Endothelin-Dependent Tone Limits Acetylcholine-Induced Dilation of Resistance Coronary Vessels After Blockade of NO Formation in Conscious Dogs

Zhi Ming, Robert Parent, Éric Thorin, Michel Lavallée

Abstract—Nitric oxide (NO) impairs endothelin (ET) formation and/or action in isolated vessels. We hypothesized that ET may magnify the consequences of NO formation blockade on receptor-operated dilation of resistance coronary vessels in conscious dogs. In conscious instrumented dogs, graded intracoronary (IC) doses of acetylcholine (ACh) were delivered before IC administration of Nω-nitro-L-arginine methyl ester (L-NAME), after L-NAME, and after L-NAME plus IC bosentan, an ETₐ/ETₐ receptor blocker. Before L-NAME, ACh (100 ng · kg⁻¹ · min⁻¹) increased coronary blood flow (CBF) by 43±4% from 47±6 mL · min⁻¹. After L-NAME, ACh failed to increase CBF (−3±2% from 50±7 mL · min⁻¹; P<0.01) after the addition of bosentan. Bosentan alone (without L-NAME) did not alter CBF responses to ACh. Blockade of ETₐ (Ro 61-1790) but not ETₐ (Ro 46-8443) receptors partially restored CBF responses to ACh after L-NAME. Myocardial immunoreactive ET levels in the perfusion territories of the circumflex and left anterior descending coronary arteries did not differ. ETₐ-dependent tone magnified the inhibitory effects of blockade of NO formation on receptor-operated dilation to ACh in resistance coronary vessels. Presumably, stimulated NO release has an inhibitory action on endogenous ET production and/or action at the level of resistance coronary vessels. (Hypertension. 1998;32:844-848.)

Key Words: endothelium-derived factors ■ endothelin ■ acetylcholine ■ endothelium ■ microcirculation

Endogenous endothelin (ET) has limited influence on peripheral hemodynamics in general and on the coronary vascular bed in particular under normal physiological conditions in various animal species. Albeit of little consequence in dogs and rats, ET receptor blockade has significant effects in conscious animals. Thus, the production of cGMP decreased thrombin-induced ET release, whereas nitroglycerin and 3-morpholinosydnonimine (SIN-1) antagonized thrombin-induced ET release. Thus, the production of ET is blunted by agents targeting the NO/cGMP pathway. In the same connection, a receptor-operated ET release triggered by acetylcholine (ACh) becomes apparent when NO formation is impaired, such as in hypercholesterolemia and atherosclerosis. A cross-talk between NO/cGMP and ET may also explain the reversal of acute pressor responses caused by arginine analogues (NO formation blockers) with selective ETₐ or mixed ETₐ/ETₐ receptor antagonists.

Taken together, these data led us to hypothesize that the influence of ET may become more important after suppression of NO formation. Consequently, ET may magnify the extent to which arginine analogues limit receptor-operated NO-dependent dilation of resistance coronary vessels. Therefore, the effects of ACh on coronary blood flow (CBF) were examined in conscious dogs before and after blockade of NO formation with and without blockade of ETₐ and ETₐ receptors.

Methods

Instrumentation

Under general anesthesia with sodium pentobarbital (30 mg/kg IV), artificial ventilation, and sterile conditions, 18 mongrel dogs (30±1 kg) underwent a left thoracotomy at the fifth intercostal space and were instrumented as previously described. In addition, pacing wires were sutured to the right ventricular outflow tract and connected to an external stimulator (model 5320, Medtronic).

Protocols

Experiments were initiated 2 to 4 weeks after surgery in conscious healthy dogs pretreated with indomethacin (5.0 mg/kg IV). Intracoronary (IC) infusions of 30.0, 100.0, and 300.0 ng · kg⁻¹ · min⁻¹ acetylcholine chloride (Sigma Chemical Co) were performed.
until a steady state was reached, ie, 4 to 6 minutes after the beginning of the infusion.

