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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

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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_4. Recently, it has been postulated that activated NADPH oxidase provides the priming event ("kindling" radicals) which may lead to NOS uncoupling and production of $O_2^{\bullet-}$ 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_A receptors in vascular smooth muscle cells to produce vasoconstriction and ET_B receptors on endothelial cells to produce vasodilation. The balance between ET_A and ET_B 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; Bousette and Giaid, 2003; Schiffrin, 2005). Both ET-1 and $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production in isolated pulmonary arterial smooth muscle cells is blocked by the actions of an ET_A receptor antagonist. Li et al. (2003) have shown that an ET_A receptor antagonist blocks the $O_2^{\bullet-}$ production in rat carotid arteries. The ET_B 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 $O_2^{\bullet-}$ in the vasculature, thus far, no studies have examined the role of NOS in ET-1 mediated $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ 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_2PO_4 1.8, $MgSO_4$ 1.17, $NaHCO_3$ 14.9, dextrose 5.5, EDTA 0.26, $CaCl_2$ 1.6.

Measurement of ET-1 mediated $O_2^{\bullet-}$ 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_4 (3 μM), BQ-123 (cyclo [D-Trp-D-Asp-Pro-D-Val-Leu]; selective ET_A 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_B 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^{TM} 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 $O_2^{\bullet -}$, xanthine/xanthine oxidase, in the presence and absence of SOD.

In situ detection of ET-1 mediated $O_2^{\bullet -}$ production. Vessels were incubated for 4 hours at 37°C in either PSS (basal) or ET-1 (1 μM) 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 μM). Aortic rings were frozen in Tissue-Tek OCT media, cut on a cryostat (Leica Cyrostat CM3000) into 30 μm thick sections and mounted on glass slides. Slides were protected from light and incubated in dihydroethidine (DHE; 1 μM) 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 $O_2^{\bullet -}$ 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. Plansfield, 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 μM in endothelium-intact and denuded tissue. The endothelium-denuded segments appear to have a higher $O_2^{\bullet-}$ production at 0.01 μM 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±5%, while in the ET-1-treated vessels, the increase in force was 118±11% (figure 5). The NADPH oxidase inhibitor, apocynin, significantly decreased the maximal ET-1 induced vasoconstriction (68±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±18% increase in force). Apocynin and sepiapterin had no effect on vehicle-treated vessels (75±8% and 87±9% increase in force, respectively).

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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_A and ET_B receptors since both receptor subtypes have been reported to increase $O_2^{\bullet-}$ (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_A and ET_B receptors on smooth muscle and endothelial cells which produce the so-called "kindling" radicals to further cause oxidation of BH_4 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 $O_2^{\bullet-}$ production and vasoconstriction

ET-1 is a known vasoconstrictor, thus we hypothesized that $O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ that mediate the increased vasoconstriction, NADPH oxidase and uncoupled NOS. As previously discussed, BH_4 is a critical factor in mediating $O_2^{\bullet-}$ 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_4 in the aortic tissue. Sepiapterin is a substrate in the salvage pathway for the synthesis of BH_4 (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_B 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_A 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 $O_2^{\bullet-}$ production as well as producing a hypertensive state (Elmarakby et al, 2005). The NADPH oxidase inhibitor, apocynin, reduced vascular $O_2^{\bullet-}$ production without affecting the hypertension. Although the mechanisms of endothelial dysfunction may be unknown, it appears that $O_2^{\bullet-}$ 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_4. 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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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 ± 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

