Endothelin-1 accentuates the proatherosclerotic effects associated with C-reactive protein

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Objectives: The proinflammatory marker C-reactive protein has been demonstrated to play a role in the development of atherosclerosis. Endothelin-1 and nitric oxide homeostasis is crucial for normal vasomotor function, limiting inflammatory activation and maintaining a nonthrombogenic endothelial surface. In addition to its vasoactive properties, endothelin-1 is also an inflammatory cytokine. We have previously demonstrated that C-reactive protein impairs endothelial cell nitric oxide production. Protein kinase C, an important signal transducer within the cell, is involved in several cellular responses to external stimuli. We therefore sought to determine whether endothelin-1 exposure modulates C-reactive protein's effects on nitric oxide production via protein kinase C.

Methods: Endothelial cells were incubated with C-reactive protein (200 μ g), endothelin-1 (100 nM), C-reactive protein + endothelin-1, or phosphate-buffered saline solution (control) for 24 hours. After exposure, endothelial nitric oxide synthase expression was determined in addition to total nitric oxide production and protein kinase C translocation and activity.

Results: Endothelial nitric oxide synthase protein expression was reduced following incubation with C-reactive protein and endothelin-1 treatment compared with baseline by 40% and 45%, respectively ($P = .04$); however, no additive effects were seen with coinubation. C-reactive protein produced a 47% decrease in nitric oxide production compared with control. Coinubation with endothelin-1 resulted in a synergistic 70% reduction in nitric oxide production ($P = .001$). C-reactive protein exposure inhibited translocation of protein kinase C α compared with control ($P = .01$). Furthermore, coinubation of C-reactive protein with endothelin-1 led to a synergistic inhibition of protein kinase C α translocation ($P = .01$). C-reactive protein exposure reduced protein kinase C activity by 40% compared with control ($P = .02$), although coinubation with endothelin-1 had a synergistic reduction in activity ($P = .02$).

Conclusions: Our results indicate that endothelin-1 exposure accentuated C-reactive protein's impairment of endothelial nitric oxide production via synergistic inhibition of protein kinase C α translocation and activity. Our investigations suggest that endothelin-1 inhibition and protein kinase C stimulation may provide a novel therapeutic strategy to improve vascular nitric oxide homeostasis and mitigate the proatherosclerotic effects of C-reactive protein.

Endothelial dysfunction, an initiator of atherosclerosis, is manifested by altered nitric oxide (NO) and endothelin-1 (ET-1) homeostasis. Impaired NO release precedes the development of atherosclerosis and serves to reinforce vascular pathology once established. ET-1 is one of the most potent endogenous

Abbreviations and Acronyms

CRP	= C-reactive protein
eNOS	= endothelial nitric oxide synthase
ET-1	= endothelin-1
HSVEC	= human saphenous vein endothelial cell
M/C	= membrane-to-cytosolic (ratio)
NO	= nitric oxide
PKC	= protein kinase C
PMA	= phorbol 12-myristate 13-acetate

vasoconstrictors¹ and mediates a host of responses including endothelial dysfunction, vasomotor contraction, leukocyte activation, and cellular proliferation.^{2,3} Diminished production of NO and exaggerated release of ET-1 are believed to be key initiators of endothelial injury.³ Impaired NO homeostasis, a hallmark of endothelial dysfunction, not only reflects the degree of endothelial cell injury but also is an active component of the atherosclerotic process. Reduced NO release results in heightened endothelial cell thrombogenicity, loss of inflammatory cell inhibition, and altered vasomotor function. The deleterious effects of NO impairment finally culminate in the development of atherosclerosis. Several investigators have demonstrated that ET-1 adversely affects endothelial function as well as outcomes following cardiac surgery.³⁻⁷ We have previously shown that ET-1 results in endothelial dysfunction following cardiac transplantation and antagonism with bosentan abrogated this effect.⁵ Recent evidence suggests that elevated levels of ET-1 may impair NO production.^{8,9} However, the mechanisms by which elevated levels of ET-1 reduce NO production in endothelial cells have yet to be determined. Therefore, the injured endothelial cell has altered ET-1 and NO homeostasis, resulting in vasomotor impairment, immune activation, and atherosclerosis.

