Objective—Coronary endothelial dysfunction is associated with an increase in cardiac events. Hypercholesterolemia (HC) and hypertension (HT) are both associated with endothelial dysfunction, and their coexistence is associated with an increased incidence of cardiac events in epidemiological studies. However, pathogenic mechanisms are poorly understood. Here we studied the effects of coexisting HC and HT on coronary endothelial function.

Methods and Results—Four groups of pigs were studied after 12 weeks of a normal diet (N, n=9), a 2% HC diet (HC, n=9), HT (achieved by unilateral renal artery stenosis, n=8), or HC+HT (n=6). Coronary endothelial function was tested, in epicardial arteries and arterioles, by using organ chamber techniques. Oxidative stress was measured in coronary artery tissue. Vasodilatory response to bradykinin and calcium ionophore was significantly impaired in animals with HC+HT compared with each risk factor alone (P<0.05 for both). In animals with coexistent HC and HT, the increase in oxidative stress was more pronounced compared with each risk factor alone (P<0.05). Furthermore, chronic antioxidant supplementation significantly improved coronary artery vasoreactivity.

Conclusions—These results suggest that HC and HT have a synergistic deleterious effect on coronary endothelial function, associated with increased oxidative stress. This interaction may contribute to the increased incidence of coronary heart disease and cardiac events seen when HC and HT coexist. (Arterioscler Thromb Vasc Biol. 2003;23:885-891.)

Key Words: animal models ▪ coronary circulation ▪ oxidant stress ▪ risk factors

Atherosclerosis-related coronary disease, in its early stages, is characterized by coronary endothelial dysfunction, potentially leading to an increased incidence of cardiac events. Hypercholesterolemia (HC) and hypertension (HT) are major risk factors for atherosclerosis, and their combination is associated with a yet greater increase in the incidence of cardiac events. Thus, it becomes increasingly important to understand the pathogenic mechanisms of their interaction. Individually, both diet-induced HC and HT have been associated with abnormalities in coronary endothelial function. Although the effect of each risk factor has been studied individually, whether their coexistence has a synergistic deleterious effect on coronary endothelial function remains poorly understood.

One of the mechanisms that might be activated in both HC and HT and might hinder coronary vascular function is a shift in scavenging activity and redox status, a state known as increased oxidative stress. Increased vascular oxidative stress can be characterized by increased production of reactive oxygen species (ROS) and pro-oxidant reactions and decreased levels of endogenous antioxidants and endogenous radical scavenger systems, as well as an increase in oxidizability, which might also impair vascular function. Furthermore, increased oxidative stress can also have deleterious effects on other pathways, such as the nitric oxide (NO) pathway. Thus, these mechanisms might conceivably play a role in the interaction between HC and HT to impair vascular function.

Elucidation of the deleterious mechanisms by which HC and HT interact could advance our understanding of the pathophysiology of coronary atherosclerosis and its early clinical manifestations. Thus, the present study was designed to investigate the hypothesis that the combination of experimental HC and HT would enhance the impairment of coronary endothelial function induced by each risk factor alone and that these effects would be associated with abnormalities in the oxidative status milieu.

Methods

Animal Preparation

All animal procedures were reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee. Female juvenile domestic crossbred pigs (50 to 55 kg) were randomized to 4 groups. One group received a normal diet (N, n=9) while a second group was placed on an atherogenic diet of 2% cholesterol and 15% lard by weight (TD 93296, Harlan Teklad; HC, n=9). In a third group, renal artery stenosis was achieved by placement of a local-
irritant stent, with the subsequent development of renovascular hypertension (HT, n = 8). In the last group (HC + HT, n = 6), HC and HT were induced simultaneously. Plasma lipid profiles (Roche) and plasma renin activity (PRA) were determined after 12 weeks of diet and/or intervention in all 4 groups. After completion of the diet and/or interventions, euthanasia was performed by intravenous administration of pentobarbital sodium (Sleepaway, Fort Dodge Laboratories), and tissue was harvested for in vitro studies.