**Combined Blockade of NO Formation and ET<sub>A</sub>/ET<sub>B</sub> Receptors**

ACh infusions were performed in 7 dogs before administration of N\(^{-}\)-nitro-L-arginine methyl ester (L-NAME, Sigma) and after L-NAME (50.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) for 12 minutes\(^1,2\)) with and without bosentan (Ro 47-0203/001, Hoffmann-La Roche Ltd), a blocker of ET<sub>A</sub>/ET<sub>B</sub> receptors.\(^{11}\) Bosentan was injected at an IC dose of 30.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) for 10 minutes plus 1.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) thereafter. The effects of bosentan on ACh-induced CBF responses were also examined in the absence of L-NAME (n=7). Adequacy of ET<sub>A</sub>/ET<sub>B</sub> blockade was demonstrated in separate experiments (n=6) by the inhibition of coronary constriction elicited by an IC bolus injection of ET-1 (0.1 \(\mu\)g, American Peptide Co).

**Blockade of ET<sub>A</sub> Receptors With and Without Blockade of NO Formation**

The same strategy was used in 8 dogs to examine the effects of selective ET<sub>A</sub> receptor blockade with IC Ro 61-1790\(^{14}\) (2.5 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) for 10 minutes plus 0.25 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) thereafter; F. Hoffmann-La Roche Ltd). Adequacy of ET<sub>A</sub> receptor blockade with Ro 61-1790 (n=5) was demonstrated by smaller ET-1–induced CBF decreases.

**Blockade of ET<sub>B</sub> Receptors With and Without Blockade of NO Formation**

In 5 dogs the effects of selective ET<sub>B</sub> receptor blockade with IC Ro 46-8443\(^{13}\) (30.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) for 10 minutes plus 1.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) thereafter; F. Hoffmann-La Roche Ltd) were examined. Adequacy of ET<sub>B</sub> receptor blockade (n=5) was demonstrated by blunted CBF responses caused by IC bolus injection of sarafotoxin S6c (0.3 \(\mu\)g, American Peptide Co), a selective ET<sub>B</sub> receptor agonist.\(^{15}\)

**Myocardial Immunoreactive ET Levels**

Myocardial samples were obtained at necropsy from the circumflex artery territory in 7 dogs. Myocardial immunoreactive (IR) ET levels were measured with a radioimmunoassay procedure according to the method described earlier.\(^{17}\) The radioimmunoassay procedure was carried out according to the procedure described by the supplier of the ET-1 antibody (Peninsula). Data are reported as IR-ET in picograms per gram of wet tissue. The cross-reactivities of ET-2, ET-3, and proendothelin in this assay were 7%, 7%, and 17%, respectively.

**Data Analysis**

Data are reported as mean±SEM. Paired comparisons were performed to determine whether ACh or nitroglycerin significantly influenced baseline left ventricular pressure (LVP), first derivative of LVP over time (LV dp/dt), mean arterial pressure (MAP), heart rate (HR), and CBF under the various experimental conditions.\(^{18}\)

Simultaneous comparisons of baseline or responses to graded doses of ACh before and after L-NAME or after L-NAME with and without bosentan, Ro 61-1790, or Ro 46-8443 were performed with ANOVA for repeated measurement.\(^{10}\) For any given dose of ACh, comparisons of responses were performed with ANOVA followed by Bonferroni’s \(t\) test. Myocardial IR-ET levels were compared with \(t\) tests. Statistical significance was reached when \(P<0.05\) in all cases. All experimental procedures were approved by an ethics committee on animal care and performed in accordance with Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care publication [ISBN] 0-919087-18-3, Ottawa, 1993).

**Results**

Right ventricular pacing was performed in dogs with bradyarrhythmias during ACh delivery. Except for CBF, hemodynamic effects of ACh did not reach statistical significance.

**Figure 1.** Percent changes in CBF (Delta % from baseline) caused by IC ET-1 (0.1 \(\mu\)g) before and after IC bosentan (top; 30.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) \(\cdot\) 10 minutes + 1.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) thereafter) and before and after IC Ro 61-1790 (middle; 2.5 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) \(\cdot\) 10 minutes + 0.25 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) thereafter; F. Hoffmann-La Roche Ltd) and after IC Ro 46-8443 (bottom; 30.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) \(\cdot\) 10 minutes + 1.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\), n=5), an ET<sub>B</sub> receptor blocker. Early and late CBF changes caused by IC sarafotoxin S6c (0.3 \(\mu\)g) are reported before and after IC Ro 46-8443 (bottom; 30.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) \(\cdot\) 10 minutes + 1.0 \(\mu\)g \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\), n=5), *\(P<0.05\) vs before ET receptor blockade; †\(P<0.01\) vs before ET receptor blockade.

