Coronary Endothelial Function Is Preserved With Chronic Endothelin Receptor Antagonism in Experimental Hypercholesterolemia In Vitro

Patricia J.M. Best, Lilach O. Lerman, Juan C. Romero, Darcy Richardson, David R. Holmes, Jr, Amir Lerman

Abstract—Hypercholesterolemia is associated with increased circulating and tissue endothelin-1 immunoreactivity, decreased nitric oxide (NO) activity, and altered endothelial function. We tested the hypothesis that chronic endothelin receptor antagonism preserves endothelial function and increases NO in experimental porcine hypercholesterolemia. Pigs were randomized to 3 groups: Group 1, a 2% high-cholesterol (HC) diet alone (n=7); group 2, RO-48-5695, a combined endothelin receptor antagonist, and an HC diet (n=8); and group 3, ABT-627, a selective endothelin-A receptor antagonist, and an HC diet (n=8). Coronary epicardial and arteriolar endothelial function was determined by a dose-response relaxation to bradykinin (10^-11 to 10^-6 mol/L), in all groups and in pigs maintained on a normal diet. Plasma total oxidized products of NO (NOx) were determined by chemiluminescence at baseline and after 12 weeks. Bradykinin-stimulated coronary epicardial and arteriolar relaxation in group 1 was attenuated compared with normal-diet controls. This relaxation was normalized with endothelin receptor antagonism. Plasma NOx decreased after 12 weeks in group 1 (−74.8±5.5%). This decrease was attenuated in the endothelin receptor antagonist groups (group 2, −28.2±15.0%; group 3, −38.9±20.6%). Chronic endothelin receptor antagonism preserves coronary endothelial function and increases NO in hypercholesterolemia. This study supports a role of endothelin-1 in the regulation of NO activity and suggests a possible therapeutic role for endothelin receptor antagonists in hypercholesterolemia. (Arterioscler Thromb Vasc Biol. 1999;19:2769-2775.)

Key Words: coronary vessels ■ hypercholesterolemia ■ endothelin receptors ■ nitric oxide ■ oxidative stress

Endothelin-1 is a 21–amino acid peptide that is atherogenic and has both mitogenic and vasoconstricting properties.1–3 These effects are exerted through the 2 endothelin receptors: the endothelin-A (ET-A) receptor located on vascular smooth muscle cells, and the endothelin-B (ET-B) receptor, located on both endothelial and vascular smooth muscle cells.4,5 Both receptors may mediate enhanced coronary vasoconstriction to endothelin-1 in pathophysiological states such as experimental hypercholesterolemia, in which levels of coronary tissue and circulating endothelin-1 are increased.6–8

Besides enhanced endothelin-1 activity, hypercholesterolemia is associated with abnormal coronary endothelial function, decreased basal nitric oxide (NO) activity, and decreased coronary endothelial cell NO synthase (NOS).6,9 Attenuation in NO activity is functionally important, since NO contributes to both basal and demand-mediated coronary blood flow, antagonizes the vascular effects of endothelin-1, and is intimately linked to the regulation of endothelin-1 production.10,11 Experimental hypercholesterolemia is also associated with increased production of oxygen free radicals and increased oxidative stress.12,13 This may subsequently lead to altered bioavailability of NO or functional changes of the endothelium and has important implications in the pathogenesis of atherosclerosis.14–16 Oxidative stress also increases endothelin-1 production and release from endothelial cells in vitro.17,18 Additionally, endothelin-1 may increase oxidative stress by increasing oxygen free-radical formation from macrophages.19

Therefore, the current investigation was undertaken with the objectives to determine (1) whether chronic endothelin receptor antagonism in hypercholesterolemia preserves coronary epicardial and arteriolar endothelial function in vitro, (2) whether chronic endothelin receptor antagonism attenuates the reduction in plasma total oxidized products of NO (NOx) and coronary endothelial NOS immunostaining associated with hypercholesterolemia, and (3) whether chronic endothelin receptor antagonism decreases plasma F2-isoprostane levels, an in vivo marker of oxidative stress, in experimental hypercholesterolemia.

