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Heart-rate reduction by I_f-channel inhibition with ivabradine restores collateral artery growth in hypercholesterolemic atherosclerosis

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Aims Collateral arteries protect tissue from ischaemia. Heart rate correlates with vascular events in patients with arterial obstructive disease. Here, we tested the effect of heart-rate reduction (HRR) on collateral artery growth.

Methods and results The I_f-channel inhibitor ivabradine reduced heart rate by 11% in wild-type and 15% in apolipoprotein E (ApoE)$^{-/-}$ mice and restored endothelium-dependent relaxation in aortic rings of ApoE$^{-/-}$ mice. Microsphere perfusion and angiographies demonstrated that ivabradine did not change hindlimb perfusion in wild-type mice but improved perfusion in ApoE$^{-/-}$ mice from 40.5 $\pm$ 15.8–60.2 $\pm$ 18.5% ligated/unligated hindlimb. Heart rate reduction (13%) with metoprolol failed to improve endothelial function and perfusion. Protein expression of endothelial nitric oxide synthase (eNOS), phosphorylated eNOS, and eNOS activity were increased in collateral tissue following ivabradine treatment of ApoE$^{-/-}$ mice. Co-treatment with nitric oxide-inhibitor N (G)-nitro-L- arginine methyl ester abolished the effects of ivabradine on arteriogenesis. Following ivabradine, classical inflammatory cytokine expression was lowered in ApoE$^{-/-}$ circulating mononuclear cells and in plasma, but unaltered in collateral-containing hindlimb tissue, where numbers of perivascular macrophages also remained unchanged. However, ivabradine reduced expression of anti-arteriogenic cytokines CXCL10and CXCL11 and of smooth muscle cell markers smoothelin and desmin in ApoE$^{-/-}$ hindlimb tissue. Endothelial nitric oxide synthase and inflammatory cytokine expression were unchanged in wild-type mice. Ivabradine did not affect cytokine production in HUVECs and THP1 mononuclear cells and had no effect on the membrane potential of HUVECs in patch-clamp experiments.

Conclusion Ivabradine-induced HRR stimulates adaptive collateral artery growth. Important contributing mechanisms include improved endothelial function, eNOS activity, and modulation of inflammatory cytokine gene expression.

Keywords Heart rate • Arteriogenesis • Inflammation • Endothelium • Peripheral artery disease • Ivabradine

Introduction

Collateral artery growth is a natural defence mechanism protecting tissue from ischaemia after stenosis or occlusion of a major artery.[1] Stimulating collateral artery growth (arteriogenesis) constitutes a promising therapeutic option for patients with peripheral arterial disease (PAD). Local inflammatory signalling involving mononuclear cell accumulation and cytokine secretion is a hallmark of collateral artery growth.[2] Endothelial function, the expression of endothelial nitric oxide (NO), and optimal concentrations of reactive oxygen species are other prerequisites for arteriogenesis.[1]

High heart rate is associated with arterial rigidity and endothelial dysfunction.[3] Arterial stiffness, in turn, has recently been associated with cardiovascular events.[4] Elevated heart rate identifies patients at

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increased risk of cardiovascular complications.[5] Therefore, pharmacological reduction of heart rate by I_f-channel inhibition has been proposed as a therapeutic approach in cardiovascular medicine.

Heart rate can be lowered with beta-blockers, however, the effects of beta-blockade on tissue perfusion are discussed controversially: while it is reported to improve myocardial perfusion through its effect on heart rate,[6] collateral blood flow was shown to be impaired by beta-blockade in clinical studies,[7,8] which is particularly problematic in patients with PAD. The I_f-channel inhibitor ivabradine lowers heart rate by inhibiting sinus node activity, leaving myocardial contractility unaffected. Heart-rate reduction (HRR) by I_f-channel inhibition has recently been shown to restore endothelial function and reduce oxidative stress and atherosclerosis in mouse models of hypercholesterolaemia and endothelial dysfunction.[9] The present study therefore tested the hypothesis whether HRR by ivabradine improves vascular function and stimulates collateral artery growth in a model of hindlimb ischaemia.

Methods

Animal experiments

Animal experiments were approved by the local authorities. Animals were treated with ivabradine, metoprolol-succinate, or N (G)-nitro-L- arginine methyl ester (L-NAME) with or without ivabradine. Six weeks after treatment initiation, all animals underwent unilateral double femoral artery ligation. Heart rate and blood pressure were measured by a computerized tail-cuff system. Tension recording of aortic rings was performed as described.[9]

Assessment of collateralization

Mice underwent collateral-dependent perfusion measurements of the lower extremities 7 days after femoral artery ligation using fluorescent microspheres as described.[10] Post-mortem angiograms of mouse hindlimbs were produced using Microfil®.

Gene and protein expression analysis

mRNA expression of mm18S rRNA, mmCCL2, mmCCR2, mmIL6, and mmTNFalpha was assessed from mononuclear cells. Similarly, RT-PCR of mm18SrRNA, mmCCL2, mmCCR2, mmTNFalpha, mmIL6, mmDesmin, mmSmoothelin, mmABRA, mmKLF2, mmiNOS, mmnNOS, mmAngiopoietin1, mmVEGF, mmTGFbeta, mmCXCL10, and mmCXCL11 was performed of hindlimb tissue. Immunoblotting for eNOS and phospho-eNOS was performed. Plasma inflammatory cytokines were measured by flow cytometry-based cytokine bead array. Histological sections were prepared from hindlimb tissue. Capillary counts, perivascular macrophages, eNOS, and desmin were stained immunohistochemically.

Measurements of nitric oxide synthase expression and activity

NO was measured in adductor tissue and systemically in whole blood using electron paramagnetic resonance (EPR) in a Bruker spectrometer. In hindlimb tissue, eNOS activity was measured using a radioactive [^{3}H]-arginine-citrulline assay.