Vascular Wall Histomorphometry
Hematoxylin and eosin–stained coronary artery histological cross sections obtained from the 4 different groups were used for morphometric analysis with a computer image analysis program. The intimal and medial layers were defined by the borders of the internal and external elastic laminas, respectively. Areas for each region were traced and calculated in square millimeters. To correct for vessel size, areas were normalized for lumen area.

Vascular Endothelial Function

Epicardial Vessels
Arterial rings were prepared as previously described. In brief, hearts from the 4 groups of pigs were placed into cold, modified Krebs-Ringer bicarbonate solution (control solution). Rings of tissue 2 to 3 mm long were dissected, transferred to organ chambers with 25 mL of control solution, and oxygenated with 94% O₂ and 6% 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 20 mmol/L KCl at baseline, at 2, 4, and 6 g of tension, each time after the KCl had been washed out. At 6 g of tension, all vessels were exposed to substance P (10⁻⁶ mol/L, Sigma), an endothelium-dependent vasodilator, to verify the functional integrity of the vascular endothelium. All chambers were then washed out with the control solution.

After an equilibration period of 30 minutes, rings were precontracted with 10⁻⁴ mol/L endothelin-1 (Phoenix Pharmaceuticals), and then the response to the endothelium-dependent vasodilator bradykinin (10⁻⁷ to 10⁻⁵ mol/L, Sigma) was obtained. In additional vascular rings from each group, a dose response to the non–receptor-mediated, endothelium-dependent vasodilator calcium ionopore A23187 (10⁻⁷ to 10⁻⁵ mol/L, Sigma) was obtained. A dose-response curve to sodium nitroprusside (10⁻⁷ to 10⁻⁴ mol/L; SNP) was used to test the nonendothelial vasorelaxation response. Complete relaxation of each ring was tested, at the end of each experiment, by exposure to 10⁻⁵ mol/L papaverine.

Small Arteries
Coronary vasomotor tone in the small arteries was determined with previously described methods. In brief, segments 2 to 3 mm long of the secondary branch of the left circumflex artery, which were in diameter, were dissected, transferred to an arteriograph, and then mounted onto microcannulas (Living System Instrumentation). The arteriograph was placed on a microscope (Diaphot-TMD, Nikon), which had a video camera connected to the viewing tube. The signal obtained was electronically processed, and both the inner diameter and wall thickness were measured and recorded.

Vessels were precontracted with 10⁻⁴ mol/L endothelin-1, and the response to substance P (10⁻⁷ to 10⁻⁵ mol/L) was then recorded. The endothelium-independent response was tested by exposure to 10⁻⁵ mol/L papaverine (Sigma).

Nitric Oxide Pathway
To assess NO pathway activity, cGMP production was measured in smooth muscle cells in response to exogenous NO donation, as previously described. In brief, in epicardial vascular rings from each group, the endothelium was carefully removed, and the rings were placed in an organ chamber filled with Krebs’ solution. After 1 hour of incubation, 146 μL of 3-isobuthyl-1-methylxanthine (10⁻⁴ mol/L) and 100 μL of indomethacin (10⁻⁵ mol/L) were added to the solution in the organ chamber for 30 minutes. Later, samples were randomized to either standards (controls) or treated with diethylamine (DEA, an NO donor; 10⁻⁴ mol/L for 1 minute, and all samples were then shock-frozen. Samples were prepared as previously described and mounted on a scintillator counter, and cGMP production was then measured. In addition, to evaluate the functional contribution of the NO pathway, relaxation responses to DEA (10⁻⁴ to 10⁻³ mol/L) were obtained in endothelium-denuded epicardial rings from each group.

