Elevated Endothelin-1 Levels Impair Nitric Oxide Homeostasis Through a PKC-Dependent Pathway

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Background—Endothelin-1 (ET-1) plays an important role in the maintenance of vascular tone and pathological states such as ischemia/reperfusion (I/R) injury, coronary vasospasm, and cardiac allograft vasculopathy. We assessed the effects of elevated ET-1 levels as seen after I/R to determine if ET-1 modulates nitric oxide (NO) production via the translocation of specific protein kinase C (PKC) isoforms.

Methods and Results—Human saphenous vein endothelial cells (HSVECs) (n=8) were incubated with ET-1 or phosphate-buffered saline (PBS) for 24 hours. NO production was determined in the supernatant by measuring nitrate/nitrite levels. Protein expression of endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), caveolin-1 and PKC were determined. Lastly, PKC translocation and activity were assessed after exposure to the drug of interest. HSVECs exposed to ET-1 displayed decreased NO production. PKC inhibition reduced NO production, whereas PKC activation increased production. NO production was maintained when HSVECs exposed to ET-1 were treated with the PKC agonist, PMA. eNOS protein expression was reduced after ET-1 treatment. PKC inhibition also downregulated eNOS protein expression, whereas PMA upregulated expression. ET-1 exposure led to a significant increase in PKCδ and PKCe translocation compared with control, whereas translocation of PKCa was inhibited. ET-1 exposure significantly reduced overall PKC activity compared with control.

Conclusions—Our study demonstrates that high levels of ET-1 impair endothelial NO production via an isoform-specific PKC-mediated inhibition of eNOS expression. ET-1 antagonism with bosentan stimulates translocation of PKCα and leads to increased PKC activity and NO production. ET-1 antagonism may provide a novel therapeutic strategy to improve vascular homeostasis. (Circulation. 2006;114[suppl I]:I-319–I-326.)

Key Words: endothelin-1 ■ endothelial dysfunction ■ nitric oxide ■ protein kinase C

The endothelium plays a key role in vascular homeostasis through the release of autocrine and paracrine substances. In addition to vasodilation, a healthy endothelium is anti-atherogenic because of its inhibition of platelet aggregation, smooth muscle cell proliferation, and leukocyte adhesion. Endothelial dysfunction is the initiator of several pathological states such as atherosclerosis, transplant coronary artery disease, and primary cardiac allograft dysfunction. Local vascular control depends on a balance between endothelium-derived vasodilators and constrictors. Endothelial dysfunction is a systemic process that develops in response to chronic inflammation, ischemia, and reperfusion and other risk factors. It is manifested by a diminished production/availability of nitric oxide (NO) with coexisting alterations in endothelium-derived contracting factors such as endothelin-1 (ET-1). Such an imbalance predisposes the vasculature to increased tone, altered geometry/ remodeling, thrombosis, inflammation, oxidation, and proliferation. Evidence suggests that impaired NO release precedes the development of atherosclerosis and serves to reinforce vascular pathology once established. NO is the most important vasodilator and maintains basal vascular smooth muscle tone and resistance to atherosclerosis. In addition to being the main determinant of tone, NO opposes the actions of potent endothelium-derived constrictors such as ET-1, angiotensin (A), and reactive oxygen species.

ET-1 is one of the most potent endogenous vasoconstrictors and mediates a host of responses including endothelial dysfunction, vasomotor contraction, leukocyte activation and cellular proliferation. Additionally, it augments the vascular actions of other vasoactive substances such as A-II, norepinephrine, and serotonin. ET-1 exerts its biological effects via 2 endothelin receptors (ETα and ETβ). Diminished production of NO and exaggerated release of ET-1 are believed to be key initiators of endothelial injury. Several investigators have demonstrated that ET-1 adversely affects endothelial function as well as outcomes after cardiac surgery. 3,7–10 We have previously shown that ET-1 results in
endothelial dysfunction after cardiac transplantation and antagonism with Bosentan abrogated this effect. Previous investigators have demonstrated that ET-1 modulates endothelial nitric oxide synthase (eNOS) expression as well as NO production. Elevated ET-1 levels have also been implicated in the development of transplant coronary disease.

We hypothesized that elevated levels of ET-1 impair endothelial cell NO production via a PKC-mediated change in eNOS expression and activity. We also propose that Bosentan, an ET-1 antagonist, can prevent ET-1 induced injury and restore normal vascular homeostasis.

Methods
All authors have read and agree to the manuscript as written. The authors had full access to the data and take full responsibility for their integrity.

