Heart Rate Reduction by Ivabradine Reduces Oxidative Stress, Improves Endothelial Function, and Prevents Atherosclerosis in Apolipoprotein E–Deficient Mice

Florian Custodis, MD*; Magnus Baumhäkel, MD*; Nils Schlimmer, MS; Franka List, MS; Christoph Gensch, MS; Michael Böhm, MD; Ulrich Laufs, MD

Background—Elevated heart rate is associated with increased cardiovascular morbidity. We hypothesized that selective heart rate reduction may influence endothelial function and atherogenesis and tested the effects of the I(f) current inhibitor ivabradine in apolipoprotein E–deficient mice.

Methods and Results—Male apolipoprotein E–deficient mice fed a high-cholesterol diet were treated with ivabradine (10 mg·kg⁻¹·d⁻¹) or vehicle for 6 weeks (n=10 per group). Ivabradine reduced heart rate by 13.4% (472±9 versus 545±11 bpm; P<0.01) but did not alter blood pressure or lipid levels. Endothelium-dependent relaxation of aortic rings was significantly improved in ivabradine-fed animals (P<0.01). Ivabradine decreased atherosclerotic plaque size in the aortic root by >40% and in the ascending aorta by >70% (P<0.05). Heart rate reduction by ivabradine had no effect on the number of endothelial progenitor cells and did not alter aortic endothelial nitric oxide synthase, phosphorylated Akt, vascular cell adhesion molecule-1, or intercellular adhesion molecule-1 expression but decreased monocyte chemotactic protein-1 mRNA and exerted potent antioxidative effects. Ivabradine reduced vascular NADPH oxidase activity to 48±6% and decreased markers of superoxide production and lipid peroxidation in the aortic wall (P<0.05).

The in vivo effects of ivabradine were absent at a dose that did not lower heart rate, in aortic rings treated ex vivo, and in cultured vascular cells. In contrast to ivabradine, treatment with hydralazine (25 mg·kg⁻¹·d⁻¹ for 6 weeks) reduced blood pressure (15%) but increased heart rate (37%) and did not improve endothelial function, atherosclerosis, or oxidative stress.

Conclusions—Selective heart rate reduction with ivabradine decreases markers of vascular oxidative stress, improves endothelial function, and reduces atherosclerotic plaque formation in apolipoprotein E–deficient mice. (Circulation. 2008;117:2377-2387.)

Key Words: atherosclerosis ■ endothelium ■ heart rate ■ pharmacology ■ tachycardia

Epidemiological studies have shown that elevated heart rate represents a risk factor for cardiovascular morbidity both in primary prevention and in patients with hypertension, coronary artery disease, and myocardial infarction.¹⁻⁵ Increased heart rate and reduced heart rate variability have been shown to be associated with coronary plaque rupture and subclinical inflammation in healthy middle-aged and elderly subjects.⁶,⁷ Experimental data suggest that sustained elevations of heart rate may play a role in the pathogenesis of coronary atherosclerosis. For example, Beere et al⁸ showed in 1984 that lowering heart rate through ablation of the sino-atrial node in cynomolgus monkeys fed an atherogenic diet decreased coronary atherosclerosis. In later studies, they reported a positive correlation between heart rate and plaque formation at the carotid bifurcation region of monkeys.⁹ However, the underlying mechanisms and the therapeutic consequences are only partially understood.

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The pacemaker current I₀ plays a central role in determining spontaneous activity of the sinus node. Ivabradine, a selective inhibitor of the I₀ channel, reduces resting and exercise heart rates without affecting cardiac contractility or blood pressure.¹⁰,¹¹ Ivabradine exerts antianginal and anti-ischemic effects in patients with stable coronary disease. Clinical trials revealed improved exercise tolerance, increased time to exercise-induced ischemia, and reduced frequency of ambient angina attacks after I₀ channel inhibi-

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Ivabradine given orally to mice (10 mg/kg body weight per day) reduces heart rate without influencing left ventricular contractile function and therefore is used as a tool to study the effects of heart rate on vascular biology.14 We hypothesized that selective heart rate reduction by ivabradine may improve vascular function. Therefore, the aim of this study was to determine the effect of ivabradine on endothelial function, atherosclerotic lesion formation, and parameters of vascular oxidative stress in cholesterol-fed apolipoprotein E–deficient (ApoE−/−) mice.

