Vascular dysfunction of venous bypass conduits is mediated by reactive oxygen species in diabetes: Role of endothelin-1

Adviye Ergul, Jeanette Schultz Johansen, Catherine Strømhaug, Alex K. Harris, Jimmie Hutchinson, Amany Tawfik, Ali Rahimi, Edward Rhim, Bryan Wells, R. William Caldwell, Mark P. Anstadt

Clinical and Experimental Therapeutics Program, University of Georgia College of Pharmacy (AE, AKH, JH), Vascular Biology Center (AE, AT, MPA), Departments of Surgery (AR, ER, BW, MPA) and Pharmacology and Toxicology (RWC), Medical College of Georgia, Augusta, Georgia and University of Tromsø Institute of Pharmacy, Tromsø, Norway (JSJ, CS)
Running title: ET-1 increases oxidative stress in coronary bypass conduits

Address correspondence to: Adviye Ergul, MD, PhD
Medical College of Georgia
Clinical Pharmacy CJ-1020
1120 15th Street
Augusta, Georgia 30912
Tel: 706-721-4915
Fax: 706-721-3994
E-mail: aergul@mail.mcg.edu

Text pages 15
Tables 2
Figures 6
References 38
Abstract word count 201
Introduction word count 485
Discussion word count 1581

ABBREVIATIONS: CABG, coronary artery bypass grafting; *O₂*, superoxide; ET-1, endothelin-1; SV, saphenous vein; DHE, dihydroethidium; PEG-SOD, polyethylene glycol conjugated superoxide dismutase; ROS, reactive oxygen species; NO, nitric oxide; H₂O₂, hydrogen peroxide; ONOO⁻, peroxynitrite; Ach, acetylcholine; PMSF, phenylmethylsulfonyl fluoride; PE, phenylephrine; DPI, diphenyleneiodonium.
ABSTRACT

Diabetes is associated with increased risk for complications following coronary bypass grafting surgery (CABG). Augmented superoxide (•O₂⁻) production plays an important role in diabetic complications by causing vascular dysfunction. The potent vasoconstrictor endothelin-1 (ET-1) is also elevated in diabetes and following CABG. However, the effect of ET-1 on •O₂⁻ generation and/or vascular dysfunction in bypass conduits remained unknown. Accordingly, this study investigated basal and ET-1-stimulated •O₂⁻ production in bypass conduits and determined the effect of •O₂⁻ on conduit reactivity. Saphenous vein (SV) specimens were obtained from nondiabetic (n=24) and diabetic (n=24) patients undergoing CABG. Dihydroethidium (DHE) staining and NAD(P)H oxidase activity assays (5,380 ± 940 vs 16,362 ± 2,550 RLU/µg) demonstrated increased basal •O₂⁻ levels in the diabetes group (p<0.05). Plasma ET-1 levels were associated with elevated basal •O₂⁻ levels and treatment of conduits with exogenous ET-1 further increased •O₂⁻ production and augmented vasoconstriction. Furthermore, vascular relaxation was impaired in the diabetic group (75% vs 40%), which was restored by •O₂⁻ scavenger superoxide dismutase (SOD). These findings suggest that ET-1 causes bypass conduits dysfunction via stimulation of •O₂⁻ production in diabetes. Novel therapies that attenuate •O₂⁻ generation in bypass conduits may improve acute and late outcome of CABG in diabetic patients.
Introduction

In addition to being a risk factor for coronary artery disease, diabetes increases the risk of post-operative vasospasm, graft stenosis and other vascular complications following coronary bypass grafting surgery (CABG) (Stewart et al., 2000). This, in part, explains why diabetic patients suffer a high incidence of unfavorable cardiac events in the postoperative period (Cohen et al., 1998). Adverse CABG outcomes in diabetic patients are most likely to be multifactorial but properties of bypass conduits may directly contribute to vascular dysfunction both in the acute and late phase. Excess generation of reactive oxygen species (ROS), especially $\cdot \text{O}_2^-$, can alter vascular function not only by reducing nitric (NO) bioavailability and thereby impairing the vasorelaxation response acutely, but also generating hydrogen peroxide ($\text{H}_2\text{O}_2$) and cytotoxic peroxynitrite (ONOO$^-$). These molecules can directly stimulate growth of vascular smooth muscle cells, which may contribute to graft stenosis (Griendling and FitzGerald, 2003b; Griendling and FitzGerald, 2003a). ONOO$^-$ can alter function of biomolecules by protein nitration and lipid peroxidation (Turko et al., 2001) as well as causing single-strand DNA breakage and endothelial cell apoptosis (Soriano et al., 2001), all of which might contribute to graft stenosis in the late phase. Guzik et al. demonstrated that enhanced $\cdot \text{O}_2^-$ generation in bypass conduits of diabetic patients is mediated by NAD(P)H oxidase and uncoupled nitric oxide synthase (eNOS) (Guzik et al., 2000; Guzik et al., 2002). However, the effect of increased $\cdot \text{O}_2^-$ generation in diabetes on contractile properties of bypass conduits is not clear.