Endothelial Cell Cultures
Cell culture experiments were performed on human saphenous vein endothelial cells (HSVECs) cultured in 10-cm diameter dishes at 37°C and 5% CO2 in medium MCDB-131 (VEC Technologies), containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells passed 2 to 4 times and aged between 14 to 30 days from the time of primary culture were used for this study. We examined the effects of our interventions on eNOS/inducible NOS (iNOS)/caveolin-1 expression, NO production, isoform specific PKC translocation, and PKC activity. Cells were treated with 100 nM of ET-1 (Sigma, Canada), Bosentan (10 μmol/L, courtesy of Actelion Pharmaceuticals Ltd, Switzerland) or ET-1 + Bosentan (n = 16 per group) for 30 minutes or 24 hours. Cells treated with inactive vehicle (0.1% DMSO or phosphate-buffered saline) were used as controls. The dose of ET-1 was chosen to completely bind the ETα and ETβ receptors and is well above postischemic levels previously demonstrated in either the myocardial interstitium or plasma.

To modulate the effects of PKC, we used 2 antagonists with different mechanisms of action, calphostin C (200 nM), and chelerythrine (1 μM) (Sigma, Canada). To stimulate PKC activity, the PKC agonist Phorbol 12-myristate 13-acetate (PMA) (10 nm) (Sigma, Canada) was used.

NO Production
Cell culture supernatants were collected at 30 minutes and 24 hours after treatment. NO production was detected spectrophotometrically by measuring its final stable equimolar degradation products, nitrite and nitrate. Total nitrite was quantified after the reduction of all nitrates with nitrate reductase. After the conversion of nitrate to nitrite, total nitrite was determined spectrophotometrically at 540 μM by using the Griess reaction. Total nitrite concentration was calculated from a standard curve constructed over the linear range of the assay and expressed as μmol/L per milligram protein. The amount of NO produced was normalized against total cellular protein and assessed using the Bradford method.

Protein Expression
Western immunoblotting was performed using chemiluminescence detection. The expression of iNOS, eNOS, and caveolin-1 were determined with the use of protein-specific monoclonal antibodies (BD Biosciences, Canada). Samples were separated using 4% stacking and 10% running tris-glycine sodium dodecyl sulfate-polyacrylamide electrophoresis gels. Gels were then transferred to polyvinylidene fluoride membranes. Blocking was performed for 1 hour at room temperature. The blots were stained with monoclonal IgG at a dilution of 1:2500 for 12 hours at 4°C, washed, and then incubated with secondary antibody for 1 hour at room temperature. Comparisons between groups were performed using densitometric analysis corrected for β-actin expression. No differences in β-actin protein expression were seen between groups at any time point.

PKC Translocation/Activity and eNOS Translocation
Determination of eNOS and isoform specific PKC translocation was performed by analysis of cytosolic and membrane fractions of HSVECs to calculate a membrane-to-cytosolic (M/C) ratio. After treatment for 30 minutes or 24 hours, cells were collected with 1 mL of ice-cold TRIS-buffered saline containing protease inhibitors. Harvested cells were then centrifuged for 10 minutes at 4°C. After centrifugation, the cells were resuspended in TRIS-buffered saline, sonicated on ice, and re-centrifuged for 10 minutes as above. The resultant supernatant represented the soluble cytosolic fraction. The pellet was resuspended in cytoskeletal buffer (150 mmol/L NaCl, 30 mmol/L HEPES, 1 mmol/L PMSF, 5 mmol/L EDTA, and 1% Triton X-100) and centrifuged to yield the membrane fraction in the supernatant. Both fractions were divided into 2 aliquots (one for protein determination and the other for Western blot analysis) and then frozen in liquid nitrogen and stored at −80°C until ready for analysis. Purity of the cytosolic and membrane fractions was determined by confirming the absence of membrane bound Ca2+/ATPase (cysotolic) or β-actin (membrane). Our preliminary studies indicated that only the α, δ, ε, γ, and α PKC isoforms are present in this cell population. Therefore, each blot was stained with these isoform specific antibodies. Blots were then scanned using a commercially available software program (BioRad, Calif) and each band assessed densitometrically.

Similarly, for measurement of PKC activity, cells were harvested as described after the treatment of interest. After centrifugation for 5 minutes at 100 000g, cells were washed in 50 μL of TRIS-buffered saline twice. Cells were sonicated on ice and extracted. After sonication, cell extracts were processed and then added to a commercially available PKC assay kit (Calbiochem, Calif) to determine protein-adjusted activity. Activity was determined colorimetrically at 490 nm and is displayed as arbitrary units per mg of protein (U/mg).