Redox Status
Coronary artery tissue activities of oxygen-radical scavenger enzymes were determined spectrophotometrically. Homogenates in potassium phosphate buffer, pH 7.4, containing 10 mol/L deferoxamine, 0.03% butylated hydroxytoluene, and 2% ethanol equilibrated with nitrogen (to reduce auto-oxidation), were centrifuged at 1000g for 15 minutes at 4°C to remove nuclei and tissue debris. The supernatant was centrifuged again at 30 000g for 35 minutes at 4°C. Levels of the endogenous antioxidants vitamins E and C in coronary artery tissue were measured by high-performance liquid chromatography, as previously described. Superoxide dismutase (in its 2 forms, CuZn-SOD and Mn-SOD), catalase, and glutathione peroxidase activity were determined spectrophotometrically, as previously described. Enzyme activity was normalized for protein content.

Immunohistochemistry for nitrotyrosine (NT; to evaluate production of peroxynitrite consequent to reaction between superoxide anion and NO) and for the lectinlike receptor for oxidized LDL (LOX-1) were performed in epicardial coronary arteries with the use of paraffin and frozen, embedded tissue, respectively. Positively charged slides were dried at 37°C for 1 hour, fixed in acetone for 10 minutes at 4°C, and air-dried for 60 minutes. Endogenous peroxidase activity was blocked by placing the slides in 1.5% hydrogen peroxide and 50% absolute methanol for 10 minutes and then rinsing. The Vectastain Elite ABC kit (Vector Laboratories, Inc) for mouse IgG was used, following the vendor’s instructions. Monoclonal antibodies to mouse NT residues (Cayman, 1:20) and to LOX-1 (diluted 1:280 in phosphate-buffered saline) served as primary antibodies, as previously described. The tissue was stained by using the Vector NovaRED substrate kit (Vector Laboratories, Inc) for 10 minutes, followed by counterstaining with hematoxylin and mounting with aqueous mounting medium. Histological sections were examined with a computer-aided image-analysis program (Metamorph, Meta Imaging Series 4.6). In each representative slide, immunostaining was quantified and expressed as a percentage of staining of the total surface area, and the results from all fields were averaged. For the analysis of LOX-1 immunostaining, 2 independent, blinded observers reviewed the sections and graded the staining, based on a previously described semiquantitative scoring system, as either no staining (0) or as positive staining in <25% (1), 25% to 50% (2), 50% to 75% (3), or >75% (4) of the endothelial or epithelial cells. The observers’ scores were averaged for each of the slides.

Antioxidant Supplementation
To further explore the role of oxidative stress in coexistent HC and HT, additional animals with combined HC and HT received long-term daily antioxidant supplementation (vitamin E 100 IU/kg and vitamin C 1000 mg). We and other investigators have previously shown that this combination of vitamins provides effective blockade of the endogenous oxidative stress system. After 12 weeks of diet and intervention, animals were killed and coronary artery vasoreactivity studies were performed, as described before. Vasoreactivity responses to increasing doses of bradykinin and calcium ionopore A23187 were obtained in epicardial arteries, as described before. In additional vascular rings, the dose response to endothelin-1 and SNP was measured to test the contractile response and the endothelium independent-vasoreactivity, respectively.

Statistical Analysis
Data are expressed as mean ± SEM or as percent change from maximal contraction (in vitro vascular reactivity). Within each group, repeated measurements were analyzed with a repeated-
measures ANOVA followed by the Bonferroni t test or the unpaired Student’s t test between groups. Statistical significance was accepted for a probability value <0.05. Synergy was tested by linear regression with an interaction term (statistical program JMP 4.0).

**Results**

**Serum Cholesterol, Systemic Hemodynamics, and Morphometric Analysis**

In both groups fed the HC diet for 12 weeks, total and LDL cholesterol levels were significantly and similarly higher than N or HT pigs (Table 1). Mean arterial pressure was significantly increased in both groups of HT animals (HT and HC+HT) compared with N and HC animals (Table 1). PRA was similar among all 4 groups of animals (N, 0.63±0.3; HC, 0.57±0.2; HT, 0.29±0.1; and HC+HT, 0.30±0.2; ANOVA, P=NS).