ET-1 is a potent vasoactive peptide with mitogenic properties. Elevated plasma levels of ET-1 have been reported in atherosclerosis and diabetes as well as during and
following CABG surgery (Lerman et al., 1991). CABG results in a biphasic increase in circulating ET-1 levels, which is associated with a complex recovery in the immediate postoperative period. Higher plasma ET-1 levels are associated with longer intensive care unit stay (Dorman et al., 2000). Furthermore, there is a positive correlation between conduit sensitivity to ET-1 and prolonged need for vasodilator support with nitroglycerin (Bond et al., 2001). These observations provide evidence that increased ET-1 levels may negatively impact outcome in CABG patients. Yet, the mechanism by which ET-1 alters bypass conduit function in diabetes remains to be determined.

Li et al. found that ET-1 stimulates \( \cdot \text{O}_2^- \) production via activation of NAD(P)H oxidase in an experimental model of hypertension (Li et al., 2003). In contrast, ET-1-mediated excess generation of \( \cdot \text{O}_2^- \) in human smooth muscle cells is primarily of mitochondrial origin (Touyz et al., 2004). Therefore, mechanisms of ET-1-mediated \( \cdot \text{O}_2^- \) production may be different in experimental models vs humans and further varies in disease states. In order to better understand the vasoactive processes that might influence function of CABG conduits, this study aimed to 1) determine whether and to what extent \( \cdot \text{O}_2^- \) affects reactivity of bypass conduits, and 2) determine the effect of ET-1 on \( \cdot \text{O}_2^- \) production in CABG conduits in diabetes and identify the mechanisms involved.
Methods

Tissue collection. The study protocol was approved by the Human Assurance Committee at the Medical College of Georgia. Informed consent was obtained from each patient enrolled in this study prior to surgery. Blood samples (3 ml) were collected into EDTA-tubes prior to surgery before the induction of anesthesia and plasma samples were stored at -80°C until the day of the assays. Saphenous vein (SV) specimens were obtained from diabetic (n=24) and nondiabetic (n=24) patients undergoing CABG. SV samples represented the remaining distal segments following each procedure. Venous specimens were placed in cold Dulbecco’s Modified Eagle Medium and kept on ice. Specimens were transferred to the laboratory where the surrounding fat was carefully removed. The specimens were then rinsed in sterile saline, cut into 3 mm segments and immediately snap-frozen or used in contractility experiments. To determine the effect of ET-1 on superoxide generation, vessels segments were incubated with 10 or 100 nM ET-1 for 30 min and then frozen. The patients’ pre-operative co-morbidities and demographics were recorded and entered into a database (Table 1). Medications of the control and diabetic patients were similar with the exception of the hypoglycemic agents.

Vascular reactivity. After the fat tissue was carefully removed, SV was cut into 2 to 3 mm rings and the rings were mounted at the optimal diastolic tension (2 g) in 5-ml tissue baths containing oxygenated Krebs buffer maintained at pH 7.4 and 37°C. Isometric contractions were recorded with digital force transducers that are integrated with a myograph data acquisition system (Danish Technologies, Denmark). After the rings were equilibrated for 1 h, rings were challenged with 70 mM KCl to assess the
viability. At plateau, the integrity of endothelium was tested by 100 nM acetylcholine (Ach). In endothelium-intact vessels, the dose-response curves were generated using progressive concentrations of ET-1 (1-200 nM), and the contractile response obtained with each concentration of ET-1 were expressed as % of tension generated by 70 mM KCl per gram of tissue. To determine the role of $^\bullet{O}_2^-$ in modulating the contractile response, additional rings were incubated with 150 U/ml polyethylene glycol (PEG)-conjugated SOD for 30 min after equilibration step and dose response curve to ET-1 was generated in the presence of PEG-SOD. In order to determine whether the augmented contractility is specific for ET-1, in a subset of patients (n=6), contractile response to 1 $\mu$M phenylephrine (PE) was assessed. For vascular relaxation studies, after washing and equilibration for at least 30 min, rings were precontracted with 10 nM ET-1 for 30 min followed by a dose response curve for Ach (1 nM-10 $\mu$M). The vasodilatation induced was expressed as % relaxation of ET-1-generated contractile response. Similar to ET-1 dose response curve experiments, vasorelaxation experiments were performed in the presence of PEG-SOD as described above.