Assessment of oxidative stress

To assess collateral superoxide production *in situ*, hindlimb sections from apolipoprotein E (ApoE)$^{-/-}$ mice were stained with dihydroethidium (DHE) and 8-dihydroxy-guanosine (8-dOHG).

In vitro effects of ivabradine

Patch-clamp experiments assessing membrane potential of human umbilical vein endothelial cells (HUVECs) after pre-incubation and stimulation with 20 μM ivabradine, respectively, were performed as previously described.[11]

Direct effects of ivabradine *in vitro*

Protein expression of eNOS and phospho-eNOS was measured in HUVECs after stimulation with increasing concentrations of ivabradine for 24 h. mRNA expression of IL6, TNFalpha and CCL2 was assessed in THP1 mononuclear cells after direct stimulation for 4 h or after 20 h pre-incubation with ivabradine and 4 h stimulation with lipopolysaccharide (LPS) *in vitro*.

Statistical analysis

Data are presented as mean ± standard deviation. Intergroup comparisons between three or more groups were performed using one-way analysis of variance (ANOVA) and Bonferroni *post hoc*. For intra-group comparisons, paired *t*-tests were performed. A *P*-value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS Version 18.0.0.

See online Supplementary material for a detailed description of the methods.

Results

Ivabradine selectively reduces heart rate

After 6 weeks of treatment, ivabradine reduced resting heart rate by 80 ± 19 beats per min (b.p.m.) (−11%, $P < 0.001$) in wild-type mice ($n = 20$ in both groups). Similarly, heart rate was reduced by 86 ± 21 b.p.m. (−15%, $P < 0.001$) in ApoE$^{-/-}$ mice ($n = 30$ in both groups). Addition of L-NAME did not influence HRR by ivabradine [$n = 10$, reduction by 87 ± 23 b.p.m. (−16%, $P < 0.001$)]. Metoprolol ($n = 8$) reduced heart rate by 67 b.p.m. (−13%, $P < 0.001$) in ApoE$^{-/-}$ mice (*Figure 1A*). There was no difference between heart rates of wild-type and ApoE$^{-/-}$ mice at baseline or after treatment. Systolic and diastolic blood pressures remained unchanged by treatment with ivabradine or metoprolol, resulting in an unchanged pulse pressure (*Figure 1B–C*). L-NAME treatment alone or in addition to ivabradine increased systolic and diastolic blood pressure (*Figure 1D*) without an effect on heart rate.

HRR by I_f-channel inhibition restores endothelial function in ApoE$^{-/-}$ mice

Hypercholesterolemic mice have impaired endothelial function.[9] Organ bath experiments of aortic rings ($n = 12–16$ per group) showed attenuated endothelial-dependent relaxation upon increasing concentrations of carbachol in ApoE$^{-/-}$ mice when compared with the wild-type mice ($P < 0.05$). Heart rate reduction using ivabradine had no effect in wild-type mice, but restored endothelial function in ApoE$^{-/-}$ mice. Beta-blockade did not change carbachol-induced relaxation significantly. Endothelium-independent vasodilation induced by glycerolnitrate was unchanged in ApoE$^{-/-}$ when compared with the wild-type mice and not affected by ivabradine or metoprolol (Supplementary material online, *Figure S1*).

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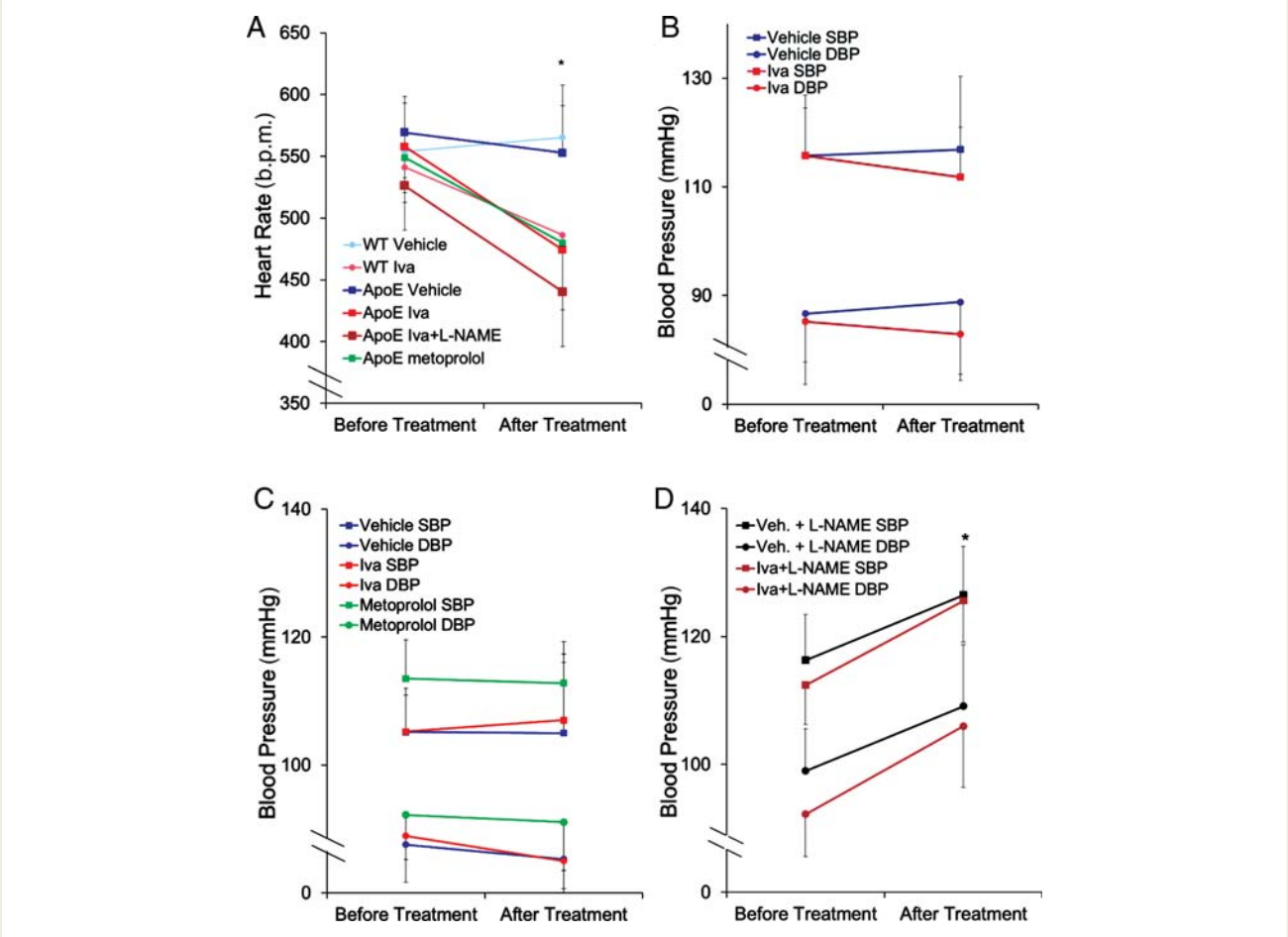