The media-to-lumen ratio in animals with either HC or HT alone was similar to N animals (N, 1.08±0.10; HC, 0.93±0.10; and HT, 1.03±0.10; P=NS). However, when HC and HT were combined, the media/lumen ratio was significantly higher compared with N animals (HC+HT, 1.28±0.10; P=0.03 compared with N animals). There was no difference in the intima-to-lumen ratio among the 4 groups (N, 0.105±0.0025; HC, 0.092±0.013; HT, 0.121±0.035; and HC+HT, 0.113±0.029; all P=NS compared with N).

**Vascular Endothelial Function**

**Epicardial Vessels**

The response of the epicardial vessels to cumulative concentrations of endothelin-1 was similar among the 4 groups studied (maximal contraction in N, 10.5±1 g tension; HC, 10.6±1.2; HT, 10.9±1.0; and HC+HT, 11.2±1.7), and there was no difference in the precontraction obtained with endothelin-1 among the 4 groups (ANOVA, P=NS).

The vasorelaxation response to bradykinin in epicardial vessels of HC or HT pigs was significantly attenuated compared with N animals (Figure 1). Furthermore, when HC and HT coexisted, the impairment in coronary vasorelaxation was significantly more pronounced (Figure 1). The vasorelaxation response to increasing doses of the endothelium-dependent vasodilator calcium ionophore A231187 was similar among N, HC, and HT animals (Figure 2). However, when HC and HT were combined, the vasorelaxation response to the calcium ionophore was almost abolished (Figure 2), showing a synergistic deleterious effect between HC and HT (P<0.05 for the interaction term). There was no difference in the vasorelaxation to the nonendothelium-dependent vasodilator SNP among the 4 groups (maximal relaxation: N, 17.9±4.1%; HC, 15.2±2.7%; HT, 12.5±5.7%; and HC+HT, 16.3±2.6%; ANOVA, P=0.97 among groups).

**Small Arteries**

There was no difference at baseline in the mean diameter of small arteries among the 4 experimental groups (N, 318±16 μm; HC, 339±36; HT, 328±20; and HC+HT, 326±22). There was no difference in the maximal vasoconstriction to endothelin-1 among the 4 groups. Small arteries from HC or HT animals exposed to substance P showed a blunted vasodilatory response compared with N pigs (Figure 3). Furthermore, when the 2 risk factors were combined, the blunted vasodilatory response was more pronounced and significantly different compared with HT and HC as well as with N animals (Figure 3). The maximal relaxation to papaverine in N animals (145±18 μm) was similar to that in HC, HT, and HC+HT (HC, 158±21 μm; HT, 119±23; and HC+HT, 159±21; ANOVA, P=0.3).

**TABLE 1. Lipid Profiles (Total and LDL Cholesterol), Mean Arterial Pressure (MAP), and Heart Rate (HR) in Normal, HC, HT, and HC+HT Pigs**

<table>
<thead>
<tr>
<th></th>
<th>Total Cholesterol, mmol/dL</th>
<th>LDL, mmol/dL</th>
<th>MAP, mm Hg</th>
<th>HR, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.73±0.14</td>
<td>0.94±0.14</td>
<td>93.4±3.5</td>
<td>84±5</td>
</tr>
<tr>
<td>HC</td>
<td>8.82±1.43*</td>
<td>6.26±1.18*</td>
<td>95.8±4.0</td>
<td>74±5</td>
</tr>
<tr>
<td>HT</td>
<td>1.69±0.16</td>
<td>0.91±0.09</td>
<td>119.4±10.3†</td>
<td>81±4</td>
</tr>
<tr>
<td>HC+HT</td>
<td>7.20±0.54*</td>
<td>5.63±0.61*</td>
<td>117.7±8.0†</td>
<td>77±7</td>
</tr>
</tbody>
</table>

Results are expressed as the mean±SEM.