**DHE fluorescence.** *In situ* superoxide generation was evaluated in vascular cryosections with the oxidative fluorescent dye dihydroethidium (DHE). Cryosections (16 $\mu$m) were incubated with DHE (2 $\mu$mol/L) in PBS, with or without PEG-SOD (150 U/ml), which was added 30 min. prior to staining. Fluorescence images were obtained with a BioRad MRC 1024 scanning confocal microscope. For each slide, at least five images from different sections of the slide were captured and average staining intensity with Metamorph Software was calculated.

**NADPH oxidase activity.** The tissue was stored at - 80º till it was pulverized using
liquid nitrogen, and further homogenized in Tris-HCl buffer (50mM Tris-HCl, 1mM phenylmethylsulfonyl fluoride (PMSF), 1µg/ml aprotinin, 1µg/ml leupeptin, 1µg/ml pepstatin). The homogenate was centrifuged for 20 min at 4000 rpm and 4 °C. The supernatant was further centrifuged in an ultracentrifuge for 45 min at 60,000 rpm (g) at 4 °C. The supernatant was saved as the cytosolic fraction and the pellet was resuspended in 100 µl buffer as the particulate membrane fraction. Superoxide was measured using luminol derivative L-012, which has been recently shown to be more sensitive than lucigenin and not to undergo redox recycling (Daiber et al., 2004). Briefly, 10 µg total protein (cytosolic or membrane fraction) was added to a total volume of 300 µl Krebs–Hepes buffer supplemented with 10 µM NADH, 200 µM L-012 and 100 µM sodium orthovanadate in a 96 well microplate luminometer. Background was read for 5 min before adding the protein. The luminometer was programmed to read each well for 5-10 sec per min in 15 minutes. Counts normalized with background subtraction were expressed as relative light units per minute per µg protein (RLU/min.µg). Because L-012 is light sensitive all work with L-012 was done in the dark. The specificity of L-012 for \( \cdot O_2^- \) was confirmed by addition of SOD (150 U/ml), which decreased the luminescence to background levels in cell free experiments as well as in measurements with tissue homogenates.

**Measurement of ET-1.** The amount of ET-1 in the plasma was determined using an enzyme-linked immunoassay kit (ELISA), specifically designed for direct measurement of plasma ET-1 (American Research Products, Belmont, MA) as we previously described (Ergul et al., 1996).
**Western analysis.** Protein levels and membrane translocation of cytoplasmic NAD(P)H oxidase subunits p47phox and p67phox (Santa Cruz Biotechnology, Inc.) were determined by immunoblotting using specific antibodies. Vascular extracts (20 µg) were diluted to the appropriate loading concentration in sample buffer containing 0.1 M Tris-HCl, 4% SDS and 0.01% bromophenol blue, and loaded onto a 10% SDS-polyacrylamide gel. Samples were then separated at 40 mA using a Tris-glycine running buffer (0.2 M Tris-base, 0.2 M glycine, pH 6.8, and 0.1% SDS). The separated samples were transferred to a nitrocellulose membrane in Tris-glycine transfer buffer supplemented with 20% methanol. The immunoblots were blocked for 1 h in blocking grade powdered goat milk (5%) diluted in 0.2M Tris-base, 1.4 M NaCl, 0.1% Tween 20 and 0.02% NaN₃, followed by incubation with the primary antibodies for 24 h for p47phox and 48 h for p67phox. After incubation with the secondary antibody (goat anti rabbit IgG from Santa Cruz Biotechnology Inc), the bands corresponding to p47phox or p67phox were visualized using Supersignal West Pico chemiluminescent substrate development kit. Equal protein loading in each gel was verified by immunoblotting for beta-actin.

**Data analysis.** The immunoblots were analyzed by densitometric scanning. The data were compared by multiple ANOVA or by Student’s t-test where appropriate. Results are given as mean ± SEM. An alpha level of p<0.05 was considered to be statistically significant.
Results

Altered vascular reactivity of CABG conduits in diabetes. The ability to contract and relax in response to agonist stimulation is an important determinant of the performance of CABG conduits. Since ET-1 is the most potent contractile agent and is also elevated during and after CABG surgery, we investigated the contractile response in SV conduits obtained from diabetic (n=15) and nondiabetic (n=15) patients. As shown by the data on Figure 1 and Table 2, maximum response to ET-1 was significantly higher in the diabetic group (167 ± 9 %) than in the nondiabetic group (124 ± 7 %) without a significant difference in the sensitivity (EC_{50}) to ET-1 (48 ± 11 nM vs 43 ± 12 nM). In a subset of experiments (n=7), preincubation of bypass conduits with PEG-SOD reduced the maximum contraction to ET-1 from 167 ± 9% to 139 ± 5% and 124 ± 7% to 112 ± 3% in the diabetic and nondiabetic groups, respectively, and this was significant in the diabetic group (p<0.05). Maximum response to PE (% contraction of 70 mM KCl) was slightly higher in the diabetic group (133 ± 11 vs 112 ± 9 %) and preincubation with PEG-SOD did not affect the response.