Figure 1 Ivabradine (Iva) reduces heart rate but not blood pressure. Treatment with the I_f-channel inhibitor Iva reduces heart rate by 11% in wild-type mice and by 15% in apolipoprotein E (ApoE)$^{-/-}$ mice, respectively. Metoprolol reduced heart rate by 13% in ApoE$^{-/-}$ mice (*A*). Blood pressure was unaffected in the wild-type (*B*) and apolipoprotein E$^{-/-}$ mice (*C*) following treatment with Iva or metoprolol. L-NAME co-treatment with Iva increased systolic and diastolic blood pressure (*D*).

Heart rate reduction stimulates arteriogenesis

One week after femoral artery ligation, there was a non-significant reduction in perfusion restoration after treatment with ivabradine [62.1 ± 25.5 (vehicle, $n = 10$ in all groups) vs. 55.5 ± 17.3% (ivabradine), $P = 0.329$ in wild-type mice]. Untreated ApoE$^{-/-}$ mice displayed reduced perfusion restoration after femoral artery ligation [40.5 ± 15.8 (ApoE$^{-/-}$, $n = 10$ in all groups) vs. 62.1 ± 25.5% (wild-type), $P = 0.040$]. In ApoE$^{-/-}$ mice, HRR with ivabradine restored collateral artery growth (60.2 ± 18.5 vs. 40.5 ± 15.8% perfusion restoration, $P = 0.041$). In contrast, HRR using metoprolol did not change perfusion restoration in ApoE$^{-/-}$ mice (40.4 ± 12.7%, $P = 1.0$ compared with vehicle) (*Figure 2A*).

In ApoE$^{-/-}$ mice, non-quantitative Microfil® angiographies depicted increased hindlimb collateralization upon treatment with ivabradine 3 and 7 days after femoral artery ligation. Typical corkscrew appearance of pre-existent collateral anastomoses across the adductor muscle was observed more strongly in ligated hindlimbs after treatment with ivabradine than after vehicle treatment (*Figure 2B*).

No change in capillary density after HRR

Capillary density in the upper or lower limb was not affected by ivabradine. Capillary/fibre ratios in the right peroneus muscle were not increased by treatment with ivabradine in wild-type mice (Supplementary material online, *Figure S2*). Similarly, in the adductor muscle, capillary/fibre ratio did not differ between vehicle and ivabradine-treated ApoE$^{-/-}$ or wild-type mice. Apolipoprotein E$^{-/-}$ mice demonstrated moderately lower capillary/fibre ratios than wild-type mice independent of treatment (Supplementary material online, *Figure S2*). Growth factor analysis showed unchanged levels of VEGF, TGFbeta, and angiopoietin mRNA, supporting the finding of unchanged capillarization.

Inflammatory cytokines are modulated by ivabradine

Inflammatory cytokine gene expression in isolated mononuclear cells tended to be higher in ApoE$^{-/-}$ than in wild-type mice and were reduced by ivabradine-induced HRR in ApoE$^{-/-}$ mice. In detail, ivabradine-treatment decreased gene expression of MCP1 (gene expression relative to 18S) (vehicle: 6.62 ± 3.78;