**Methods**

**Animals and Procedures**

Animal experiments were conducted in accordance with institutional guidelines and the German animal protection law. Male C57/B16 mice (wild-type) and male ApoE−/− mice (C57/B16 genetic background; Charles River Laboratories, Sulzfeld, Germany) were used for this study. The animals were maintained in a 22°C room with a 12-hour light/dark cycle and received drinking water ad libitum. All mice were fed a high-fat, cholesterol-rich diet for 6 weeks that contained 21% fat, 19.5% casein, and 1.25% cholesterol (Western-type diet, Smiff, Soest, Germany) starting at 12 weeks of age. Randomly selected ApoE−/− mice were treated with Western-type diet chow pellets supplemented with commercially available ivabradine (Procoralan, Servier, Neuilly-sur-Seine, France) 10 and 2 mg/kg body weight per day. For indicated mice, Nω-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich, Taufkirchen, Germany) was added to the drinking water (50 mg/kg body weight per day). An additional group of age-matched ApoE−/− mice was treated with the vasodilator hydralazine 25 mg/kg body weight per day (Sigma) via drinking water.15 Heart rate and blood pressure were measured by a computerized tail-cuff system (BP-2000, Visitech Systems, Apex, NC) in conscious animals. Mice were trained for 3 consecutive days in the prewarmed tail-cuff device to accustom them to the procedure, followed by measurements of heart rate and systolic blood pressure every other day. During each procedure, 20 measurements were obtained and averaged for each individual animal. The mean values of all analyses were used for comparisons. Cholesterol levels were determined by routine chemical methods.

**Endothelium-Dependent Vasodilatation**

The detailed preparation and the protocol of the tension recording of aortic rings are described in the online Data Supplement and was performed as described.15,16

**Polymerase Chain Reactions**

The primer sequences and polymerase chain reaction (PCR) protocols are listed in the online Data Supplement.16,17

**Staining of Atherosclerotic Lesions and Morphometric Analysis**

Samples were sectioned on a Leica (Wetzlar, Germany) cryostat (10 μm), starting at the apex and going through the aortic valve area into the ascending aorta. At least 25 consecutive sections per animal were stained with Oil Red O and used for analysis. Lucia Measurement Version 4.6 software (Nikon, Düsseldorf, Germany) was used to measure lipid-staining area and total area of the histological sections. The online Data Supplement gives details.15,16

**Western Blot Analysis**

Immunoblotting for endothelial nitric oxide synthase (eNOS; BD 610296), phosphorylated eNOS (p-eNOS; BD 612706), phosphorylated Akt (p-Akt; sc 7985-R), and β-actin (sc 1615-R), to control for equal protein loading, with an enhanced chemiluminescence kit (Amersham, Uppsala, Sweden) was performed as described.16,17

**Fluorescence-Activated Cell Sorter Analysis and Culture of Spleen-Derived Endothelial Progenitor Cells**

Blood and bone marrow were processed as described.17–20 The viable lymphocyte population was analyzed for Sca-1-FITC (E13-161.7, BD PharMingen, San Diego, Calif) and VEGFR-2-PE (Fik-1) (Avas12a1, BD PharMingen). Isotype-identical antibodies (Becton Dickinson, Franklin Lakes, NJ) served as controls in every experiment. Spleen mononuclear cells were isolated and cultured on fibronectin (Sigma) as described.17–20 After 7 days in culture, endothelial progenitor cells (EPCs) were identified by uptake of 1,1'-dioctadeyl-3,3,3',3'-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (DiLDL; 2.4 μg/ml; CellSystems, St Katharinen, Germany) and staining with FITC-labeled Giffonia (bandeiraea) simplicifolia lectin I (lectin; 10 μg/ml) (Vector Laboratories, Burlingame, Calif).

**Measurement of Lipid Peroxidation**

Aortic tissue was homogenized in PBS (pH 7.4) containing butylated hydroxytoluene (4 mmol/L). Lipid hydroperoxides were determined with the Lipid Peroxidation Assay Kit II (Calbiochem, Gibbstown, NJ) and expressed as micromoles per microgram of protein.16

**Measurement of Vascular Superoxide Production and NADPH Oxidase Activity**

Superoxide release in intact aortic segments was determined by L-012 chemiluminescence.16,21 To assess vascular superoxide production in situ, dihydroethidium fluorescence of aortic tissue sections was used. Aortas from each treatment group were processed in parallel; images were acquired with identical acquisition parameters and were analyzed and stored digitally (ImageJ 1.37v by W. Rasband, National Institutes of Health, Baltimore, Md).16 NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay.16,22 Details are given in the Data Supplement.