Impaired vascular relaxation is a well-described phenomenon of endothelial dysfunction in diabetes. In order to determine whether and to what extent the dilatory response of SV conduits is blunted in diabetes, endothelium-dependent vasorelaxation was assessed using Ach. Maximum relaxation (R_{max}) was significantly lower in the SV specimens obtained from diabetic patients (46% vs 70% in nondiabetic patients). Presence of PEG-SOD significantly improved the vasorelaxation response in diabetics (Fig. 2).
Increased basal and ET-1-mediated vascular \( \cdot \)O\(_2\)\(^{-} \) production in diabetes. Since the vascular reactivity experiments provided evidence for involvement of \( \cdot \)O\(_2\)\(^{-} \) in vascular dysfunction in diabetes, \( \cdot \)O\(_2\)\(^{-} \) production was determined using DHE fluorescence. The basal \( \cdot \)O\(_2\)\(^{-} \) levels were significantly higher in the diabetic group as evidenced by increased DHE fluorescence (Fig. 3), which was inhibited by the application of PEG-SOD providing evidence that DHE staining is due to \( \cdot \)O\(_2\)\(^{-} \). In light of the results of the vascular reactivity experiments showing that \( \cdot \)O\(_2\)\(^{-} \) augments ET-1-mediated vasoconstriction, effect of ET-1 on \( \cdot \)O\(_2\)\(^{-} \) production was also investigated. Vascular rings were stimulated with 100 nM ET-1 for 30 min, which caused a significant increase in \( \cdot \)O\(_2\)\(^{-} \) formation in both groups (Fig. 3B). Lower concentrations of ET-1 (10 nM) also increased DHE fluorescence by 27% in the diabetic group (data not shown).

To further investigate sources of \( \cdot \)O\(_2\)\(^{-} \) generation in bypass conduits, NAD(P)H oxidase activity in the cytosolic and membrane fractions of vascular homogenates was examined using chemiluminescent dye L-012. Basal superoxide production in the membrane fractions in nondiabetic and diabetic groups was 5,380± 940 and 16,362 ± 4,551 RLU/min.µg, respectively (Fig. 4A). Presence of nonspecific (100 µM diphenyleneiodonium-DPI) and relatively specific (30 µM apocynin) inhibitors of NAD(P)H attenuated \( \cdot \)O\(_2\)\(^{-} \) generation by approximately 50%. Interestingly, L-NNA inhibited O\(_2\)\(^{\cdot} \) by 33% in diabetic but not in nondiabetic samples suggesting that NOS, which may be uncoupled in the diabetic specimens, contributes to O\(_2\)\(^{\cdot} \) generation. The \( \cdot \)O\(_2\)\(^{-} \) scavenger tiron (10 mM) reduced NAD(P)H oxidase-mediated increase in \( \cdot \)O\(_2\)\(^{-} \) levels in the diabetic group to comparable levels in the nondiabetic group. As expected
NAD(P)H oxidase activity in the cytosolic fractions was only slightly higher than background levels (~150-200 RLU/min.µg) in both diabetic or nondiabetic groups.

In order to determine the effect of ET-1 on NAD(P)H oxidase activity, SV samples (n=5/group) were incubated with 100 nM ET-1 for 30 min and oxidase activity was then measured. As shown in Figure 4B, ET-1 had no effect on membrane NAD(P)H oxidase activity in either group. ET-1 stimulation did not alter the low cytosolic oxidase activity in either group.

Plasma ET-1 levels. Systemic ET-1 levels in the preoperative period were significantly higher in the diabetic group (Fig. 5). In order to examine the relationship between ET-1 levels and basal superoxide production, linear regression analysis was performed. A positive linear relationship was observed between plasma ET-1 and NAD(P)H activity with r=0.72 and p<0.001. However, incubation of specimens with a combination of ETA (BQ-123) and ETB (BQ-788) antagonists did not have an effect on basal NAD(P)H activity in either group (data not shown).