Accumulating evidence supports the concept that inflammation plays a central role in the genesis of atherosclerosis and its complications.^{10,11} Chronic inflammation results in endothelial dysfunction and facilitates the interactions between modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall (Figure 1).^{10,11} This inflammatory process ultimately leads to the development and progression of atherosclerosis.^{10,11} The inflammatory marker C-reactive protein (CRP) has been shown to predict myocardial infarction, stroke, and vascular death in a variety of settings.¹⁰⁻²⁰ This acute-phase reactant has also been demonstrated to predict future coronary events and also to portend the vulnerability of an atherosclerotic lesion and the likelihood of plaque rupture.^{10-13,15,17-20} CRP augments the production of key inflammatory cytokines such as interleukin-6 and directly impairs NO homeostasis.²¹ Verma and colleagues²¹ demonstrated that CRP is not only a risk factor but also an active player in atherosclerosis by impairing NO production, resulting in endothelial dysfunction with its associated negative consequences. The mechanisms underlying this impairment in NO production remain unknown. Furthermore, whether ET-1 enhances the potential proatherosclerotic effects of CRP remains unclear.

Protein kinase C (PKC) plays an important role in several signaling pathways and is a family of kinases composed of at least 12 isozymes. Several investigators have demonstrated that ET-1 alters specific PKC isoform translocation and activity.^{22,23} We hypothesized that elevated levels of CRP and ET-1 impair endothelial cell NO production via an isoform-specific PKC-mediated change in endothelial NO synthase (eNOS) expression and activity. We also propose that ET-1 accentuates CRP-induced injury and that bosentan, an ET-1 antagonist, may prevent both ET-1- and CRP-induced endothelial dysfunction and restore normal vascular NO homeostasis.

Figure 1. A, Normal vascular NO homeostasis. B, Chronic inflammation results in endothelial dysfunction and facilitates the interactions between modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall, resulting in decreased NO production leading to impaired endothelial function and vasoconstriction. These same factors also increase ET-1 production, further promoting vasoconstriction and vasomotor impairment. C, Our hypothesis that CRP alters PKC activity leading to NO impairment.

Materials and Methods

Endothelial Cell Cultures

Human saphenous vein endothelial cells (HSVECs) were cultured in 10-cm diameter dishes at 37°C and 5% CO₂ in medium MCDB-131 (VEC Technologies, Rensselaer, NY) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells passaged 2 to 4 times and aged between 14 and 30 days from the time of primary culture were used for this study. We examined the effects of our interventions on eNOS expression, NO production, isoform-specific PKC translocation, and PKC activity. Cells were treated with CRP (25 µg/mL, Calbiochem, San Diego, Calif), 100 nM of ET-1 (Sigma, Oakville, Ontario, Canada), ET-1 + CRP, or phosphate-buffered saline solution (control, n = 12 per group) for 24 hours. ET-1 antagonism was achieved with the use of bosentan 10 µM (courtesy of Actelion Pharmaceuticals Ltd, Allschwil, Switzerland). We employed 2 PKC antagonists with different mechanisms of action, calphostin C (200 nM) and chelerythrine (1 µM; Sigma). Chelerythrine inhibits the adenosine triphosphate binding site, whereas calphostin C interferes with the lipid cofactor-binding site of PKC. The PKC agonist phorbol 12-myristate 13-acetate (PMA; 10 nM; Sigma) was used to stimulate PKC activity. The concentrations of these agents have been previously validated and published by our laboratory.²⁴

NO Production

Cell culture supernatants were collected following treatment. NO production was detected spectrophotometrically by measuring its 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 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.²⁵

Protein Expression

Western immunoblotting was performed using chemiluminescent detection and protein-specific monoclonal antibodies for eNOS (BD Biosciences, Mississauga, Ontario, Canada). Samples were separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis. Gels were then transferred to polyvinylidene fluoride membranes. Comparisons between groups were performed using densitometric analysis (BioRad, Hercules, Calif) corrected for β-actin expression.

PKC Translocation/Activity

Determination of isoform-specific PKC translocation was performed by analysis of cytosolic and membrane fractions of HSVEC to calculate a membrane-to-cytosolic (M/C) ratio using Western blot analysis. Purity of the cytosolic and membrane fractions was determined by confirming the absence of membrane bound Ca-ATPase (cytosolic) or β-actin (membrane). Our preliminary studies indicated that only the α, δ, ε, ι, and λ PKC isoforms are present in this cell population. Therefore, each blot was stained with these isoform-specific antibodies.

For measurement of PKC activity, a commercially available PKC assay kit (Calbiochem) was used 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 means ± SD. Statistical comparisons were made by analysis of variance followed by Dunn's multiple contrast test to identify differences between various treatments. Exact *P* values are provided for each comparison. Statistical analysis was performed with the SAS statistical software program version 8.2 (SAS Institute, Inc, Cary, NC).

