Donor Simvastatin Treatment Abolishes Rat Cardiac Allograft Ischemia/Reperfusion Injury and Chronic Rejection Through Microvascular Protection

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Background—Ischemia/reperfusion injury may have deleterious short- and long-term consequences for cardiac allografts. The underlying mechanisms involve microvascular dysfunction that may culminate in primary graft failure or untreatable chronic rejection.

Methods and Results—Here, we report that rat cardiac allograft ischemia/reperfusion injury resulted in profound microvascular dysfunction that was prevented by donor treatment with peroral single-dose simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase and Rho GTPase inhibitor, 2 hours before graft procurement. During allograft preservation, donor simvastatin treatment inhibited microvascular endothelial cell and pericyte RhoA/Rho-associated protein kinase activation and endothelial cell–endothelial cell gap formation; decreased intragraft mRNA levels of hypoxia-inducible factor-1α, inducible nitric oxide synthase, and endothelin-1; and increased heme oxygenase-1. Donor, but not recipient, simvastatin treatment prevented ischemia/reperfusion injury–induced vascular leakage, leukocyte infiltration, the no-reflow phenomenon, and myocardial injury. The beneficial effects of simvastatin on vascular stability and the no-reflow phenomenon were abolished by concomitant nitric oxide synthase inhibition with N-nitro-L-arginine methyl ester and RhoA activation by geranylergyl-1, pyrophosphate supplementation, respectively. In the chronic rejection model, donor simvastatin treatment inhibited cardiac allograft inflammation, transforming growth factor-β1 signaling, and myocardial fibrosis. In vitro, simvastatin inhibited transforming growth factor-β1–induced microvascular endothelial-to-mesenchymal transition.

Conclusions—Our results demonstrate that donor simvastatin treatment prevents microvascular endothelial cell and pericyte dysfunction, ischemia/reperfusion injury, and chronic rejection and suggest a novel, clinically feasible strategy to protect cardiac allografts. (Circulation. 2011;124:1138-1150.)

Key Words: endothelium ▪ inflammation ▪ ischemia ▪ microcirculation ▪ transplantation

Restoration of compromised blood flow is vital in vascular occlusion, but paradoxically, tissue reperfusion is often accompanied by significant morbidity and mortality. This is especially evident in heart transplantation in which ischemia/reperfusion injury (IRI) may result in primary graft failure and the development of untreatable chronic rejection.1,2–3 Clinically feasible organ preservation strategies are needed to limit early IRI, to blunt subsequent pathological immunological and tissue remodeling responses, and to prolong cardiac allograft recipient survival.

Clinical Perspective on p 1150

Endothelial cell (EC) dysfunction is a hallmark of IRI4–6 and the development of cardiac allograft fibrosis and arteriosclerosis, manifestations of chronic rejection.2,3,7 IRI5 and several IRI-related factors such as hypoxia, thrombin, vascular endothelial growth factor, and RhoA GTPase activation are involved in EC-EC gap formation and compromised endothelial stability.6,8–10 Dysfunctional ECs also predispose the patients to thrombosis, leukocyte adhesion, and vasoconstriction and are involved in pathological fibroproliferation.3,7,11 They also directly participate in myocardial fibrosis through endothelial-to-mesenchymal transition (EndMT) in which transforming growth factor (TGF)-β1 induces EC transdifferentiation to fibroblasts.11 Pericytes are perivascular cells that cover endothelial tubes in small arterioles, capillaries, and postcapillary venules.
Percytes communicate with the underlying ECs, share the same basement membrane, and regulate the microvascular tone\textsuperscript{12} possibly mediated by RhoA GTPase activation.\textsuperscript{13,14} Percyte dysfunction may result in microvascular hypertension, pathological angiogenesis, increased capillary permeability,\textsuperscript{12} and the no-reflow phenomenon after IRI.\textsuperscript{15}

Statins are cholesterol-lowering drugs that are widely used in preventing cardiovascular diseases.\textsuperscript{16} They also have important cholesterol-independent vasculoprotective effects mediated largely through the inhibition of Rho GTPases, important regulators of cell cytoskeleton and intracellular signaling.\textsuperscript{16,17} Simvastatin is commonly begun shortly after transplantation because of its beneficial effects against chronic rejection and on long-term survival.\textsuperscript{18,19} Here, we investigated whether preoral simvastatin treatment of rat cardiac allograft donors 2 hours before allograft procurement has vasculoprotective and immunomodulatory effects on IRI and subsequent pathological tissue remodelling.