Tissue levels and translocation of NAD(P)H oxidase subunits in diabetes. NAD(P)H oxidase consists of 2 membrane, p22phox and p91phox (nox-1 or nox-4 homologs in vascular smooth muscle cells) and 3 cytosolic subunits, p40phox, p47phox and p67phox, which are translocated to the membrane upon activation. Therefore, tissue levels of p47phox and p67phox subunits were assayed in the cytosolic and pellet (membrane) fractions in order to determine whether ET-1 promotes translocation of these subunits and thereby activation of NAD(P)H oxidase (Fig. 6). The p47phox subunit was detected primarily in the cytosolic fraction and protein levels were slightly higher in the diabetic group. ET-1 stimulation increased protein levels in the cytosolic
fraction but did not promote the translocation of this subunit to the membrane. Bands corresponding to p67phox subunit were detected in the cytosolic fractions but higher molecular weight bands around 90 kDa (possibly p22phox and p67phox complex) were also found in the membrane fractions. There was no difference in the protein expression level of this subunit between the study groups. Furthermore, ET-1 stimulation (100 nM for 30 min) did not affect the protein expression nor the translocation to the membrane.
Discussion

Major findings of this study are that ET-1 causes increased contraction as well as impaired vasorelaxation of saphenous vein conduits and excess generation of ROS contributes to ET-1-mediated vascular dysfunction. We also identify that diabetic patients have increased basal $\cdot O_2^-$ production in bypass conduits, which is associated with increased plasma ET-1 levels. While NAD(P)H oxidase plays an integral role in $\cdot O_2^-$ production under basal conditions, ET-1-stimulated $\cdot O_2^-$ generation in vitro does not appear to involve activation of NAD(P)H oxidase. These results suggest that ET-1 mediates vascular dysfunction of saphenous vein conduits and provide important evidence suggesting that redox-sensitive mechanisms may contribute to increased complication rates observed in diabetic CABG patients.

ET-1, a potent vasoactive peptide, has been shown to mediate vasoconstriction of the pulmonary and systemic circulation and influence myocardial contractility (Wenzel et al., 1998; Fleisch et al., 2000). All these effects are relevant to patients undergoing CABG. Bond and colleagues reported that circulating ET-1 levels increase during CABG surgery in a biphasic manner (Bond et al., 2001). Furthermore, prolonged pharmacologic management and lengthened intensive care unit are associated with elevated plasma ET-1 and increased bypass conduit sensitivity to ET-1 (Dorman et al., 2000). However, whether and to what extent ET-1 contributes to vascular dysfunction in high-risk patients remained unknown. In light of the observations that increased ET-1 levels are associated with diabetic complications (Hattori et al., 1991) and that diabetic patients are more prone to postoperative complications (Herlitz et al., 2000), we specifically investigated plasma ET-1 levels and ET-1-mediated graft reactivity in
diabetic patients. Results of this study demonstrate that vasoconstrictor response to ET-1, not to PE, is heightened in the saphenous vein specimens from diabetic patients. Incubation of vessels with O$_2^{-}$ scavenger PEG-SOD improved contractile response providing evidence that O$_2^{-}$ augments ET-1-mediated vasoconstriction. Vasorelaxation capacity of graft conduits is equally important since many CABG patients require vasodilator treatment. In the current study, we demonstrate that endothelium-dependent vasorelaxation of bypass conduits preconstricted with ET-1 is impaired in diabetic patients via excess generation of O$_2^{-}$. Although the impact of altered conduit reactivity on postoperative outcome measures was not studied, increased ROS may influence vascular function by several pathways. First, O$_2^{-}$ immediately reacts with NO generating ONOO$^{-}$ and reducing NO bioavailability. Since NO is one of the major pathways involved in vasorelaxation, this may directly impair the dilator response in patients with diabetes. Second, ONOO$^{-}$ can oxidize tetrahydrobiopterin (BH$_4$), an important cofactor for eNOS, and this will lead to decreased NO and increased superoxide formation by eNOS. Third, ONOO$^{-}$ may cause nitration of downstream proteins involved in vasorelaxation such as large conductance Ca$^{2+}$-activated (BK$_{Ca}$) and ATP-dependent (K$_{ATP}$) potassium channels. Liu et al. showed that ONOO$^{-}$ inhibits BK$_{Ca}$ channels resulting in reduced vasorelaxation of coronary arterioles and this reduced activity is associated with strong staining for nitro tyrosine in this vascular bed (Liu and Gutterman, 2002). Another group recently reported that vasodilator responses to K$^{+}$ channel openers are reduced in two experimental models of insulin resistance and increased O$_2^{-}$ production is responsible for impaired relaxation (Erdos et al., 2004).
Therefore future studies are needed to specifically investigate the regulation of K⁺ channels by ROS in human bypass graft conduits.