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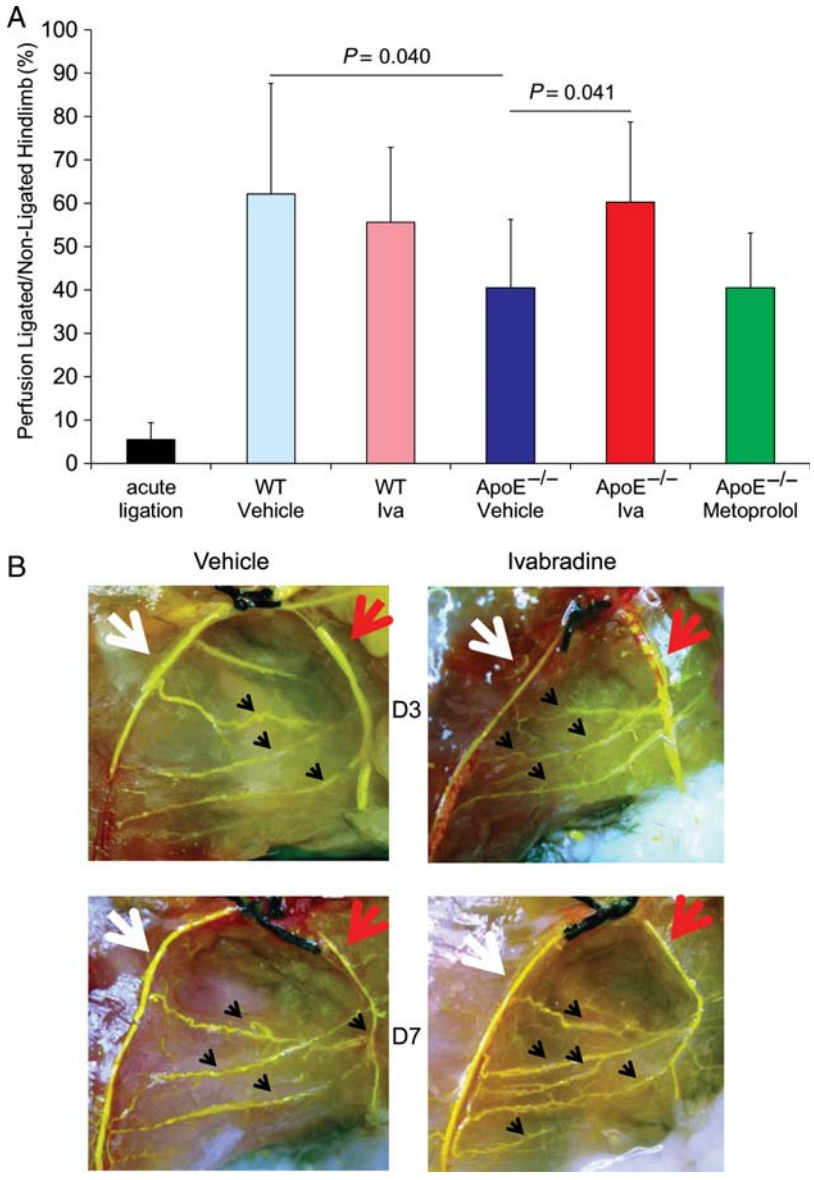

Figure 2 Heart-rate reduction improves perfusion restoration. Microsphere perfusion under conditions of maximal vasodilation (*A*) showed that adaptive collateral artery growth in the wild-type mice restored perfusion to 62.0 ± 25.5%. Natural perfusion restoration was hampered in apolipoprotein E (ApoE)$^{-/-}$ mice (40.5 ± 15.8%). Six week treatment with ivabradine (Iva) remained without effect in the wild-type mice, but restored perfusion to the level of wild-type mice in ApoE$^{-/-}$ mice (60.2 ± 18.5%). Heart rate reduction using metoprolol remained without an effect on perfusion in ApoE$^{-/-}$ mice (40.4 ± 12.7%). In representative Microfil angiographies, an increase in number and size and more corkscrew-like appearance of collateral arteries (black arrows) can be appreciated after treatment with Iva (*B*).

ivabradine: 0.44 ± 0.44; $P = 0.006$), CCR2 (the MCP1 receptor) (vehicle: 6.00 ± 3.27; ivabradine: 0.72 ± 0.45; $P = 0.004$), IL6 (vehicle: 8.55 ± 3.96; ivabradine: 0.72 ± 0.73; $P = 0.045$), and TNFalpha (vehicle: 5.07 ± 1.12; ivabradine: 0.31 ± 0.22; $P = 0.034$) ($n = 10$ per group; *Figure 3A–D*). Similarly, plasma levels of inflammatory cytokines MCP1, IL6, and TNFalpha were higher in ApoE$^{-/-}$ than in wild-type mice and reduced in the former only ($n = 10$ per groups for both wild-type and ApoE$^{-/-}$ mice, *Figure 3E–G*). mRNA expression of the same inflammatory markers were found unaltered by ivabradine in hindlimb tissue after femoral artery ligation or sham-operation ($n = 10$ per group (Supplementary material online, *Figure S3*). Numbers of peri-collateral macrophages were not different between vehicle and ivabradine-treated ApoE$^{-/-}$ and wild-type mice (*Figure 4A–C*).

In contrast to the above-mentioned 'classical' inflammatory markers, gene expression of anti-arteriogenic, interferon-beta-induced cytokines CXCL10 and CXCL11 was downregulated following HRR by ivabradine in ApoE$^{-/-}$ mice but remained unaffected in C57Bl/6 mice [$n = 10$ for all groups, CXCL10 5.05 ± 4.99 (vehicle) vs. 0.57 ± 0.32 (ivabradine) gene expression