Results

CRP and ET-1 Attenuate NO Release

Treatment with CRP resulted in a 47% decrease in NO production compared with control (Figure 1, A). Exposure to ET-1 also reduced NO production (*P* = .001), although coincubation with CRP led to a further 70% reduction in NO release (*P* = .001; Figure 2, A). Bosentan treatment was effective in attenuating the effects of ET-1 on NO production; however, bosentan did not mitigate the effects of CRP. HSVEC exposed to bosentan alone demonstrated a significant increase in NO production by approximately 20% (*P* = .001).

Effect of PKC on Nitrate/Nitrite Production

Exposure to the PKC inhibitors calphostin C and chelerythrine significantly reduced NO release (*P* < .001; Table 1). Coincubation of ET-1 or CRP with our PKC inhibitors did not demonstrate any further reduction in NO production (Table 1). HSVEC treated with PMA demonstrated increased NO release compared with control and significantly abrogated the effects of both CRP and ET-1 (Table 1).

CRP and ET-1 effect on eNOS Protein Expression

Exposure to CRP or ET-1 markedly downregulated eNOS protein expression by approximately 40% and 45% compared with control (*P* = .004; Figure 2, B). Coincubation demonstrated no added reduction in eNOS protein expression (Figure 2, B). Furthermore, PKC antagonism showed a significant reduction in eNOS protein expression (Table 1). No interactive effects were seen between PKC inhibitors and ET-1 or CRP (Table 1). PKC activation with PMA resulted in a significant upregulation of eNOS protein expression compared with control and completely abolished both CRP- and ET-1-induced downregulation of eNOS expression (Table 1). Figure 1, B shows that bosentan treatment prevented ET-1-induced downregulation, although it had no effect on CRP-induced reduction of eNOS expression.

PKC Isoforms

We assessed our cell cultures for the presence of several PKC isoforms (α, β, γ, δ, ε, λ, ι, ζ). We found the presence of only α, δ, ε, ι, and λ isoforms in our HSVECs. No

Figure 2. A, NO production in HSVECs following treatment. The presence of CRP and ET-1 significantly reduced NO production. Coincubation of CRP with ET-1 resulted in a greater decrease in NO release. Bosentan treatment prevented ET-1-induced impairment in NO release but did not prevent the effects of CRP. B, Quantitative Western blot analysis following 24-hour exposure demonstrated that CRP, ET-1, and CRP + ET-1 exposure downregulated eNOS protein expression compared with control. Treatment of HSVEC with bosentan abrogated ET-1 inhibition of eNOS protein expression while having no effect on CRP-induced downregulation.

significant differences in PKC α and PKC ι M/C ratios (translocation to the membrane) were seen between groups. PKC δ , ϵ , and λ isoforms demonstrated significant changes following treatment (Figure 3, A, B, C).

CRP exposure resulted in no change in PKC δ translocation but increased PKC ϵ translocation ($P < .02$; Figure 3, A, B). The translocation of PKC λ was significantly reduced following CRP treatment ($P < .01$; Figure 3, C). ET-1 exposure resulted in increased PKC δ and PKC ϵ translocation compared with control and CRP ($P < .05$; Figure 3, A, B). Examination of the PKC λ isoform demonstrated that ET-1 significantly reduced the M/C ratio (suggesting inhibition of translocation) compared with control ($P < .01$;

Figure 3, C). Coincubation of CRP with ET resulted in a greater increase in PKC δ translocation while reducing the M/C ratio for PKC λ (Figure 3, B, C).

PKC inhibition resulted in a significantly reduced M/C ratio for PKC δ and PKC ϵ in control and in treated cells (CRP, ET, and CRP + ET; Table 2). PKC λ translocation was impaired following PKC inhibition only in the control groups ($P < .05$). PKC activation resulted in significantly greater translocation to the membrane of all PKC isoforms in all our treatment groups except for PKC δ in the CRP + ET group (Table 2).

The effects of bosentan on HSVEC PKC translocation were significant. Bosentan demonstrated the ability to abrogate both ET-1- and CRP-induced effects on PKC δ and attenuated CRP's effect on PKC ϵ translocation ($P < .05$; Table 2). HSVEC exposed to bosentan with and without ET-1 demonstrated increased translocation of PKC λ compared with control ($P < .05$). Bosentan minimally inhibited CRP-induced effects on PKC λ and abolished the effects of ET-1 when coincubated with CRP (Table 2).

