Treatment with endothelin-receptor antagonists increases NOS activity in hypercholesterolemia

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Taner, C. Burcin, Sandra R. Severson, Patricia J. M. Best, Amir Lerman, and Virginia M. Miller. Treatment with endothelin-receptor antagonists increases NOS activity in hypercholesterolemia. J Appl Physiol 90: 816–820, 2001.—In experimental hypercholesterolemia, endothelium-dependent relaxations decrease, as does endothelial immunoreactivity for nitric oxide (NO) synthase (NOS; eNOS) in coronary arteries. Systemic levels of NO also decrease with concomitant elevations in systemic circulating levels of endothelin (ET)-1. Chronic treatment of hypercholesterolemic pigs with ET-receptor antagonists increases circulating NO and improves endothelium-dependent relaxations. Mechanisms causing these increases are not known. Therefore, experiments were designed to test the hypothesis that chronic administration of ET-receptor antagonists to hypercholesterolemic pigs increases NO production through increases in NOS activity. Female juvenile pigs were fed a 2% cholesterol atherogenic diet and were randomly allocated to receive no treatment (controls), a selective ETα-receptor antagonist (ABT-624), or a combined ETα + ETβ-receptor antagonist (RO-48-5695) daily for 12 wk. After 12 wk, endothelial cells from thoracic aorta were prepared for measurement of eNOS mRNA or eNOS activity. Total cholesterol, low-density-lipoprotein cholesterol, and concentrations of ET-1 were significantly higher in all three groups at 12 wk compared with baseline levels. Circulating plasma-oxidized products of NO (NOx) increased with ET-receptor blockade. NOS mRNA was similar among groups. Total and Ca-dependent NOS activity was significantly higher in aortic endothelial cells from the ETα + ETβ-treated pigs compared with those treated with ETα antagonist alone. These results suggest that changes in systemic NOx after chronic inhibition of ETα + ETβ receptors in hypercholesterolemia may result from posttranscriptional changes in NOS.

atherosclerosis; endothelial cells; endothelin-1; lipidemia; messenger ribonucleic acid

THE BALANCE BETWEEN endothelium-derived nitric oxide (NO) and endothelin-1 (ET-1) may contribute to altered vasomotor function in pathophysiological states, including hypercholesterolemia and atherosclerosis (11). For example, in experimental hypercholesterolemia, circulating levels of ET-1 increase, whereas circulating levels of plasma oxidized products of NO (NOx) decrease (2, 3). At the level of the coronary arteries, endothelium-dependent relaxations are reduced, as is immunoreactivity for endothelial NO synthase (NOS; eNOS) (2, 13). These observations, taken together with reductions in contractions to infusion of the arginine analog Nω-monomethyl-L-arginine, provide indirect evidence that activity of NO synthase (NOS) decreases with hypercholesterolemia (13). Chronic inhibition of hypercholesterolemic pigs with antagonists for ETα receptors or combined inhibition of ETα plus ETβ receptors increases circulating levels of NO and restores endothelium-dependent relaxations and immunostaining for NOS in coronary arteries of pigs (2, 3). Whether endothelin-receptor antagonists affect changes in NOS at the transcriptional or posttranscriptional level is unclear. Therefore, experiments were designed to extend observational studies of changes in NOx and NOS immunostaining by directly determining changes in mRNA for endothelial NO and enzyme activity in the setting of chronic hypercholesterolemia and endothelin-receptor blockade. It was hypothesized that chronic endothelin-receptor blockade in hypercholesterolemia would increase activity of eNOS.

METHODS

Animals. All study procedures using 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. Female juvenile domestic crossbred pigs (23–35 kg) were placed on an atherogenic diet of 2% cholesterol and 15% lard by weight (TD-93296; Harlan Teklad, Madison, WI; Table 1) for 12 wk (11). The mean nitrate concentration is 8.1 parts/million (range 0.1–22), and nitrite concentration is 1.8 parts/million (range 0.1–6.9). Animals were assigned to one of three groups. They either did not receive any medications (control group), or were treated with oral ABT-627 (Abbott Laboratories, Abbott Park, IL), an ETα receptor antagonist, on a weight-adjusted scale to maintain a dose of 4 mg·kg⁻¹·day⁻¹, or were treated with oral RO-48-5696 (Hoffman-LaRoche, Basel, Switzerland), a combined ETα plus ETβ-receptor antagonist, on a weight-adjusted scale every 3