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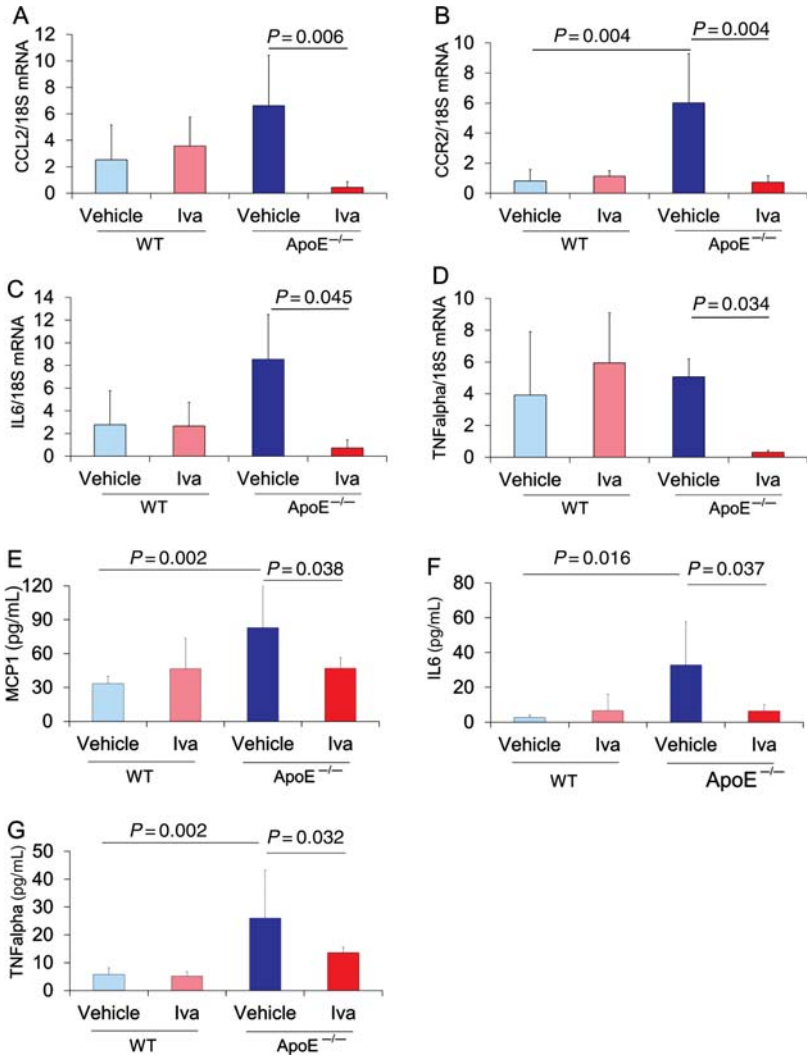

Figure 3 Downregulation of inflammatory cytokines in apolipoprotein E (ApoE)$^{-/-}$ mice. In isolated peripheral blood mononuclear cells of ApoE$^{-/-}$ mice, mRNA expression of the inflammatory cytokines CCL2 (MCP1) (*A*), CCR2 (*B*), IL6 (*C*) and TNFalpha (*D*) tended to be higher compared with wild-type mice and was markedly reduced after heart-rate reduction with ivabradine (Iva). Similarly, inflammatory cytokine protein levels in plasma were higher in ApoE$^{-/-}$ than in C57Bl/6 mice and were reduced by Iva (*E*–*G*).

ligated/unligated hindlimb, $P = 0.047$ and CXCL11 3.85 ± 1.80 (vehicle) vs. 1.69 ± 1.70 (ivabradine) gene expression ligated/unligated hindlimb, $P = 0.049$] (*Figure 4D–E*).

Optimal concentrations of reactive oxygen species are necessary for collateral artery growth. *In situ* detection of superoxide production was performed by DHE staining ($n = 7$ per group) in ApoE$^{-/-}$ mice. Fluorescence units selectively assessed in the collateral vessels were unchanged between vehicle group and after ivabradine treatment. Immunohistochemical staining of 8-dihydroxy guanosine (8-dOHG) within collateral arteries showed no difference between vehicle and ivabradine-treated animals (data not shown).

Markers of shear stress and cyclic strain

Marker genes for laminar shear stress (iNOS, KLF2, ABRA) were not increased by treatment with ivabradine. In contrast, expression of genes involved in vascular smooth muscle cell (VSMC) phenotype regulation was strongly affected by HRR. Cytoskeletal desmin was decreased in ApoE$^{-/-}$ mice following treatment with ivabradine ($64 \pm 20\%$ of vehicle, $P = 0.041$, $n = 10$ per group). Using immunohistochemistry ($n = 7$ per group), desmin expression was found particularly in VSMC. Quantitatively, HRR reduced desmin in VSMC to $70 \pm 17\%$ of vehicle [18.3 ± 3.6 (vehicle) vs. 12.8 ± 3.0 (ivabradine) fluorescence units, $P = 0.032$]. mRNA expression of smoothelin, another cytoskeletal protein specific for a contractile VSMC phenotype, was reduced to $46 \pm 27\%$ of vehicle, $P = 0.046$. In C57Bl/6 mice, HRR using ivabradine did not affect smooth muscle cell marker expression (*Figure 5*).

Ivabradine-induced heart-rate reduction stimulates endothelial nitric oxide synthase

In ApoE$^{-/-}$ mice, western blot analysis of $n = 9$ animals per group showed an increase in expression of endothelial nitric oxide

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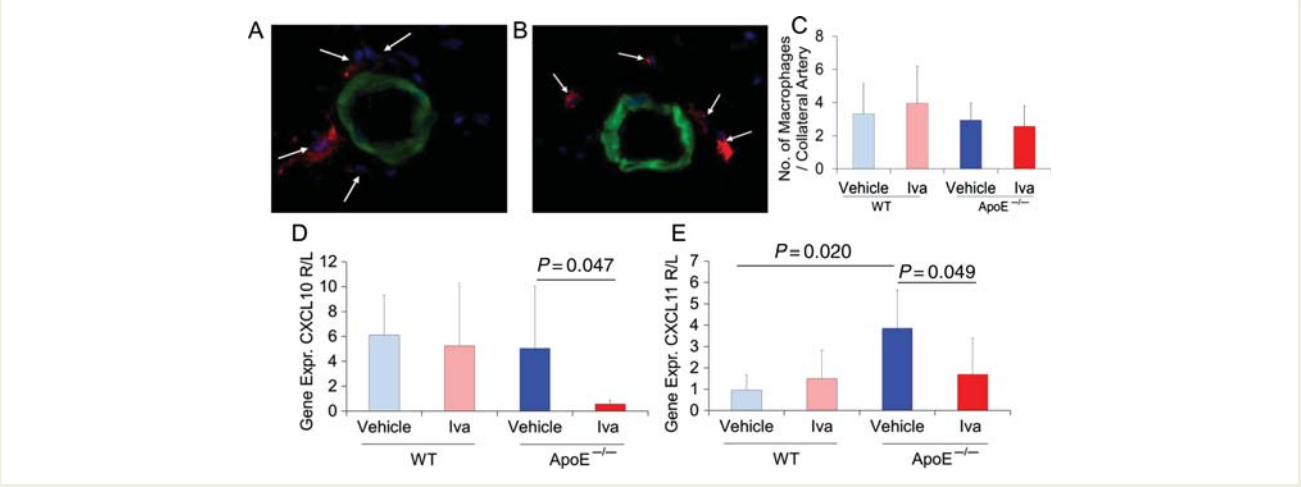