PKC Activity

Determination of PKC translocation does not necessarily correlate with cellular PKC activity, and therefore we measured direct overall PKC activity. Endothelial cells treated with CRP or ET-1 displayed a significant reduction in PKC activity ($P = .01$; Figure 4). A greater reduction was seen following coincubation of CRP with ET-1 (Figure 4). Bosentan treatment blocked the ET-1-induced effect but also resulted in enhanced PKC activity ($P < .05$; Table 1). However, bosentan did not abrogate the CRP-induced effects. As expected, HSVEC treated with calphostin C and chelerythrine demonstrated reduced PKC activity. No further effect of ET-1 on CRP was seen. PMA exposure increased PKC activity and abrogated both CRP- and ET-1-induced effects.

Discussion

CRP and ET-1 are known to result in endothelial dysfunction and are risk factors for atherosclerosis.^{5,12,13,18-20} The normally functioning endothelium is maintained by the balance between NO and ET-1. Impairment in NO production and/or increased release of ET-1 are key initiators of endothelial dysfunction and injury.³ Given the importance of a normally functioning endothelium, we sought to determine the mechanisms by which CRP impairs NO bioavailability in isolated human endothelial cells. We further aimed to determine the effect of ET-1 exposure on CRP's proatherosclerotic effects.

We have made the following observations outlined in Figure 5:

1. CRP attenuates NO production and downregulates eNOS protein expression.

TABLE 1. Summary of NO production ($\mu\text{M}/\text{mg}$) and eNOS protein expression data (% control)

	CON	CRP	ET	CRP + ET
NO production ($\mu\text{M}/\text{mg}$) (total nitrite)				
No Tx	7.3 $\pm$ 0.1	3.9 $\pm$ 0.2*	4.3 $\pm$ 0.2*	2.2 $\pm$ 0.1*§
BOS	9.0 $\pm$ 0.4*	4.3 $\pm$ 0.2*†	8.8 $\pm$ 0.4*	4.0 $\pm$ 0.1*†
Cal	1.3 $\pm$ 0.3*	1.0 $\pm$ 0.2*§	1.1 $\pm$ 0.2*§	1.3 $\pm$ 0.2*
Chel	1.5 $\pm$ 0.2*	1.1 $\pm$ 0.5*§	1.2 $\pm$ 0.5*§	1.4 $\pm$ 0.2*
PMA	11.9 $\pm$ 0.4*	11.7 $\pm$ 0.4*§	10.8 $\pm$ 0.3*§	11.5 $\pm$ 0.2*
eNOS expression (% control)				
No Tx	100	59 $\pm$ 3†	54 $\pm$ 4†	53 $\pm$ 6†
BOS	98 $\pm$ 10	60 $\pm$ 9††	93 $\pm$ 9	63 $\pm$ 11††
Cal	58 $\pm$ 5†	58 $\pm$ 3†	55 $\pm$ 3†	54 $\pm$ 4†
Chel	63 $\pm$ 6†	56 $\pm$ 6†	60 $\pm$ 8†	54 $\pm$ 7†
PMA	129 $\pm$ 10†	122 $\pm$ 10†	124 $\pm$ 15†	123 $\pm$ 8†

CON, Control; CRP, C-reactive protein; ET, endothelin-1; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; No Tx, no added treatment; BOS, bosentan; Cal, calphostin C; Chel, chelerythrine; PMA, phorbol 12-myristate 13-acetate. * $P < .001$ vs No Tx CON; † $P = .04$ vs No Tx CON; †† $P < .05$ vs BOS CON; § $P < .05$ vs No Tx CRP and ET; || $P < .05$ vs No Tx CRP + ET.

- ET-1 accentuates the CRP-induced reduction in NO release with no further downregulation of eNOS expression.
- CRP appears to selectively stimulate PKC ϵ translocation while inhibiting λ translocation with a further reduction in PKC α translocation when coincubated with ET-1.
- CRP inhibits PKC activity with a further decrease seen following ET-1 coincubation.
- Bosentan can maintain cellular NO production following ET-1 exposure but does not prevent CRP-induced impairment.
- PKC inhibition leads to a severe reduction in cellular NO production with no further reduction observed with simultaneous exposure to CRP or ET-1.
- PKC stimulation with PMA significantly increases cellular NO production and is capable of completely reversing the inhibitory effects of both CRP and ET-1 exposure.