Methods

Experimental Design

Cardiac allografts were transplanted from fully major histocompatibility complex-mismatched male Dark Agouti (DA; RT1\textsuperscript{av1}) to male Wistar Furth (RT1\textsuperscript{b}) rats (Scanburg, Stockholm, Sweden). Allografts were left without hypothermic preservation or were preserved in PBS at 4°C for 2 to 4 hours, depending on the study model. Warm ischemia was standardized to 1 hour. Microvascular RhoA activity and intragraft inflammation were analyzed by immunohistochemistry and immunofluorescence; microvascular interendothelial gaps, by biotinylated Ricinus communis lectin or transmission electron microscopy; microvascular leakage, by a modified Miles assay; perfusion, by laser Doppler monitoring and intracoronary FITC-labeled Lycopersicon esculentum (Tomato) lectin; myocardial edema, by magnetic resonance imaging; and myocardial injury, by serum cardiac troponin T. Intragraft mRNA expression was determined by real-time reverse-transcription polymerase chain reaction. In the chronic rejection model, fibrosis was analyzed by Masson trichrome staining, and arteriosclerosis was assessed morphometrically by hematoxylin-eosin and Resorcinfuchsins staining for internal elastic lamina. To prevent severe episodes of acute rejection, allograft recipients were given cyclosporine A (Novartis, Basel, Switzerland) 2.0 mg kg\textsuperscript{-1} d\textsuperscript{-1} SC for the first 7 days and 1.0 mg kg\textsuperscript{-1} d\textsuperscript{-1} thereafter. Sample sizes are given in the figure legends. The Methods section in the online-only Data Supplement gives detailed information.

Drug Administration

Peroral simvastatin (Merck Research Laboratories, Whitehouse Station, NJ) was given to the donor as a single dose (5 mg/kg) 2 hours before graft removal, to the recipient (2 mg/kg) 2 hours before reperfusion and daily thereafter (2 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}), or to both. The controls received polyethylene glycol vehicle orally. To inhibit nitric oxide synthase (NOS) activity, donors received N-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich Co, St. Louis, MO) 50 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} for 4 days before allograft removal. To block heme oxygenase-1 (HO-1) activity, zinc protoporphyrin (Porphyrin Products Inc, Logan, UT) was administered intraperitoneally (20 mg/kg) to donor rats 2 hours before allograft removal. To supplement RhoA GTPase prenylation pathway, geranylgeranylated pyrophosphate (Sigma-Aldrich) at a concentration of =1 mg/ml in methanol was administered intraperitoneally (1 mg/kg) to donor rats 2 hours before allograft removal.

Effects of Simvastatin on Transforming Growth Factor-\(\beta\)1-Induced Endothelial-to-Mesenchymal Transition In Vitro

Transforming growth factor-\(\beta\)1 10 ng/ml was used to induce EndMT in human cardiac microvascular ECs. Simvastatin was dissolved in EtOH and activated by NaOH followed by neutralization, and concentrations of 0.1, 0.5, and 1.0 \(\mu\text{mOL/L}\) were used to inhibit EndMT. Immunohistochemistry identified endothelial CD31 and zonula occludens-1 density. Real-time reverse-transcription polymerase chain reaction analysis, SDS-PAGE, and immunoblotting were performed to measure the mRNAs and protein levels of the mesenchymal markers calponin and \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA).

Statistics

The Mann--Whitney \(U\) test was used for 2-group comparisons, and the Kruskal-Wallis test with the Dunn test was used when multiple groups were compared with control. The Dunn post hoc test was applied only if the Kruskal-Wallis test demonstrated an overall statistically significant difference. For comparison in a longitudinal study, data were analyzed by repeated measures ANOVA. For survival, Kaplan–Meier analysis with log rank (Mantel-Cox) was applied. Linear regression analysis was applied to evaluate the relation of different simvastatin concentrations to the expression of mesenchymal genes in vitro. Data are given as mean±SEM for longitudinal analysis and for discrete variables and by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) for continuous variables (SPSS Statistics 15.0, SPSS Inc, Somers, NY). The outliers are shown as circles outside the box. Values of \(P<0.05\) were regarded as statistically significant.

Results

Microvascular Endothelial Cells and Percytes Express HMG-CoA Reductase in the Rat Heart

To identify the target cells of donor simvastatin treatment, we performed HMG-CoA reductase immunostainings in normal DA rat hearts. HMG-CoA reductase immunoreactivity was found mainly in cardiac vasculature (Figure 1A). Immunofluorescent double stainings showed that HMG-CoA reductase was expressed mostly in microvascular rat endothelial cell antigen-1\textsuperscript{+} (RECA-1\textsuperscript{+}) ECs and in NG2\textsuperscript{+} pericytes but not in \(\alpha\)-SMA\textsuperscript{+} smooth muscle cells or in tropomyosin\textsuperscript{+} cardiomyocytes (Figure 1A).

