Loss of Selective Endothelial Cell Vasoactive Functions Caused by Hypercholesterolemia in Pig Coronary Arteries

Richard A. Cohen, Kevin M. Zitnay, C. Christian Haudenschild, and Leslie D. Cunningham

The influence of hypercholesterolemia on the reactivity of coronary arteries was investigated after feeding a high-cholesterol diet to pigs for 9 weeks. After this duration of hypercholesterolemia, the fatty or intimal proliferative changes of atherosclerosis were not yet evident in the coronary arteries by light or electron microscopy. Changes in isometric tension were compared in isolated ring segments of coronary arteries from normal and hypercholesterolemic animals. The endothelium failed to inhibit contractions caused by 5-hydroxytryptamine in coronary arteries from hypercholesterolemic animals, but it did so in normal vessels. In contracted arteries, endothelium-dependent relaxations caused by 5-hydroxytryptamine and substance P were reduced by hypercholesterolemia. In contrast, endothelium-dependent relaxations mediated by norepinephrine acting at α₂-adrenoceptors and those caused by the calcium ionophore A23187 were unaffected. Endothelium-independent β-adrenergic relaxations caused by norepinephrine, as well as those caused by nitroprusside, and papaverine also were unaffected by hypercholesterolemia. The loss of selective endothelial cell receptor-mediated relaxation suggests that it is not the ability of the coronary artery endothelium to elaborate vasodilators, but the initiation of the coronary artery endothelial cell response to 5-hydroxytryptamine and substance P that is affected by hypercholesterolemia. Thus, during hypercholesterolemia, selective endothelial cell dysfunction giving rise to abnormal coronary artery reactivity precedes the onset of coronary artery atherosclerosis. (Circulation Research 1988;63:903–910)

By releasing endothelium-derived relaxing factor(s), the endothelium plays a critical role in the local control of blood vessels. The tonic release of relaxing factors as well as that induced by several receptor-dependent agonists is important in modulating contraction of the smooth muscle. Changes in the physiological responses of endothelial cells have been noted in atherosclerotic arteries, which suggests that vasoactivity of atherosclerotic arteries could be due to lack of the normal vasodilator influence of the endothelium. In atherosclerotic human coronary arteries, there are attenuated endothelium-dependent relaxations induced by acetylcholine, but those caused by the calcium ionophore A23187 may or may not be inhibited. In rabbits made atherosclerotic by severe hypercholesterolemia, there is an impairment of relaxations induced by acetylcholine and adenosine triphosphate, but those caused by A23187 are preserved. In pig coronary arteries made atherosclerotic by a combination of cholesterol feeding and balloon catheter-induced intimal injury, endothelium-dependent relaxations caused by 5-hydroxytryptamine (5-HT) are markedly attenuated, whereas those to A23187 are minimally affected. In monkey iliac arteries made atherosclerotic by prolonged hypercholesterolemia, endothelium-dependent relaxations caused by acetylcholine and thrombin are inhibited, whereas those to A23187 are only moderately decreased. Thus, studies of atherosclerotic arteries have demonstrated that endothelium-dependent relaxations caused by some, but not all, agonists are inhibited, which suggests a selective alteration by atherosclerosis of endothelial cell function. While it is not unexpected that atherosclerosis and the accompanying intimal thickening will affect the influence of endothelial cells on vascular smooth muscle, it is unknown if hypercholesterolemia affects the vaso-
active role of the endothelium before the onset of atherosclerosis. The purpose of this study therefore was to determine the effect of hypercholesterolemia induced for a relatively short duration and moderate degree on pig coronary artery reactivity with special emphasis on endothelium-dependent responses.

Materials and Methods

Seven domestic swine were fed a high-cholesterol diet (Table 1) modified from Nam et al.12 for a period of 8.8 ± 0.8 weeks (range, 6–12). At the time the animals were killed, the cholesterol-fed pigs weighed 26 ± 2.2 kg. Seven control pigs were fed regular pig mash for an average of 2–3 weeks, and their weights at the time of death did not differ significantly from the cholesterol-fed pigs.

Pigs were killed by exsanguination following ketamine (20 mg/kg i.m.), acepromazine (0.8 mg/kg i.m.), chloralose (75 mg/kg i.v.), and sodium heparin (80 units/kg i.v.). At the time of death significant hypercholesterolemia was present in the experimental group (601 ± 45 mg/dl) compared with the controls (94 ± 7.9, p<0.05). No significant alteration in triglycerides occurred (34 ± 4.5 vs. 34 ± 6.3 mg/dl).

