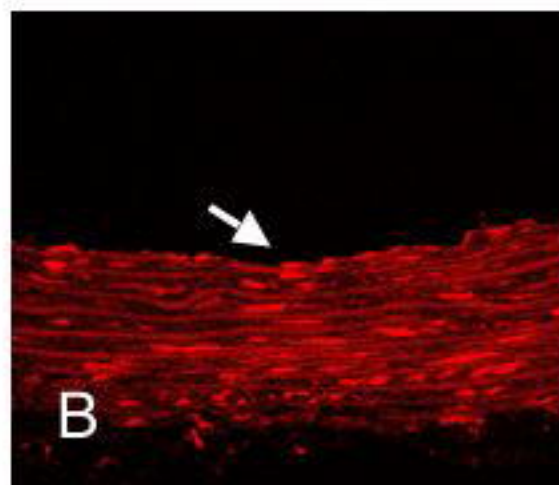
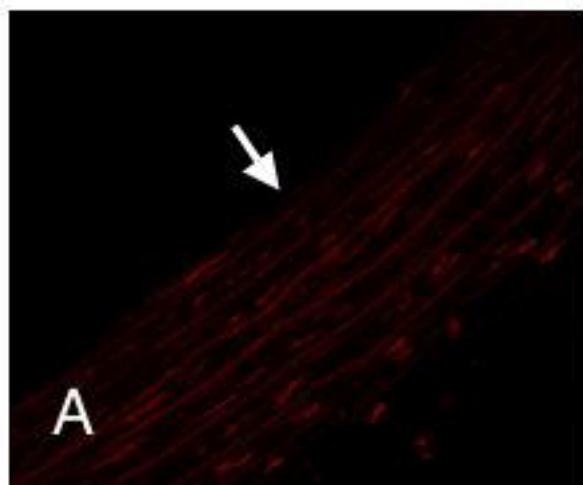


FIGURE 2



# FIGURE 3

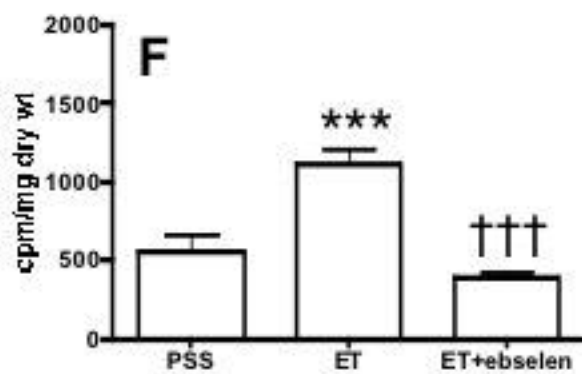
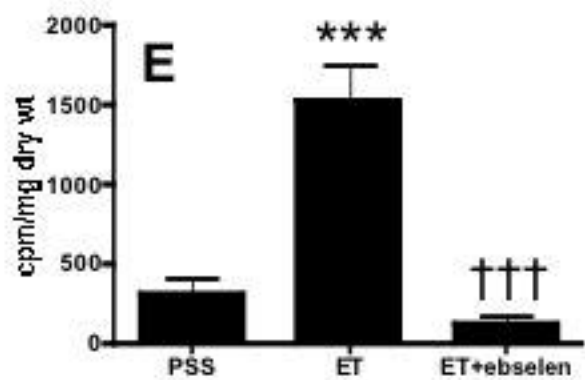
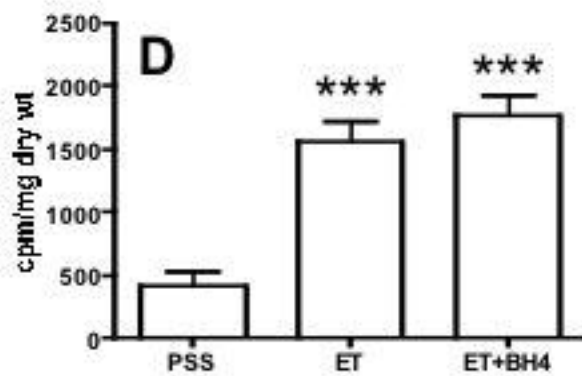
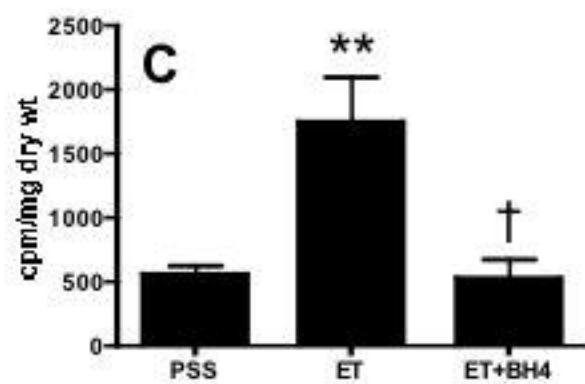
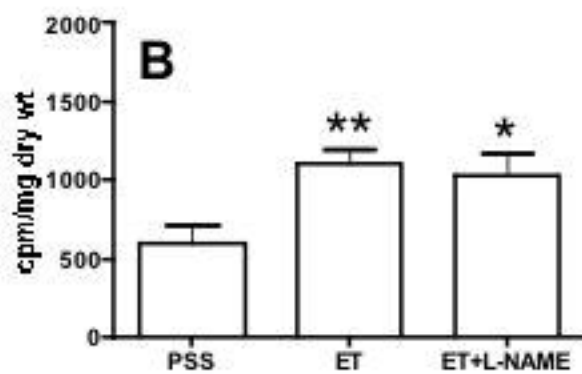
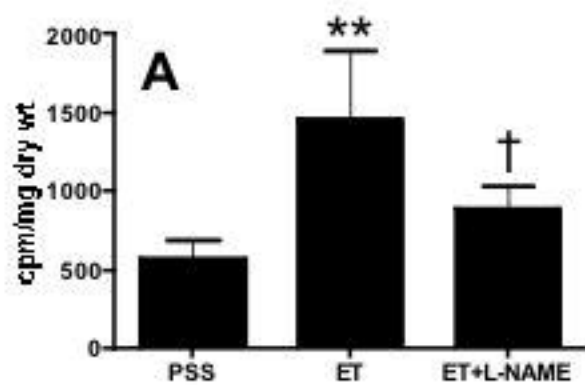