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The cDNA was next quantified by using the PCR MIMIC technique (Clontech Labs, Palo Alto, CA). A MIMIC DNA was constructed by performing two rounds of PCR amplification. In the first reaction, two composite primers, 5'-TCA ACC AGT ACT ACA GCT CCC GCA AGT GAA ATC TCC TCC G and 3'-GTG GTT GCA GAT GTA GGT CAT CTC ACG CAG TTG GTA G, were used, each containing the target gene primer sequence attached to a nucleotide strand designed to hybridize to opposite strands of a MIMIC DNA fragment. A dilution of this reaction was then amplified again by using only the gene-specific primers. The MIMIC DNA was next purified by passage through CHROMA SPIN+TE-100 columns, and the yield was calculated and diluted to 100 amol/μl. Competitive PCR amplification was next performed by titrating 1 μl of the target cDNA against serial 10-fold dilutions of the MIMIC DNA with the use of the eNOS 5'-TCA ACC AGT ACT ACA GCT CCC GCA AGT GAA ATC TCC TCC G and eNOS 3'-GTG GTT GCA GAT GTA GGT CAT CTC ACG CAG TTG GTA G. Reaction was terminated by heating to 70°C for 10 min, followed by incubation with RNase H for 20 min at 37°C. eNOS activity was determined by measuring absorbance at 260 nm in a spectrophotometer (Beckman DU 640, Fullerton, CA). DNase treatment of 1 μg of total RNA was carried out with 1 μl of DNase buffer (200 mM Tris-HCl at pH 8.4, 500 mM KCl, 20 mM MgCl₂) and 2 μl of amplification-grade DNase I (Life Technologies) for 15 min at room temperature. DNase I was then inactivated by heating to 65°C after the addition of 25 mM EDTA. First-strand cDNA synthesis was next performed (SuperScript Preamplification System, Life Technologies) by sequential reactions after the addition of 1 μl of oligo(dT)₁₂₋₁₈ primers to hybridize to 3' poly(A) tails on mRNA (70°C for 10 min) and then 7 μl of reaction mixture (2 μl 10× PCR buffer, 2 μl 25 mM MgCl₂, 1 μl dNTP mix, and 2 μl 0.1 M dithiothreitol at 42°C for 5 min) and 1 μl of Superscript II RT (42°C for 50 min). Reaction was terminated by heating to 70°C for 15 min followed by incubation with RNase H for 20 min at 37°C. Target cDNA was next amplified by PCR: 38 cycles of denaturation (94°C for 45 s), annealing (60°C for 45 s), and polymerization (72°C for 60 s). The primers used detected poly(A) tails on mRNA (70°C for 10 min followed by incubation with RNase H for 20 min at 37°C). Target cDNA was next amplified by PCR: 38 cycles of denaturation (94°C for 45 s), annealing (60°C for 45 s), and polymerization (72°C for 60 s). The primers used detected poly(A) tails on mRNA (70°C for 10 min followed by incubation with RNase H for 20 min at 37°C). Target cDNA was next amplified by PCR: 38 cycles of denaturation (94°C for 45 s), annealing (60°C for 45 s), and polymerization (72°C for 60 s). The primers used detected poly(A) tails on mRNA (70°C for 10 min followed by incubation with RNase H for 20 min at 37°C). Target cDNA was next amplified by PCR: 38 cycles of denaturation (94°C for 45 s), annealing (60°C for 45 s), and polymerization (72°C for 60 s). The primers used detected poly(A) tails on mRNA (70°C for 10 min followed by incubation with RNase H for 20 min at 37°C).
Table 2. Concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides measured in plasma from cholesterol-fed pigs at baseline and after 12 wk

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n = 17)</th>
<th>Control (n = 3)</th>
<th>ETA blocked (n = 8)</th>
<th>ET_A plus ET_B blocked (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>518.0 ± 22.1*</td>
<td>518.0 ± 22.1*</td>
<td>384.6 ± 30.4*</td>
<td>388.5 ± 54.0*</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>403.3 ± 19.4*</td>
<td>279.2 ± 24.5*</td>
<td>272.1 ± 44.0*</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>99.0 ± 4.1*</td>
<td>99.1 ± 6.3*</td>
<td>96.5 ± 9.8*</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>77.0 ± 11.8*</td>
<td>31.8 ± 5.5</td>
<td>20.0 ± 9.8</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE in mg/dl for n pigs. ET, endothelin; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Baseline values represent combined values before assignment to treatment groups. *Statistical significance from baseline, P < 0.05.

