Reduced Sarco/Endoplasmic Reticulum Ca\(^{2+}\) Uptake Activity Can Account for the Reduced Response to NO, but Not Sodium Nitroprusside, in Hypercholesterolemic Rabbit Aorta

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Background—Hypercholesterolemia (HC) impairs acetylcholine-induced relaxation but has little effect on that caused by the NO donor sodium nitroprusside (SNP), suggesting that acetylcholine releases less NO from the endothelium in HC. The relaxation to authentic NO gas, however, is also impaired in HC aortic smooth muscle, indicating an abnormal smooth muscle response. NO relaxes arteries by both cGMP-dependent and -independent mechanisms, and the response involves calcium (Ca\(^{2+}\)) store refilling via the sarco/endoplasmic reticulum calcium ATPase (SERCA). We studied the involvement of cGMP and SERCA in the smooth muscle response to NO and SNP in HC rabbit aorta.

Methods and Results—A selective guanylyl cyclase inhibitor, 1H-[1,2,4]-oxadiazole-[4,3-a]quinoxalin-1-one, eliminated SNP-induced relaxation but only partially blocked NO-induced relaxation in both normal and HC aorta. The residual relaxation to NO was still less in HC and, in both normal and HC aorta, was abolished by concomitant administration of the SERCA inhibitor cyclopiazonic acid (CPA). In contrast, CPA did not affect SNP-induced relaxation in either normal or HC aorta. SERCA activity measured by \(^{45}\)Ca\(^{2+}\) uptake was markedly decreased in HC, although SERCA2 protein expression did not change significantly.

Conclusions—These data suggest that NO-induced relaxation but not that to SNP is partially mediated by cGMP-independent Ca\(^{2+}\) uptake into sarco/endoplasmic reticulum and that reduced sarco/endoplasmic reticulum Ca\(^{2+}\) pump function can account for the impaired response to NO in HC. (Circulation. 2001;104:1040-1045.)

Key Words: hypercholesterolemia ■ nitric oxide ■ sarcoplasmic reticulum ■ muscle, smooth ■ calcium

Hypercholesterolemia (HC) impairs endothelium-dependent relaxation but has little effect on sodium nitroprusside (SNP)–induced relaxation. Therefore, it is widely accepted that HC impairs endothelial release of NO and that the smooth muscle response to nitric oxide (NO) is preserved. Previously, we used authentic NO gas solution and showed that the decreased relaxation to acetylcholine in rabbit and mouse aortic rings was mirrored by decreased relaxation to NO. The decrease in intracellular free calcium caused by NO in smooth muscle from HC aorta in primary culture is also decreased in magnitude. It is not clear why the relaxation to NO and SNP is affected differently in HC aorta, but the question of whether the release and/or the response to NO is impaired is an important one from both an experimental and a therapeutic viewpoint.

NO gas requires diffusion, which can be interfered with by oxygen, superoxide anion, and metals. SNP can release NO by 1 electron reduction at the plasma membrane of smooth muscle cells, where it might not be as susceptible as NO to scavenging. HC increases superoxide production, and although long-term treatment with antioxidants improves vascular function in HC rabbit aorta, antioxidants like glutathione ester or other scavengers of superoxide anion do not immediately normalize responses either to endothelium-dependent agents or to NO. Adenoviral transfection of superoxide dismutase (SOD) had no effect, and extended in vivo treatment with a cell-permeable form of SOD only partially improved endothelium-dependent relaxation in HC. In addition, the response to high concentrations of NO is decreased in HC, even though these concentrations have been shown to effectively scavenge superoxide anion. Therefore, it is difficult to explain the reduced response in HC by only the increased scavenging of NO.

Another possible reason for the difference in response to NO and SNP is differences in signaling pathways in aortic smooth muscle. We recently provided evidence that a principal action of NO in normal vascular smooth muscle and platelets is a reduction of intracellular free Ca\(^{2+}\) levels that occurs by cGMP-dependent and cGMP-independent mechanisms. We also proposed that NO can activate potassium...
channels and sarco/endoplasmic reticulum calcium ATPase (SERCA) by cGMP-independent means, and it may be that there are differences in the contributions of these proteins to the relaxation caused by NO and SNP. In HC rabbit carotid arteries, potassium channel–mediated relaxation to NO is not impaired in smooth muscle from HC, and therefore decreased response of potassium channels may not explain the decreased response to NO.