Methods

Animals
All study procedures with animals were reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee.

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and were designed in accordance with National Institutes of Health guidelines. Female juvenile, domestic, crossbred pigs (23 to 35 kg) (Larson Farms, Seargent, Minn) were placed on an atherogenic diet of 2% cholesterol and 15% lard by weight (TD 93296, Harlan Teklad) for 12 weeks. 

In the interim, group 1 animals (control group) did not receive any additional medications. Group 2 animals were placed on oral RO-48-5695 (Hoffmann–La Roche Ltd, Basel, Switzerland), a combined ET-A and ET-B receptor antagonist, on a weight-adjusted dose every 3 weeks to maintain a dose of 3 mg·kg⁻¹·day⁻¹. 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, Abbott Park, Ill) on a weight-adjusted scale to maintain a dose of 4 mg·kg⁻¹·day⁻¹. The dosage of ABT-627 was based on our previous studies.

At the start of the study and after 12 weeks of therapy, animals were anesthetized with ketamine and xylazine, and the external carotid artery was exposed by cutdown and cannulated with an 18G arterial sheath for blood pressure measurement.

Plasma lipid profiles (Roche) were determined at baseline and after 12 weeks of the high-cholesterol diet. Additionally, plasma F₂-isoprostanes and NOₓ. Euthanasia was performed by intravenous administration of 30 mg/kg pentobarbital sodium (Sleepaway, Fort Dodge Laboratories) for the in vitro studies. Additional control animals maintained on a normal diet were used for the in vitro studies to determine normal vascular reactivity for comparison with the 3 groups on the high-cholesterol diet.

In Vitro Endothelial Function

After euthanasia, normal hearts as well as hearts from the experimental animals were harvested for in vitro analysis of coronary epicardial relaxation in response to bradykinin.

Epicardial Vessels

As previously described, the hearts were placed into cold, modified Krebs-Ringer bicarbonate solution (control solution) of the following millimolar composition: 118.3 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, 25.0 NaHCO₃, 0.016 Ca-EDTA, and 11.1 glucose. Rings of tissue 2 to 3 mm long were dissected from the left circumflex artery, transferred to organ chambers with 25 mL of control solution (37°C, pH 7.4), and oxygenated with 95% O₂ and 5% CO₂. The tissue was suspended between 2 stirrups and connected to a strain gauge for continuous recording of isometric tension. The artery rings were equilibrated for 1 hour at a resting tension. Viability of the vessels was confirmed by a contractile response to 10⁻⁵ mol/L endothelin-1, followed by the response to cumulative concentrations of bradykinin. In 9 rings from normal-diet pigs and 7 rings from group 1 pigs, the dose response to cumulative concentrations of bradykinin was recorded. Complete relaxation of each artery was obtained by exposure to 10⁻⁴ mol/L papaverine (Sigma). Additionally, in 6 arterioles from normal-diet pigs, 10⁻⁶ mol/L L-NAME was added 20 minutes before the addition of endothelin-1, followed by the dose response to cumulative concentrations of bradykinin.

Plasma Total NOₓ

Plasma NOₓ levels were determined by chemiluminescence with a Sievers nitric oxide analyzer (model 280) as previously described.

Blood samples from each group at baseline and after 12 weeks of therapy (group 1 n=7, group 2 n=8, and group 3 n=8) were centrifuged at 2500 rpm for 20 minutes at 10°C. The supernatant was removed and stored at −70°C. The NO assay was standardized by a calibration curve using known concentrations of nitrate (0.01 μmol/L to 100 μmol/L) obtained from NaNO₃. For each sample, 4 μL of sample was placed in a reducing vessel with 5 mL of 0.1 mol/L vanadium(III)chloride, 1 mol/L HCl, and 100 μL of antimicrobial agent (Sievers) at 90°C. Each sample and standard were analyzed at least 3 times. The mean value was used for all subsequent analysis.