**Cell Culture**

Endothelial cells harvested from bovine aortas and vascular smooth muscle cells isolated from rat thoracic aorta were cultured as described.23,24 Cellular viability was determined by cell count, morphology, and trypan blue exclusion. Cellular protein content was quantified by a modified Lowry assay.

**Measurement of Intracellular Reactive Oxygen Species**

Intracellular reactive oxygen species production in vascular smooth muscle cells was measured by 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescence.23,25 Details are given in the Data Supplement.

**Statistical Analysis**

Results are presented as mean±SEM. Unpaired Student’s t test and ANOVA for multiple comparisons were used when applicable. Post hoc comparisons were performed with the Newman-Keuls test. Values of P<0.05 were considered statistically significant. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

Heart Rate, Blood Pressure, and Lipid Levels

Twelve-week-old C57/B16 (wild-type) and ApoE−/− mice were fed a Western-type diet and were randomized to oral ivabradine, hydralazine, or vehicle treatment for 6 weeks. The Table shows mean heart rates, systolic and diastolic blood pressures, and serum cholesterol concentrations after 6 weeks. Treatment with 10 mg/kg ivabradine decreased heart...
rate by $-13.2\%$ ($-73.2$ bpm; $P<0.01$ versus ApoE$^{-/-}$ vehicle), whereas systolic and diastolic blood pressures were not altered significantly. Heart rate reduction remained consistently stable throughout the whole treatment period. As control for potential effects of ivabradine independently of heart rate, age-matched ApoE$^{-/-}$ mice were fed the highest dose of ivabradine that did not result in a significant reduction in heart rate, namely $2$ mg/kg body weight, for $6$ weeks. A second control group was treated with ivabradine $10$ mg/kg body weight and the NOS inhibitor L-NAME ($50$ mg/kg body weight per day) for $6$ weeks. A third control group was treated orally with ivabradine ($10$ mg/kg body weight per day) for $6$ weeks; a lower dose of ivabradine that did not result in a significant reduction in heart rate, namely $2$ mg/kg body weight, for $6$ weeks. Treatment with ivabradine ($10$ mg/kg body weight per day) did not alter endothelial function (Figure 1A). Serum cholesterol levels were increased in ApoE$^{-/-}$ mice as expected but were not altered by the drug treatments.

**Vascular Function**

Vascular function was assessed in isolated aortic ring preparations. As expected, endothelium-dependent vasodilation was impaired in ApoE$^{-/-}$ mice. Treatment with ivabradine ($10$ mg/kg body weight per day) significantly improved endothelial function in ApoE$^{-/-}$ mice almost to the level of wild-type controls ($P<0.05$ versus ApoE$^{-/-}$ vehicle). In contrast, the lower dose of ivabradine that did not affect heart rate ($2$ mg/kg body weight per day) did not alter endothelial function (Figure 2). Cotreatment with ivabradine ($10$ mg/kg body weight per day) and L-NAME showed a trend toward decreased endothelial function compared with ivabradine alone, suggesting that prevention of eNOS uncoupling does not play a major role. Endothelium-independent vasorelaxation induced by glyceroltrinitrate and vasoconstriction induced by phenylephrine and KCl were similar in all groups.

**Atherosclerotic Lesion Formation**

The chemokine monocyte chemotactic protein-1 (MCP-1) plays a causal role in the progression from endothelial dysfunction to atherosclerotic lesion development, primarily by inducing leukocyte arrest and transendothelial migration. MCP-1 has been shown to be regulated by fluid shear stress. Reverse-transcription PCR of aortic mRNA showed a marked reduction in MCP-1 expression to $26.7\%$ (Figure 3) after treatment with ivabradine ($10$ mg/kg body weight per day for $6$ weeks; $P<0.05$). Ivabradine had no effect on the aortic mRNA expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1.

Histomorphometric analysis of atherosclerotic lesions in the aortic sinus and the ascending aorta showed that selective heart rate reduction by ivabradine significantly slows atherosclerosis. The atherosclerotic plaque area was reduced by $>40\%$ in the aortic root ($32.4\%$ versus $18.2\%$) and by $>70\%$ in the ascending aorta ($26.4\%$ versus $7.1\%$) compared with vehicle-treated mice ($n=10$ per group; $P<0.05$; Figure 3D through 3I).

