Chronic Endothelin Receptor Antagonism Preserves Coronary Endothelial Function in Experimental Hypercholesterolemia

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Background—Endothelin-1 (ET-1) is an endothelium-derived peptide that constricts coronary vessels through stimulation of the ET-A and ET-B receptors. Experimental porcine hypercholesterolemia is associated with impaired coronary endothelial function and elevated ET-1 concentrations. This study was designed to test the hypothesis that chronic endothelin receptor antagonism preserves coronary endothelial function in experimental hypercholesterolemia.

Methods and Results—Acetylcholine (10^{-6} to 10^{-4} mol/L) was serially infused into the left anterior descending coronary artery in pigs at baseline and after 12 weeks of a high-cholesterol diet. In the interim, the animals were randomized to 3 groups: Group 1 received no therapy, group 2 received 3 mg/kg per day RO 48-5695, a combined ET-A/ET-B receptor antagonist, and group 3 received 4 mg/kg per day ABT-627, a selective ET-A receptor antagonist. Percent change in coronary artery diameter, coronary blood flow, and coronary vascular resistance were calculated on the basis of quantitative coronary angiography and intracoronary Doppler. At 12 weeks, total cholesterol was significantly and similarly increased in all groups. Chronic endothelin receptor antagonism significantly increased coronary blood flow in response to acetylcholine at 12 weeks (group 1: -41.6%±10.7%, group 2: -4.7%±11.9%, group 3: 11.4%±7.4%).

Conclusions—Chronic endothelin receptor antagonism preserves coronary endothelial function in experimental hypercholesterolemia. This study supports the role for ET-1 in the pathogenesis of endothelial function. Moreover, endothelin receptor antagonists may have a therapeutic role by maintaining coronary endothelial function in pathophysiological states. (Circulation. 1999;99:1747-1752.)

Key Words: endothelin n receptors n vessels n endothelium-derived factors n hypercholesterolemia n vasculature

Endothelin-1 (ET-1) is a 21–amino acid peptide with both mitogenic and vasoconstricting properties.1,2 Two major receptors, the endothelin-A (ET-A) and the endothelin-B (ET-B) receptors, mediate these effects.3 In the vasculature, the ET-A receptor is located on vascular smooth muscle cells, whereas the ET-B receptor is found on both endothelial and vascular smooth muscle cells.4,5 The ET-A receptor produces coronary vasoconstriction that may be enhanced in hypercholesterolemia.6 The ET-B receptor on endothelial cells releases nitric oxide and causes vasodilatation, and the ET-B receptor on vascular smooth muscle cells mediates vasostriction. Although ET-B receptor activation may cause vasodilatation in physiological states, in pathophysiological states such as hypercholesterolemia, the overall effect of ET-B receptor activation may be vasoconstriction.7-9 Thus the overall effect of chronic antagonism on these receptors in hypercholesterolemia is unclear.

Porcine experimental hypercholesterolemia is associated with impaired coronary endothelial function characterized by enhanced vasoconstriction to the endothelium-dependent vasodilator acetylcholine, in association with increased ET-1 immunoreactivity. These functional changes occur before the appearance of gross histopathological changes associated with atherosclerosis.6,10,11 Given the vasoconstrictor effects of ET-1 and the rise in coronary sinus ET-1 during acetylcholine infusion in humans with coronary endothelial dysfunction, ET-1 has been implicated in the abnormal coronary vasomotor tone associated with hypercholesterolemia.4,6,10,11

In atherosclerosis, ET-1 has a pathophysiological role and may be an important factor in the progression of atherosclerosis.12,13 Moreover, endothelin receptor antagonism attenuates the histological changes associated with atherosclerosis.14,15 However, as yet there are few data on the ability of endothelin receptor antagonism to affect endothelial dysfunction. Given that the endothelial dysfunction observed in experimental hypercholesterolemia is presumed to represent an early stage of atherosclerosis,16,17 these studies were designed to test the hypothesis that chronic endothelin antagonism would preserve endothelial function in experimental porcine hypercholesterolemia.
Methods