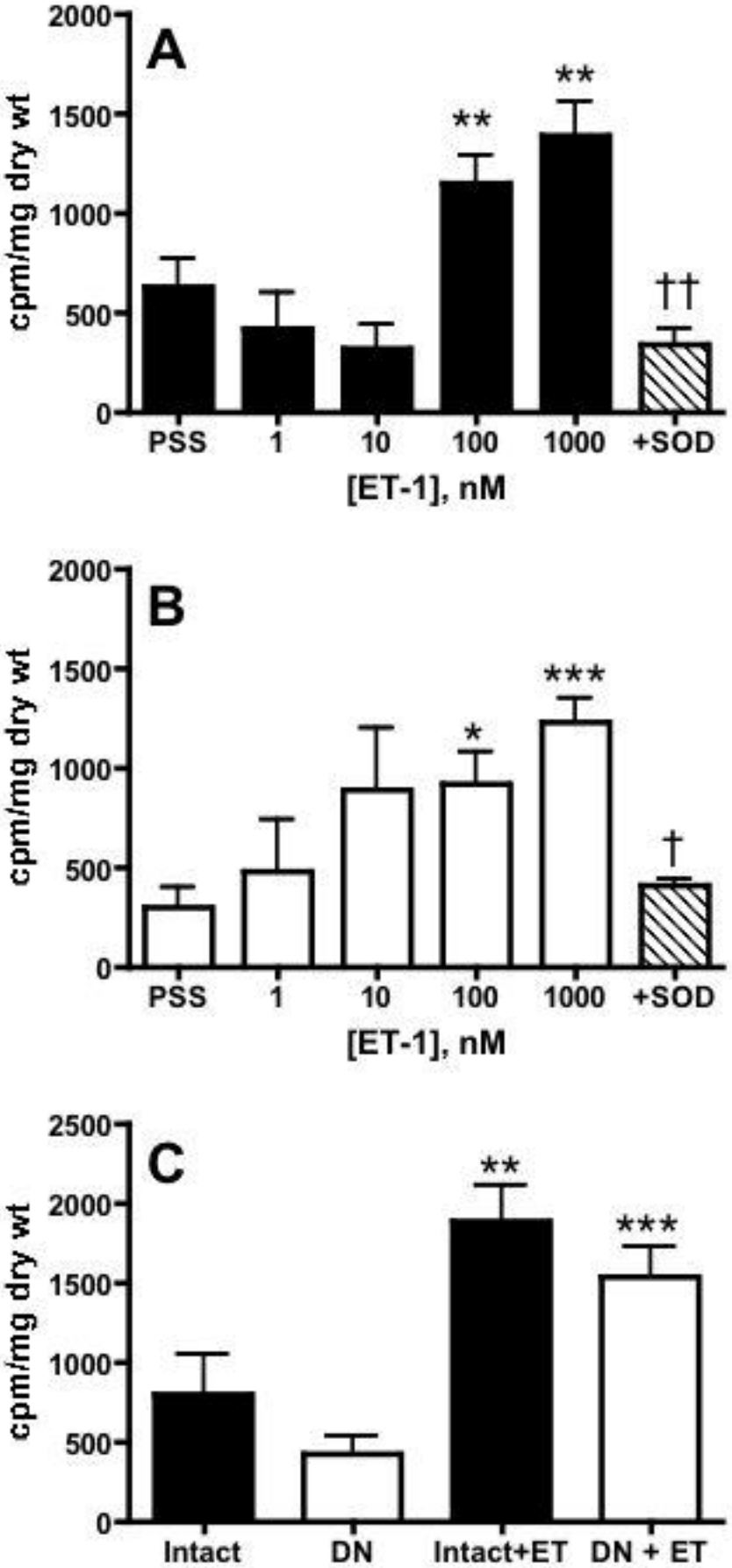
Figure 2. Dihydroethidine (DHE) staining confirms ET-1 mediated $O_2^{\bullet-}$ production. Aortic tissue incubated in (A) PSS, (B) ET-1 (1000 nM), (C) ET-1 + PEG-SOD (250 U/ml), (D) ET-1 + apocynin (300 µM). (n=4-7) Lumen indicated by arrow.

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 µM), (C, D) BH_4 (3 µM), or (E, F) ebselen (50 µM). Values are means ± 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 $O_2^{\bullet-}$ 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 ± 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 µM, n=7) or (lower panel) the NADPH oxidase inhibitor, apocynin (300 µM; n=8). Values are means ± 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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+SOD
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Intact
DN
Intact+ET
DN + ET