**Table. Heart Rate, Systolic Blood Pressure, Diastolic Blood Pressure, and Plasma Cholesterol Concentrations in Wild-Type Mice and in ApoE$^{-/-}$ Mice Fed a Western-Type Diet**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SBP, mm Hg</th>
<th>DBP, mm Hg</th>
<th>Total cholesterol, mg/dL</th>
<th>LDL cholesterol, mg/dL</th>
<th>HDL cholesterol, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>135 ± 10</td>
<td>76 ± 18</td>
<td>149 ± 10</td>
<td>16 ± 2</td>
<td>120 ± 14</td>
</tr>
<tr>
<td>Vehicle</td>
<td>134 ± 10</td>
<td>72 ± 15</td>
<td>148 ± 9</td>
<td>17 ± 1</td>
<td>120 ± 14</td>
</tr>
<tr>
<td>Iva 2</td>
<td>133 ± 10</td>
<td>70 ± 14</td>
<td>147 ± 9</td>
<td>16 ± 1</td>
<td>120 ± 14</td>
</tr>
<tr>
<td>Iva 10</td>
<td>132 ± 10</td>
<td>65 ± 13</td>
<td>146 ± 8</td>
<td>16 ± 1</td>
<td>120 ± 14</td>
</tr>
</tbody>
</table>

**DBP** indicates diastolic blood pressure; **SBP** indicates systolic blood pressure; **LDL** indicates low-density lipoprotein; and **HDL** indicates high-density lipoprotein. Mice were treated orally with ivabradine (Iva; 2 and 10 mg · kg$^{-1}$ · d$^{-1}$), L-NAME (50 mg · kg$^{-1}$ · d$^{-1}$), hydralazine (25 mg · kg$^{-1}$ · d$^{-1}$), or vehicle for 6 weeks. Heart rate, SBP, and DBP were determined every other day during the treatment period (mean ± SEM; $n=10$ per group).

* $P<0.01$ versus wild type; ** $P<0.001$ versus ApoE$^{-/-}$ vehicle; † $P<0.001$ versus ApoE$^{-/-}$ + Iva; § $P<0.01$ versus ApoE$^{-/-}$ + Iva; || $P<0.001$ versus wild type; ¶ $P<0.05$ versus ApoE$^{-/-}$ + vehicle.

![Figure 1. Heart rate and blood pressure. Effect of 6-week treatment with ivabradine (Iva; 2 and 10 mg · kg$^{-1}$ · d$^{-1}$), L-NAME (50 mg · kg$^{-1}$ · d$^{-1}$), and hydralazine (25 mg · kg$^{-1}$ · d$^{-1}$) on heart rate (A) and blood pressure (B) in ApoE$^{-/-}$ mice (mean ± SEM; $n=10$ per group). ** $P<0.01$ versus ApoE$^{-/-}$ vehicle; † $P<0.01$ versus ApoE$^{-/-}$ + Iva. SBP indicates systolic blood pressure; DBP, diastolic blood pressure.
positive spleen-derived EPCs and on EPCs in the peripheral blood and bone marrow (Figure 4A through 4C).

Endothelial nitric oxide is a key mediator of endothelial function and atherogenesis. However, reduced heart rate was not associated with a significant upregulation of eNOS mRNA in the aorta as determined by real-time PCR. Similarly, protein expression of eNOS, p-eNOS, and phosphorylated protein kinase Akt was not increased (Figure 4D through 4H).

**Vascular Oxidative Stress**

Reactive oxygen species impair vascular function. The NADPH oxidase is a major source of superoxide radicals in the vascular wall. Figure 5 shows that NADPH oxidase activity in the aorta was downregulated to 48±6% in the ivabradine-treated ApoE−/− mice (P<0.05). Vascular release of superoxide radicals was measured by L-012 chemiluminescence assays in intact aortic segments. Reactive oxygen species release was significantly decreased in ivabradine-treated mice (186.5±52.5 versus 44.2±17.2 relative light units · μg −1 · min −1; P<0.05). As a global parameter of oxidative stress, lipid peroxidation of the aortic wall was quantified. Animals with reduced heart rate displayed down-regulation of vascular lipid hydroperoxides compared with vehicle-treated littermates (0.94±0.04 versus 0.61±0.08 μmol/μg; P<0.05). In addition, in situ detection of superoxide production was performed by dihydroethidium fluorescence microscopy in aortic sections showing a marked reduction in reactive oxygen species release in ivabradine-treated ApoE−/− mice (124±7 versus 77±5 arbitrary units; P<0.01; Figure 5).