Effect of CRP and ET-1 on NO Production

Our findings demonstrate that CRP has a deleterious effect on NO homeostasis in an vitro assessment of endothelial function. ET-1 also resulted in impaired NO production and aggravated CRP's inhibitory effect on NO release. Prolonged exposure to CRP with the resultant impairment of NO production may explain its predictive value for determining atherosclerosis. Our data confirm that CRP is an active player in the pathogenesis of atherosclerosis by impairing NO homeostasis a marker of endothelial dysfunction. Elevated ET-1 levels, as seen following ischemia-reperfusion, myocardial infarction, or heart failure, may accelerate or enhance the atherosclerotic burden in these patients.

Our results are consistent with the studies by Verma and colleagues²¹ and Dong and associates,⁸ which demonstrated

that elevated CRP and ET-1 levels result in downregulation of eNOS protein expression in endothelial cells. We demonstrated that CRP-induced NO dysregulation was partly caused by changes in eNOS protein expression. eNOS protein expression was downregulated following both CRP and ET-1 treatment. In contrast to NO production, no further reduction in eNOS expression was seen with coincubation. The greater impairment in NO production seen with coincubation may be a result of decreased eNOS activity. Decreased eNOS activity may be caused by changes in regulatory kinase activity such as PKC.

Our study demonstrated that NO production is regulated by PKC. NO bioavailability is impaired following PKC inhibition, and PKC activation led to NO release. Further evidence that PKC is a player in both CRP- and ET-1-induced NO impairment is that concomitant exposure of CRP or ET-1 with PKC inhibition did not lead to a greater reduction in NO bioavailability as would be anticipated if they acted in a PKC-independent fashion. The simultaneous exposure of our endothelial cells to PMA and CRP or ET-1 resulted in increased NO production similar to exposure to PMA alone. Our data support the hypothesis that CRP and ET-1 decrease NO production through PKC inhibition. eNOS protein expression was also regulated by PKC. We found that PKC antagonism downregulated eNOS protein expression, although PKC agonists upregulated eNOS protein expression. In addition, concomitant exposure of HSVECs to CRP or ET-1 with PMA resulted in upregulation of eNOS to a level similar to PMA alone, further demonstrating that CRP- and ET-1-induced alterations in eNOS expression is PKC dependent and is fully reversible by direct PKC stimulation.

The differential roles of PKC on NO production have been observed in various animal models.²⁶⁻²⁸ Matsubara

Figure 3. A, PKC δ translocation. *Top*, Western blot of PKC δ expression in the cytosolic fractions (C) and membrane fractions (M) following treatment. *Bottom*, Quantitative Western blot analysis of PKC δ translocation. No differences were seen after CRP treatment, although ET-1 exposure resulted in a significant PKC δ translocation to the membrane compared with control. Coincubation of CRP with ET-1 resulted in a further increase in PKC δ translocation. B, PKC ϵ translocation. *Top*, Western blot of PKC ϵ expression in the cytosolic fractions (C) and membrane fractions (M) following treatment. *Bottom*, Quantitative Western blot analysis of PKC ϵ translocation. PKC ϵ was significantly translocated to the membrane following both CRP and ET-1 treatment compared with control. Coincubation demonstrated PKC ϵ translocation that was significantly greater than that of CRP alone but similar to ET-1 exposed cells. C, PKC λ translocation. *Top*, Western blot of PKC λ expression in the cytosolic fractions (C) and membrane fractions (M) following treatment. *Bottom*, Quantitative Western blot analysis of PKC λ translocation. Exposure of HSVECs to CRP and ET-1 caused a significant lowering of PKC λ M/C ratio. Coincubation of CRP with ET-1 led to further decrease in PKC λ M/C ratio.

and colleagues²⁶ in bovine aortic cells found that PKC activation decreases NO production, although Partovian and coworkers²⁷ found that PKC activation increases NO formation. Partovian and associates²⁷ found that PKC α activation increases eNOS activity in the vasculature, indicating that PKC regulation of eNOS is tissue and species dependent. Therefore, depending on species and cell type, PKC inhibition or activation can lead to NO impairment. In our study, using human endothelial cells, we demonstrated that PKC inhibition, likely the lambda isoform, decreases eNOS protein expression and NO production.