*P<0.05 compared with normal and HT.

†P<0.05 compared with normal and HC.

![Figure 1. Epicardial arterial vasorelaxation response to bradykinin in N (n=9), HC (n=9), HT (n=8), and HC+HT (n=6) pigs. P<0.05 compared with N, HC, or HT.](image1.png)

![Figure 2. Epicardial arterial vasorelaxation response to calcium ionophore in N (n=9), HC (n=9), HT (n=8), and HC+HT (n=6) pigs. P<0.05 compared with N, HC, or HT.](image2.png)
NO Production

Vasoreactivity Response to DEA

In response to the NO donor DEA, there was no difference in the vasorelaxation response of epicardial arteries among N, HC, and HT animals at any DEA dose (maximal relaxation: N, 92.5%; HC, 97.7%; and HT, 96.6%; P=NS; Figure 4). In HC+HT animals, there was a significant shift to the left of the vasorelaxation curve at DEA concentrations of 10^{-7} to 10^{-6} mol/L (Figure 4), suggesting increased sensitivity to the NO donor at lower concentrations, although the maximal vasorelaxation response was unchanged (98.5%; P=NS compared with N, HC, and HT; Figure 4).

Smooth Muscle Cell cGMP Production

Under basal conditions, there was no statistically significant difference in cGMP levels in epicardial coronary arteries of N, HC, and HT animals (ANOVA, P=NS). However, in response to exogenous NO donation, HC animals had increased smooth muscle cell cGMP production compared with N animals (109.2±11.8 vs 50.0±6.2 pg/mg protein, respectively; P<0.01), suggesting sensitization of the smooth muscle to NO. In HT animals, there was a reduction in smooth muscle cell cGMP production in response to exogenous NO donation (24.4±6 pg/mg protein), which was significantly different from N (P<0.01) and HC (P<0.01) animals. When HC and HT were combined, the smooth muscle cell cGMP production was not different from N (59.22±16.5; P=0.31 compared with N animals).

Oxidative Stress

Both HC- and HT-only pigs had decreased coronary artery tissue levels of endogenous antioxidant vitamin E compared with animals receiving the N diet, while levels of vitamin C remained unchanged (Table 2). These changes were associated with a significant decrease in endogenous activity of the intracellular radical scavenger system (Table 2). Furthermore, when these 2 risk factors were combined (HC+HT), the effects on the antioxidant vitamins as well as on scavenging activity were even more pronounced, and in the case of Cu-SOD, they achieved statistical significance (Table 2), suggesting a deleterious interaction between these 2 risk factors. Indeed, tissue levels of vitamin C, unaltered in HC and HT alone, showed a synergistic deleterious interaction when HC and HT were combined (P<0.05 for the interaction term; Table 2). In addition, there was significant production of peroxynitrite in epicardial arteries from HC+HT animals, as detected by immunostaining for NT. Whereas in HC- and HT-only animals there was a tendency toward an increase in NT immunostaining (N, 1.54±0.42; HC, 2.24±0.28; and HT, 2.30±0.43; P=0.11 and P=0.13 compared with N, respectively; Figure 5 top), in the HC+HT group the change was even more pronounced and significantly different from N, HC, or HT (HC+HT, 6.00±0.70; all P<0.01; Figure 5 top).

The scoring of LOX-1 immunostaining in the epicardial coronary endothelium (Figure 5 bottom) was significantly higher in HC (2.4±0.3; P=0.006) compared with N (1.4±0.2), whereas in HT, the difference from N did not reach statistically significant levels (1.9±0.2; P=0.08; Figure 5 bottom). Nevertheless, in HC+HT animals, immunostaining for LOX-1 (Figure 5 bottom) was markedly enhanced compared with N (3.2±0.3, P=0.0008) and with HC or HT alone (P<0.05 for both).