FIGURE 2

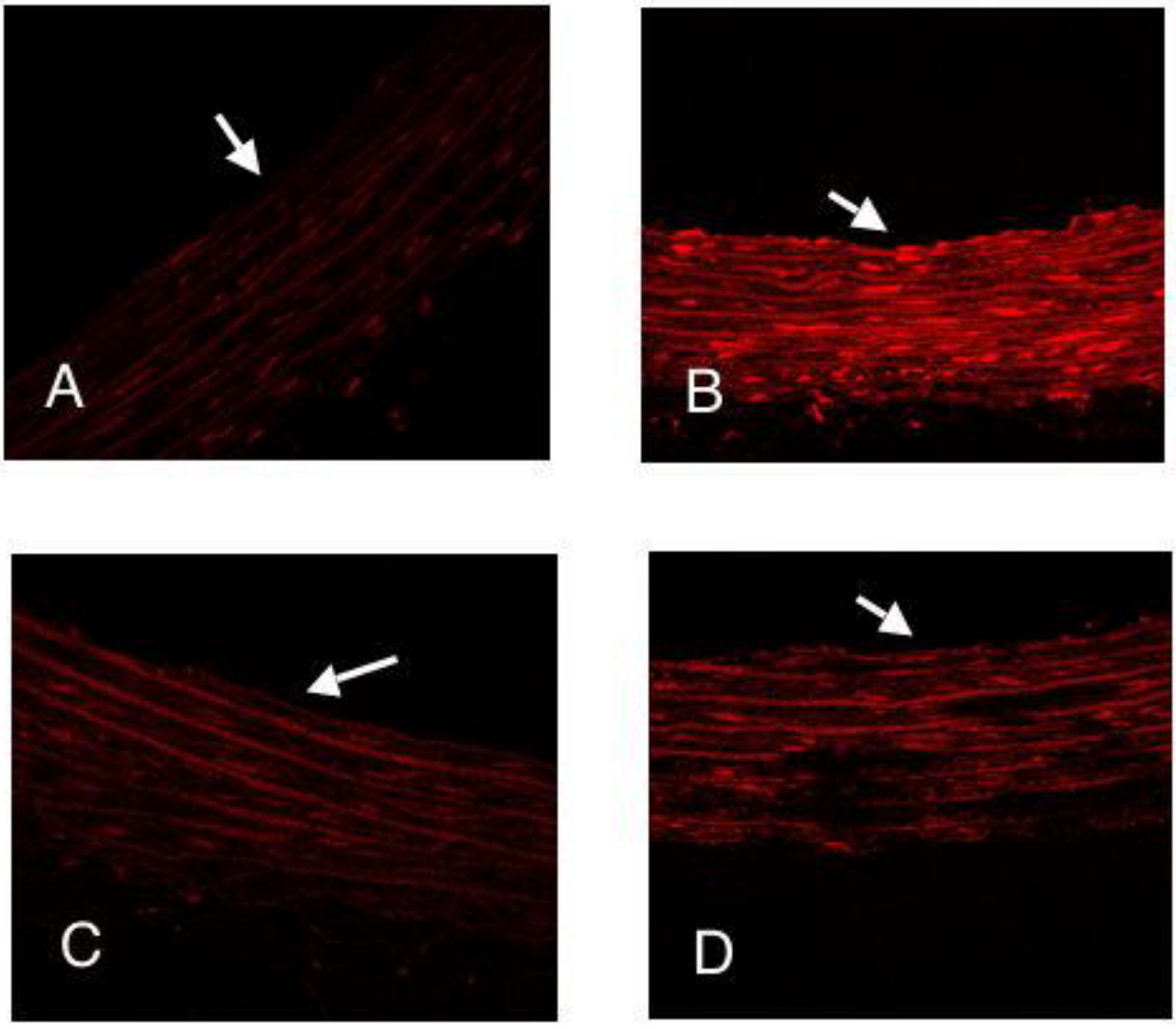


FIGURE 3