Cold and Warm Ischemia Induce Microvascular Endothelial Cell and Pericycle RhoA/ROCK Activation and Endothelial Cell–Endothelial Cell Gap Formation

To determine whether cold and warm ischemia lead to RhoA/ROCK activation, we performed immunostainings of adducin phosphorylated at Thr445 (p-adducin)\textsuperscript{20,21} in normal DA hearts and hearts subjected to 4-hour cold or to 4-hour cold and 1-hour warm ischemia. Microvascular p-adducin immunoreactivity was low in normal DA hearts (Figure 1B through D), whereas 4-hour cold and 1-hour warm ischemia resulted in prominent p-adducin immunoreactivity in capillaries (\(P<0.05\); Figure 1B), postcapillary venules, arterioles (Figure 1C), and arteries (\(P<0.05\); Figure 1D). p-Adducin immunoreactivity localized to some RECA-1\textsuperscript{+} capillary ECs but mainly to NG2\textsuperscript{+} pericytes and to \(\alpha\)-SMA\textsuperscript{+} smooth muscle cells in postcapillary venules and arteries (Figure 1B through 1D).

Transmission electron microscope analysis indicated that combined 4-hour cold and 1-hour warm ischemia markedly increased the incidence of microvascular EC-EC gaps (\(P<0.01\); Figure 1E). The presence of microvascular EC-EC gaps during cold and warm ischemia was confirmed by fluorescence microscopy of grafts that received intracoronary
Figure 1. Donor simvastatin treatment prevents microvascular RhoA/ROCK activation and endothelial cell (EC)-EC gap formation in the rat heart subjected to 4-hour cold and 1-hour warm ischemia. A, Dark Agouti rat heart microvascular HMG-CoA reductase (HMG-CoAR) immunoreactivity colocalized with rat endothelial cell antigen-1 \(^*\) (RECA-1 \(^*\)) ECs and NG2 \(^*\) pericytes but not with \(\alpha\)-smooth muscle actin–positive (SMA \(^*\)), smooth muscle cells, or tropomyosin \(^*\) cardiomyocytes. B through D, Immunoreactivity for adducin phosphorylated at Thr445 (p-adducin) as an in situ indicator of RhoA/ROCK activation. E, Microvascular EC-EC gaps (arrows) determined by transmission electron microscopy of left ventricular longitudinally cut microvascular vessels. F, Microvascular EC-EC gaps confirmed by intracoronary perfusion with EC-binding rhodamine-labeled Concavalin A (red) lectin and basement membrane-binding biotinylated Ricinus communis lectin (blue, arrows). Negative controls are shown in the insets (A and D). n=5 to 6 per group. The Kruskal-Wallis test with the Dunn test was used to compare the effect of ischemia and no ischemia (B through E). The Mann-Whitney U test was used to compare donor simvastatin treatment and no treatment with the same ischemia time (B through E). Data are given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers), and the outliers are shown as circles outside the box (B through E). Rbc indicates red blood cell. Arrows indicate EC-EC junctions. Scale bars=50 \(\mu\)m (A through D and F) and 100 nm (E). \(*P<0.05, **P<0.01, ***P<0.001.\)
perfusion of endothelium-binding rhodamine-labeled Concanavalin A lectin and basement membrane-binding biotin-labeled R. communis lectin22 (Figure 1F).

**Donor Simvastatin Treatment Prevents Microvascular RhoA/ROCK Activation and Endothelial Cell–Endothelial Cell Gap Formation During Cold and Warm Ischemia**

Next, we investigated whether peroral donor treatment with simvastatin 5 mg/kg 2 hours before graft procurement inhibits graft RhoA/ROCK activation and EC-EC gap formation. Simvastatin was absorbed and metabolized to its active form in hours in rats (simvastatin 5 mg/kg; Figure 1A in the online-only Data Supplement) and in human organ donors (simvastatin 80 mg; Figure 1B in the online-only Data Supplement). Donor simvastatin treatment decreased p-adducin immunoreactivity in capillaries (P<0.05; Figure 1B), postcapillary venules, and arterioles (P<0.05; Figure 1C), as well as in the media of arteries (P<0.05; Figure 1D), and decreased the formation of EC-EC gaps (P<0.001; Figure 1E and 1F), indicating decreased RhoA/ROCK activation and enhanced endothelial stability.