**Vascular Effects of Hydralazine**

As a control group, mice were treated with hydralazine. As expected, hydralazine treatment lowered blood pressure (99±2 mm Hg) and increased heart rate (650±8 bpm; P<0.05; the Table and Figure 1). Interestingly, hydralazine treatment did not confer a significant improvement in endothelial function despite robust blood pressure lowering (Figure 6A and 6B). Furthermore, in contrast to ivabradine treatment, hydralazine exerted only a minor effect on atherosclerosis that was not statistically significant (Figure 6C and 6D). Treatment with hydralazine showed no effect on the activity of NADPH oxidase, lipid peroxidation, or superoxide production (Figure 6E and 6G).

**Test for Potential Direct Effects of Ivabradine Independently of Heart Rate**

To investigate potential direct effects of ivabradine on endothelial function, aortic ring preparations from ApoE−/− mice fed the Western-type diet for 6 weeks were treated ex vivo with ivabradine in the organ bath. Increasing concentrations of ivabradine (0.2 to 20 μmol/L) did not induce vasorelaxation. The L-NAME–mediated vasoconstriction was not affected by the presence of ivabradine in the organ bath. Preincubation of aortic rings with ivabradine (2 μmol/L for 5 minutes) did not result in an improved vasorelaxation in the presence of carbachol. The nitroglycerin-mediated vasodila-

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**Figure 2.** Endothelial function. After 6 weeks of treatment of ApoE−/− mice with ivabradine (Iva; 2 and 10 mg · kg −1 · d −1), L-NAME, or vehicle, aortic rings were isolated, studied in organ chamber experiments, and compared with aortic rings of wild-type (WT) mice. Endothelium-dependent vasodilatation of aortic segments induced by carbachol (A, B) and endothelium-independent vasorelaxation induced by glyceroltrinitrate (C), both expressed as percent of maximal phenylephrine-induced vasoconstriction, are shown (n=10 per group; *P<0.05 vs ApoE−/− vehicle; #P<0.001 vs ApoE−/− vehicle).

**EPCs and eNOS**

Recent evidence has shown that cardiovascular function and angiogenesis are significantly modulated by circulating EPCs from the bone marrow. Cardiovascular risk factors impair EPC number and function, whereas vasculoprotection, eg, by statins and physical activity, is associated with increased numbers of EPCs. To investigate a possible effect of heart rate reduction by I(f) channel inhibition on EPCs, DiLDL/lectin-positive EPCs were expanded from spleen-derived mononuclear cells. As a second method of EPC quantification, Sca-1/VEGFR-2–positive EPCs were quantified by fluorescence-activated cell sorter analysis. However, treatment with ivabradine had no effect on DiLDL/lectin-

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**Figure 3.** Aortic oxidative stress. Aortic sections were stained for NADPH oxidase activity by dihydroethidium fluorescence microscopy, and quantified using fluorescence microscopy. The Table shows that reactive oxygen species production was significantly decreased in ivabradine-treated mice (186.5±52.5 versus 44.2±17.2 relative light units · μg −1 · min −1; P<0.05). In addition, in situ detection of superoxide production was performed by dihydroethidium fluorescence microscopy in aortic sections showing a marked reduction in reactive oxygen species release in ivabradine-treated ApoE−/− mice (124±7 versus 77±5 arbitrary units; P<0.01).
tation was not changed in the presence of ivabradine (Figure 7A through 7C).

To test whether ivabradine exerts direct cellular effects, cultured bovine aortic endothelial cells were treated with ivabradine (0.2 to 200 μmol/L for 16 hours), and protein expression was examined by Western blot analyses. As depicted in Figure 7D, expression of p-Akt, eNOS, and p-eNOS was not altered by increasing doses of ivabradine. Similarly, NADPH oxidase activity in bovine aortic endothelial cells was not altered in the presence of ivabradine (Figure 7E). To assess a potential direct antioxidative action of ivabradine, vascular smooth muscle cells were treated with ivabradine alone and in combination with angiotensin II and the NOS inhibitor L-NAME. Release of reactive oxygen species was detected by H2DCFDA fluorescence. The angiotensin II–induced free radical release was not attenuated by pretreatment with ivabradine (Figure 7F).