Figure 4 Anti-arteriogenic cytokines are reduced in hindlimb tissue, while peri-collateral macrophage number is unchanged. Representative immunohistochemical sections of vehicle (*A*) and ivabradine (*B*) treated apolipoprotein E (ApoE)$^{-/-}$ mice demonstrate pericollateral macrophages [anti-mouse macrophage antibody (red)]. Quantification of histological sections showed unchanged numbers of perivascular macrophages in both wild-type and ApoE$^{-/-}$ mice (*C*). Anti-arteriogenic cytokines CXCL10 (*D*) and CXCL11 (*E*), shown as ratios ligated (right) vs. unligated (left) hindlimb, were reduced locally in collateral-containing hindlimb tissue of ApoE$^{-/-}$ mice, but not in wild-type animals.

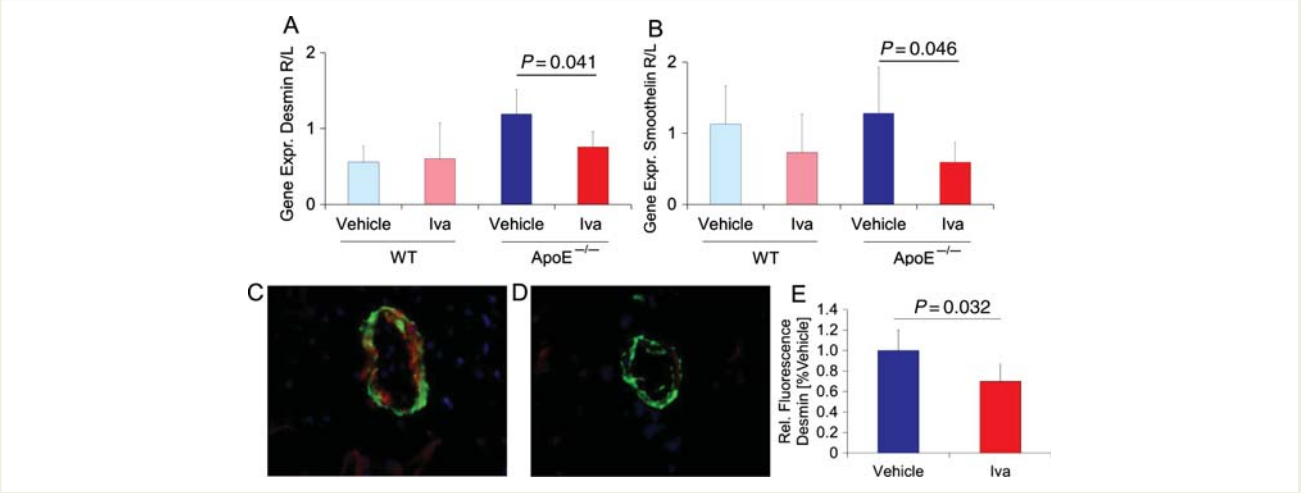

Figure 5 Desmin and smoothelin are reduced in collateral hindlimb tissue. Real-time RT-PCR of collateral-containing hindlimb tissue reveals decreased relative gene expression of desmin after femoral artery ligation in apolipoprotein E (ApoE)$^{-/-}$ mice (*A*), indicating a more proliferative smooth muscle cell phenotype following treatment with ivabradine (Iva). Smoothelin, another marker of a contractile VSMC phenotype, was also found less strongly expressed in ApoE$^{-/-}$ mice upon heart-rate reduction by ivabradine (*B*). In C57Bl/6 mice, markers of VSMC contractile phenotype were unaffected. Immunohistochemically, desmin protein is found predominantly in the vascular smooth media (red) [representative sections (*C*) (vehicle) and (*D*) (ivabradine)]. Desmin protein in collateral VSMC is found reduced after treatment with Iva (*E*).

synthase (eNOS) to 177 ± 73% in the ivabradine-treated group ($P = 0.048$, $n = 9$). Phosphorylated (Ser1177) eNOS increased to 186 ± 127%, $P = 0.018$, $n = 17$) (*Figure 6A–C*). In hindlimbs of C57Bl6 mice, eNOS and phospho-eNOS protein expressions were not changed by HRR. Direct measurements of NO availability using EPR demonstrated a non-significant increase of systemic (whole blood) NO levels (119 ± 32% of vehicle, $P = 0.22$, $n = 7$ per group). [^{3}H] citrulline arginine converting assays showed enhanced eNOS activity in collateral-containing adductor muscle following ivabradine (*Figure 6D*). Other NO synthases (nNOS, iNOS) were not expressed differently (data not shown). Immunohistochemistry demonstrated increased eNOS protein in collateral endothelium (*Figure 6E–F*). Treating ApoE$^{-/-}$ mice with the NO-inhibitor L-NAME reduced perfusion restoration after femoral artery ligation. In L-NAME-treated animals, ivabradine failed to restore perfusion restoration ($n = 10$, *Figure 6G*).