Organ Chamber Experiments

Rings of artery 5 mm long were suspended in organ chambers (25 ml) filled with physiological salt solution (PSS) of the following millimolar composition: NaCl 118.3, KCl 4.7, MgSO4 0.6, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25, Ca-EDTA 0.026, and glucose 11.0. The solution was maintained at 37°C and gassed with 20% O2-5% CO2-75% N2. The endothelium was removed from some rings by rubbing of the luminal surface with a stainless steel wire; removal of endothelium was later confirmed by absence of relaxations caused by substance P.13 The rings were mounted on stirrups connected to a strain gauge so as to measure isometric circumferential force. The rings were stretched gradually over 1 hour to 12 g resting tension that had been determined previously to be optimal tension for contraction of normal pig coronary arteries.3 During the last 15 minutes of this period, sodium nitroprusside (10^-7 M) was introduced to eliminate spontaneous active tone which is observed in rings without endothelium2 and tension was again adjusted to 12 g. The sodium nitroprusside was then removed by rinsing for 30 minutes.

Relaxations. To measure relaxations, the rings were contracted with prostaglandin F2α (PGF2α). After the PGF2α-induced contraction stabilized, increasing concentrations of 5-HT, substance P, norepinephrine, A23187, nitroprusside, or papaverine were added until a maximum response occurred. Following this, nitroprusside (10^-6 M) was added, which caused a maximal relaxation in all rings; this was assured by adding higher concentrations in some studies. Responses to each agonist were obtained in pairs of rings, one with and one without endothelium. The order of agonists was randomized. The control pigs were studied concurrently. Relaxations are expressed as a percentage of the maximal relaxation caused by nitroprusside. In some studies, the concentration causing 50% maximal relaxation (IC50) was estimated by linear regression and is reported as the logarithm.

Contractions. Contractions caused by 5-HT, PGF2α, and potassium are reported in grams. The concentration of potassium causing fifty percent of the maximum contraction (ED50) was estimated by linear regression analysis. The negative logarithm of the ED50 is reported as the pD2.14

Drugs

The following agents were used: A23187, 5-HT creatinine sulfate, norepinephrine bitartrate, papaverine, propranolol hydrochloride, PGF2α (Tris salt), sodium nitroprusside, substance P (Sigma Chemical, St. Louis, Missouri); ketanserin bitartrate (Janssen Pharmaceutica, Beerse, Belgium); methiothepin maleate (Hoffman-LaRoche, Nutley, New Jersey); prazosin hydrochloride (Pfizer, Brooklyn, New York); and rauwolscine hydrochloride (Carl Roth, KG, Karlsruhe, FRG).

Unless otherwise specified, drugs were dissolved in distilled water such that volumes of 100 μl were added to the organ chamber. Norepinephrine was dissolved in 0.1% ascorbic acid. Prazosin and methiothepin were solubilized in DMSO and diluted with water. All concentrations are the final molar concentrations that reached in the organ chamber. Potassium concentration (10, 20, 40, and 120 mM) was increased by equimolar replacement of sodium.

Data and Statistical Analysis

The data was expressed as mean ± SEM. Unless otherwise indicated, n was seven. The geometric means of the concentrations were analyzed. Statistical evaluations were by way of Student’s t test for paired comparisons of responses of rings of the same artery with or without endothelium, or for unpaired comparisons of responses of rings of arteries from normal and hypercholesterolemic animals. Values of p<0.05 were regarded as significant.

Morphology

After dissection from the heart, a ring of coronary artery from each heart was fixed by immersion in
4% formaldehyde, 1% glutaraldehyde in isotonic phosphate buffer. Paraffin sections at 4 μm were examined after staining with hematoxylin-eosin, modified trichrome, or van Gieson-elastin stains. For scanning electron microscopy, samples were dehydrated through graded alcohol, dried by the critical point method, sputter-coated with gold palladium alloy, and examined with a Jeol microscope.