Effects of CRP and ET-1 on PKC Translocation and Activity

To determine which isoforms were involved in NO regulation, we evaluated isoform-specific translocation. Our study revealed that both CRP and ET-1 exposure resulted in membrane translocation of the epsilon isoforms, although the M/C ratio of the lambda isoform was reduced, suggesting inhibited translocation. Coincubation of CRP with ET-1 led to a greater increase in PKC ϵ translocation and further reduced PKC λ translocation. Because translocation does not always correlate with activity, we measured PKC activ-

TABLE 2. Summary of PKC translocation and activity (U/mg) data

	CON	CRP	ET	CRP + ET
PKCδ M/C ratio				
No Tx	1.17 $\pm$ 0.10	1.21 $\pm$ 0.11	2.53 $\pm$ 0.23*	3.34 $\pm$ 0.25*¶
BOS	1.18 $\pm$ 0.13	1.15 $\pm$ 0.16	1.27 $\pm$ 0.15	1.33 $\pm$ 0.06**
Cal	0.68 $\pm$ 0.15*	0.49 $\pm$ 0.12*	0.54 $\pm$ 0.13*	0.45 $\pm$ 0.10***
Chel	0.45 $\pm$ 0.11*	0.41 $\pm$ 0.08*	0.47 $\pm$ 0.23*	0.43 $\pm$ 0.14***
PMA	2.87 $\pm$ 0.23*	2.98 $\pm$ 0.21*	3.14 $\pm$ 0.17*	3.54 $\pm$ 0.20***
PKCϵ M/C ratio				
No Tx	1.23 $\pm$ 0.12	1.48 $\pm$ 0.05†	1.92 $\pm$ 0.17†	1.97 $\pm$ 0.08†§
BOS	1.38 $\pm$ 0.15	1.58 $\pm$ 0.11†	1.33 $\pm$ 0.10	1.62 $\pm$ 0.17†
Cal	0.97 $\pm$ 0.28†	0.96 $\pm$ 0.23†§	0.98 $\pm$ 0.16†§	1.06 $\pm$ 0.10†††
Chel	0.98 $\pm$ 0.13†	0.90 $\pm$ 0.18†§	0.93 $\pm$ 0.07†§	0.91 $\pm$ 0.12†††
PMA	1.91 $\pm$ 0.12†	1.97 $\pm$ 0.14†	2.01 $\pm$ 0.09†	.99 $\pm$ 0.03†
PKCλ M/C ratio				
No Tx	0.72 $\pm$ 0.05	0.36 $\pm$ 0.07‡	0.44 $\pm$ 0.08‡	0.13 $\pm$ 0.04‡‡‡
BOS	1.05 $\pm$ 0.03	0.54 $\pm$ 0.09‡	1.09 $\pm$ 0.07	0.52 $\pm$ 0.08‡§§
Cal	0.36 $\pm$ 0.21‡	0.34 $\pm$ 0.07‡	0.30 $\pm$ 0.14‡	0.14 $\pm$ 0.23‡
Chel	0.43 $\pm$ 0.06‡	0.38 $\pm$ 0.14‡	0.34 $\pm$ 0.17‡	0.15 $\pm$ 0.17‡
PMA	2.07 $\pm$ 0.19‡	2.13 $\pm$ 0.15‡	2.55 $\pm$ 0.20‡	2.22 $\pm$ 0.21‡
PKC activity (U/mg)				
No Tx	4.90 $\pm$ 0.10	2.90 $\pm$ 0.20*	3.90 $\pm$ 0.10*	2.00 $\pm$ 0.10*¶
BOS	6.40 $\pm$ 0.44*	3.40 $\pm$ 0.38*	5.90 $\pm$ 0.58*	2.20 $\pm$ 0.20*¶
Cal	0.91 $\pm$ 0.21*	0.88 $\pm$ 0.20*	0.88 $\pm$ 0.39*	0.72 $\pm$ 0.22*
Chel	0.89 $\pm$ 0.10*	0.86 $\pm$ 0.10*	0.88 $\pm$ 0.18*	0.84 $\pm$ 0.16*
PMA	9.40 $\pm$ 0.16*	9.10 $\pm$ 0.27*	9.00 $\pm$ 0.38*	8.80 $\pm$ 0.32*

CON, Control; CRP, C-reactive protein; ET, endothelin-1; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; No Tx, no added treatment; BOS, bosentan; Cal, calphostin C; Chel, chelerythrine; PMA, phorbol 12-myristate 13-acetate. *PKC δ : $P < .01$ vs No Tx CON; †PKC ϵ : $P < .02$ vs No Tx CON; ‡PKC λ : $P < .01$ vs No Tx CON; §PKC ϵ : $P < .05$ vs No Tx CRP and ET; ||PKC δ : $P < .05$ vs CRP; ¶PKC δ : $P < .05$ vs No Tx CRP and ET; **PKC δ : $P < .05$ vs CRP + ET; ††PKC ϵ : $P < .05$ vs No Tx CRP + ET; ‡‡PKC λ : $P < .05$ vs No Tx CRP + ET; §§PKC λ : $P < .05$ vs No Tx CRP + ET.

ity in our cell cultures. We determined that cellular PKC activity is reduced following both CRP and ET-1 exposure, suggesting that inhibition of PKC λ activity is the likely mechanism by which CRP and ET-1 impairs NO production.