FIGURE 4

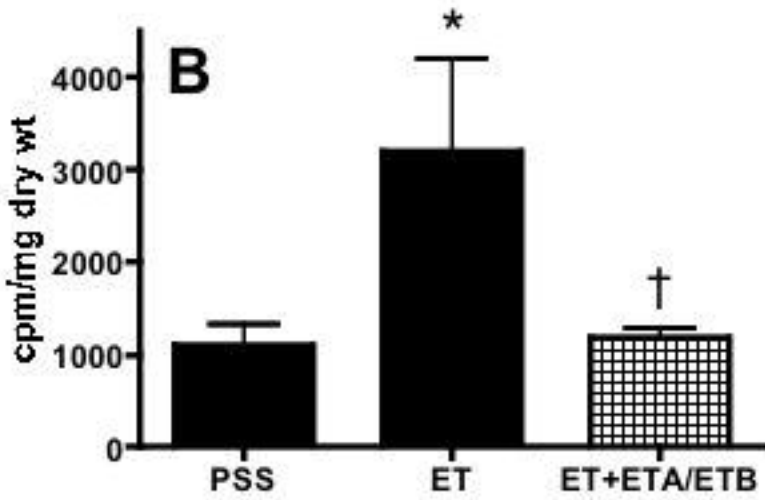
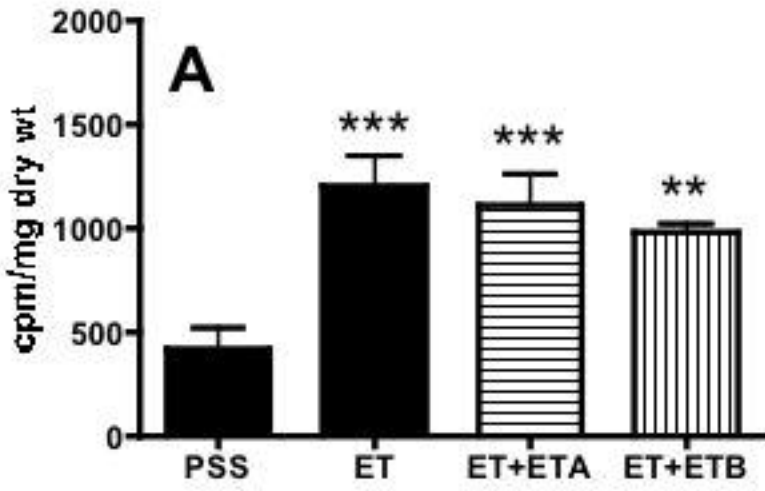


FIGURE 5