Results

Spontaneous Tone and Contractions Caused by PGF$_{2\alpha}$, 5-HT, and Potassium

Following stretching to the optimal resting tension for contraction in the presence of nitroprusside (10$^{-7}$ M), rinsing coronary artery rings denuded of endothelium from normal and hypercholesterolemic pigs resulted in spontaneous increases in tone. The tone developed in rings from normal pigs was significantly greater than in rings from hypercholesterolemic pigs (5.4±0.9 vs. 2.3±0.7 g). In neither group did rings with endothelium develop tone.

PGF$_{2\alpha}$ was used to bring the total tension of rings with and without endothelium to similar levels. Higher concentrations of PGF$_{2\alpha}$ were required to do this in rings with endothelium than in rings denuded of endothelium from both normal and hypercholesterolemic pigs (Table 2). There was no significant difference in the concentrations used for rings of arteries from normal and hypercholesterolemic pigs.

At these concentrations, PGF$_{2\alpha}$ caused contractions of arterial rings with or without endothelium from hypercholesterolemic animals that averaged significantly less than those of normal rings.

In coronary arteries from both normal and hypercholesterolemic pigs contracted with PGF$_{2\alpha}$, 5-HT (10$^{-8}$ to 10$^{-5}$ M) caused further contractions. In normal pigs, these contractions were of significantly greater magnitude in rings with intact endothelium, while in coronary arteries from hypercholesterolemic animals, the contractions caused by 5-HT were not different in rings with or without endothelium (Figure 1). In rings without endothelium, the contractions caused by 5-HT (3.2×10$^{-7}$ M and 10$^{-6}$ M) were significantly greater in normal coronary arteries than in those from hypercholesterolemic pigs (Figure 1).

Contractions caused by potassium (10–120 mM) were not significantly different in coronary artery rings denuded of endothelium from normal and hypercholesterolemic pigs (Table 3).

Relaxations Caused by 5-HT, Substance P, Norepinephrine, A23187, Nitroprusside, and Papaverine

In the presence of ketanserin (10$^{-6}$ M), coronary artery rings with endothelium relaxed in response to 5-HT (10$^{-4}$ to 10$^{-5}$ M); these relaxations were significantly less in arteries from hypercholesterolemic animals (Figure 2). In both normal and hypercholesterolemic pigs, methiothepin (10$^{-6}$ M) blocked the relaxations caused by 5-HT. 5-HT caused minimal

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**Table 2. PGF$_{2\alpha}$-Induced Contraction of Coronary Arteries From Normal and Hypercholesterolemic Pigs**

<table>
<thead>
<tr>
<th>Endothelium</th>
<th>Concentration</th>
<th>Contraction (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>With</td>
<td>−5.63±0.07</td>
</tr>
<tr>
<td></td>
<td>Without</td>
<td>−6.30±0.10*</td>
</tr>
<tr>
<td>Hypercholesterolemic</td>
<td>With</td>
<td>−5.52±0.06</td>
</tr>
<tr>
<td></td>
<td>Without</td>
<td>−6.42±0.13*</td>
</tr>
</tbody>
</table>

Data are mean±SEM (n=7) of the concentrations (logM) of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) used to contract pig coronary artery rings and the resulting contractions (g). *For arteries from both normal and hypercholesterolemic animals, a significantly lower concentration of PGF$_{2\alpha}$ was required in rings denuded of endothelium to yield equal contractions to those in rings with endothelium. 1PGF$_{2\alpha}$-induced contractions of arterial rings with or without endothelium from hypercholesterolemic animals were significantly less than those of normal rings.

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**Table 3. Response of Normal and Hypercholesterolemic Pig Coronary Artery to Potassium**

<table>
<thead>
<tr>
<th>Potassium concentration (mM)</th>
<th>pD$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.75±0.02</td>
</tr>
<tr>
<td>20</td>
<td>1.73±0.06</td>
</tr>
<tr>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

*Responses to 10, 20, and 40 mM potassium are expressed as percent of maximal contraction to potassium (120 mM) which was 22.0±2.4 g and 18.0±2.4 g for normal and hypercholesterolemic pig arteries, respectively. The pD$_2$ is the log of the molar concentration causing 50% maximal contraction. There were no significant differences in responses to potassium of normal and hypercholesterolemic pig coronary arteries whether expressed as percent of maximum or in grams tension.*
or no relaxation in rings of normal or hypercholesterolemic coronary arteries denuded of endothelium.

Coronary artery rings with endothelium from hypercholesterolemic pigs relaxed significantly less to substance P (3x 10^{-12} to 3x 10^{-10} M) than did those from normal pigs (Figure 3). In both normal and hypercholesterolemic pigs, coronary artery relaxations caused by substance P were abolished in rings denuded of endothelium.