Endothelial NOS Immunostaining

Frozen sections of tissue were cut in 5-μm sections and mounted on positively charged slides. The slides were dried at 37°C for 1 hour and fixed in acetone for 10 minutes at 4°C. The slides were air dried for 30 minutes. Endogenous peroxidase activity was blocked by placing the slides in 1.5% H₂O₂ and 30% absolute methanol for 10 minutes and then rinsed. The slides were pretreated with 0.25% SDS for 10 minutes. To block nonspecific binding sites, the tissue was incubated with 5% goat serum (Dako/PBS/Tween 20 for 10 minutes). Monoclonal antibodys to endothelial NOS (Transduction Laboratories) were then added (400 μL of a 0.25 μg/mL dilution) and incubated overnight at 4°C. The slides were rinsed, incubated for 30 minutes with biotinylated goat anti-mouse IgG diluted 1:400 and 2% normal swine serum, rinsed, incubated with peroxidase-labeled streptavidin diluted 1:500 for 30 minutes, and rinsed. Next, the tissue was stained for 15 minutes with 3-amino-9-ethyl carbazole solution and rinsed. To enhance nuclear detail, the slides were counterstained with hematoxylin. Three independent observers who were blinded to the different treatment groups reviewed the tissue sections.

The presence of staining was quantified on the basis of a scoring system as previously described: 0, no staining; 1, positive staining in <25% of the endothelial cells per slide; 2, positive staining in 25% to 75% of the endothelial cells; and 3, positive staining in >75% of the endothelial cells. The scores for each of the observers were averaged for each of the slides, and the average score for each of the groups was then calculated.

F₂-Isoprostanes

Blood samples from 5 pigs in each of the 3 groups at baseline and after 12 weeks of therapy were collected in EDTA tubes, and the plasma was stored at −80°C until the time of the assay. The total levels of 8-isoprostaglandin F₂ were measured with an enzyme immunoassay kit (EIA, Cayman). Before the enzyme immunoassay, alkaline hydrolysis was used. Plasma samples were purified by Sep-Pak C-18 columns (Waters) before analysis. The samples,
Lipid Profiles, Total NOx, and F₂-isoprostanes at Baseline and After 12 Weeks of a High-Cholesterol Diet in the Control Group

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>12 Weeks</th>
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<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>89.3±2.8</td>
<td>446.4±53.1*</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>50.0±3.1</td>
<td>317.7±47.4*</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>35.0±2.5</td>
<td>83.7±7.4*</td>
</tr>
<tr>
<td>Total NOx, μmol/L</td>
<td>53.0±12.2</td>
<td>11.0±1.9*</td>
</tr>
<tr>
<td>F₂-isoprostanes, pg/mL</td>
<td>84.2±6.8</td>
<td>238.0±29.7*</td>
</tr>
</tbody>
</table>

*p<0.05.

tracer, and antiserum were added to wells precoated with mouse monoclonal antibody. The plates were washed to remove all unbound reagents. Ellman’s reagent (containing the substrate to acetylcholinesterase) was added to the wells. The intensity of the distinct yellow color produced by this enzymatic reaction was determined with a spectrophotometer at 405 nm.

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 between groups, or the Mann-Whitney rank-sum test. Statistical significance was achieved with a value of P<0.05.