**Donor Simvastatin Treatment Decreases Hypoxia-Inducible Factor-1α, Inducible Nitric Oxide Synthase, and Endothelin-1 and Increases Heme Oxygenase-1 mRNA During Cold and Warm Ischemia**

Real-time reverse-transcription polymerase chain reaction analysis showed that 4-hour cold and 1-hour warm ischemia significantly decreased inducible NOS (iNOS) (P<0.01; Figure 2A), vascular endothelial growth factor (P<0.001; Figure 2A), and HO-1 (P<0.05; Figure 2B) mRNA levels, whereas it increased heat shock protein 27 mRNA levels (P<0.05; Figure 2B) in the rat heart. Donor simvastatin treatment significantly decreased mRNA levels of hypoxia-inducible factor-1α (HIF-1α; P<0.05; Figure 2A), its downstream iNOS (P<0.05; Figure 2A), and vasoconstrictive endothelin-1 (P<0.05; Figure 2B) and increased the expression of protective HO-1 (P<0.05; Figure 2B). Simvastatin treatment resulted in a 50% reduction in EC HIF-1α and endothelin-1 mRNA levels in vitro (Table I in the online-only Data Supplement).

**Cold and Warm Ischemia Induce Vascular Leakage, the No-Reflow Phenomenon, and Myocardial Edema During Reperfusion**

To determine the effect of allograft reperfusion on microvascular permeability and perfusion, DA hearts with or without 4-hour cold ischemia were transplanted to Wistar Furth recipients using a standardized 1-hour warm ischemia. In a vascular permeability assay, 4-hour cold ischemia significantly increased Evans blue dye extravasation 30 minutes after reperfusion (P<0.001; Figure 3A). This was accompanied by a prominent no-reflow phenomenon with diminished numbers of perfused capillaries determined by intracoronary injection of endothelium-binding FITC-labeled L. esculentum lectin 30 minutes after reperfusion (P<0.01; Figure 3B). The loss of microvascular perfusion was not due to widespread intravascular thrombosis (Figure II in the online-only Data Supplement).

Furthermore, serial laser Doppler measurements from the apex of normal DA hearts and cardiac allografts with 4-hour cold ischemia (Figure 3C) showed a prominently diminished microvascular perfusion early after cardiac allograft reperfusion (15 mL·min⁻¹·100 g⁻¹ tissue 1 to 30 minutes after reperfusion) that gradually increased (30 mL·min⁻¹·100 g⁻¹ tissue 1 hour after reperfusion) toward normal DA heart values (53±0.7 mL·min⁻¹·100 g⁻¹ tissue), indicating that IRI results in impaired microvascular perfusion early after reperfusion that gradually resolves over time.

To assess myocardial edema, we used cardiac allograft magnetic resonance imaging to analyze myocardial transverse relaxation time (T2) value 4 hours after reperfusion. T2 >40 milliseconds is considered an indicator of edema.⁵ The T2 median value of normal DA hearts was 32 milliseconds (Figure 3D, dashed line), whereas that of cardiac allografts with 4-hour cold ischemia was 55 milliseconds, indicating myocardial edema (Figure 3D).

**Donor Simvastatin Treatment Prevents Vascular Leakage, the No-Reflow Phenomenon, and Myocardial Edema in During Reperfusion**

To investigate the effect of donor simvastatin treatment on microvascular dysfunction, donor DA rats were given simva-
Simvastatin 5 mg/kg via a nasogastric tube 2 hours before allograft removal. Donor simvastatin treatment prevented Evans blue dye extravasation (P<0.001; Figure 3A), maintained normal myocardial capillary perfusion 30 minutes after reperfusion (P<0.01; Figure 3B), resulted in rapid restoration of microvascular perfusion to normal levels as early as 1 minute after reperfusion (P<0.001; Figure 3C), and led to normal myocardial T2 values 4 hours after reperfusion (P<0.05; Figure 3D). These results indicate that donor simvastatin treatment offers immediate and profound microvascular protection after cardiac allograft reperfusion.

**Donor But Not Recipient Simvastatin Treatment Prevents Ischemia/Reperfusion Injury**

We next treated the donor (5 mg/kg), the recipient (2 mg/kg), or both with simvastatin. Six hours after reperfusion, the 4-hour cold ischemia increased serum cardiac troponin T levels (P<0.01; Figure 4A) and the number of allograft-infiltrating ED-1+ macrophages (P<0.05; Figure 4B) and myeloperoxidase+ neutrophils (P<0.01; Figure 4B) compared with allografts without 4-hour cold ischemia. Donor simvastatin treatment and combined donor and recipient simvastatin treatment decreased serum cardiac troponin T levels (P<0.05, P<0.01; Figure 4A) and the number of allograft-infiltrating ED1+ macrophages (P<0.01, P<0.05; Figure 4B) and myeloperoxidase+ neutrophils (P<0.05, P<0.01; Figure 4B). A range of donor simvastatin treatment doses (0.5, 2.0, and 5.0 mg/kg) and donor pravastatin 5.0 mg/kg treatment inhibited IRI (Figure IIIA and IIIB in the online-only Data Supplement). In contrast, recipient simvastatin treatment alone did not have significant effects on serum cardiac troponin T levels (Figure 4A) or on inflammation (Figure 4B).