Antioxidant Supplementation

HC+HT animals that received long-term antioxidant supplementation had similar increases in total and LDL cholesterol levels (10.5±1.58 and 7.67±1.25 mmol/L, respectively) and mean arterial pressure (122.2±3.4 mm Hg) when compared with N animals (all P<0.05). The media-to-lumen ratio (0.84±0.09) was similar to that observed in N animals (P=0.3) and significantly different from untreated HC+HT animals (P<0.01).

There was significant improvement, albeit not normalization, in the epicardial coronary artery vasoreactivity response to both bradykinin and calcium ionophore A23187 (Figure 6). As expected, dose responses to SNP and endothelin were comparable to those observed in N and HC+HT animals (data not shown).

Discussion

This study demonstrates, for the first time, that the combination of experimental HC and HT exacerbates the coronary endothelial dysfunction induced by each risk factor alone. The effects on endothelial function were accompanied by a shift of the oxidative status balance toward a pro-oxidant state. This study supports a potential mechanism for an interaction between HC and HT in the progression of coronary endothelial dysfunction and atherosclerosis.
The early stages of atherosclerosis are characterized by coronary endothelial dysfunction. HC and HT, 2 major risk factors for atherosclerosis, have both been individually associated with coronary endothelial dysfunction.3–5 Clinical epidemiological studies have shown that the combination of these 2 major risk factors leads to an increase in cardiac events, even in the absence of significant coronary artery disease.1,23 Thus, the mechanism of interaction between these 2 risk factors has attracted considerable attention. However, little is known about the impact of coexisting HC and HT in the early stages of atherosclerotic disease. Our study addresses this very important question and demonstrates that coexistence of short-term (12-week), experimental HC and HT exacerbates the impairment in coronary vascular function induced by each risk factor alone, underscoring crosstalk between these 2 independent cardiovascular risk factors that may potentially contribute to an increased incidence of cardiac events.

Diet-induced HC alone has been associated with coronary artery endothelial dysfunction.3,17 Diet-induced HC is associated with impairment in endothelium-dependent coronary vasorelaxation, both at the epicardial3,17 and the microvascular17,24 level, associated with a decrease in NO bioavailability.3 Moncada et al25 suggested that to compensate for the decrease in NO bioavailability, the vascular wall becomes "sensitized" to NO. This is evidenced by elevated smooth muscle cell levels of cGMP, the principal second messenger of NO, in response to exogenous NO donation. The current study is in accord with this previous observation and demonstrates that this decrease in NO bioavailability, with consequent endothelial dysfunction, is also associated with enhanced generation of cGMP by the smooth muscle cells in response to exogenous NO donation.

HT has also been associated with coronary endothelial dysfunction.26 This study corroborates these observations and also demonstrates that these changes occurred both at the epicardial and microvascular level. Furthermore, it also suggests that in addition to endothelial dysfunction, HT might also exert a direct deleterious effect on the smooth muscle cell, as evidenced by the poor response in cGMP production in response to exogenous NO donation. Indeed, it has been shown that angiotensin II might induce direct deleterious effects on the smooth muscle cell.

### Table 2.

<table>
<thead>
<tr>
<th>Scavenger Enzyme</th>
<th>Superoxide Dismutase</th>
<th>Endogenous Antioxidant</th>
<th>Vitamin E</th>
<th>Vitamin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuZn-SOD, mL/g</td>
<td>Mn-SOD, mL/g</td>
<td>Catalase, mL/g</td>
<td>Glut-Perox, mL/g</td>
<td>Vitamin E, mg/mg</td>
</tr>
<tr>
<td>Normal</td>
<td>8.5±0.5</td>
<td>2.4±0.0</td>
<td>20.5±1.4</td>
<td>75.3±2.7</td>
</tr>
<tr>
<td>HC</td>
<td>7.3±0.2†</td>
<td>2.0±0.1†</td>
<td>15.4±1.3*</td>
<td>59.9±4.4†</td>
</tr>
<tr>
<td>HT</td>
<td>7.3±0.2†</td>
<td>2.3±0.1</td>
<td>15.5±1.0*</td>
<td>69.0±1.5*</td>
</tr>
<tr>
<td>HC+HT</td>
<td>6.7±0.2‡</td>
<td>1.9±0.1†</td>
<td>13.2±1.5*</td>
<td>56.4±2.8†</td>
</tr>
</tbody>
</table>