Our data revealed that ET-1 coincubated with CRP resulted in the greatest reduction in PKC activity, which corroborates the effect of ET-1 on CRP-induced NO im-

pairment. The inhibition of eNOS protein expression was similar in all treatment groups; therefore, differences in NO production were caused by changes in eNOS activity. We speculate that eNOS activity is regulated by specific PKC isoforms. The results of our studies suggest that CRP and ET-1 reduces PKC λ translocation and overall PKC activity, leading to decreased eNOS activity and impaired NO production.

ET-1 has been extensively studied and found to typically increase PKC translocation in various cell types.^{22,23,29-32} Dlugosz and colleagues²² demonstrated that ET-1 exposure results in PKC δ and PKC ϵ translocation in mesangial cells. McNair and associates³¹ data showed that PKC ϵ is activated by ET-1 exposure in coronary smooth muscle. Our findings are consistent with previous results demonstrating PKC ϵ and PKC δ translocation following ET-1 exposure. Our observation that ET-1 reduces PKC λ translocation in human endothelial cells is a novel finding. We speculate that unlike lower levels of ET-1, which normally results in G protein S activation leading to PKC translocation, higher levels of ET-1 also activate G protein I, which leads to PKC inhibition. Further studies are required to demonstrate the mechanisms behind ET-1-induced PKC inhibition.

Figure 4. HSVEC PKC activity. CRP and ET-1 caused a significant decrease in PKC activity compared with control with a further reduction seen following coincubation.

Figure 5. A, B, Human endothelial cell exposed to CRP or ET-1 results in decreased NO production by reducing PKC λ activity and translocation leading to eNOS downregulation. C, The concomitant exposure of ET-1 with CRP further reduces endothelial cell NO release by enhancing PKC λ inhibition and increased eNOS downregulation.

Effects of ET-1 Antagonism

Our study also sought to determine whether endothelin blockade with bosentan can prevent ET-1- and CRP-induced impairment in NO homeostasis. The beneficial effects of ET-1 antagonism have previously been observed following ischemia and reperfusion and in cardiac transplantation.^{5,33-35} We have previously demonstrated that bosentan enhances endothelial protection during cardiac allograft storage.⁵ In atherosclerotic mice, Barton and co-workers³⁵ also showed that ET-1 blockade restores NO-mediated endothelial function. Li and associates³⁶ have demonstrated that ET-1 antagonism may attenuate CRP-induced endothelial dysfunction. They showed that ET-1 blockade reduces CRP-induced upregulation of lectinlike oxidized low-density lipoprotein receptor-1.³⁶ Our study examined the effect of ET-1 antagonism on CRP-induced impairment of NO homeostasis. However, ET-1 antagonism does not appear to preserve NO production following CRP exposure. When bosentan was given to the CRP + ET groups, the incremental deleterious effect of ET-1 on NO production was eliminated. In our experiments, bosentan failed to inhibit CRP's effect on eNOS expression; however, it completely abrogated ET-1-induced downregulation. Bosentan exposure resulted in increased PKC activity as well as greater PKC λ translocation compared with CRP, ET-1, and control. Bosentan's effects seem to further support the position that PKC λ is the key isoform involved in NO regulation. In support of this conclusion is the fact that bosentan results in increased NO production although only increases PKC λ translocation and PKC activity.