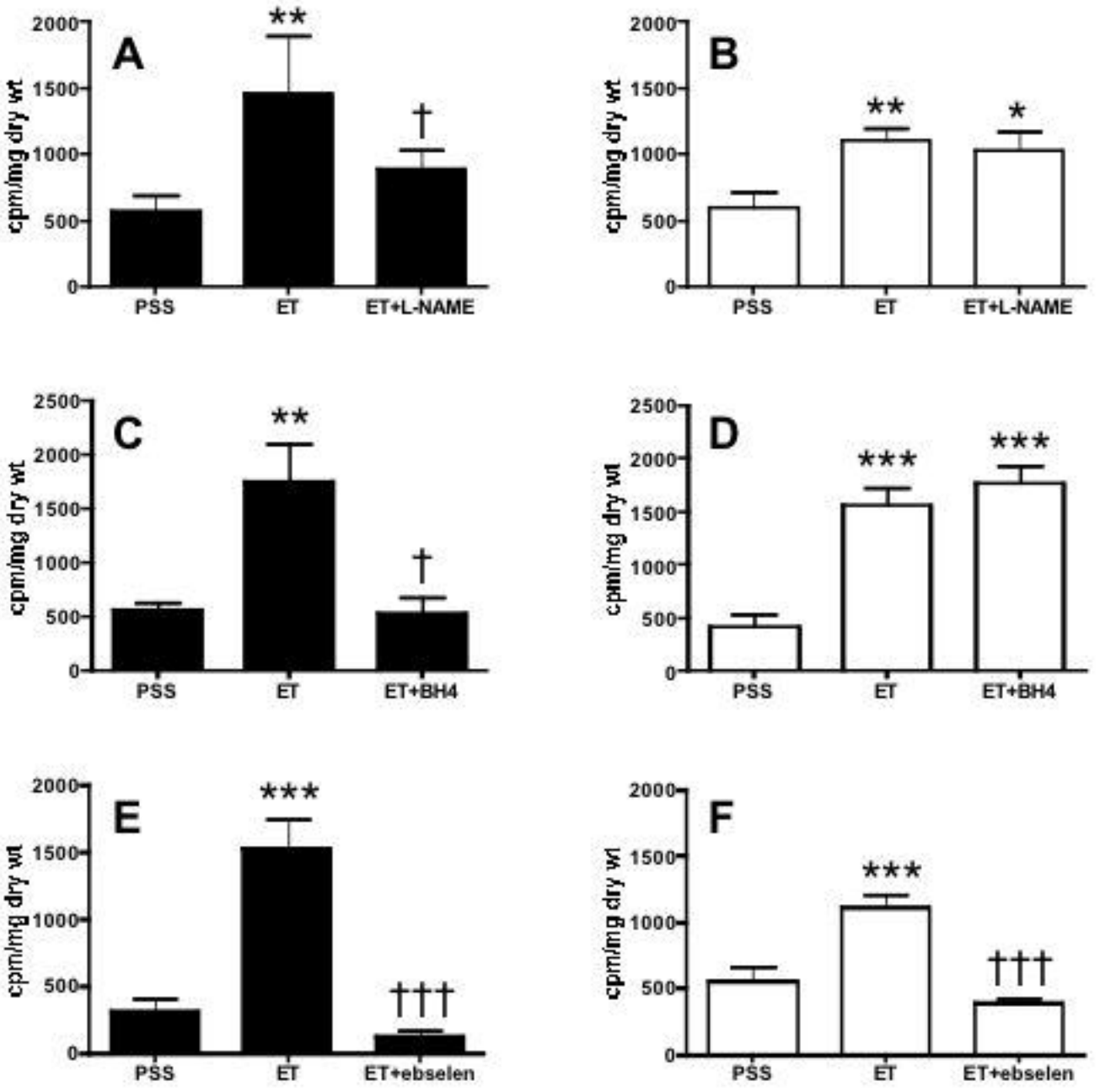
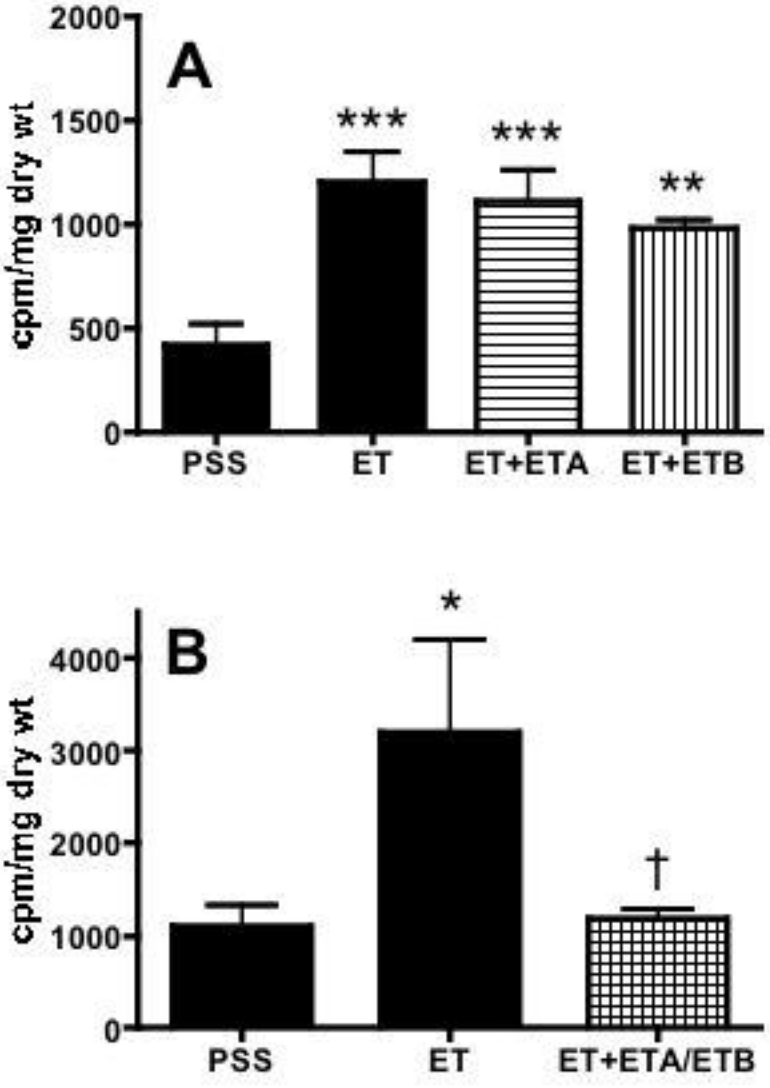