Results are expressed as the mean±SEM of 18 different experiments (normal=3, HC=6, HT=4, and HC+HT=5).

Glut-Perox indicates glutathione-peroxidase.

*P<0.05 compared with normal.
†P<0.05 compared with HT.
‡P<0.02 compared with Normal, HC, or HT.

**Figure 5.** Representative immunostaining for NT (top) and LOX-1 (bottom) in distal left anterior descending coronary arteries of normal, HC, HT, and HC+HT pigs.

**Figure 6.** Epicardial arterial vasorelaxation response to bradykinin (top) and calcium ionophore (bottom) in normal (N), HC (HC), HT (HT), and HC+HT pigs that received long-term antioxidant supplementation. *P<0.05 compared with normal; †P<0.05 compared with HC+HT.
As typical for the chronic phase of HT,\textsuperscript{28} systemic angiotensin II production was not increased (reflected by PRA) in our model of HT. However, local (or tissue) production and activity of angiotensin II cannot be excluded. Moreover, coexistence of experimental HC and HT has been previously demonstrated to induce or accelerate atherosclerotic changes in the kidney\textsuperscript{9,29,30} and the rat aorta.\textsuperscript{31,32} Our study confirms these findings and demonstrates that the combination of HC and HT is associated with alterations in arterial wall structure. However, the functional impact of the combination of these 2 risk factors on coronary vascular reactivity remains undefined. This study addresses this issue and demonstrates that the combination of HC and HT is associated with greater impairment of coronary endothelial function than each risk factor alone, suggesting synergism between them in the coronary circulation. The impairment was observed in response to both receptor- and non–receptor-mediated, endothelium-dependent vasodilators (bradykinin and calcium ionophore A23187, respectively). The mechanisms for the impaired endothelial function observed in combined HC and HT might well be multifactorial. This study suggests that impairment of the NO pathway might be of critical importance in this model, as shown by impaired relaxations to endothelium-dependent vasodilators. Although cGMP has been recognized as the main intracellular mechanism of NO activation,\textsuperscript{33,34} other pathways have also been proposed to play a role.\textsuperscript{35,36} Indeed, Weisbrod et al\textsuperscript{35} previously showed that even after selective blockade of cGMP, there was a significant decrease in intracellular calcium and vasorelaxation in aortic rings. We have extended these observations and have shown that even in the presence of impaired cGMP production (induced by both HC and HT), the vasoreactivity response to NO donation was not blunted, thus suggesting that additional pathways (cGMP independent) might play a role in this experimental setting. Because vasoreactivity to an NO donor might express the concerted action of many different mechanisms, whereas measurement of smooth muscle cGMP production might only represent 1 step in this pathway, a certain discrepancy between the measurement of cGMP production and vasoreactivity response to exogenous NO donation might occur. We recognize such a discrepancy, which might constitute a limitation of the current study. In this study, the impairment in coronary vascular reactivity occurred at both the epicardial and the microvascular level, representing the wide extent of this effect that is likely to be functionally consequential.