Statistical Analysis
All results are presented as mean±SD. Statistical comparisons were made by analysis of variance (ANOVA) followed by Dunn’s multiple contrast test to identify differences between various treatments or by the Mann-Whitney U test for nonparametric data. Exact probability values are provided for each comparison.

Results
Endothelin-1 Attenuates NO Release
Figure 1 depicts the dose-response effect of ET-1 on HSVEC NO production in culture medium after 24 hours of exposure to treatment. Basal NO production was unaffected at low concentrations of ET-1, whereas exposure to 1 nM of ET-1 significantly increased NO production (P<0.01). In contrast, higher doses of ET-1 (≥1 nM) led to a reduction in total nitrite levels. (P<0.01).

Figure 2 depicts the effect of our treatments on NO production after 30-minute or 24-hour exposure. HSVECs
exposed to ET-1 (100 nM) for 30 minutes resulted in a slight but significant reduction in total nitrite levels compared with control, whereas the 24-hour treatment resulted in a 42% reduction (P<0.001). Bosentan treatment attenuated the effect of ET-1 on NO production. HSVECs exposed to Bosentan alone demonstrated a significant increase in NO production after 30 minutes of treatment with an increase of ~20% after 24 hours. (P<0.001).

**Effect of PKC on Nitrate/Nitrite Production**
Exposure to the PKC inhibitors calphostin C and chelerythrine significantly reduced NO release from HSVECs (P<0.001) after 30 minutes with a further reduction after 24 hours (Figure 2). Co-incubation of ET-1 with PKC inhibitors did not demonstrate any synergistic reduction in NO production. HSVECs treated with the PKC agonist, PMA, demonstrated increased NO release compared with control and significantly abrogated the effects of ET-1 after both 30-minute and 24-hour exposure.

**PKC Isoforms**
We assessed our cell cultures for the presence of several PKC isoforms (α, β, γ, δ, ε, η, θ, λ). Only the α, δ, ε, η, and λ were present in our HSVECs. PKCα was expressed solely on the membrane fraction, whereas PKCε was expressed exclusively in the cytosolic fraction. No significant differences in M/C ratios (translocation to the membrane) were seen between groups for PKCα and PKCε. PKCδ, ε, and λ isoforms demonstrated significant changes after treatment (Figures 3, 4, 5).

ET-1 exposure for 30 minutes resulted in a significant translocation of both PKCδ and PKCε isoforms to the membrane (P<0.05); 24-hour exposure to ET-1 resulted in a further increase in PKCδ and PKCε translocation compared with 30 minutes of exposure (P<0.05; Figures 3a, 3b, 4a, 4b). Examination of the PKCα isoform demonstrated that ET-1 significantly reduced the M/C ratio (suggesting inhibition of translocation) compared with control (P<0.001) (Figure 5a, 5b).

PKC inhibition with calphostin C and chelerythrine resulted in a significantly reduced M/C ratio for PKCδ after 30-minute and 24-hour exposure in control and ET-1–treated cells (Figures 3 to 5). PKC inhibition impaired translocation of PKCα only in the control groups (P<0.05), whereas translocation of PKCε was reduced only in the ET-1 treated group (P<0.05). PKC activation with PMA resulted in translocation to the membrane of PKCα in both the control and ET-1 treatment group (P<0.05), whereas only increasing PKCδ and PKCε translocation in the control group.

The effects of Bosentan on HSVEC PKC translocation were significant. Bosentan demonstrated the ability to abrogate ET-1 induced effects on PKCδ and PKCε translocation at both 30 minutes and 24 hours (P<0.05) (Figures 3, 4). HSVECs exposed to Bosentan with and without ET-1 dem-
ET-1 exposure to ET-1 caused PKC translocation. PKC inhibition for 30 minutes and 24 hours reduced PKC activity at 30 minutes and a greater reduction in activity after 24 hours of exposure. No synergistic effect was seen with ET-1. PMA exposure increased PKC activity and abrogated the ET-1–induced effects (Figure 6).