Animals
All study procedures that involved animals were reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee and were designed in accordance with the National Institutes of Health Guidelines. After the baseline in vivo study (see below), juvenile domestic crossbred pigs (23 to 35 kg) were placed on an atherogenic diet of 2% cholesterol and 15% lard by weight (TD 93296, Harlan Teklad) for 12 weeks. In the interim, the pigs were randomized to 3 groups. Group 1 (control group) did not receive any additional drug therapy (n=7). Group 2 animals (n=9) were placed on oral RO 48-5695 (Hoffmann-La Roche Ltd), a combined ET-A and ET-B receptor antagonist, on a weight-adjusted scale every 3 weeks to maintain the dose at 3 mg/kg per day. RO 48-5695 is a potent, orally available nonpeptide endothelin receptor antagonist that is a derivative of bosentan. RO 48-5695 has a balanced affinity for both the ET-A and ET-B receptors (IC_50 ET-A receptor 0.7 nmol/L and IC_50 ET-B receptor 5 nmol/L). The dosage of RO 48-5695 was determined on the basis of preliminary studies by Hoffmann-La Roche. Group 3 animals (n=8) were placed on ABT-627 (Abbott Laboratories) on a weight-adjusted scale to maintain a dose of 4 mg/kg per day. ABT-627 is a potential orally active, nonpeptide ET-A receptor selective antagonist that is the active R, S isomer of the racemic compound A-127727. A-127727 has been fully characterized and has a binding K for the ET-A receptors approximately 2000-fold greater than for the ET-B receptors. 

Plasma Assays
Arterial blood was collected during the initial baseline period for the in vivo studies. Total cholesterol, HDL, LDL, and triglyceride levels were measured in the Mayo Clinic ImmunoChemistry Laboratory (Roche Diagnostic Systems). Plasma ET-1 was determined by radioimmunoassay using an endothelin-1,2 [E-2] assay system (Amersham) as previously described.

In Vivo Experiments
Each animal was fasted overnight before the day of the study but allowed ad libitum access to tap water. Animals were initially anesthetized with 30 mg/kg ketamine and 5 mg/kg xylazine, and additional anesthesia was given by intravenous infusion of a solution containing ketamine (5 g/L) and xylazine (7.5 mg/L) to maintain a constant level of anesthesia. The external or internal jugular vein was then exposed by cutdown and cannulated with a 7F venous sheath. A flow-directed thermodilution catheter was advanced through the venous sheath for the measurement of pulmonary artery pressure (PAP), pulmonary capillary wedge pressure (PCWP), and cardiac output (CO). The external carotid artery was also exposed by cutdown and cannulated with an 8F arterial sheath. After injecting 10,000 units of heparin intravenously, followed by a intravenous infusion of 1000 units of heparin per hour, an 8F Judkins left coronary guiding catheter was used to engage the left main coronary artery. A 2.2F coronary infusion catheter (Ultrasound, SciMed Life System) was advanced over a 0.014-in Doppler guide wire (Cardiometrics) and positioned into the proximal or mid portion of the left anterior descending coronary artery. All infusions (see below) were delivered at a rate of 1 mL/min through the coronary infusion catheter. Before and after each drug was delivered, mean arterial pressure (MAP), heart rate (HR), and CO were measured. The CO was measured in triplicate, and the average was used for all subsequent analysis. Average peak velocity (APV) obtained from on-line analysis of Doppler parameters of intracoronary flow velocities was recorded at each time point. At each time interval, selective coronary angiography was then performed for the measurement of coronary artery diameter (Cor Diam). The angles, skew, rotation, and table height were kept constant during the procedure. Cor Diam within 5 mm distal to the tip of the of the Doppler wire was measured by a blinded observer with the use of a computer-based imaging system, as previously described. Coronary blood flow (CBF) was calculated from the Doppler-derived time-velocity integral and vessel diameter, as previously described: 

\[ \text{CBF} = \frac{(\pi/2)(\text{APV})(\text{PCWP})}{\text{Cor Diam}} \]

Systemic vascular resistance (SVR) was calculated as MAP/CO, and coronary vascular resistance (CVR) was determined by the formula CVR = (MAP – PCWP)/CBF.