Results
Control Group
After 12 weeks of the high-cholesterol diet, total cholesterol significantly increased (the Table). This was associated with a significant increase in HDL and LDL, whereas triglycerides did not change significantly. In this group after 12 weeks, there was an increase in mean arterial blood pressure (99±6 mm Hg versus 134±5 mm Hg, P=0.05). This increase in mean arterial blood pressure occurs with the increasing size of the animal and regardless of whether the pigs were fed the normal diet or the high-cholesterol diet. In epicardial coronary vessels removed from the hypercholesterolemic pigs, the in vitro response to bradykinin was attenuated compared with that in the normal-diet pigs (maximal relaxation 70.80±6.22% versus 88.66±2.26%, respectively; P<0.05; Figure 1). There was no difference in the epicardial vessels from normal-diet pigs compared with vessels from normal-diet pigs in their response to endothelin-1 (normal endothelin-1 precontraction 11.09±0.79 g tension, group 1 endothelin-1 precontraction 8.67±0.86 g tension; P=0.06) or KCl (data not shown). Additionally, preincubation of the vessels with L-NMMA did not alter the magnitude of the precontraction with endothelin-1 (with L-NMMA 9.62±1.37 g tension versus without L-NMMA 11.09±0.79 g tension; P=0.42). The endothelin-dependent coronary vasorelaxation in response to the maximal dose of bradykinin (10⁻⁶ mol/L) was significantly attenuated in the hypercholesterolemic, porcine epicardial vessels (n=9 rings) compared with the normal vessels (n=7 rings). L-NMMA significantly attenuated the response to bradykinin in the normal vessels (maximal relaxation: 38.66±6.47% with L-NMMA versus 88.66±2.26% without L-NMMA; P<0.001) and in the high-cholesterol vessels (maximal relaxation: 59.78±13.49% with L-NMMA versus 70.80±6.22% without L-NMMA; P=0.022). Endothelin-independent vasorelaxation to sodium nitroprusside (10⁻⁹ to 10⁻⁴ mol/L) was not attenuated with the high-cholesterol diet (maximal relaxation: normal diet 75.79±4.54%, group 1 75.00±6.78%). The response of the epicardial vessels to cumulative concentrations of endothelin-1 was not altered in rings from pigs fed the high-cholesterol diet compared with the control rings from pigs on the normal diet (maximal contraction compared with 60 mmol/L KCl in the normal vessels: 131.14±12.84%, group 1: 116.78±9.01%; P=0.378). The in vitro arteriolar response to bradykinin at the higher concentrations (10⁻⁷ to 10⁻⁵ mol/L, Figure 2) similarly showed an attenuated endothelium-dependent relaxation in the high-cholesterol pigs (n=6 rings) compared with the normal-diet pigs (n=7 rings). The arteriolar response to bradykinin was again significantly attenuated with the addition of L-NMMA in arterioles from normal-diet pigs (78.42±4.08% with L-NMMA versus 96.57±1.34% without L-NMMA; P<0.001).

In group 1, plasma NOx values significantly decreased after 12 weeks of the high-cholesterol diet compared with baseline (P=0.01; the Table). This decrement was associated with an overall decrease in immunoreactivity for NOS in coronary vessels after 12 weeks of the high-cholesterol diet compared with pigs on the normal diet (2.8±0.1 versus 1.2±0.4; P<0.01; Figure 3).

Plasma F₂-isoprostane levels significantly increased in the control group after 12 weeks of the high-cholesterol diet (the Table). In previous studies in our laboratory, normal-diet pigs have been shown to not have alterations in plasma F₂-isoprostane levels after 12 weeks.

Endothelin Receptor Antagonist Groups
After 12 weeks of the high-cholesterol diet, total cholesterol significantly increased in both of the endothelin receptor antagonist groups compared with baseline. There was no difference in cholesterol levels (total cholesterol, HDL, or LDL) between the 3 groups at baseline and after 12 weeks. Additionally, the lipid profile in the normal-diet control group used for the organ chamber experiments was similar to all 3 groups at baseline (total cholesterol 83.6±7.15 mg/dL). In
both group 2, treated with the combined ET-A/ET-B receptor antagonist, and group 3, treated with the selective ET-A receptor antagonist, the normal increase in blood pressure seen in the control group was attenuated (104±6 mm Hg and 108±2 mm Hg, respectively, compared with 134±5 mm Hg in group 1).