PKC Activity
Determination of PKC translocation does not necessarily correlate with cellular PKC activity. ET-1 exposure significantly reduced PKC activity compared with control after both 30 minutes and 24 hours (P=0.01) (Figure 6). Bosentan treatment not only blocked the ET-1–induced effect but also resulted in enhanced PKC activity (P<0.05) (Figure 6). HSVECs treated with calphostin C and chelerythrine demonstrated reduced PKC activity at 30 minutes and a greater reduction in activity after 24 hours of exposure. No synergistic effect was seen with ET-1. PMA exposure increased PKC activity and abrogated the ET-1–induced effects (Figure 6).

eNOS, iNOS, Caveolin-1 Expression, and eNOS Translocation
There was no effect observed on eNOS, iNOS and caveolin-1 expression in HSVECs exposed for 30 minutes to ET-1 (Figure 7a). However, 24-hour exposure markedly reduced eNOS protein expression by 46±4% compared with control (P=0.001) (Figure 7b). Furthermore, PKC antagonism showed a significant reduction in eNOS protein expression. No interactive effects were seen with PKC inhibitors and ET-1. PKC activation with PMA did result in a significant upregulation of eNOS protein expression compared with control and completely abolished the ET-1–induced reduction of eNOS expression (Figure 7b). Figure 7b also demonstrates that Bosentan treatment prevented ET-1–induced downregulation of eNOS. Expression of both iNOS and caveolin-1 were unaffected after 24-hour treatment with ET-1 and was similar in all treatment groups (Figure 7a).

eNOS is active when it is in the cytosol and not sequestered by the protein caveolin-1 on the membrane. Table 1 displays the eNOS M/C ratio after treatment with ET-1 and Bosentan. ET-1 resulted in a higher M/C ratio after 30-minute and 24-hour exposure (1.84±0.05, 1.75±0.06) compared with control (1.11±0.001) (P<0.05). Bosentan treatment abrogated ET-1 sequestration of eNOS at both time points.

Discussion
ET-1 plays an important role in normal vessel homeostasis and is associated with adverse outcomes after cardiac surgery.3,7–10 Endothelial dysfunction is implicated in the development of coronary vasospasm, unstable angina, myocardial infarction, atherosclerosis, and transplant coronary disease.3,6,8,13,14,19 The normally functioning endothelium maintains ET-1 and NO in balance. Impairment in NO production and/or increased release of ET-1 are key initiators of endothelial dysfunction and injury.7 Given the importance of a normally functioning endothelium we sought to determine the mechanisms by which high levels of ET-1 impairs NO bioavailability in isolated human endothelial cells.

We have made the following observations: (1) isolated HSVECs exposed to ET-1 demonstrate a dose-dependent change in nitric oxide production. At levels of 10 nM and above, there is a significant reduction in cellular NO production; (2) ET-1 blockade with Bosentan can maintain cellular NO production; (3) PKC inhibition leads to a severe reduction in cellular NO production with no synergistic effect observed with simultaneous exposure to ET-1; (4) PKC stimulation with PMA significantly increases cellular NO production and is capable of completing reversing the inhibitory effects of ET-1 exposure; (5) ET-1 appears to selectively stimulate PKCα and ε translocation while inhibiting λ translocation; and (6) ET-1 exposure results in decreased eNOS expression and an increase in the membrane to cytosolic ratio, suggesting increased binding to membrane-bound caveolin.

Effect of ET-1 on NO Production
Our study demonstrated that ET-1 has a concentration-dependent effect on endothelial cell NO production. Elevated levels of ET-1 resulted in a dose-dependent decrease in NO bioavailability. Although several investigators have reported ET-1 induced changes in NO production, it remains unclear if physiological levels of ET-1 increase or decrease NO bioavailability.12,15,20 Elegant studies by Spinale et al have demonstrated a 4-fold increase in myocardial interstitial ET-1 levels after cardiopulmonary bypass.21 However, even at peak levels postoperatively, myocardial ET-1 levels were only 50 fmol/L and in plasma these levels were <20 fmol/L. At doses of 100 pmol/L, we observed no effect of ET-1 on cellular NO
production. 1 nmol/L resulted in higher NO production, whereas 10 nmol/L inhibited NO production. As in previous studies, a dose of 100 nmol/L was chosen to completely bind both the ET₁ and ET₂ receptors.²²

ET-1 also induced a time-dependent decrease in NO production. As seen in our study, 30 minutes of exposure decreased NO levels compared with control with a greater reduction after 24 hours. The acute effects of ET-1 impairment in NO bioavailability may explain poor outcomes acutely after coronary bypass surgery or cardiac transplantation.⁹ Elevation of ET-1 early after transplantation results in endothelial dysfunction, inability to wean off cardiopulmonary bypass, and allograft failure.⁹ Increases in ET-1 have also been demonstrated after myocardial infarction to increase infarct size.¹⁷,¹⁸ Prolonged exposure to ET-1 with the resultant greater impairment of NO production may lead to atherosclerosis, transplant coronary disease and pulmonary hypertension.³,⁴,¹²–¹⁵,¹⁹,²⁰,²³