Initial hemodynamic, angiographic, and Doppler measurements were obtained followed by a 20-minute infusion of normal saline to allow for stabilization of the animal, and then measurements were repeated. Normal saline was infused during 20-minute washout periods between drug infusion. Acetylcholine (10⁻⁴, 10⁻³, 10⁻⁴ mol/L) (Sigma) was selectively infused into the left anterior descending artery for 5 minutes at each dose. After equilibration at each dose, repeated measurements were obtained. Additionally, after 20-minute washout periods between medications and after repeated baseline measurements were taken, 50 μg nitroglycerin (Abbott Laboratories) over 3 minutes was selectively infused through the intracoronary route in the left anterior descending artery.

Statistical Analysis
Data are expressed as mean±SEM. Within each group, repeated measurements were analyzed with repeated-measures ANOVA followed by the Bonferroni t test or by Student’s paired t test, unpaired t test, or Mann-Whitney rank sum test. Statistical significance was achieved at a value of P<0.05.

Results

Plasma Assays
After 12 weeks of a high-cholesterol diet, total cholesterol significantly increased in all 3 groups compared with baseline (Table). This was associated with a significant increase in HDL and LDL, without a change in triglycerides. Cholesterol levels did not differ between the groups at baseline or at 12 weeks.

After 12 weeks, plasma ET-1 concentrations significantly increased in all 3 groups. Chronic oral ET-A/ET-B receptor antagonism resulted in a significantly higher plasma ET-1 concentration at 12 weeks compared with the untreated control group and the selective ET-A antagonist group (Table).

In Vivo
The Table summarizes the basal systemic and coronary hemodynamic parameters of the pigs before any drug infusions at baseline and after 12 weeks of high-cholesterol feeding in groups 1 to 3 in the anesthetized state. There was no difference in HR, SVR, and CVR among the groups. After 12 weeks of the cholesterol diet, there was a mild but significant increase in mean arterial pressure in group 1. CO and CBF significantly increased in all 3 groups after 12 weeks. This trend toward increased CBF after 12 weeks is consistent with the increasing size of the pig and has been seen in pigs on both normal and high-cholesterol diets.

Figure 1 demonstrates the dose-response effect of acetylcholine on CBF at baseline and after 12 weeks of the high-cholesterol diet in the control pigs and the endothelin
receptor antagonist groups at 12 weeks. There was no difference in the dose-response effect of acetylcholine on CBF at baseline in any of the groups. There was a significant decrease in CBF with infusion of acetylcholine at all 3 concentrations in group 1 at 12 weeks compared with groups 2 and 3 at 12 weeks and with group 1 at baseline. Figure 2 illustrates the percent change in Cor Diam, CBF, and CVR in the baseline studies and after 12 weeks of therapy in all 3 groups. Acetylcholine administration at a dose of $10^{-5}$ mol/L in animals that received no drug therapy (group 1) minimally decreased Cor Diam at baseline, with a greater effect at 12 weeks ($P<0.05$). CBF decreased more at 12 weeks compared with baseline ($-11.55\%$ vs $-41.6\%$, respectively, $P<0.01$) and CVR increased more at 12 weeks ($18.2\%$ vs $135.2\%$, $P<0.05$). In the combined ET-A/ET-B receptor antagonist group (group 2), the maximal dose of acetylcholine decreased Cor Diam at baseline as was seen in group 1, but this effect was normalized in group 2, which received the combined endothelin receptor antagonist ($P=0.75$, baseline vs 12 weeks). In this group, CBF in response to acetylcholine was not different between baseline and after 12 weeks of cholesterol feeding ($P=0.38$, baseline vs 12 weeks). CVR in response to acetylcholine also was not different after 12 weeks of cholesterol feeding ($P=0.30$). In group 3, which received the selective ET-A receptor antagonist, Cor Diam also did not decrease in response to acetylcholine after 12 weeks of cholesterol feeding ($P=0.75$). Additionally, in group 3, the CBF and CVR response to intracoronary administration of acetylcholine was maintained at 12 weeks ($P=0.03$ for CBF and $P=0.008$ for CVR). In both group 2 and group 3, there was a significant difference in the maximal CBF and CVR response to acetylcholine compared with group 1 at 12 weeks ($P<0.05$ for all groups). There was no difference at 12 weeks in Cor Diam ($P=0.67$), CBF ($P=0.28$), and CVR ($P=0.53$) in response to acetylcholine between group 2 and group 3.