Individually, HC and HT have also been associated with an imbalance in oxidant status toward a more pro-oxidant state.\textsuperscript{7,8,13,37,38} An increased oxidative stress state can be characterized by increased production of ROS and decreased levels of both natural body antioxidants and cellular scavenger enzymes.\textsuperscript{7,15,17} We have previously shown that individually, both HC and HT are characterized by decreased levels of antioxidants as well as increased levels of oxidative stress markers, both reflecting a more pro-oxidant state.\textsuperscript{7,8} This study corroborates these findings and extends them by showing that HC and HT might increase oxidative stress through different pathways. HC induces a more pronounced decrease in glutathione peroxidase, compared with that seen in HT alone, associated with increases in LOX.\textsuperscript{39} The differences in LOX-1 in HT alone found in our study, compared with previous reports,\textsuperscript{40} likely reflect different stages of the disease and the degrees of HT response observed. Most important, when HC and HT coexisted, there was an even greater effect, suggesting an interaction between them. We observed a similar synergistic effect in the production of NT (a product of the reactions between NO and ROS). We have also shown a marked pro-oxidant shift in the systemic and renal circulation owing to the interaction of HC and HT.\textsuperscript{15,16} This study extends these observations to the interaction that exists between HC and HT in the coronary circulation and shows that the increase in the pro-oxidant milieu is more pronounced than with each risk factor alone. The important role played by increased oxidative stress is further underscored by the improvement in coronary artery vasoreactivity observed when animals with HC+HT received long-term antioxidant supplementation. The lack of normalization of the vasorelaxation response likely reflects the activation of additional mechanisms that might not be affected by antioxidant supplementation. Chronic increases in oxidative stress in conjunction with inflammatory changes are likely to be responsible for the alterations in arterial wall structure observed in our study, as evidenced by the normalization in structure seen when HC+HT animals received antioxidant supplementation. This interaction might at least be partly responsible for the deleterious milieu that results in altered coronary vascular structure and function and might ultimately increase cardiac events. Heitzer et al\textsuperscript{23} have shown that increased oxidative stress (as suggested by improvement in the peripheral endothelial function response to vitamin C) was associated with increased incidence of cardiac events. The authors further suggested that increased oxidative stress might be independently predictive of cardiac events. Our study might support a role for long-term antioxidant therapy in pathophysiological states associated with increased oxidative stress, such as the early stage of atherosclerosis.

The term endothelial dysfunction commonly refers to alterations in vasoreactivity. However, the function of the endothelial layer extends beyond that of vasodilatation and includes anti-inflammatory and antithrombotic properties, as well as regulation of vascular permeability and modulation of vascular growth.\textsuperscript{41} The common pathway for these alterations might be an imbalance in oxidative mechanisms, as shown in our study. Most importantly, impairment in endothelial properties, as manifested by alterations in coronary vasoreactivity, might have significant clinical implications in predicting cardiac events and clinical outcomes.\textsuperscript{1,42}

Identifying the time course of each of these risk factors in the development and progression of endothelial dysfunction and atherosclerosis might be of critical importance in assessing not only the stage of the disease but also in targeting medical therapy. Because cardiovascular risk factors (such as HC and HT) are indolent and may be present for a long time before they are identified, clinical studies have not been able to delineate the exact time course of these risk factors in the development and progression of disease. Because HC and HT were induced in this study, we were able to address this important question and describe the early interaction between HC and HT and the critical role they play in the exacerbation of myocardial vascular dysfunction observed when these 2 risk factors coexist.

In summary, the present study demonstrates that the combination of HC and HT is associated with a greater impairment of
coronary endothelial function than each risk factor alone. In addition, this impairment is associated with a similar alteration in oxidative stress. This deleterious interaction might play a role in the increased incidence of cardiac events seen when these 2 risk factors coexist.

Acknowledgments
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References
Hypercholesterolemia and Hypertension Have Synergistic Deleterious Effects on Coronary Endothelial Function

Martin Rodriguez-Porcel, Lilach O. Lerman, Joerg Herrmann, Tatsuya Sawamura, Claudio Napoli and Amir Lerman

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