In rings with or without endothelium, the relaxations caused by norepinephrine (10^{-8} to 3x 10^{-6} M) were not significantly different between coronary arteries from normal and hypercholesterolemic pigs (Figure 4, upper panel). In the presence of propranolol (10^{-6} M) and prazosin (5x 10^{-7} M), norepinephrine still relaxed rings with endothelium from normal and hypercholesterolemic pigs similarly (Figure 4, lower panel). The relaxations caused by norepinephrine (10^{-8} to 3x 10^{-6} M) in the presence of propranolol and prazosin were abolished after removing the endothelium from arteries of both normal and hypercholesterolemic pigs (Figure 4, lower panel). As in normal arteries, the further addition of rauwolscine (3x 10^{-7} M) blocked the endothelium-dependent relaxations caused by norepinephrine (10^{-8} to 3x 10^{-6} M) in rings from hypercholesterolemic pigs (Figure 4, lower panel).

There were no significant differences in the endothelium-dependent relaxations induced by A23187 (10^{-8} to 10^{-6} M, Figure 5) of coronary arteries from normal and hypercholesterolemic pigs.

Relaxations caused by nitroprusside or papaverine of coronary artery rings without endothelium from normal and hypercholesterolemic pigs were not significantly different. The log IC_{50} for nitroprusside was 8.1 ±0.2 (n=7) in arteries from normal pigs and 7.8 ±0.1 (n=5) in those from hypercholesterolemic pigs (p>0.1). The log IC_{50} for papaverine was −6.0 ±0.2 (n=4) in arteries from normal pigs and −6.1 ±0.1 (n=5) in those from hypercholesterolemic pigs (p>0.2).

Morphology

By light microscopy high cholesterol diet of this duration and degree had no noticeable atherogenic effects on the coronary arteries. Specifically, foam cells were not observed on regular sections or fat stained frozen sections, and there was no intimal thickening (Figure 6). Scanning electron microscopy showed a continuous layer of endothelial cells with rare gaps and some microvillous surface changes (Figure 7). Comparable minimal alterations in the endothelial cell surface were observed in similarly fixed coronary artery rings of three control pigs.

Discussion

Effect of Hypercholesterolemia on Contractions of Pig Coronary Artery

Pig coronary arteries without endothelium spontaneously develop myogenic tone which may be due to the endogenous synthesis of contractile prostaglandins, since it is prevented by cyclooxygenase inhibitors. The decreased generation of myogenic tone in arteries from hypercholesterolemic pigs is therefore consistent with the smaller contractions caused by exogenously added PGF_{2\alpha}. There is a reduction in other receptor-mediated contractions of...
Influence of Hypercholesterolemia on Endothelium-Dependent Coronary Artery Relaxations

Relaxations caused by several agonists were used to assess functional abnormalities of the pig coronary artery endothelium in cholesterol-fed animals. In pig\textsuperscript{8,9,11} and dog\textsuperscript{12,13} coronary arteries, 5-HT causes relaxations that are dependent on the presence of an intact endothelium and are mediated by 5-HT-like receptors which may be blocked by methysergide or methiothepin.\textsuperscript{14,15} In pig coronary arteries, the endothelium-mediated relaxations are observed only after preventing contractions mediated by 5-HT\textsubscript{2} receptors on the smooth muscle.\textsuperscript{16} Reduction of the relaxations caused by 5-HT in coronary arteries from hypercholesterolemic pigs suggests an impairment of the production of or response of the smooth muscle to endothelium-derived relaxing factor(s). An impairment of function is also suggested by the lack of the ability of the endothelium of arteries from hypercholesterolemic pigs to reduce 5-HT-induced contractions of the smooth muscle observed in the absence of ketanserin.

Substance P also causes endothelium-dependent relaxations of pig coronary arteries by releasing endothelium-derived relaxing factor(s).\textsuperscript{3,4} Although a significant reduction in effectiveness of substance P was seen in arteries from cholesterol-fed animals, the reduction was less marked than that for 5-HT.