With this background, this study tested the differences in signaling pathways used by NO and SNP to relax the normal and HC rabbit aorta. The results indicate that differences in the dependence of NO and SNP on guanylyl cyclase and signaling pathways used by NO and SNP to relax the normal and HC rabbit aortas.

**Isometric Tension Measurement**
The detailed methods were previously reported. Rings from the midportion of the descending aorta were used for this study. In some rings, the endothelium was removed mechanically. Rings were mounted at optimal resting tension (6 g). Some rings without endothelium were incubated with either thapsigargin (10 μM) and either 1 H-[1,2,4]-oxadiazole-[4,3-a]quinoxalin-1-one (ODQ: 10 μM/L), cyclopiazonic acid (CPA: 20 μM/L), or both for 1 hour. A saturated NO gas solution (Matheson) was prepared by adding 100 μM/L NO or 10 μM/L SNP to elevate [Ca2+]i, and either 1 μM/L NO or 10 μM/L SNP was added when [Ca2+]i level reached an equilibrium after the peak rise caused by angiotensin II or CPA.

**Cell Culture and Intracellular Free Calcium Measurement**
Methods for primary cell culture of rabbit aortic smooth muscle from normal and HC rabbits were previously reported. Cells on coverslips were loaded with fura 2 acetoxy-methyl ester in PSS and fluorescence was measured. Intracellular free calcium ([Ca2+]i) was monitored by the ratio of absorbance at 340/380 nm. Cells were stimulated with 0.1 μM/L angiotensin II or 20 μM/L CPA to elevate [Ca2+]i, and either 1 μM/L NO or 10 μM/L SNP was added when [Ca2+]i, level reached an equilibrium after the peak rise caused by angiotensin II or CPA.

**Western Blot Analysis**
The remaining rabbit aortic arch was homogenized in Tris-sucrose buffer with 1% Triton X-100 and protease inhibitors. After centrifugation at 14 000 rpm for 15 minutes at 4°C, the supernatant was mixed with Laemmli buffer and applied to 7.5% SDS-PAGE gels (10 μg protein/lane) for separation. Proteins were transferred to nitrocellulose paper, and the membrane was incubated with anti-SERCA 2 (Affinity Bioreagents, clone I1D8, 1:2500, overnight) or α-actin (Sigma, 1:5000, 2 hours) antibody. The secondary anti-mouse IgG (Pierce, 1:50 000) was incubated, and bands were detected by enhanced chemiluminescence.

**Statistical Analysis**
Relaxations were analyzed by the maximum response after each concentration was added and were expressed as a percentage of the contraction to phenylephrine. Data are expressed as mean±SEM. Statistical evaluation of the concentration-response curves and the time course of Ca2+ uptake was performed with a repeated-measures ANOVA (SAS). The bands on Western blots were quantified by densitometry (Molecular Analyzer). Exponential half-time (τ) was measured by estimating the rate of the initial decrease in [Ca2+]i, caused by NO. Differences between groups of relaxation to individual concentrations of NO, exponential half-times, band densities, and SERCA activity were analyzed with Student’s t test. A value of P<0.05 was considered to be statistically significant.

**Results**
HC impaired acetylcholine-induced relaxation in endothelium-intact arteries (Figure 1A). In endothelium-denuded arteries, HC impaired NO-induced relaxation by ~50% throughout a range of concentrations (ANOVA P<0.001,
Figure 1. (A) ACh-, (B) SNP-, and (C) NO-induced relaxation in normal and HC rabbit aorta and (D) effect of SOD and EUK-8 on NO-induced relaxation in HC. Concentration-dependent relaxations to ACh (A: normal, n = 7; HC, n = 10), SNP (B: normal, n = 6; HC, n = 10), and NO (C: normal, n = 13; HC, n = 12) were determined. In endothelium-denuded arteries, NO-induced relaxation was not changed. *ANOVA, P < 0.05. A and C. D. Incubation with SOD (n = 5; 150 U/L) or EUK-8 (n = 5; 10 μmol/L) had no significant effect on NO-induced relaxation in HC (n = 9).