**Adequacy of ET Receptor Blockade**

The effects of IC ET-1 or sarafotoxin S6c on CBF before and after bosentan, Ro 61-1790, and Ro 46-8443 were examined in preliminary experiments and are reported in Figure 1. Combined ET<sub>A</sub>/ET<sub>B</sub> receptor blockade led to substantial reductions of ET-1–induced decreases in CBF. ET<sub>B</sub> receptor blockade blunted the early increases and the late decreases in CBF caused by sarafotoxin S6c.

**Combined Blockade of NO Formation and ET<sub>A</sub>/ET<sub>B</sub> Receptors**

Hemodynamic responses elicited by L-NAME and L-NAME+bosentan are reported in Table 1. Bosentan given after L-NAME did not cause further hemodynamic effects.

Before L-NAME, IC administration of 100 ng \(\cdot\) kg\(^{-1}\) \(\cdot\) min\(^{-1}\) ACh (ACh 100) increased CBF by 43±4% (\(P<0.01\),
After L-NAME, Ach failed to increase CBF (-3±2%). After the addition of bosentan, CBF responses to Ach were partially restored (+10±2%, P<0.01) and greater than after L-NAME alone (P<0.05). Overall, L-NAME abolished Ach-induced CBF increases that were partially restored after bosentan (Figure 2).

Except for a slight increase in LVP (from 102±2 to 107±3 mm Hg, P<0.01), bosentan alone had no other significant hemodynamic effects. Ach 100 increased CBF by 33±6% (P<0.01) before bosentan and by 34±5% (P<0.01) thereafter. For all doses of Ach examined, CBF responses did not differ before and after bosentan.

**TABLE 1. Baseline Hemodynamics Before L-NAME, After L-NAME, and After L-NAME+Bosentan (n=7)**

<table>
<thead>
<tr>
<th>Hemodynamic Variable</th>
<th>Before L-NAME</th>
<th>After L-NAME</th>
<th>L-NAME + Bosentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBF, mL/min</td>
<td>49±4</td>
<td>52±4</td>
<td>51±4</td>
</tr>
<tr>
<td>LVP, mm Hg</td>
<td>107±2</td>
<td>115±3*</td>
<td>108±2</td>
</tr>
<tr>
<td>LV dP/dt, mm Hg/s</td>
<td>2285±77</td>
<td>2167±94</td>
<td>2045±99</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>89±3</td>
<td>103±3*</td>
<td>94±2</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>94±5</td>
<td>87±6</td>
<td>87±7</td>
</tr>
</tbody>
</table>

*P<0.01 vs previous treatment.

**TABLE 2. Baseline Hemodynamics Before L-NAME, After L-NAME, and After L-NAME+Ro 61-1790 (n=8)**

<table>
<thead>
<tr>
<th>Hemodynamic Variable</th>
<th>Before L-NAME</th>
<th>After L-NAME</th>
<th>L-NAME + Ro 61-1790</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBF, mL/min</td>
<td>43±2</td>
<td>44±2</td>
<td>40±2*</td>
</tr>
<tr>
<td>LVP, mm Hg</td>
<td>119±2</td>
<td>132±3†</td>
<td>118±3†</td>
</tr>
<tr>
<td>LV dP/dt, mm Hg/s</td>
<td>2967±159</td>
<td>2837±164</td>
<td>2637±175*</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>94±2</td>
<td>108±3*</td>
<td>92±3†</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>76±3</td>
<td>68±3*</td>
<td>72±4</td>
</tr>
</tbody>
</table>

*P<0.05 vs previous treatment; †P<0.01 vs previous treatment.