Effects of ET-1 on NOS Protein Expression
We found that 24-hour exposure to ET-1 led to decreased eNOS protein expression. Dong et al also demonstrated that elevated ET-1 results in downregulation of eNOS protein expression in endothelial cells and Wedgwood et al demonstrated that high ET-1 concentrations reduce NOS activity and expression in smooth muscle cells.¹²,¹⁵ The inhibition of eNOS expression was abrogated by Bosentan treatment and/or PKC stimulation with PMA. Cellular localization of eNOS after ET-1 exposure appears to be shifted toward the membrane while bosentan treatment appears to preserve eNOS in its active cytoplasmic form. Because caveolin-1 expression is unchanged by ET-1, eNOS translocation is likely caused by increased binding to caveolin-1.

Effects of ET-1 on PKC Activity
We found that NO production is regulated by PKC. We demonstrated that PKC inhibition resulted in a significant reduction in NO bioavailability, whereas PKC activation led to NO release. The concomitant exposure to ET-1 and PKC inhibition did not lead to a synergistic decrease in NO bioavailability as would be anticipated if ET-1 acted in a PKC-independent fashion. The simultaneous exposure of our endothelial cells to PMA and ET-1 increased NO production similar to exposure to PMA alone. Our data support the hypothesis that ET-1 decreases NO production through PKC inhibition. eNOS protein expression was also regulated by PKC. We found that PKC antagonism downregulated eNOS protein expression, whereas PKC agonists upregulated eNOS protein expression. ET-1–induced downregulation of eNOS protein expression was only seen after 24 hours of exposure, indicating that NO impairment seen after 30 minutes of treatment is as a result of decreased eNOS activity and not through changes in protein expression. In addition, concomitant exposure of HSVEC to ET-1 and PMA resulted in upregulation of eNOS to a level similar to PMA alone, further demonstrating that ET-1–induced alterations in eNOS expression is PKC-dependent and is fully reversible by direct PKC stimulation. To determine which isoforms were involved in
ET-1 regulation, we evaluated isoform specific translocation. Our study revealed that ET-1 exposure resulted in membrane translocation of the delta and epsilon isoforms, whereas the M/C ratio of the lambda isoform was reduced suggesting inhibited translocation. Because translocation does not always correlate with activity, we measured PKC activity in our cell cultures. We determined that cellular PKC activity is reduced after ET-1 exposure indicating that PKC\(\lambda\) is the likely isoform that regulates ET-1 effects on NO production. ET-1–induced alterations in NO homeostasis can also be a result of increased caveolin-1 expression, leading to greater eNOS-caveolin-1 interaction and inhibition of eNOS. ET-1 and PKC modulation does not affect caveolin-1 expression. NO production can also be regulated by increasing eNOS–caveolin-1 binding. Table 1 demonstrates that ET-1 exposure increases eNOS expression on the membrane fraction. The higher M/C ratio with ET-1 evident after both 30-minutes and 24-hour exposure result in inhibition of eNOS because sequestered eNOS is inactive. The acute effect of ET-1 on eNOS cellular location explains the decreased NO production observed after 30 minutes of exposure and further confirms the conclusion that ET-1 decreases eNOS activity. We also assessed whether ET-1 results in iNOS induction. However, neither ET-1 nor PKC modulation induced iNOS.

**Figure 5.** PKC\(\lambda\) translocation. A, The presence of ET-1 for 30 minutes resulted in a significant lowering of PKC\(\lambda\) M/C ratio (increased cytosolic expression). Treatment with PKC inhibitors significantly reduced the M/C ratio in the control group while having no effect on the ET-1 treated cells. PMA resulted in a significantly higher M/C ratio in both the control and ET-1 groups. Bosentan exposure in addition to attenuating ET-1–induced effects also resulted in a significantly higher translocation of PKC\(\lambda\). B, Exposure of HSVECs to ET-1 for 24 hours caused a significant lowering of the PKC\(\lambda\) M/C ratio. PKC inhibition for 24 hours lowered the M/C ratio, whereas PMA increased the M/C ratio. HSVECs exposed to Bosentan and ET-1 blocked ET-1 induced effects, whereas treatment of cells with Bosentan alone also resulted in an increased PKC\(\lambda\) M/C ratio.