Vascular sources that augment reactive species generation include NAD(P)H oxidase, xanthine oxidase, NOS, lipoxygenases and mitochondrial respiratory chain enzymes (Guzik et al., 2000; Brownlee, 2001; Guzik et al., 2002; Aliciguzel et al., 2003). NAD(P)H oxidase, a membrane associated enzyme, is a major source of •O₂⁻ production in the vasculature (Guzik et al., 2000; Guzik et al., 2002; Etoh et al., 2003; Kitada et al., 2003; Endemann and Schiffrin, 2004). Similar to neutrophil (phagocytic) oxidase, vascular enzyme is also composed of multiple subunits (Taniyama and Griendling, 2003). Cytoplasmic subunits (p40phox, p47phox and p67phox) are translocated to the membrane where they associate with the membrane subunits p22phox and gp91phox (or homologs nox1 and nox4) to form the active enzyme. There is recent evidence that novel homologues of p47phox (p41nox or NOXO1) and p67phox (p51nox or NOXA1) can substitute for p47phox and p67 phox (Banfi et al., 2003; Takeya et al., 2003; Griendling, 2004). Guzik et al. demonstrated that there is enhanced production of NAD(P)H oxidase-mediated •O₂⁻ in the bypass conduits (both internal mammary artery and saphenous veins) obtained from patients with diabetes and this is accompanied by increased expression of the subunit proteins (Guzik et al., 2000; Guzik et al., 2002). In contrast to our and Guzik's findings, Huraux and colleagues reported no differences in superoxide levels or vasorelaxation in internal mammary artery segments obtained from patients with identified cardiovascular risk factors (Huraux et al., 1999) but they did not specifically investigate the effect of diabetes, which may explain the difference in their findings. Results of our current study confirm that NAD(P)H oxidase
activity is significantly higher in bypass conduits obtained from diabetic patients. Apocynin inhibits \( \cdot O_2^- \) generation by blocking the subunit association of NADPH oxidase but if the subunit complex is already formed, it does not inhibit free radical formation. Since basal levels were measured without any stimulation, we suspect there is significant NADPH complex already formed and that is why apocynin and DPI failed to inhibit NAD(P)H oxidase activity completely. Consistent with increased basal activity, we detected complexes of cytoplasmic and membrane subunits in the membrane fractions of vascular homogenates. We also found that plasma ET-1 levels are significantly higher in the diabetic patients and this is associated with enhanced superoxide production in diabetic patients. However, incubation of vessels with mixed ET receptor antagonists did not reduce the increased basal activity detected in the specimens obtained from diabetic patients. One possible explanation is that elevated ET-1 levels might have already stimulated downstream targets of ROS production in diabetes and thus blockade of ET receptors \textit{in vitro} does not have an effect on basal NAD(P)H oxidase activity.

Based on our results with modulation of ET-1-mediated vascular reactivity by \( \cdot O_2^- \) and elevated plasma ET-1 levels in diabetic patients undergoing CABG, we asked whether ET-1 stimulates ROS generation in graft conduits and identify the mechanisms involved. DHE staining of SV specimens stimulated with ET-1 demonstrated increased \( \cdot O_2^- \) production in both patient groups, which was more pronounced in the diabetic group. Li et al demonstrated that ET-1 increases vascular \( \cdot O_2^- \) generation via NAD(P)H oxidase (Li et al., 2003). Based on the findings by Guzik (Guzik et al., 2000; Guzik et al., 2002) and Li, we hypothesized that ET-1 induces \( \cdot O_2^- \) production by promoting the
translocation of p47phox and p67phox cytoplasmic subunits of NAD(P)H oxidase to the membrane compartment and activating the oxidase as seen by angiotensin II (Ang II) stimulation (Touyz et al., 2002). In order to test this hypothesis, we measured oxidase activity in SV specimens stimulated with ET-1 as well as determining the protein levels of p47phox and p67phox in cytoplasmic and membrane fractions prepared from bypass conduits stimulated with ET-1. To our surprise, there was no change in NAD(P)H oxidase activity in ET-1-stimulated tissue in either the diabetic or nondiabetic group. ET-1-stimulation also failed to cause a shift of p47phox and p67phox from the cytoplasm to membrane. In light of the recent evidence that NOXO1 and NOXA1 can substitute for p47phox and p67phox, respectively, one possibility is that ET-1 may stimulate these novel homologues and not conventional cytoplasmic subunits of NAD(P)H oxidase. Due to lack of specific antibodies for these homologues, we could not investigate this possibility. A recent study by Touyz et al reported that Ang II and ET-1 regulate MAP kinases through different redox-dependent pathways in human vascular smooth muscle cells (Touyz et al., 2004). While Ang II induces NAD(P)H oxidase-mediated ROS, ET-1 predominantly stimulates the generation of mitochondrial-derived superoxide suggesting that mechanisms of ET-1-mediated $\cdot$O$_2$ production might be different in human tissue and in disease states. These results provide support for our findings and warrant future studies focusing on the role of mitochondria in ET-1-mediated ROS generation.