In baseline studies in group 1, nitroglycerin increased CBF $114.1\%\pm31.2\%$ without a significant effect on Cor Diam ($-0.4\%\pm1.6\%$). There was no significant change in response to nitroglycerin after 12 weeks (CBF: $132\%\pm38.9\%$, Cor Diam: $3.7\%\pm1.0\%$, $P>0.05$ for all). There was no difference in response to nitroglycerin among the groups.

**Discussion**

The principal finding of the current study is that chronic endothelin-receptor antagonism of either the ET-A receptor or combined ET-A and ET-B receptors in experimental porcine hypercholesterolemia results in preserved coronary endothelial function in vivo. This supports the role of endothelin receptor antagonism in impeding the development of vasomotor abnormalities associated with hypercholesterolemia and supports a therapeutic role for endothelin receptor antagonists in pathophysiological states associated with altered endothelial function.

Prior studies indicate that ET-1 may in part mediate vasomotor dysregulation in hypercholesterolemia. However, the exact role of the different receptors in endothelial...
dysfunction has not been established. The current study suggests that ET-1 is critically important in the development of endothelial dysfunction in experimental hypercholesterolemia and that blockade of the endothelin axis through either a combined ET-A/ET-B receptor antagonist or a selective ET-A receptor antagonist preserves endothelial function in porcine experimental hypercholesterolemia without affecting cholesterol levels.

The preservation of endothelial function may have functional significance. We have recently reported that altered coronary endothelial function is associated with myocardial perfusion defects suggestive of myocardial ischemia.\textsuperscript{27} Additionally, Fuster and colleagues\textsuperscript{16} suggest that vascular functional abnormalities preceding the presence of morphological changes in the vascular wall may represent the earliest detectable stage of atherosclerosis. Thus preservation of endothelial function may serve as an important mechanism to prevent both progression of atherosclerosis and myocardial ischemia.

There are several potential mechanisms for the beneficial effect of chronic endothelin receptor antagonism on endothelial function including alterations in coronary hemodynamics caused by the vasoactive properties of ET-1 inhibition, antiatherosclerotic effects of endothelin receptor antagonists, and augmentation of nitric oxide and non–nitric oxide pathways. We have previously shown that the acute inhibition of either the ET-A receptor or both the ET-A and ET-B receptors did not attenuate acetylcholine-induced epicardial vasoconstriction in experimental hypercholesterolemia.\textsuperscript{11} Thus these previous studies suggest that the acute effects of endothelin receptor antagonists on coronary hemodynamics do not account for improved endothelial function with chronic endothelin receptor antagonism. Furthermore, previous studies failed to demonstrate a beneficial effect on endothelial function with chronic lowering of blood pressure except with therapies that have specific interactions with vascular remodeling.\textsuperscript{28,29} Thus the chronic systemic and hemodynamic effects of endothelin receptor antagonism alone cannot account for the improvement in endothelial function, although these effects may contribute to the beneficial effects of these agents.