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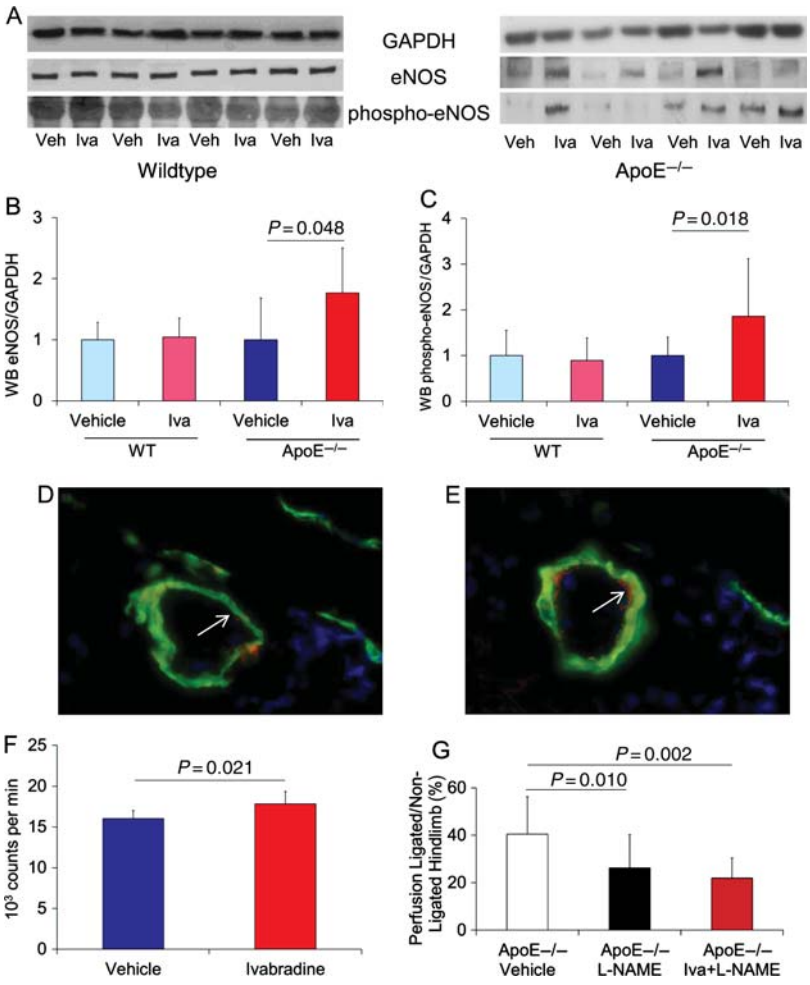

Figure 6 Endothelial nitric oxide synthase (eNOS) expression and function. Ivabradine upregulated expression of eNOS and phosphorylated eNOS (*A*). Quantification shows no change of eNOS protein in the wild-type mice following ivabradine (Iva), but in apolipoprotein E (ApoE)$^{-/-}$ mice (*B*). Phosphorylated eNOS also remained unchanged in the wild-type mice but increased in ApoE$^{-/-}$ mice (*C*). Immunohistochemistry showed eNOS to be found predominantly in the collateral artery endothelium. Representative images confirm stronger expression of eNOS protein in ivabradine-treated (*E*) than in vehicle-treated animals (*D*). [^{3}H] arginine-citrulline assays showed increased eNOS activity in hindlimbs of ivabradine-treated ApoE$^{-/-}$ mice (*F*). Treating ApoE$^{-/-}$ mice with L-NAME abrogated the positive effects of Iva on perfusion restoration as shown by microsphere perfusion measurements after femoral artery ligation (*G*).

Assessment of direct effects of ivabradine *in vitro*

Neither direct application of ivabradine on current-clamped HUVECs nor pre-incubation with ivabradine for 24 h had an effect on the membrane potential of these cells (Supplementary material online, *Figure S4*).

Stimulation of THP1 mononuclear cells with ivabradine *in vitro* did not change mRNA expression profile of the inflammatory cytokines that were found downregulated in mononuclear cells *in vivo*. Similarly, pre-incubation of THP1 cells with ivabradine did not affect the effect of stimulation with LPS. Treatment of endothelial cells (HUVECs) with ivabradine *in vitro* had no effect on the protein expression or activation of eNOS (Supplementary material online, *Figure S5*).

Discussion

In this study, HRR using the I_f-channel inhibitor ivabradine enhanced hindlimb arteriogenesis and increased eNOS expression, NO availability, and endothelium-dependent relaxation in a murine hindlimb model of endothelial dysfunction. HRR by beta-blockade did not exert positive effects on endothelial function and collateralization. In ApoE$^{-/-}$ mice, classical inflammatory cytokine expression was reduced systemically by ivabradine but remained unchanged locally in collateral tissue, where anti-arteriogenic cytokines CXL10 and CXCL11 were down-regulated. Ivabradine induced a more proliferative VSMC phenotype in collateral arteries of ApoE$^{-/-}$ mice. *In vitro*, the I_f-channel inhibitor did not affect eNOS or inflammatory cytokine expression.

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I_f-channel inhibition by ivabradine restored aortic endothelial function in hyperlipidemic mice to the level of healthy wild-type mice. These findings are in agreement with previous observations that have identified anti-oxidative and anti-inflammatory effects as the underlying mechanisms of the beneficial effect of ivabradine on vascular pathophysiology.[9] Arteriogenesis is associated with both similar and distinct features compared with endothelial function. Nitric oxide production is central to both processes.[12] The relevance of NO for collateral artery growth has been elucidated in recent studies.[13,14] In this study, endothelium-dependent vasorelaxation was improved by HRR. eNOS and phospho-eNOS protein expression in collateral tissue increased upon ivabradine treatment. Direct measurements of NO showed an increase in availability after HRR. ROS production as measured by DHE bromide and 8-dihydroxy-guanosine stainings *in situ* selectively within collateral arteries remained unchanged. We speculate that the small absolute difference in NO activity translates into a functional meaningful difference because the collateral endothelium makes up only a small percentage of cells of the musculature. Qualitative immunohistochemistry showed that eNOS protein is present in the collateral artery endothelium and that the endothelial expression is increased in the animals with slower heart rate. Importantly, co-treatment with the NO inhibitor L-NAME abrogated the positive effect of ivabradine on collateral artery growth. In summary, our data support that eNOS is an important contributing mediator in the ivabradine-induced increase of collateralization. However, we cannot exclude the relevance of heart-rate dependent regulation of signalling independent of eNOS.