After 12 wk, plasma ET-1 concentrations significantly increased from a mean baseline value of 3.4 ± 0.1 pg/ml (n = 17) in all three groups (Table 3). Circulating NOx decreased significantly with cholesterol feeding to ~80% of baseline control levels (52.3 ± 9.4 nmol/ml, n = 17) (2). This decrease in circulating NOx was attenuated by ~50% with ETA plus ETB-receptor antagonism (Table 3).

**Quantitation of mRNA for eNOS.** Quantitative RT-PCR performed on RNA extracted from the aortic endothelial cells showed no significant differences in the mRNA levels for eNOS among groups [control: 0.0250 ± 0.0125 amol/μl (n = 3); ETA blocked: 0.0424 ± 0.017 amol/μl (n = 8); ETA plus ETB blocked: 0.03708 ± 0.0197 amol/μl (n = 6); Fig. 1; P value for the exact version of the Kruskal-Wallis test was 0.9420].

**NOS activity.** Citrulline accumulation from total and calcium-dependent NOS enzyme was increased in cells from cholesterol-fed pigs treated with ETA plus ETB antagonists compared with those treated with ETA-receptor antagonists alone (Fig. 2). Calcium-independent NOS enzyme accumulation was similar in all three groups [control: 144.8 ± 78.2 pmol·mg protein⁻¹·h⁻¹ (n = 3); ETA blocked: 116.3 ± 87.1 pmol·mg protein⁻¹·h⁻¹ (n = 8); ETA plus ETB blocked: 127.6 ± 50.6 pmol·mg protein⁻¹·h⁻¹ (n = 6)].

Table 3. Venous plasma concentrations of endothelin-1 and NOx from cholesterol-fed pigs after 12 wk of treatment

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 3)</th>
<th>ETA Blocked (n = 8)</th>
<th>ETA and ETB Blocked (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelin-1, pg/ml</td>
<td>5.5 ± 0.3</td>
<td>5.2 ± 0.1</td>
<td>8.8 ± 0.4*</td>
</tr>
<tr>
<td>NOx, nmol/ml</td>
<td>14.2 ± 1.2</td>
<td>16.8 ± 1.5</td>
<td>22.4 ± 2.6</td>
</tr>
</tbody>
</table>

Values are means ± SE for n pigs. NOx, plasma-oxidized products of nitric oxide. *Statistical difference from control at 12 wk, P < 0.05.

**DISCUSSION**

Previous studies have demonstrated that high-cholesterol feeding of pigs increases plasma cholesterol and ET-1 with concomitant decreases in plasma NOx and endothelium-dependent relaxations in coronary arteries (2, 3, 13). Furthermore, chronic treatment of hypercholesterolemic pigs with endothelin-receptor antagonists improves endothelium-dependent relaxations of coronary arteries and attenuates decreases in plasma NOx (2, 13). These observations suggest indirectly that endothelin-receptor antagonism increases activity of NOS (2, 3). Results of the present study extend these observations to provide direct evidence that combined chronic antagonism of ETA and ETB receptors results in increased eNOS enzyme activity (as defined by citrulline accumulation at a single time point) in pigs with hypercholesterolemia. Increases in eNOS activity would account in part for increases in plasma NOx in animals with combined endothelin-receptor antagonism and improved endothelium-dependent relaxation (3). It is important to note that...
compared with those from pigs treated with a selective ET A antagonist using modification of methods previously described (17). Values ETA plus ET B antagonist compared with the group activity was significantly greater in the group receiving oxidative stress (3, 9).

prostaglandin or adrenomedullin (15), or changes in release of other endothelium-derived factors such as regulatory systems associated with production or re- phoinosital as occurs in hypertension (24), autocrine changes in intracellular regulation of calcium by phos- in message at earlier time points after treatment. Indeed, 12 wk of treatment may represent a “steady- state” condition and may not be indicative of changes for eNOS, as only a single time point was studied. 

increases in NOS activity were due to an increase in calcium-dependent activity and not the calcium-indepen- dent activity typically defining activity of inducible NOS. This probably represents, in part, postranscriptional regulation of the enzyme, as mRNA for eNOS was similar between the tissue from animals receiving ET A and ET A plus ET B antagonists. However, NOS activity was significantly greater in the group receiving ET A plus ET B antagonist compared with the group receiving only ET A-receptor antagonists. These data should not be interpreted to mean that endothelin-receptor antagonists would not influence transcription for eNOS, as only a single time point was studied. Indeed, 12 wk of treatment may represent a “steady-state” condition and may not be indicative of changes in message at earlier time points after treatment.