**Combined Blockade of NO Formation and ET\textsubscript{A} Receptors**

Ro 61-1790 given after L-NAME caused significant decreases in LVP, LV dP/dt, MAP, and CBF but did not significantly alter HR, as reported in Table 2. Before L-NAME, IC Ach 100 increased CBF by 43±6% (P<0.01). After L-NAME, CBF responses were abolished (-4±3%). Ro 61-1790 given after L-NAME augmented (P<0.05) CBF responses to Ach 100 (+13±1%, P<0.01). Overall, L-NAME abolished Ach-induced CBF increases that were partially restored by Ro 61-1790, as reported in Figure 3.

**Figure 2.** Percent changes in 7 dogs in CBF and coronary resistance caused by IC Ach (30.0, 100.0, and 300.0 ng·kg⁻¹·min⁻¹) before IC L-NAME (50.0 μg·kg⁻¹·min⁻¹·12 minutes), after L-NAME, and after L-NAME+IC bosentan (30.0 μg·kg⁻¹·min⁻¹·10 minutes+1.0 μg·kg⁻¹·min⁻¹), a mixed ET\textsubscript{A}/ET\textsubscript{B} receptor blocker. L-NAME abolished Ach-induced changes in CBF and coronary resistance that were partially restored after bosentan. *P<0.01 vs before L-NAME (ANOVA).

**Figure 3.** Percent changes in 8 dogs in CBF and coronary resistance caused by IC Ach (30.0, 100.0, and 300.0 ng·kg⁻¹·min⁻¹) before IC L-NAME (50.0 μg·kg⁻¹·min⁻¹·12 minutes), after L-NAME, and after L-NAME+IC Ro 61-1790 (2.5 μg·kg⁻¹·min⁻¹·10 minutes+0.25 μg·kg⁻¹·min⁻¹), an ET\textsubscript{A} receptor blocker. L-NAME abolished Ach-induced changes in CBF and coronary resistance that were partially restored after Ro 61-1790. *P<0.01 vs before L-NAME (ANOVA).
Discussion

Our data highlight an important feature of ET activity displayed by resistance coronary vessels, i.e., a receptor-operated cross-talk between NO and ET. ET antagonists failed to increase baseline CBF with and without normal background NO formation. A significant contribution of ET to receptor-operated dilation became apparent only after blockade of NO formation. Combined ET\(_A/ET\_B\) or selective ET\(_A\) receptor blockade partially restored ACh-induced CBF responses after L-NAME. Therefore, an ET-dependent process magnified the inhibitory effects of L-NAME on ACh-induced CBF responses. In contrast, ET-dependent effects failed to intervene in ACh-induced CBF responses when NO formation was intact, consistent with the suppression of ET production or action by NO.

We were concerned about the possibility that instrumentation of the proximal circumflex coronary artery may have influenced ET activity in the distal vascular territory. Myocardial IR-ET levels did not differ between the circumflex and left anterior descending perfusion territories, consistent with normal intrinsic ET activity that follows the instrumentation procedure. The site from which ET was derived in the present experiments cannot be directly inferred on the basis of tissue IR-ET measurements because a variety of cells are able to produce ET.\(^\text{20}\) An endothelial production of ET was most likely involved since these cells are the primary target of ACh and L-NAME. In fact, in large epicardial canine coronary arteries, endothelial denudation abolished ACh-induced dilation, whereas L-NAME constricted large coronary arteries through an endothelium-dependent process.\(^\text{21}\)

ET receptors involved in limiting CBF responses to ACh after L-NAME were of the ET\(_A\) subtype in our study. In contrast, ET\(_B\) receptors were not involved. ET\(_B\) receptors have been associated with NO and prostacyclin release from the endothelium.\(^\text{22,23}\) However, ET\(_B\) receptors can trigger significant constriction of resistance coronary vessels in vivo through a direct action on smooth muscle cells.\(^\text{16}\) Conceivably, differences in the threshold endogenous ET-1 levels required to elicit ET\(_B\) versus ET\(_A\)-dependent responses may explain why ET\(_A\) receptors were primarily involved in the present study.