Despite the fact that it was less potent than 5-HT or substance P in causing endothelium-dependent relaxations, norepinephrine caused normal relaxations of coronary arteries from hypercholesterolemic pigs that were blocked by the \(\alpha_2\)-adrenoceptor antagonist rauwolscine.\textsuperscript{3,19} The normal \(\alpha_2\)-adrenoceptor-mediated relaxation indicates that the impairment in the endothelium-dependent response to 5-HT and substance P in cholesterol-fed pigs is specific for those agonists rather than affecting responses mediated by all endothelial cell receptors.

Coronary smooth muscle in cholesterol-fed pigs because contractions of rings without endothelium caused by 5-HT were also of less magnitude. The arteries from hypercholesterolemic animals could develop similar responses to a direct smooth muscle contractile stimulus since the sensitivity to and magnitude of contractions caused by potassium-induced depolarization did not differ in the two groups of animals. Therefore, it is unlikely that arteries from hypercholesterolemic animals had decreased prostaglandin and 5-HT-induced contractions due to dissimilar passive mechanical characteristics. While the mechanism of the reduced contractions is unknown and merits further investigation, moderate decreases in the magnitude of agonist-induced contractions have been observed in the aortas of hypercholesterolemic rabbits.\textsuperscript{7,8}
A23187 initiates endothelium-dependent relaxations by inducing calcium influx into endothelial cells, bypassing the initial steps in endothelial cell activation mediated by receptors. Normal relaxations caused by the ionophore suggest that endothelial cells from hypercholesterolemic pigs can produce normal amounts of relaxing factor(s), which in turn initiates a normal smooth muscle relaxation.

Tonic release of relaxing factors from endothelium inhibits myogenic tone and PGF$_2$-$\alpha$-induced contractions of pig coronary artery. That these contractions were inhibited to a similar extent as those of normal arteries suggests that the endothelium of coronary arteries of cholesterol-fed pigs also tonically releases relaxing factor(s) in normal quantities.

Normal relaxations of arteries from cholesterol-fed arteries mediated by direct smooth muscle relaxants nitroprusside, papaverine, and norepinephrine (in the absence of propranolol), as well as by endothelial cell $\alpha_2$-adrenoceptors and A23187, indicate that differences in the magnitude of the contractions caused by PGF$_2$-$\alpha$ were not responsible for the differences observed in relaxations to 5-HT and substance P.

The mechanism by which hypercholesterolemia selectively inhibited endothelial cell function in the...
The present study is unknown but could involve changes in the numbers of endothelial cell receptors for 5-HT and substance P or in the steps leading to activation of endothelial cells initiated by those particular receptors that are susceptible to hypercholesterolemia. The three receptor agonists employed in this study and A23187 are all likely to share a common mechanism of relaxation by activation of smooth muscle guanylate cyclase where the relaxations are all inhibited by methylene blue (authors' unpublished observations). 5-HT and substance P do distinguish themselves in that their transmembrane signal transduction is known to be dependent on phosphatidylinositol turnover to increase cell calcium. Cellular activation by way of α2-adrenoceptors and A23187 are by contrast strictly dependent on the influx of extracellular calcium. The recent demonstration that exposure of the rabbit aorta in vitro to low density lipoproteins rapidly inhibits the endothelium-dependent relaxation caused by acetylcholine suggests that the dysfunction observed in the present study could be due to a direct interaction of elevated levels of lipoproteins with endothelial cells. As low density lipoproteins have also been shown to activate cells by way of phosphatidylinositol metabolism, interference with this pathway would provide a possible mechanism by which responses to 5-HT and substance P are selectively inhibited by hypercholesterolemia.

In the present study, pig coronary arteries were studied at a time when hypercholesterolemia had produced no abnormal structural alterations; the minimal changes observed in the endothelial cells were also observed in similarly fixed specimens from normal pigs. Thus, the changes in coronary artery endothelium-dependent responses observed are due to hypercholesterolemia without atherosclerosis. Furthermore, the abnormalities in specific endothelium-dependent vasoactive functions observed in this study suggest that the similar abnormalities in previous studies of atherosclerotic human and animal arteries could represent changes that preceded the development of atherosclerosis. Therefore, rather than reflecting atherosclerosis per se, the changes in endothelial cell vasoactive function induced by hypercholesterolemia may implicate early changes in endothelial cell function important in the development of atherosclerosis.

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References


KEY WORDS  •  endothelial cell  •  hypercholesterolemia  •  coronary artery  •  substance P  •  5-hydroxytryptamine  •  prostaglandin F₂α.
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