Donor and combined donor and recipient simvastatin treatment significantly increased allograft heat shock protein...
Donor (D), but not recipient (R), simvastatin treatment prevents ischemia-reperfusion injury in cardiac allografts 6 hours after reperfusion. A, Serum cardiac troponin T (s-TnT) levels (dashed line shows TnT levels in nontransplanted Dark Agouti hearts). B, The number of allograft-infiltrating inflammatory cells by immunohistochemistry. C and D, Allograft mRNA expression by real time reverse-transcription polymerase chain reaction. The results were normalized to 18S rRNA and are presented as the ratio to mRNA expressed in allografts without donor simvastatin treatment and without cold ischemia. n=6 to 10 (A and B) and n=5 (C and D) per group. The Mann-Whitney U test was used to compare the effect of ischemia with no ischemia. The Kruskal-Wallis test with the Dunn test was used to compare simvastatin treatment of donors, recipients, or both donors and recipients and no treatment with the same ischemia time (A through C). Data are given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers), and the outliers are shown as circles outside the box (A through D). Ang indicates angiopoietin; HIF-1α, hypoxia-inducible factor-1α; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; TGF-β1, transforming growth factor-β1; ET-1, endothelin-1; HO-1, heme oxygenase-1; and HSP, heat shock protein. *P<0.05, **P<0.01.

Beneficial Effects of Donor Simvastatin Treatment on Microvasculature Stability and Perfusion Are Mediated Through Nitric Oxide and RhoA During Ischemia/Reperfusion Injury

We next investigated whether endothelial NOS,23 HO-1,24 and RhoA activation through prenylation25 mediated the protective effects of simvastatin. Inhibition of NOS by donor L-NAME administration reversed the antipermeabil-

ity (P<0.01; Figure 5A) and antiinflammatory (P<0.05; Figure 5D and 5E) effects achieved with donor simvastatin treatment. Inhibition of HO-1 by donor zinc protoporphyrin administration did not result in significant changes in permeability, inflammation, or serum troponin T levels (Figure 5A through 5D). Supplementation of RhoA GTPase prenylation pathway by concomitant geranylgeranyl pyrophosphate reversed the beneficial effects of donor simvastatin treatment on capillary perfusion (P<0.05; Figure 5B and 5F). These results indicate that NO and the RhoA prenylation pathway participate in microvascular stability and perfusion after donor simvastatin treatment, respectively.

Donor Simvastatin Treatment Has Sustained Antiinflammatory Effects in Immunosuppressed Rat Cardiac Allografts

We next investigated the effect of donor, recipient, and both donor and recipient simvastatin treatment on cardiac allograft inflammation 10 days after transplantation in cyclosporine A–immunosuppressed recipients. In the re-

27 (P<0.05; Figure 4D) and decreased dendritic cell maturation marker CD86 mRNA levels (P<0.001; Figure IVC in the online-only Data Supplement). In addition, combined donor and recipient treatment significantly decreased allograft mRNA levels of profibrotic basic fibroblast growth factor and TGF-β1 (P<0.05; Figure 4D), vasoconstrictive endothelin-1 (P<0.05; Figure 4D), and hyaluronan synthase-1 (P<0.01; Figure IVA in the online-only Data Supplement). In addition, donor and combined donor and recipient simvastatin treatment significantly decreased allograft nuclear HIF-1 immunoreactivity (P<0.01; Figure V in the online-only Data Supplement).
recipient simvastatin groups, simvastatin was given before the operation and daily thereafter. Donor simvastatin treatment and combined donor and recipient simvastatin treatment decreased the number of vascular cell adhesion molecule-1–positive postcapillary venules (P<0.01; Figure 6A) and the number of graft-infiltrating ED1+ macrophages, CD4+ T cells, and OX62+ dendritic cells (P<0.05; Figure 6B) in cardiac allografts. These results show that inhibition of IRI by single-dose donor simvastatin treatment has sustained antiinflammatory effects.