Our data imply that ET should be readily available for release through a receptor-operated mechanism. Although earlier studies reported that de novo ET synthesis accounts for ET release in vitro,\(^\text{5}\) the existence of an endogenous pool of ET in cells targeted by ACh could better account for ET-dependent responses elicited over brief periods of ACh delivery. In the same connection, only preformed ET could account for the rapid onset of pressor responses to arginine analogues sensitive to ET blockers.\(^\text{1,3,10}\) Consistent with this possibility, a recent report identified ET-1–containing vesicles isolated from bovine aortic endothelial cells.\(^\text{24}\)

The partial reversal of the inhibitory effect of L-NAME on ACh-induced CBF increases by ET receptor blockade implies that an active dilator process resistant to blockade of NO formation intervened. Prostacyclin, another endothelium-derived relaxing factor, was not involved because our experiments were conducted after indomethacin treatment. Aside from an incomplete blockade of NO formation after

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**Figure 4.** Percent changes in 5 dogs in CBF and coronary resistance caused by IC ACh (30.0, 100.0, and 300.0 ng · kg\(^{-1}\) · min\(^{-1}\)) before IC L-NAME (50.0 µg · kg\(^{-1}\) · min\(^{-1}\)×12 minutes), after L-NAME, and after L-NAME+IC Ro 46-8443 (30.0 µg · kg\(^{-1}\) · min\(^{-1}\)×10 minutes +1.0 µg · kg\(^{-1}\) · min\(^{-1}\)), an ET\(_B\) receptor blocker. L-NAME abolished ACh-induced changes in CBF and coronary resistance that were not further altered after Ro 46-8443. *P<0.01 vs before L-NAME (ANOVA).

Except for a slight decrease in CBF (from 46±3 to 43±3 mL · min\(^{-1}\), P<0.01), Ro 61-1790 had no other significant hemodynamic effects in the absence of L-NAME. ACh 100 increased CBF by 37±5% (P<0.01) before Ro 61-1790 and by 40±6% (P<0.01) thereafter. For all doses of ACh examined, CBF responses did not differ before and after Ro 61-1790.

**Combined Blockade of NO Formation and ET\(_A\) Receptors**

Ro 46-8443 given after L-NAME had no significant hemodynamic effects.

Before L-NAME, IC ACh 100 led to steady-state increases (P<0.01) in CBF by 65±6%. After L-NAME, CBF responses to ACh were blunted (−1±5%). Ro 46-8443 given after L-NAME failed to alter CBF responses to ACh 100 (−1±4%). Overall, L-NAME abolished ACh-induced CBF increases and Ro 46-8443 had no further effects, as reported in Figure 4.

**Myocardial IR-ET Levels**

Similar myocardial IR-ET levels were detected in the perfusion territories of the circumflex (202±72 pg/g tissue) and the left anterior descending (272±101 pg/g tissue) coronary arteries.
L-NAME, ACh-induced CBF increases after L-NAME+ET receptor blockade may involve an alternate pathway leading to the formation of an endothelium-dependent hyperpolarizing factor (EDHF). This factor has been reported to account for L-NAME- and indomethacin-resistant dilation to endothelium-dependent agents.

Although our strategy of IC drug delivery allowed us to minimize systemic hemodynamic effects, a decrease in baseline MAP caused by Ro 61-1790 given after L-NAME could not be avoided. Conceivably, this decrease in MAP may have influenced our measurements of ACh-induced CBF responses thereafter. However, ACh-induced CBF responses were magnified by bosentan given after L-NAME without significant changes in MAP. Thus, the effects of ETα1 receptor blockade after L-NAME on CBF responses to ACh could not be primarily related to an altered hemodynamic baseline caused by Ro 61-1790.

In conclusion, ETα1-dependent tone magnified the inhibitory effects of blockade of NO formation on ACh-induced dilation of resistance coronary vessels. This receptor-operated cross-talk between NO and ET was revealed after blockade of NO formation and did not intervene under baseline conditions. Presumably, stimulated NO release has an inhibitory action on endogenous ET production and/or action at the level of resistance coronary vessels. This process may contribute to further impairment of endothelium-dependent coronary dilation to ACh observed in patients with an altered endothelial function.

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References

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