FIGURE 4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PSS
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PSS
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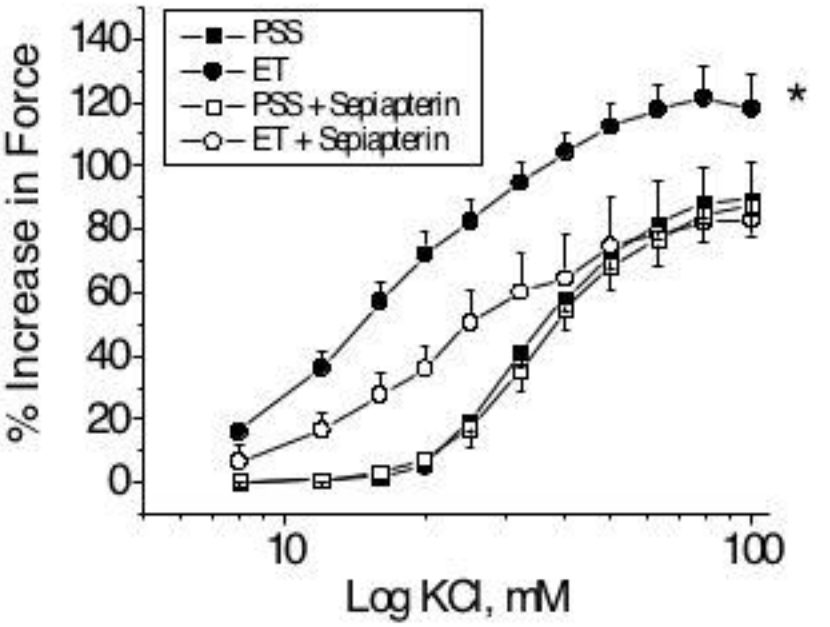
ET+ETA/ETB

FIGURE 5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PSS + Sepiapterin
ET + Sepiapterin
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100
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40
20
0
10
100
Log KCl, mM
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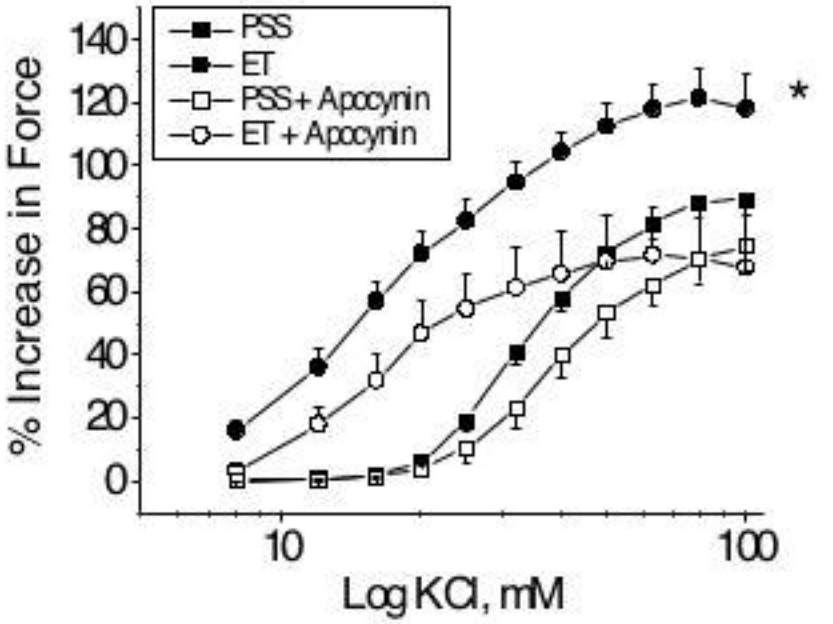
PSS
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PSS + Apocynin
ET + Apocynin
% Increase in Force
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Log KCl, mM
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