Figure 5. The beneficial effects of donor (D) simvastatin treatment on microvascular permeability and perfusion are mediated through nitric oxide (NO) and inhibition of the RhoA GTPase pathway, respectively. Donor Dark Agouti rats received simvastatin and either the NO synthase inhibitor N-nitro-l-arginine methyl ester (L-NAME) or the heme oxygenase-1 inhibitor zinc protoporphyrin (ZnPP) or geranylgeranyl pyrophosphate (GGPP) supplementation before allograft removal. A. Extravasated Evans blue dye 30 minutes after reperfusion. B and F. The density of perfused vessels 30 minutes after reperfusion. C. Serum troponin T (s-TnT) levels 6 hours after reperfusion. D and E. The number of allograft-infiltrating inflammatory ED-1+ macrophages 6 hours after reperfusion. n=6 to 7 per group. The Kruskal-Wallis test with the Dunn test was used to compare the effect of L-NAME, ZnPP, or GGPP with donor simvastatin treatment (A through D). Data are given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers), and the outliers are shown as circles outside the box (A through D). Scale bars=100 μm. MPO indicates myeloperoxidase. *P<0.05, **P<0.01, ***P<0.001.
Donor Simvastatin Treatment Decreases Cardiac Allograft Fibroproliferative Activity and Transforming Growth Factor-β1 Signaling

Because inflammation and TGF-β1 signaling play a pathological role in myocardial fibrosis,26 cardiac allografts were stained with fibroblast marker prolly-4-hydroxylase and phosphorylated Smad2 (p-Smad2), which indicates TGF-β1 activation. At 10 days after transplantation, numerous prolly-4-hydroxylase+ fibroblasts (Figure 7A) and p-Smad2+ cells (Figure 7B) were present in the border zone of fibrotic areas in allografts. Simvastatin treatment of donors and both donors and recipients decreased the number of allograft prolly-4-hydroxylase+ fibroblasts (P<0.05, P<0.01; Figure 7A). Donor simvastatin treatment significantly decreased the number of allograft p-Smad2+ cells (P<0.01; Figure 7B). These results indicate that donor simvastatin treatment has sustained effects on allograft TGF-β1 signaling and initiation of fibrotic pathways.

Simvastatin Decreases Transforming Growth Factor-β1-Induced Endothelial-to-Mesenchymal Transition of Human Cardiac Microvascular Endothelial Cells

We next investigated whether simvastatin interferes with TGF-β1–induced EndMT by stimulating cultured human cardiac microvascular ECs with TGF-β1 in the presence of different concentrations of simvastatin. Indeed, TGF-β1 induced a clear morphological change characterized by human cardiac microvascular EC filopodia formation and loss of cell-to-cell contact at 3 days. In addition, the tight junction protein zonula occludens-1 was clearly lost from EC surfaces (Figure 7C). In addition, TGF-β1 markedly increased mRNA levels of mesenchymal genes calponin and α-SMA (Figure 7D), as well as calponin protein levels, at 6 days (Figure 7E), indicating EndMT.

Simvastatin inhibited human cardiac microvascular EC filopodia formation, cell detachment, and relocation of zonula occludens-1 induced by TGF-β1 (Figure 7C), and a concentration-dependent reduction was noted in the human cardiac microvascular EC calponin (R²=0.751, P=0.005; Figure 7D) and α-SMA (R²=0.623, P=0.020; Figure 7D) mRNA levels and calponin protein levels (Figure 7E). These findings indicate that inhibition of EndMT may directly contribute to the antifibrotic effects of simvastatin.

Treatment of Both Donor and Recipient With Simvastatin Has Beneficial Effects on Long-Term Survival and the Development of Cardiac Allograft Fibrosis and Arteriosclerosis

Next, we investigated the long-term effects of simvastatin treatment on cardiac allograft inflammation, survival, capillary density, fibrosis, and arteriosclerosis using a chronic rejection model with cyclosporine A background immunosuppression. Because our preliminary studies indicated that the 4-hour cold ischemia resulted in poor 56-day allograft survival (∼30%), the grafts were subjected to 2-hour cold ischemia. At 56 days, the 2-hour cold ischemia increased allograft CD4+ T-cell density (P<0.05; Figure 8A), reduced cardiac allograft survival from 86% (54±2 days) to 44% (47±4 days; Figure 8B), decreased myocardial RECA-1+ capillary density (P<0.05; Figure 8C), and enhanced cardiac allograft arteriosclerosis (P<0.05; Figure 8G) compared with allografts without the 2-hour cold ischemia.