Limitations of this study must be recognized. All patients had coronary artery disease. Although vascular tissue specimens used in this study were considered relatively healthy and utilized for bypass conduits, we cannot differentiate the impact of coronary artery disease on these ROS-generating systems in our study population.
Second, we only evaluated saphenous vein conduits since the amount of internal mammary artery, another commonly used bypass conduit, is quite limited. Third, all the patients were receiving pharmacotherapy for comorbid conditions. Although medications, age and comorbid conditions appear to be similar in both groups, in the diabetes group there were more African American patients and the number of subjects in each group is small for multivariate analyses. Complex drug interactions, ethnic differences and effects of concurrent diseases on oxidative stress markers remain potential confounding factors. Nevertheless, the findings of this study demonstrate that ET-1 alters bypass conduit reactivity in part by stimulating excess formation of ROS in diabetes. This study also provides novel evidence that ET-1 induces $^\cdot$O$_2^-$ via a non-NAD(P)H dependent mechanism in the venous grafts. While acute modulation of the tone of bypass graft conduits by $^\cdot$O$_2^-$ may be critical for the pharmacological management of postoperative vasospasm, long-term consequences may be increased proliferative response to vascular injury as shown in an experimental venous bypass grafts (West et al., 2001). Since the long-term results of CABG are limited by stenosis and subsequent occlusion of SV grafts, the most commonly utilized conduit for these operative procedures (Angelini and Newby, 1989; Cox et al., 1991; Bryan and Angelini, 1994; Davies and Hagen, 1995; Davies et al., 1998), ET receptor antagonism may offer a therapeutic benefit especially in high-risk diabetic patients.
References

oxidoreductase and antioxidant enzymes in different tissues of diabetic rats. *J Lab
Clin Med* **142**:172-177.

Angelini GD and Newby AC (1989) The future of saphenous vein as a coronary artery


Bond BR, Dorman BH, Clair MJ, Walker CA, Pinosky ML, Reeves ST, Walton S, Kratz
during and after cardiopulmonary bypass: association to graft sensitivity and


Bryan AJ and Angelini GD (1994) The biology of saphenous vein graft occlusion:

30-day mortality after coronary artery bypass grafting in patients with versus without
diabetes mellitus. Israeli Coronary Artery Bypass (ISCAB) Study Consortium. *Am J
Cardiol* **81**:7-11.

Cox JL, Chiasson DA and Gotlieb AI (1991) Stranger in a strange land: the
pathogenesis of saphenous vein graft stenosis with emphasis on structural and


Footnotes

This work was supported by grants to Adviye Ergul from American Heart Association Scientist Development Grant, American Diabetes Association and NIH (R15HL076236).
FIGURE LEGENDS

**Fig. 1.** Cumulative dose-response curves to ET-1 in saphenous vein bypass conduits obtained from diabetic and nondiabetic patients. Vasoconstrictor response to ET-1 is increased in diabetic patients (n=15) and incubation of conduits with *O₂⁻* scavenger PEG-SOD (n=7) partially attenuates augmented vasoconstriction. Results are given as mean ± SEM. **p<0.001 diabetic vs nondiabetic, *p<0.05 basal vs + PEG-SOD.**

**Fig. 2.** Cumulative dose-response curves to Ach in saphenous vein bypass conduits obtained from diabetic and nondiabetic patients. Endothelium-dependent vasorelaxation response is impaired in diabetic patients and incubation of conduits with *O₂⁻* scavenger PEG-SOD restores the dilator ability to levels observed in nondiabetic patients. *p<0.05 diabetic vs nondiabetic or diabetic + PEG-SOD.

**Fig. 3.** (A) Representative confocal microscopy images of SV conduits obtained from diabetic and nondiabetic patients with or without ET-1 stimulation and (B) image analysis of all cases (n=6 in each group). Fluorescent intensity is markedly increased in all layers by ET-1 stimulation and this effect is more prominent in the diabetes group. +PEG-SOD denotes serial DHE-stained sections incubated with PEG-conjugated SOD (150 U/ml). **p<0.001 diabetic vs nondiabetic or diabetic + ET-1.**

**Fig. 4.** (A) Measurement of NAD(P)H oxidase-mediated superoxide generation in the particulate fractions of conduit homogenates in the absence and presence of oxidase
inhibitors apocynin and DPI, NOS inhibitor L-NNA and superoxide scavengers tiron and PEG-SOD (n=15/per group). (B) Effect of ET-1 on conduit \( \cdot O_2^\cdot \) production. Vessels were treated with ET-1 (100 nM) for 30 min and then homogenized. 10 µg membrane protein was added to Kreps–Hepes buffer supplemented with 10 µM NADH, 200 µM L-012 and 100 µM sodium orthovanadate and luminescence was recorded per second for 15 min. Counts normalized with background subtraction were expressed as RLU/min.µg.