**Discussion**

The novel finding of the study is the marked reduction in vascular oxidative stress associated with an improvement in endothelial function and inhibition of atherosclerotic plaque formation after selective heart rate reduction with the I(f) channel inhibitor ivabradine in ApoE−/− mice.

ApoE−/− mice fed a Western-type diet represent a well-characterized model of hyperlipidemia-induced endothelial dysfunction. Endothelial dysfunction precedes vascular diseases in several vascular beds, including coronary and peripheral artery disease and ischemic stroke. Ivabradine treatment improved endothelium-dependent vasodilatation in the absence of blood pressure or lipid lowering. Improvement in endothelial function was associated with a significant reduction in atherosclerotic plaque volume despite persistent severe hypercholesterolemia. We propose that the main mechanism by which ivabradine exerts these effects is the reduction in heart rate because previous animal studies have shown that a reduction in heart rate by other means, eg, ablation of the sinoatrial node in cynomolgus monkeys, beneficially affects atherogenesis.

The circadian regulation of heart rate affects cardiovascular morbidity. Therefore, it will be important to characterize the modulation of circadian rhythms by ivabradine treatment. Modification of the hemodynamic environment by accelerated heart rate is likely to contribute to enhanced atherosclerosis in certain parts of the vascular tree. In regions of the vasculature where flow reversal and shear stress oscillation dominate flow pattern, high heart rate is associated with expanded periods of low shear stress levels, potentially facilitating atherosclerotic lesion formation. In elastic arteries, acute increases in heart rate can be accompanied by reductions in arterial compliance and distensibility. Those
hemodynamic and tensile effects contribute to the propensity for plaque formation in vessels exposed to high heart rate. Here, we show that improvement in endothelial function and significant slowing of atherogenesis can be achieved by ivabradine, an oral treatment that is well tolerated in mice and humans.13,14

Treating mice with ivabradine at the highest dose that did not reduce heart rate had no effect on endothelial function. Exposure of isolated aortic rings from ApoE−/− mice to ivabradine in the organ bath did not alter vasodilation. Furthermore, ivabradine showed no effect in cultured endothelial and cultured vascular smooth muscle cells. Taken together, these control experiments show that a direct effect of ivabradine on vascular cells is unlikely and support the reduction of heart rate as the primary mechanism of action.

A pharmacological agent that selectively and permanently increases heart rate is not available. We therefore treated ApoE−/− mice with hydralazine as a control group. Hydralazine acts as a potent arteriolar dilator that stimulates a reflective increase in pulse rate.39 At the same time, hydralazine lowers blood pressure. Decreased systolic and diastolic blood pressures lead to improved endothelial function and reduced atherogenesis in animal models and humans. Interestingly, despite a robust reduction in systolic and diastolic blood pressures in our model, hydralazine did not significantly improve endothelial function or the extent of atherosclerosis. Similarly, hydralazine showed no effect on several parameters of vascular oxidative stress. These data are in agreement with the hypothesis that an increase in heart rate may be associated with negative vascular effects that outweigh the beneficial effects of blood pressure lowering.

Endothelial nitric oxide is a key mediator of endothelial function and is selectively upregulated by steady laminar shear stress.17,32,40 However, in contrast to our expectations, the profound improvement in endothelial function by ivabrad-
dine was not associated with upregulation of aortic eNOS mRNA expression. Furthermore, Western blot analysis did not show an upregulation of eNOS or p-eNOS protein nor an increase of p-Akt expression. A potential effect of ivabradine on NOS uncoupling was ruled out by the control experiments with L-NAME in vivo, in isolated aortic rings, and in cell cultures. Therefore, it appears that modulation of eNOS expression is not the primary mechanism. The observed beneficial effects are mediated, at least in part, by the eNOS-independent antioxidative effects of ivabradine.