**Figure 6.** Simvastatin treatment of donors (D), recipients (R), and both donors and recipients prevents adaptive immune responses in cardiac allografts 10 days after transplantation. Recipients received cyclosporine A background immunosuppression to avoid severe acute rejection. A, Immunohistochemical stainings of vascular cell adhesion molecule-1 (VCAM-1) on postcapillary venules and (B) endothelial nitric oxide synthase (eNOS) in the cross-section of human cardiac microvascular ECs with TGF-β1-induced EndMT by stimulating cultured human cardiac microvascular ECs with TGF-β1. VCAM-1+ vessels/mm2 and eNOS+ cells/mm2 were decreased in simvastatin treatment compared with NO TREATMENT. Scale bars=50 μM. MPO indicates myeloperoxidase. *P<0.05, **P<0.01, ***P<0.001.
Figure 7. Simvastatin treatment decreases cardiac allograft fibroproliferative activity and transforming growth factor-β1 (TGF-β1) signaling at 10 days and TGF-β1-induced endothelial-to-mesenchymal transition in vitro. A, The number of cardiac allograft fibroblast marker prolyl-4-hydroxylase immunoreactive cells. B, The activation of TGF-β1 pathway analyzed by phosphorylated Smad2 immunoreactivity. Pathological fibroproliferation was profound in perifibrotic areas of nontreated cardiac allografts (arrows, inset). C, Human cardiac microvascular endothelial cells (ECs) were stimulated with TGF-β1 (10 ng/mL) in the presence of different concentrations of simvastatin. Simvastatin reversed the TGF-β1–induced morphological changes in filopodia formation and loss of cell-to-cell contact and prevented the loss of tight junction associated protein zonula occludens-1 (ZO-1) from the EC surface at 3 days. D, mRNA expression levels of the mesenchymal marker proteins calponin and α-smooth muscle actin (α-SMA) and (E) protein levels of calponin were inhibited by simvastatin in a concentration-dependent manner. n=5 per group (A and B). Arrow indicates perifibrotic borderline. The Mann-Whitney U test was used to compare the effects of ischemia and no ischemia. The Kruskal-Wallis test with the Dunn test was used to compare simvastatin treatment of donors (D), recipients (R), or both donors and recipients and no treatment with the same ischemia time (A and B). Linear regression analysis was applied to evaluate the relation of different simvastatin concentrations to expression of mesenchymal genes in TGF-β1–stimulated cells (D). Data are given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers), and the outliers are shown as circles outside the box (A and B). Dashed line shows expression in cells without TGF-β1 (D). Scale bars=50 μm. *P<0.05, **P<0.01.
Donor and combined donor and recipient simvastatin treatment decreased the number of allograft-infiltrating ED1
macrophages, myeloperoxidase+ neutrophils, and CD4+ T cells (P<0.05; Figure 8A and Figure VI in the online-only Data Supplement) and reduced myocardial fibrosis (P<0.05; Figure 8E and 8F). Treatment of both donors and recipients with simvastatin also significantly prolonged cardiac allograft survival (P<0.05; Figure 8B), increased RECA-1+ capillary density (P<0.01; Figure 8C and 8D), and reduced cardiac allograft arteriosclerosis (P<0.05; Figure 8G and 8H). Our findings thus indicate that combining the beneficial early effects of donor simvastatin treatment with daily recipient simvastatin treatment gives the best long-term protection for cardiac allografts.

**Discussion**

Similar to the response-to-injury hypothesis on atherosclerosis,27 our present findings highlight the importance of early injury to cardiac allografts in the development of subsequent pathological responses. A clinically relevant 4-hour cold ischemia followed by 1-hour warm ischemia increased allograft cardiomyocyte injury and inflammation 6 hours after reperfusion of cardiac allografts. This early injury had sustained effects on the development of chronic rejection, as
shown in previous experimental transplantation studies. Interestingly, cold and warm ischemia resulted in cardiac allograft microvascular RhoA/ROCK activation and formation of EC-EC gaps and led to profound microvascular permeability and the no-reflow phenomenon after reestablishment of circulation. Thus, our findings support the critical role of microvascular injury and dysfunction in cardiac allograft IRI and in the development of chronic rejection.

It is important that administration of a single dose of simvastatin to cardiac allograft donors prevented IRI and had beneficial long-term consequences (main findings of the study are summarized in the Table). The rapid effects of peroral administration of simvastatin to the allograft donor only 2 hours before graft removal may have important implications because this time frame fits the window of organ donor treatment in clinical transplantation and makes bench-to-bedside studies feasible. The short-term effects of statins are supported by previous clinical observations on the beneficial effects of peroral statin administration hours before percutaneous coronary intervention, although most studies reporting IRI protection are performed with long-term statin administration for days, weeks, or months before IRI.

A multitude of evidence in our study indicates that the protective effects of donor simvastatin treatment on microvasculature were cholesterol-independent pleiotropic effects. First, the cholesterol levels of rats were low with a low-cholesterol diet, and the levels were not affected by simvastatin treatment (see Results in the online-only Data Supplement). Second, HMG-CoA reductase expression was detected mainly in cardiac microvasculature. Third, donor, but not recipient, simvastatin treatment effectively blunt the IRI. Fourth, donor simvastatin treatment resulted in beneficial microvascular effects in that it stabilized the endothelium during allograft preservation and reversed the profound microvascular EC permeability and the no-reflow phenomenon after reperfusion. Fifth, simvastatin prevented microvascular RhoA/ROCK activation, the pathway considered central in the pleiotropic effects of statins in both experimental and clinical studies.