\*p<0.05 diabetic vs nondiabetic or tiron.

Fig.5. Plasma ET-1 levels were significantly higher in diabetic CABG patients as compared to nondiabetic patients (n=15/group, p<0.05). Linear regression analysis of plasma ET-1 and basal NAD(P)H activity shown in Fig. 4 indicated a positive correlation with r=0.72 and p<0.001.

Fig. 6. (A) A representative immunoblot of cytoplasmic subunits p47phox and p67phox in bypass conduits stimulated with ET-1. Cytoplasmic (C) and particulate (P) fractions prepared from SV specimens obtained from diabetic and nondiabetic CABG patients were separated on SDS-PAGE and blotted with antibodies against p47phox or p67phox. Densitometric analyses of all the specimens (n=4) analyzed for p47phox protein expression are summarized in panel B.
Table 1. Patient demographics and list of medications

<table>
<thead>
<tr>
<th></th>
<th>Diabetic</th>
<th>Nondiabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>59 ± 2.2</td>
<td>56 ± 2.5</td>
</tr>
<tr>
<td>Race (African-American/Caucasian)</td>
<td>9/9</td>
<td>1/16</td>
</tr>
<tr>
<td>Sex (Female/Male)</td>
<td>8/10</td>
<td>5/12</td>
</tr>
<tr>
<td>Blood glucose (mg/dL mean ± SD)</td>
<td>174 ±16</td>
<td>105 ±18</td>
</tr>
<tr>
<td>Body surface area (m², mean ± SD)</td>
<td>2 ±0.1</td>
<td>2 ±0.3</td>
</tr>
<tr>
<td>SBP (mm-Hg)</td>
<td>146 ± 7</td>
<td>142 ± 7</td>
</tr>
<tr>
<td>DBP (mm-Hg)</td>
<td>75 ± 5</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>127 ± 13</td>
<td>129 ± 8</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>42 ± 3</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca²⁺ channel blockers</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>ACE inhibitors/ All receptor blockers</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>β-blockers</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Cholesterol lowering drugs</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Diuretics</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Antiaggreant therapy</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>Antidepressant therapy</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Vasodilators</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Oral agents/insulin</td>
<td>8/14</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2. Sensitivity and maximum response (% contraction or relaxation) in venous bypass conduits of diabetic and nondiabetic patients

<table>
<thead>
<tr>
<th></th>
<th>Diabetic</th>
<th>Nondiabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC₅₀ (nM)</td>
<td>Maximum response</td>
</tr>
<tr>
<td>Contraction (% 70 mM KCl contraction)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1 contraction</td>
<td>48 ± 11</td>
<td>167 ± 9*</td>
</tr>
<tr>
<td>ET-1 + PEG-SOD contraction</td>
<td>63 ± 20</td>
<td>139 ± 5**</td>
</tr>
<tr>
<td>Relaxation (% 10 nM ET-1 contraction)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ach relaxation</td>
<td>65 ± 10</td>
<td>46 ± 6***</td>
</tr>
<tr>
<td>Ach + PEG-SOD relaxation</td>
<td>113 ± 13</td>
<td>68 ± 4***</td>
</tr>
<tr>
<td>KCl contraction (mN)</td>
<td>10.3 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.001 diabetic vs nondiabetic, **p<0.05 diabetic vs diabetic + PEG-SOD, ***p<0.05 diabetic vs nondiabetic or diabetic+ PEG-SOD
Fig. 1

Maximum contraction (% 70 mM KCl)

- ○ Diabetic
- △ Nondiabetic
- ● Diabetic + PEG-SOD
- ▲ Nondiabetic + PEG-SOD

ET-1 (Log M)

*,**
Fig. 2
A.

Diabetic

B.

Nondiabetic

Fig. 3
This article has not been copyedited and formatted. The final version may differ from this version.
Fig. 5
Fig. 6

Nondiabetic

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>+ ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>p47phox</td>
<td>C, P</td>
<td>C, P</td>
</tr>
<tr>
<td>p67phox</td>
<td>C, P</td>
<td>C, P</td>
</tr>
</tbody>
</table>

Diabetic

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>+ ET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>p47phox</td>
<td>C, P</td>
<td>C, P</td>
</tr>
<tr>
<td>p67phox</td>
<td>C, P</td>
<td>C, P</td>
</tr>
</tbody>
</table>

Optical density (pixels)

- p47phox
- p67phox

- Nondiabetic
- Diabetic