The present study demonstrates a potential mechanism behind CRP- and ET-1-induced injury and suggests possible therapeutic strategies. Although bosentan treatment was beneficial in reducing ET-1 injury as well as eliminating the added effect of ET-1 on CRP's proatherosclerotic effect, it was unable to block CRP-induced endothelial cell injury

assessed by decreased NO production. Bosentan may be beneficial in those patients who have both elevated CRP and ET-1 levels, such as patients having cardiac transplant or heart failure. Caution must be taken with bosentan, as we previously found that administration of bosentan can result in severe vasodilation and hypotension, rendering its clinical use problematic. Similarly, despite widespread evidence that ET-1 levels are elevated in congestive heart failure, large randomized clinical trials such as The ENABLE trials (Endothelin Antagonist Bosentan for Lowering Cardiac Events in Heart Failure) also demonstrated a higher rate of sudden death, potentially due to hypotension-related arrhythmias. Therefore, care must be taken with the use of ET-1 antagonism, especially in patients with heart failure. Hence, determining the mechanism of benefit for ET-1 blockade may permit the development of novel targeted therapies without the side effects associated with a nonselective antagonist. The effects of CRP are not mitigated by endothelin antagonism alone. We suggest that PKC modulation may be the optimal strategy to reduce the atherosclerotic burden in patients with elevated acute phase reactants such as CRP and ET-1. The results of these investigations have obvious clinical implications for patients with acute coronary syndrome, post-coronary artery bypass graft patency, and transplant vasculopathy. Further studies are required to assess the potential benefits of PKC modulation in preventing endothelial dysfunction.

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Discussion

Dr Y. Joseph Woo (Philadelphia, Pa). The authors should be congratulated on a great body of work and, as usual, great graphics from Toronto. Are venous endothelial cells the same as arterial endothelial cells, and are they an adequate surrogate if you are using this as a model for allograft vasculopathy and other sorts of arterial problems?

Dr Danny Ramzy (Toronto, Canada). Thank you for the question. We have actually performed experiments looking at that question. Between human saphenous vein endothelial cells, human aortic endothelial cells, and human coronary endothelial cells, once you pass the first passage, and all experiments are performed beyond P1, they all behave the same way as ET-1 and other vasoconstrictors and soluble factors. So in terms of using saphenous vein versus aortic cells, the experiments will yield the same results.

Dr Mark J. Krasna (Baltimore, Md). Dr Ramzy, could you tell us a little bit about bosentan? It sounds very exciting. Has it been used clinically in your group in any other arena yet?

Dr Ramzy. It has been used clinically. It is approved primarily for primary pulmonary hypertension. It reduces mortality and does reduce pulmonary arterial pressures. It has been used in patients with heart failure, with a few of the studies actually showing harm, likely due to its induction of hypotension. The patient with heart failure died from sudden death, likely from arrhythmia due to

hypotension. So other than in that setting, it has been used safely in patients who can be monitored closely or who have adequate blood pressure. The main problem is the blood pressure.

Dr Krasna. Have either of those groups had any relationship between atherosclerosis and bad outcomes and use of bosentan?

Dr. Ramzy. It has been looked at mostly in allograft vasculopathy in terms of as a surrogate of atherosclerosis, and it does reduce the burden in terms of amount of disease and the degree of stenosis.

Dr John G. Byrne (Nashville, Tenn). What do you think are the principal clinical applications of these findings?

D. Ramzy. The clinical applications are severalfold. In terms of atherosclerosis, the reduction of CRP and ET-1 does reduce both the atherosclerotic burden and outcomes such as myocardial infarction. From our results in terms of protein kinase C, we could more specifically target the mechanisms by which CRP results in atherosclerosis and endothelial dysfunction, both in the transplant setting and in atherosclerosis, and especially following transplantation where there is a high degree of vein restenosis, which, if we can modulate basically soluble inflammatory markers like ET-1

and CRP with bosentan or targeted therapy, we could reduce graft restenosis and improve survival and quality of life.

Dr Byrne. Are you talking about a vein graft?

Dr Ramzy. Vein graft.

Dr Turki Albacker (Montreal, Canada). Just for my curiosity, for the clinical application of this study, what do you think about the level of CRP in a patient posttransplant and do you think it is high enough to cause atherosclerosis so you can target it with your therapy? Thanks.

Dr Ramzy. There are several studies looking at CRP posttransplant. There are a group of patients who do have elevated levels of CRP, so any level above 3 $\mu\text{g/mL}$ does result in poor outcome. There is actually a recent study that shows any levels above 1.06 $\mu\text{g/mL}$ result in poor outcome. So a slight increase in CRP is able to result in worse outcomes in terms of earlier development of allograft vasculopathy and worse disease, and it has been also shown if you reduce this level, there is a trend toward improvement. Of course, the concentration we used was 25 μg , which is in the intermediate level. We have done studies previous to that. Any level above 3 $\mu\text{g/mL}$ does result in endothelial dysfunction.

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