Figure 2. Effect of ODQ on (A) NO-, (B) SNP-, and (C) 8-bromo-cGMP–induced relaxation in HC. Guanylyl cyclase inhibitor ODQ (10 μmol/L, 1 hour) reduced NO-induced relaxation partially (A: normal, n = 6; HC, n = 10). ODQ-resistant relaxation remained significantly decreased in HC aorta. SNP-induced relaxation is nearly blocked with ODQ in both normal and HC aortic rings (B: normal, n = 5; HC, n = 10). 8-Bromo-cGMP–induced relaxation was not significantly different between normal and HC rings (C: normal, n = 5; HC, n = 10), suggesting that cGMP-dependent mechanisms are preserved but cGMP-independent mechanisms are inhibited in HC. *ANOVA P < 0.05 vs normal, †ANOVA P < 0.05 normal vs HC after incubation with ODQ.

Incubation of HC aorta for 1 hour with SOD or EUK-8 had no effect on NO-induced relaxation (Figure 1D).

A guanylyl cyclase inhibitor, ODQ (10 μmol/L), only partially inhibited NO-induced relaxation in both normal and HC arteries (Figure 2A). This concentration of ODQ completely blocked the rise in cGMP by either NO or SNP in normal arteries (Reference 11 and unpublished observations). The residual cGMP-independent relaxation to NO was also significantly decreased in HC (Figure 2A). Conversely, SNP-induced relaxation was nearly abolished by ODQ (Figure 2B), suggesting that SNP-induced relaxation is almost completely dependent on cGMP. Both SNP- and 8-bromo-cGMP–induced relaxation did not change in HC (Figure 2C), suggesting that cGMP-dependent relaxation is preserved in HC.

Previously, we reported that NO accelerated Ca2+ refilling into sarco/endoplasmic reticulum via SERCA. Therefore, we applied a SERCA inhibitor, CPA (20 μmol/L), and measured the relaxation to NO and SNP. CPA significantly inhibited NO-induced relaxation both in normal and to a smaller extent in HC aorta (Figure 3A). In contrast, CPA did not significantly alter SNP-induced relaxation in either normal or HC aorta, suggesting that SNP-induced relaxation does not depend on SERCA function (Figure 3B). CPA nearly abolished the ODQ-resistant relaxation up to 1 μmol/L NO in both normal and HC aorta (Figure 3C), suggesting that the cGMP-independent relaxation to NO is dependent on SERCA function.

Because these observations provided evidence that the contribution of SERCA to NO-induced relaxation is decreased in HC rabbit aorta, SERCA activity was measured directly in aortic homogenates by 45Ca2+ uptake. An irreversibly SERCA inhibitor, thapsigargin, was applied in this assay to measure SERCA-dependent uptake. Thapsigargin-sensitive 45Ca2+ uptake was decreased significantly, from 20±2.2 to 10±2.7 nmol · mg−1 · min−1 (P = 0.01, Figure 4A and 4B). Thapsigargin-insensitive 45Ca2+ uptake was not significantly different in normal and HC rabbit aortic homogenates (1.2±0.2 versus 2.1±0.7 nmol · mg−1 · min−1, P = 0.24, Figure 4A), indicating that the change in thapsigargin-sensitive uptake reflects a specific difference in SERCA activity.

Figure 5A shows a typical recording of the decrease in [Ca2+]i caused by NO or SNP. CPA blocked the decrease in [Ca2+]i, caused by NO in cultured rabbit aortic smooth muscle from normal and HC rabbits. The rate of the decrease in [Ca2+]i, caused by NO, represented by the exponential halftime, increased significantly, by 4-fold, in HC compared with normal smooth muscle (1.44±0.20 seconds, n = 6, versus 5.93±1.69 seconds, n = 7). Figure 5B shows the effect of ODQ and CPA on the decrease in [Ca2+]i, to NO or SNP in normal rabbit aortic cells. ODQ almost completely abolished the response to 10 μmol/L SNP but had no significant effect on the response to 1 μmol/L NO. CPA blocked the decrease in [Ca2+]i, caused by NO or SNP.

Because NO-induced relaxation, the decrease in [Ca2+]i, and 45Ca2+ uptake mediated by SERCA were reduced in HC rabbit aorta, the expression of SERCA protein was examined by Western blot (Figure 6A). Densitometry showed that the expression of SERCA 2 did not change significantly (8.0±0.5 U, n = 5, versus 6.5±1.0 U, n = 7, P = 0.21). The
expression of α-actin, another abundant smooth muscle protein, was also not significantly altered (13±1.7 U versus 14±2.0 U, P=0.84). The ratio of SERCA 2 to α-actin expression as detected by Western blot also did not significantly change in HC (0.7±0.2 versus 0.6±0.2, P=0.78, Figure 6B).