The antiatherosclerotic effects of endothelin receptor antagonists may be another potential mechanism for the preservation of endothelial function seen in this study. ET-1 is an important mitogenic peptide that alters vascular remodeling.\textsuperscript{30,31} It is increased in patients with atherosclerotic risk factors including hypercholesterolemia and is increased locally within atherosclerotic plaques.\textsuperscript{10,32,33} This peptide also mediates the response to vascular injury and promotes restenosis after balloon catheter injury.\textsuperscript{13,34} Moreover, selective ET-A receptor antagonism decreases the development of atherosclerosis caused by cholesterol feeding, and chronic ET-A receptor antagonism inhibits neointimal hyperplasia after both balloon and stent injury.\textsuperscript{15,20,35} Because endothelial dysfunction is considered a manifestation of early atherosclerosis, it may be speculated that the chronic endothelin receptor antagonists improve coronary endothelial function by attenuating the atherosclerotic process.

Another possible mechanism for the beneficial effects of chronic endothelin receptor antagonism on coronary vasomotor function may be through alterations in the relative balance between vasoconstricting and vasorelaxing factors. This balance is altered in pathophysiological states, and restoration of
this balance may mediate a protective effect.\textsuperscript{36–38} One of the most important vasodilators is nitric oxide, which antagonizes the effects of ET-1.\textsuperscript{22} We have recently reported that porcine experimental hypercholesterolemia is characterized by an enhanced vasoconstrictor response to ET-1 and decreased endogenous nitric oxide activity.\textsuperscript{4} Moreover, previous studies suggest that endothelin receptor antagonism increases nitric oxide.\textsuperscript{39} Additionally, our studies suggest that nitric oxide activity is augmented with chronic endothelin receptor antagonism by the preservation of the response to acetylcholine in vivo. Thus augmentation of the relative effects of vasodilating and antimitogenic factors such as nitric oxide may be one mechanism by which chronic endothelin receptor antagonism preserves endothelial function.

Chronic angiotensin-converting enzyme inhibition also improves endothelial function in pathophysiological states.\textsuperscript{29,40} Furthermore, nitric oxide is an important mediator of the structural and functional effects of angiotensin-converting enzyme inhibitors.\textsuperscript{28,41} The renin-angiotensin system is intimately linked to the endothelin axis. Angiotensin augments ET-1 secretion and ET-1 stimulates the conversion of angiotensin I to angiotensin II.\textsuperscript{42,43} Angiotensin-converting enzyme inhibitors block these effects and attenuate ET-1 production in vivo.\textsuperscript{44} Possible mechanisms for the preservation of endothelial function by chronic endothelin receptor antagonists include many of the same mechanisms as those suggested for chronic angiotensin-converting enzyme inhibitors, including augmentation of nitric oxide. Endothelin receptor antagonists may also decrease the effects of angiotensin through the interactions of these 2 systems.

This study used both a selective ET-A receptor antagonist and a combined ET-A/ET-B receptor antagonist. As has been previously demonstrated, combined endothelin receptor antagonism in our study significantly increased ET-1 levels at 12 weeks compared with the control group and the selective ET-A antagonist group.\textsuperscript{45} Additionally, selective ET-A antagonism did not increase plasma ET-1 levels. Thus, this supports the idea that the ET-B receptor was inhibited only in the combined endothelin receptor antagonist group. This study also demonstrates that both combined ET-A/ET-B receptor antagonism and selective ET-A receptor antagonism are effective in preserving coronary endothelial function and supports the role of ET-A receptor antagonism in preserving endothelial function in hypercholesterolemia. Furthermore, this study did not show any added benefit of ET-B receptor antagonism.

In summary, this study demonstrates for the first time that chronic endothelin receptor antagonism prevents the development of coronary endothelial dysfunction in vivo in an experimental porcine model. These data support a role of ET-1 in the regulation of coronary vascular tone and endothelial function and suggest a possible therapeutic role for endothelin receptor antagonists in preserving endothelial function in pathophysiological states, such as hypercholesterolemia.

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