Recent evidence has shown that cardiovascular function and angiogenesis are significantly modulated by circulating EPCs derived from bone marrow. EPCs have been shown to enhance angiogenesis, to promote vascular repair, and to improve endothelial function.17,29–31 Circulating EPCs are regulated, and vascular risk factors have been shown to
reduce EPCs. Therefore, we hypothesized that a reduction in heart rate may increase the number of EPCs. However, we did not observe a regulation of EPCs using both EPC culture and fluorescence-activated cell sorter analysis and looking at spleen-, blood-, and bone marrow–derived EPCs. As with any negative result, technical limitations can never be completely excluded; however, other interventions such as physical exercise show clear effects in the same animal model, making a large contribution of EPCs to the observed effects less likely.

The increased release and production of reactive oxygen species is one of the key events in the pathogenesis of endothelial dysfunction and atherosclerosis. Treatment with ivabradine potently inhibited vascular oxidative stress, providing an explanation for the observed effects on vascular function. Superoxide derived from the vascular NADPH-oxidase complex has been shown to impair endothelial function and to promote atherogenesis. Iivabradine-induced reduction in heart rate was associated with marked inhibition of NADPH-oxidase activity and superoxide release, which was confirmed histologically by dihydroethidium staining. In addition, vascular lipid peroxidation as a global marker of oxidative stress was significantly decreased. Taken together, the data of these 4 different assays show a robust antioxidative effect of ivabradine on the vasculature.

Consistent with these findings, ivabradine treatment resulted in a potent downregulation of MCP-1 expression. MCP-1 provides a link between endothelial dysfunction and atherosclerotic lesion formation by inducing leukocyte arrest and transendothelial migration. MCP-1 has been shown to be regulated by fluid shear stress and cyclic strain. Interestingly, cyclic strain–induced MCP-1 expression in both endothelial and vascular smooth muscle cells involves superoxide anion production, which is reduced by ivabradine. This may explain the selective regulation of MCP-1 compared with intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 that is controlled predominantly by eNOS.

Figure 6. Vascular effects of hydralazine. Effects of treatment of ApoE/ mice with hydralazine (25 mg · kg⁻¹ · d⁻¹ PO for 6 weeks) or vehicle on endothelium-dependent vasodilatation (A), endothelium-independent vasorelaxation (B), and atherosclerotic lesion formation in the aortic sinus (C) and the ascending aorta (D). Effects of hydralazine on aortic NADPH oxidase activity (E), lipid hydroperoxides (F), and superoxide production in situ (G) determined by dihydroethidium (DHE) fluorescence (n=6 to 10 per group; P=NS). WT indicates wild type.
Conclusions

Chronic heart rate reduction by ivabradine leads to inhibition of vascular oxidative stress, endothelial dysfunction, and atherosclerotic lesion formation in ApoE<sup>−/−</sup> mice regardless of blood pressure and plasma cholesterol levels. The experiments support the potential of heart rate reduction as an intervention to improve endothelial function and to attenuate progression of atherosclerosis in vascular prevention, in
addition to the symptomatic treatment of angina pectoris. Pharmacological inhibition of the I\(_f\) current may represent a novel intervention to prevent endothelial dysfunction that should be tested in a prospective clinical investigation.

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**Disclosures**
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**References**


CLINICAL PERSPECTIVE

Epidemiological studies have shown that elevated heart rate is a risk factor for cardiovascular morbidity. Experimental data suggest that sustained elevations of heart rate may play a role in the pathogenesis of coronary atherosclerosis. Ivabradine, a selective inhibitor of the I(f) channel, reduces resting and exercise heart rates without an effect on cardiac contractility. In the present study of apolipoprotein E–deficient mice fed a high-cholesterol diet, ivabradine reduced heart rate without affecting blood pressure or lipid levels. Endothelium-dependent relaxation of aortic rings was significantly improved in ivabradine-fed animals, and ivabradine decreased the development of atherosclerotic lesions. The main underlying molecular observation was a potent antioxidative effect of ivabradine that was mediated by the reduction of heart rate. There was no evidence for a heart rate–independent action of the drug. In summary, long-term heart rate reduction by ivabradine leads to inhibition of vascular oxidative stress, endothelial dysfunction, and atherosclerotic lesion formation in apolipoprotein E–deficient mice, regardless of blood pressure and plasma cholesterol levels. The experiments support the potential of heart rate reduction as an intervention to improve endothelial function and to attenuate progression of atherosclerosis in vascular prevention in addition to the symptomatic treatment of angina pectoris. Pharmacological inhibition of the I(f) current may represent a novel intervention to prevent endothelial dysfunction that should be tested in a prospective clinical investigation.
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