Pleiotropic effects of ivabradine on cardiomyocytes have been reported, their role in vascular tissue is still under debate.[15] The lack of an effect of HRR by beta-blockade on vascular growth in our study could suggest a specific effect of the I_f-channel inhibitor. However, in contrast to I_f-channel inhibition, beta-blockade is known to exert vasoconstrictor effects on the peripheral circulation,[16] and we did not observe a positive effect of HRR by metoprolol on endothelium-dependent vasorelaxation. These data are in agreement with a recent study demonstrating that metoprolol succinate, in contrast to nebivolol, did not improve endothelium-dependent vasorelaxation in spite of a similar decrease in heart rate in a mouse model of myocardial infarction.[17] Similarly, improvement of endothelial function in aortae and corpora cavernosa of $ApoE^{-/-}$ mice was observed following treatment with nebivolol but not metoprolol.[18] In contrast to nebivolol, metoprolol was not found to release NO which is important for the improvement of endothelial function. Potentially, differences in endothelium-dependent vasorelaxation could have contributed to distinctive effects of ivabradine or beta-blockade not only at the level of the collateral arteries, but also at the site of the collateral-supplying arteries (external iliac artery). Resulting differences in perfusion pressure might have contributed to an ivabradine but not metoprolol-induced increase in vascular growth. To assess potential direct, heart-rate independent effects of ivabradine, *in vitro* experiments were performed on endothelial and mononuclear cells. Ivabradine did not affect eNOS protein expression or activation, did not influence inflammatory cytokine production, and did not prevent the pro-inflammatory effect of LPS stimulation. A relevant effect on endothelial ion currents was excluded by patch-clamp experiments. Differential effects of I_f-channel inhibition and beta-blocker cannot exclude heart rate independent effects of ivabradine. Yet, decreasing heart rate without a negative effect on endothelial function is an important mechanism of the beneficial effects of ivabradine on the peripheral circulation.

Earlier studies have shown a positive effect of HRR on coronary flow.[19] Here, angiogenesis (capillary sprouting) has to be distinguished from arteriogenesis (growth of pre-existent collateral arteries). Increased coronary angiogenesis depends on enhanced myocardial stretch due to increased diastolic filling and subsequent secretion of angiogenic growth factors VEGF and TGFbeta,[20] and stimulation of coronary collateral artery growth by HRR is driven by an increase of laminar shear stress in a prolonged diastole.[19] In contrast to these observations in the coronary circulation, the influence of heart rate on vascular pathophysiology in the peripheral circulation has not been characterized in detail. Peripheral collateral artery growth is of particular relevance for patients with PAD. In contrast to the coronary circulation, flow and pressure waves in the peripheral circulation are less affected by myocardial contraction and duration of diastole. In the physiological range, stroke volume will be elevated when heart rate is reduced to keep cardiac output constant (cardiac output = stroke volume $\times$ heart rate).[21] Enhanced stroke volume increases pulse pressure wave in the peripheral circulation, resulting in enhanced cyclic strain.[22] Cellular strain is known to stimulate VSMC proliferation, which is essential for collateral artery growth. Ivabradine downregulates desmin, a cytoskeletal proteins associated with a stable, contractile phenotype of VSMC[23] and smoothelin, a marker of a contractile phenotype of VSMC.[24]

An interesting finding of the study was the reduction of systemic inflammatory cytokines in $ApoE^{-/-}$ mice. To our knowledge, this is the first report on increased vascular growth during systemic reduction of inflammation. Anti-inflammatory effects may also have been mediated be increased NO bioavailability, as suggested by a recent study of angiogenesis.[25] Our data support earlier findings of an anti-atherosclerotic effect of ivabradine.[9] Interestingly, recent evidence points toward an anti-arteriogenic effect of certain inflammatory mediators.[10,26] Particularly patients with PAD could benefit from an anti-inflammatory, anti-atherosclerotic, pro-arteriogenic therapy. Translation of therapeutic approaches to stimulate vascular growth to clinical practice has hitherto been difficult because of their proinflammatory effects and the concomitant negative effects on atherosclerosis.[27] Increased heart rate is known to be associated with systemic inflammation.[28] One potential explanation is the hypothesis that ivabradine-induced HRR may prevent cholesterol-induced arterial stiffness, thereby restoring local hemodynamic forces essential for vascular growth.

The effect of eNOS on arteriogenesis has been discussed controversially. While some authors show eNOS to be important for flow rather than active growth of collateral arteries,[14] others demonstrate its role in collateral artery proliferation.[13] Our microsphere perfusion measurements under conditions of maximal vasodilation exclude confounding vasodilatory processes during the assessment of collateral artery growth. As a limitation we cannot exclude, however, that improved collateral function (due

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to enhanced eNOS activity) in the first hours and days after femoral artery ligation has eventually led to increased arterial structural growth. In the coronary circulation, one would expect bradycardia to stimulate laminar shear stress via prolongation of diastole. Our study investigated the effects of HRR in the peripheral circulation, where markers of laminar shear stress could not be found. However, decreased heart rate increases arterial distensibility by decreasing stiffness,[3,29] thus enhancing cyclic stretch as a pro-arteriogenic mechanism.

In conclusion, HRR using ivabradine restores peripheral arteriogenesis in a murine hindlimb model, improving NO availability and endothelial function, modulating systemic and local inflammatory cytokine expression, and modifying collateral VSMC phenotype. These data identify a role of heart rate in the regulation of vessel growth in the peripheral vasculature.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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