Posttranscriptional regulation of eNOS could include changes in intracellular regulation of calcium by phos- phoinosital as occurs in hypertension (24), autocrine regulatory systems associated with production or re-lease of other endothelium-derived factors such as prostaglandin or adrenomedullin (15), or changes in oxidative stress (3, 9).

Fig. 2. Nitric oxide synthase enzyme activity in aortic endothelial cells of pigs treated with ETA plus ETB antagonists. However, NOS activity was significantly greater in the group receiving ET A plus ET B antagonist compared with the group receiving only ET A-receptor antagonists. These data should not be interpreted to mean that endothelin-receptor antagonists would not influence transcription for eNOS, as only a single time point was studied. Indeed, 12 wk of treatment may represent a “steady-state” condition and may not be indicative of changes in message at earlier time points after treatment.

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It is unlikely that changes in circulating NOx repre- sented changes in dietary intake of nitrates and nitrates. All pigs received the same diet, and pigs with the highest plasma NOx (those treated with ET A plus ET B antagonist) actually weighed less than the control pigs, which would suggest less dietary intake of nitrates and nitrites.

A shortcoming of this study is that NOS activity was determined in aortic rather than coronary endothelial cells. Coronary arteries from these animals were used for functional studies (2, 3). Heterogeneity in distribu- tion of NO and ET-1 throughout the vasculature is well recognized. Although NOS activity and mRNA were not measured in coronary arterial endothelial cells, it should be pointed out that changes in circulating NOx and ET-1 probably represent mean production and secretion from several vascular beds. Increases in NOS activity in aortic endothelial cells of animals treated with the ET A plus ET B antagonists are consistent with increases in systemic concentrations of NOx and increases in functional expression of endothelium-depen- dent responses observed in the coronary arteries (2, 3). Therefore, chronic endothelin-receptor antagonism is likely to affect endothelin receptors and NOS activity throughout the vasculature.

ET A receptors are located on vascular smooth muscle cells, whereas ET B receptors are located on both endo- thelium and vascular smooth muscle cells (12, 21, 23). Stimulation of ET B receptors has been associated with release of NO from the endothelium in experimental animals and humans (4, 5, 8, 10, 20, 22). Therefore, it is unclear how antagonism of ET B receptors would act to maintain NOx in the setting of hypercholesterol- emia. In hypercholesterolemia, there is an enhanced vasoconstrictor response to the selective ET B agonist sarafotoxin in the coronary microcirculation (14). In support of this observation, there was a trend, albeit statistically insignificant, for increases in both ET B receptor affinity and number in conduit coronary ar- teries with hypercholesterolemia (14). Although the receptor-binding assay did not differentiate receptors on the smooth muscle or endothelial cells, data suggest that there are changes in ET B receptors in hypercholesterol- emia. Clearly, additional experiments are needed to better define regulation of endothelin-receptor subtypes, their distribution, affinity for both ago- nists and antagonists, and intracellular signaling pathways in hypercholesterolemia.

Antagonism of ET A plus ET B receptors but not ET A receptors alone also increased plasma ET-1. This result is consistent with previous studies demonstrating a negative feedback between the stimulation of ET B receptors and the half-life of exogenously administered ET-1 (6). The increase in ET-1 concentrations in ETA plus ET B-blocked animals suggests that ET B-receptor binding may be an important mechanism in clearance of endogenous ET-1. The relationship between clearance of ET-1 and regulation of NOS remains to be defined. Chronic administration of exogenous ET-1 has been shown to increase NO-dependent reactivity of resistance vessels in rats (7).
In summary, in hypercholesterolemia, plasma ET-1 increases, whereas plasma NOx decreases. Chronic antagonism of ET\textsubscript{A} and ET\textsubscript{B} receptors attenuates the decrease in plasma NOx associated with hypercholesterolemia. The greatest restoration in plasma NOx was observed during simultaneous blockade of both ET\textsubscript{A} plus ET\textsubscript{B} receptors, a treatment that further elevated circulating concentrations of ET-1. After 12 wk of treatment, mRNA for eNOS was similar between animals treated with the ET\textsubscript{A}-receptor antagonist alone and those treated with ET\textsubscript{A} plus ET\textsubscript{B} antagonist. However, because NOS activity increased only with ET\textsubscript{A} plus ET\textsubscript{B} antagonist, this suggests that ET\textsubscript{B} receptors are associated with posttranscriptional regulation of NOS in hypercholesterolemia.

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REFERENCES