**Figure 6.** HSVEC PKC activity. ET-1 caused a significant decrease in PKC activity after 30 minutes, with a further reduction after 24 hours compared with control. The exposure to PKC inhibitors resulted in a lowering of HSVECs PKC activity, whereas PMA increased activity. Bosentan exposure blocked ET-1 reduction in PKC activity and increased PKC activity compared with control.
Effects of ET-1 Antagonism With Bosentan

Our study also sought to determine whether endothelin blockade with Bosentan prevented ET-1–induced endothelial injury. NO production assessments demonstrated that Bosentan had the ability to increase basal NO release in addition to abrogating ET-1 induced effects. This was seen both acutely and with prolonged exposure. Bosentan also attenuated ET-1–induced downregulation of eNOS expression while maintaining an M/C ratio similar to control. Furthermore, Bosentan treatment inhibited ET-1–induced translocation of PKCδ and PKCe. PKCα demonstrated greater translocation to the membrane after Bosentan exposure compared with ET-1 and control. PKC activity was also measured after Bosentan exposure and revealed that both 30 minutes and 24 hours of treatment increased PKC activity. Bosentan demonstrated the simultaneous ability to attenuate ET-1–induced endothelial dysfunction and improve NO homeostasis. These effects of Bosentan seem to further support the position that PKCα is the key isoform involved in NO regulation. In support of this conclusion: (1) PKC inhibition/activation decreased/increased NO production; (2) the presence of Bosentan results in increased NO production, whereas only increasing PKCα translocation and PKC activity; (3) ET-1 increased cytosolic expression of PKCα while decreasing PKC activity; and (4) PKC inhibition/activation translocated PKCα on activation and increased cytosolic expression on antagonism in the control group.

The beneficial effects of ET-1 antagonism have previously been observed after ischemia and reperfusion, in pulmonary
hypertension, and in transplantation.\textsuperscript{9,22,24,25} Gonon et al demonstrated that NO mediates the myocardial protective effects of Bosentan during ischemia and reperfusion.\textsuperscript{24} Our data indicate that Bosentan’s protective effects also extend to the endothelium. Barton et al also showed that ET-1 blockade restores NO-mediated endothelial function in atherosclerotic mice.\textsuperscript{25} We have previously demonstrated that Bosentan enhances both myocardial and endothelial protection during cardiac allograft storage.\textsuperscript{9} The present study demonstrates the mechanism behind ET-1 injury and the putative beneficial effect of Bosentan treatment.

**Study Limitations**

Our study provides evidence that ET-1 impairs NO homeostasis in an in vitro model through a PKC-dependent pathway. Our use of a supraphysiologic dose of ET-1 may not be entirely representative of what occurs in vivo. The potential beneficial effects of ET-1 antagonism with Bosentan in the clinical setting are tentative and require further study. Although our study demonstrated the role of PKC in ET-1–induced endothelial injury, it does not exclude the involvement of alternative signaling pathways.

**Summary**

In conclusion, prolonged treatment of HSVECs to elevated levels of ET-1 decreases NO bioavailability through the modulation of PKC translocation and activity. HSVEC exposure to ET-1 decreased PKC activity (PKC\textsuperscript{\theta}) and eNOS expression, whereas ET-1 antagonism with Bosentan restored the NO–ET-1 balance. ET-1 antagonism may prove to be a novel therapeutic strategy to improve vascular homeostasis after ischemia/reperfusion, heart failure, and cardiac transplantation. We have previously demonstrated the beneficial effects of Bosentan after ischemia and reperfusion.\textsuperscript{9} Our study demonstrated that this benefit may be as a result of improved vessel homeostasis and that PKC plays a critical role in this balance. Targeted PKC modulation may prove to be a superior strategy as it has the potential to augment the beneficial effects of ET-1 antagonism.

**Sources of Funding**

This work was supported by the Heart and Stroke Foundation of Ontario (grant NA 5868), the Canadian Institutes for Health Research, the Thoracic Surgery Foundation for Research and Education, and the Tailored Advanced Collaborative Training in Cardiovascular Science for Research Fellows. D.R. is a Research Fellow of the TSFRE and TACTICS. V.R. is a CIHR New Investigator.

**Disclosures**

None.

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Circulation. 2006;114:I-319-I-326
doi: 10.1161/CIRCULATIONAHA.105.001503

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