There was no difference in the response of the epicardial vessels in any of the groups to endothelin-1 (group 2 endothelin-1 precontraction 8.40±1.48 g tension; *P*=0.87 versus group 1; group 3 endothelin-1 precontraction 11.33±0.98 g tension; *P*=0.07 versus group 1) or KCl (data not shown). The attenuated endothelium-dependent vasorelaxation of the hypercholesterolemic epicardial vessels was normalized when either the combined ET-A/ET-B receptor antagonist (n=6, group 2) or the selective ET-A receptor antagonist (n=6, group 3) (maximal relaxation 93.25±4.94% and 86.50±4.67%, respectively) was given chronically in combination with the high-cholesterol diet (Figure 1). The in vitro arteriolar studies (mean size of the vessel was 392±17 μm) showed a similar trend as observed in the epicardial vessels. The attenuated endothelium-dependent relaxation to bradykinin in the high-cholesterol pigs was normalized in both the combined ET-A/ET-B receptor antagonist group (n=8) and in the selective ET-A receptor antagonist group (n=7, Figure 2).

After 12 weeks, the decrease in plasma NOx observed in the control group was significantly attenuated in both group 2 and group 3 (Figure 4). Similarly, there was an increase in the presence of enzyme immunoreactivity for endothelial NOS in the coronary arteries obtained from the endothelin antagonist groups (groups 2 and 3, 2.1±0.3 and 2.3±0.3) compared with the group on the high-cholesterol diet alone (group 1, 1.2±0.4; Figure 3).

Compared with group 1, chronic endothelin receptor antagonism also significantly attenuated the increase in plasma F2-isoprostanes from baseline in both the combined endothelin receptor antagonist group, from 108.0±9.7 to 158.4±15.9 pg/mL at 12 weeks, and the selective ET-A
Endothelin-1 is a potent vasoconstrictor and atherogenic peptide with enhanced immunoreactivity in atherosclerotic tissue. Furthermore, increased endothelin-1 is associated with increased plasma NOx and increased coronary epicardial and arteriolar endothelial function. This was associated with increased plasma NOx and increased coronary immunoreactivity for constitutive NOS. Finally, these data show that chronic endothelin receptor antagonism attenuates the increase in F2-isoprostanes in porcine hypercholesterolemia. This study supports a role for endothelin in the progression of coronary atherosclerosis in experimental porcine hypercholesterolemia.

Endothelin-1 is a potent vasoconstrictor and atherogenic peptide with enhanced immunoreactivity in atherosclerotic tissue. Furthermore, increased endothelin-1 is associated with most atherosclerotic risk factors. Thus, this peptide may be part of a common mechanism in the evolution of coronary atherosclerosis. In atherosclerosis, endothelin-1 alters vascular remodeling by inhibiting apoptosis, promoting proliferation of smooth muscle cells and fibroblasts, and acting as a chemoattractant factor and activator of macrophages.

In addition to its direct atherosclerotic effects, endothelin-1 may in part mediate its effects through decreased NO production. NO antagonizes both the atherogenic and vasoconstricting effects of endothelin-1. Furthermore, the regulatory mechanisms of these vasoactive factors interact. NO decreases both the production and the release of endothelin-1 from the vascular endothelium. Endothelin-1 in turn modulates NO production through inhibition of NOS. NO also modulates both the number and affinity of ET-A receptors. In disease states such as hypercholesterolemia and atherosclerosis, an imbalance between NO and endothelin-1 activity is detected and may contribute to both vasomotor abnormalities and vascular remodeling. Thus, determining the effect of chronic endothelin receptor antagonism on NO may demonstrate a mechanism for the antiatherosclerotic effect.

Our study demonstrates that in hypercholesterolemia, chronic endothelin receptor antagonism partly preserves the metabolic products of NO, as determined by total NOx, suggesting that the amount of NO produced is partly preserved. This was associated with an attenuated decrease in coronary endothelial NOS immunoreactivity and preserved coronary endothelial function. The response to bradykinin was similar between the high-cholesterol group and the normal group with L-NMMA, suggesting that the attenuated response in the high-cholesterol group may be due to decreased NO bioavailability in the epicardial vessels and arterioles. Thus, this suggests that preservation of bradykinin-stimulated vascular relaxation in the endothelin antagonist groups is also associated with preserved agonist-stimulated NO release. We have recently demonstrated that experimental hypercholesterolemia is characterized by enhanced coronary vasoconstriction to endothelin-1 in vivo without a change in endothelin receptor density. This was associated with a decrease in endogenous coronary NO activity. Therefore, preservation of NO production could potentially attenuate the enhanced vasoconstrictor effects of endothelin-1. Furthermore, endothelial function has been proposed as a marker for early atherosclerosis. These data suggest that I of the mechanisms for the antiatherosclerotic effects of chronic endothelin receptor antagonism may be through the effects on NO.