Discussion

Our results suggest that differences in signaling mechanisms account for the fact that relaxation of aortic smooth muscle to NO but not SNP is impaired by HC. The relaxation of aortic smooth muscle to SNP, which is nearly entirely blocked by

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**Figure 3.** Effect of CPA on relaxation of rabbit aorta to (A) NO- and (B) SNP-induced relaxation and (C) ODQ-resistant relaxation to NO. CPA (20 μmol/L, 1 hour) significantly decreased NO-induced relaxation in normal and to a lesser extent in HC aortic rings (A: Normal, n=6; HC, n=9). Normal and HC rabbit aorta relaxed similarly to SNP, and CPA had no significant effect on response (B: normal, n=5; HC, n=10). CPA significantly inhibited ODQ-resistant response to NO in normal but not in HC aorta. After coincubation with ODQ and CPA, difference between normal and HC was eliminated (C: normal, n=6; HC, n=9). *ANOVA P<0.05 vs normal.

**Figure 4.** A, Effect of HC and thapsigargin on 45Ca2+ uptake in homogenates of normal and HC rabbit aorta. Total uptake (nmol/mg protein) in paired samples of normal (n=9) and HC (n=9) rabbit aorta treated or not with thapsigargin (TG: 10 μmol/L) is shown over 60 minutes (*ANOVA P<0.05 vs normal). B, After rate of total uptake at 60 minutes was calculated, values in presence of TG were subtracted from those in its absence to give TG-sensitive uptake (nmol · mg protein2 · min−1). *Values of total and TG-sensitive uptake were significantly less than normal in samples of HC rabbit aorta (*t test P<0.05 vs normal).

**Figure 5.** A, [Ca2+]i was measured by fura 2 fluorescence in aortic smooth muscle cell monolayers from normal and HC rabbits. Cells were stimulated with 0.1 μmol/L angiotensin II to elevate [Ca2+], and NO (1 μmol/L) was added to decrease [Ca2+]. Tracing shows [Ca2+]i level (solid line) after NO addition and calculated first-order exponential decay (dashed line). In response to NO, exponential half-time (τ) was significantly greater (P<0.05) in cells from cholesterol-fed rabbits than in those from normal rabbits. B, In normal aortic smooth muscle cells, decreases in [Ca2+]i caused by NO (1 μmol/L) and SNP (10 μmol/L) were prevented by blocking SERCA with CPA (20 μmol/L). In contrast, ODQ (10 μmol/L) prevented decrease in [Ca2+]i caused by SNP but not NO.

**Figure 6.** Effect of HC on rabbit aortic expression of SERCA 2 and α-actin. A, Western blots (IB) of samples of normal (n=5) and HC (n=7) rabbit aortic homogenates. Estimated positions of proteins are indicated (kDa). B, Densitometry analysis shows no significant difference in expression of SERCA2/α-actin.
ODQ, was not significantly affected by HC, suggesting that cGMP-dependent relaxation is preserved. This is further indicated by the fact that relaxation caused by 8-bromo-cGMP was normal in HC. NO, however, relaxes the aorta by an additional cGMP-independent mechanism in the presence of a concentration of ODQ sufficient to prevent the rise in cGMP caused by NO.11 Under these conditions, the relaxation to NO was still reduced in HC. This suggests that a cGMP-independent mechanism mediated by NO is impaired in HC. Although HC can reduce arterial guanylyl cyclase activity at a later stage of HC,6 increases in cGMP in the carotid artery15 or aorta (unpublished studies) caused by NO were normal at this stage of HC. cGMP has numerous potential molecular targets that can mediate a decrease in intracellular calcium and cause relaxation, including L-type Ca\(^{2+}\) channels,18 potassium channels,19 inositol 1,4,5-triphosphate receptors,20 phospholamban,21 and myosin light chain phosphatase.22 The normal relaxation to SNP and 8-bromo-cGMP would indicate that the mechanisms involved are preserved in HC. These findings suggested to us that another mechanism that is cGMP-independent is impaired in HC and may account for the decreased relaxation to NO.