Besides increased endothelin-1, experimental hypercholesterolemia is also associated with increased vascular production of oxygen free radicals. This leads to augmented oxidation of LDL, which appears to be pivotal in the formation and progression of atherosclerosis through multiple pathways, including enhanced cellular LDL uptake and proinflammatory effects. Oxidative stress also promotes atherosclerosis by increasing endothelin-1 production and inactivation of NO by oxygen free radicals. However, through the same scavenger mechanism, NO can inhibit lipid peroxidation and the formation of F2-isoprostanes. Moreover, endothelin may increase oxidative stress by increasing oxygen free-radical formation from macrophages. Thus, oxidative...
tive stress promotes an atherogenic environment of increased endothelin-1, decreased NO, and increased lipid peroxidation. In addition to the atherosclerotic effects, oxidative stress can also alter vascular tone. In part, this effect may be mediated through the alteration in the balance of NO and endothelin-1. Thus, oxidative stress increases the atherosclerotic process and increases vascular tone.

Our study demonstrates that in hypercholesterolemia, chronic inhibition of endothelin receptors by either combined ET-A/ET-B receptor antagonism or by selective ET-A receptor antagonist attenuates the increase in circulating F_2-isoprostane concentrations, an in vivo marker of endogenous lipid peroxidation. This suggests that not only may oxidative stress alter endothelin-1 production but also that endothelin-1 may alter oxidative stress. Furthermore, decreased oxidative stress with subsequent increased NO bioavailability and decreased lipid peroxidation may be 1 of the mechanisms for the antiatherosclerotic effects of endothelin receptor antagonists. In addition, this study shows that decreased oxidative stress is associated with normalization of bradykinin-stimulated endothelium-dependent vasodilation in vitro. This effect may in part be due to preservation of NO production and decreased formation of vasoconstricting substances, including F_2-isoprostanes. Despite this potential mechanism, we cannot rule out the possibility that the chronic hemodynamic effects of endothelin receptor antagonists may in part mediate some of these effects. Chronic endothelin antagonists lower blood pressure in both normal-diet pigs and, in the current study, in pigs fed a high-cholesterol diet. The improvement in endothelial function seen with chronic endothelin antagonists is unlikely to be due to acute hemodynamic effects of endothelin receptor antagonism, since we have previously demonstrated that acute endothelin receptor antagonism does not attenuate hypercholesterolemia-induced endothelial dysfunction. Furthermore, this study demonstrated that acute endothelin receptor antagonism does not alter endothelial function in normal-diet pigs.

To determine the relative importance of each receptor type in the hypercholesterolemic state, this study used both a combined ET-A/ET-B receptor antagonist and a selective ET-A receptor antagonist. Because the ET-B receptor is functionally coupled to NOS, one could speculate that blockade of this receptor might be deleterious. However, we and others have previously demonstrated that in pathophysiological states, the ET-B receptor predominantly mediates vasocostriction. In our current study, blockade of the ET-B receptor as part of the combined ET-A/ET-B receptor blockade showed similar effects as blockade of the ET-A receptor alone. Thus, this study did not show any added benefit of ET-B receptor antagonism in hypercholesterolemia.

In summary, the present study demonstrates that chronic endothelin receptor antagonism in experimental hypercholesterolemia can preserve coronary endothelial function, augment NO production, and decrease oxidative stress. These data support a role of endothelin-1 in the regulation of NO production and suggest a possible therapeutic role for endothelin receptor antagonists in pathophysiological states.

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References


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