In other studies, we have presented evidence that calcium uptake into intracellular stores by SERCA is normally involved in mediating the decrease in intracellular Ca\(^{2+}\) and relaxation of the aorta to NO and that the response is at least partially cGMP-independent.11,12 CPA reduced NO-induced relaxation in both normal and HC aorta, and the effects of thapsigargin were similar to those of CPA (unpublished observations). In contrast, SNP-induced relaxation was not affected by CPA, suggesting that the largely cGMP-dependent relaxation to SNP does not rely on SERCA. This suggests that the difference in the ability of HC aorta to relax to NO and SNP is related to impaired SERCA function. Moreover, after concomitant administration of ODQ and CPA, the difference in relaxation to NO between normal and HC aorta was eliminated. These data suggest that it is primarily the cGMP-independent relaxation to NO that relies on SERCA activity and that impairment of this mechanism accounts for the decreased response to NO in HC. Indeed, our data showing that SERCA-dependent \(^{45}\)Ca\(^{2+}\) uptake activity is reduced are compatible with this hypothesis.

Moreover, HC decreased the exponential half-time to decrease [Ca\(^{2+}\)], in primary cultured aortic smooth muscle cells. Aortic smooth muscle has 3 major [Ca\(^{2+}\)], extrusion mechanisms: SERCA, plasma membrane Ca\(^{2+}\) ATPase, and Na\(^+\)/Ca\(^{2+}\) exchanger. We blocked the latter 2 mechanisms without causing any change in the decrease in [Ca\(^{2+}\)], caused by NO, whereas blocking SERCA drastically reduced the response.13 This suggests that a decrease in SERCA function in HC can explain the slower [Ca\(^{2+}\)] response to NO in HC rabbit aortic smooth muscle.

The molecular mechanisms by which NO increases calcium uptake by SERCA are not fully understood. Smooth muscle SERCA can be regulated by protein kinase G phosphorylating phospholamban, providing a cGMP-dependent mechanism to increase its activity.23 SERCA also was shown to be S-nitrosylated, providing a potential cGMP-independent mechanism of regulation by NO.23 This may explain why we found that the reductions in [Ca\(^{2+}\)], caused by both NO and SNP were both blocked by CPA but that ODQ blocked the reduction in [Ca\(^{2+}\)], caused by SNP but not NO. In contrast to the reduction in [Ca\(^{2+}\)], caused by SNP, relaxation caused by the NO donor was unaffected by blocking of SERCA, indicating that other mechanisms account for the relaxation. The most likely mechanism is the cGMP-dependent phosphorylation of myosin light chain phosphatase, which can account for relaxation without a decrease in [Ca\(^{2+}\)].22 These data indicate that relaxation caused by NO and SNP differ substantially in their dependence both on a SERCA-mediated decrease in [Ca\(^{2+}\)], and on cGMP.

The small, insignificant decrease in SERCA expression can hardly account for the \(\approx 50\%\) decrease in SERCA-dependent \(^{45}\)Ca\(^{2+}\) uptake observed in HC. Decreased function of SERCA could be accounted for by abnormal function of the molecule itself or of molecules or substrates that regulate it. One possibility is that SERCA function is impaired by oxidative modification. Hydrogen peroxide or hypochlorous acid, both agents that are increased in HC, have been reported to inhibit SERCA function.24,25 HC increases superoxide generation that can inactivate NO.6 SOD or EUK-8, however, had no effect on NO-induced relaxation in HC aorta. This is compatible with previous data showing that adenovirus-mediated overexpression of SOD in the aorta of hypercholesterolemic rabbits had no effect on endothelium-dependent relaxation.8 These findings suggest that rapid removal of superoxide anion may not be enough to recover NO responsiveness in HC. This suggests that another mechanism, such as inactivation of SERCA, can explain the reduced NO response of smooth muscle in HC.

SNP is widely used as an endothelium-independent NO donor in studies of endothelial function because it is assumed to act similarly to NO itself. Our data suggest that NO and SNP rely on different mechanisms for relaxation. Whereas reduced SERCA function accounts for the impaired reduction in [Ca\(^{2+}\)], and relaxation to NO in HC, the relaxation to SNP remains normal because SERCA is not required for the cGMP-dependent relaxation caused by the NO donor. Whereas SNP is a good therapeutic agent, perhaps because it bypasses impaired SERCA function in diseased arteries, NO itself may be a better agent with which to judge whether or not the smooth muscle response to NO is normal in diseased arteries.

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