In addition to microvascular ECs, the surrounding pericytes have an important regulatory role in physiological and pathological microvascular functions. Our present results suggest that simvastatin controls vascular permeability and the no-reflow phenomenon in cardiac allograft IRI by regulating microvascular EC integrity and pericyte contraction, respectively. Inhibition of NOS with L-NAME reversed the beneficial effect of donor simvastatin treatment on vascular permeability but had no effect on capillary perfusion. This supports the role of simvastatin and endothelial NOS in the regulation of endothelial integrity and indicates that the no-reflow phenomenon in our study was not due to edema-induced capillary collapse. In addition, the lack of intravascular thrombosis in the cardiac allografts suggests that there must be additional mechanisms behind the observed myocardial perfusion defects. Interestingly, HMG-CoA reductase was expressed in microvascular pericytes, and supplementation of the geranylgeranyl pyrophosphate pathway, which is important for RhoA activation, recapitulated the no-reflow phenomenon. Previous studies show that sustained microvascular pericyte contraction impairs capillary reflow after cerebral ischemia and that RhoA modulates the contractile phenotype of pericytes. Thus, our results present the novel possibility that statins control the no-reflow phenomenon through regulation of pericyte contraction. Importantly, we also found that donor simvastatin treatment increased cardiac allograft HO-1 and heat shock protein 27 expression, which may also participate in the protective effects of statins in IRI.

Interestingly, we found that simvastatin decreased the mRNA levels of HIF-1α in rat hearts. Previously, statins have been shown to decrease HIF-1α DNA binding in ECs and smooth muscle cells and HIF-1α immunoreactivity in pig arteries. However, to the best of our knowledge, this is the first study to show that simvastatin decreases HIF-1α mRNA.

### Table. Summary of the Main Findings of the Study

<table>
<thead>
<tr>
<th>Phase</th>
<th>Time Point</th>
<th>Simvastatin Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preservation ex vivo</td>
<td>4-h cold + 1-h warm ischemia</td>
<td>Decreased microvascular permeability and RhoA activation, HIF-1α, iNOS, and ET-1 mRNA expression and increased HO-1 mRNA expression in preserved hearts</td>
</tr>
<tr>
<td>Reperfusion injury</td>
<td>30 min to 6 h after reperfusion</td>
<td>Decreased microvascular leakage and leukocyte infiltration (mediated by NOS), increased tissue perfusion (mediated by RhoA), and decreased myocardial injury (possibly mediated by HO-1 and HSP27) in cardiac allografts</td>
</tr>
<tr>
<td>Alloimmune response</td>
<td>10 d after transplantation</td>
<td>Decreased microvascular VCAM-1 expression, leukocyte infiltration, fibroproliferative activity, and TGF-β1 signaling in cardiac allografts</td>
</tr>
<tr>
<td>Chronic rejection</td>
<td>56 d after transplantation</td>
<td>Decreased leukocyte infiltration, fibrosis, and arteriosclerosis and increased myocardial capillary density and cardiac allograft survival</td>
</tr>
<tr>
<td>In vitro</td>
<td>3 and 6 d after stimulation</td>
<td>Decreased TGF-β1–induced EndMT in HMVEC culture</td>
</tr>
</tbody>
</table>

RhoA indicates ras homolog gene family member A; EC, endothelial cell; HIF-1α, hypoxia-inducible factor-1α; iNOS, inducible nitric oxide synthase; ET-1, endothelin-1; HO-1, heme oxygenase-1; HSP27, heat shock protein 27; VCAM-1, vascular cell adhesion molecule-1; TGF-β1, transforming growth factor-β1; EndMT, endothelial-to-mesenchymal transition; and HMVEC, human cardiac microvascular endothelial cell.
Mechanistically, these effects may be attributed to RhoA inhibition by simvastatin because RhoA regulates HIF-1α mRNA expression in renal cell carcinoma and trophoblast cells. Recent observations show that ECs contribute directly to myocardial fibrosis in a process of EndMT. A similar phenomenon, epithelial-to-mesenchymal transition, occurs in kidney fibrosis; it involves TGF-β1, RhoA, hypoxia, and HIF-1α and is inhibited by statins. Here, we found that donor simvastatin treatment reduced cardiac allograft TGF-β1 activation and fibrosis and that simvastatin also inhibited TGF-β1-induced EndMT in vitro. Our results thus support the findings that cardiac allograft EC dysfunction may directly participate in myocardial fibrosis through EndMT. In addition, our novel finding that simvastatin may directly participate in myocardial fibrosis through inhibited TGF-β1 activation and fibrosis and that simvastatin also inhibited TGF-β1-induced EndMT in vitro. Our results thus support the findings that cardiac allograft EC dysfunction may directly participate in myocardial fibrosis through EndMT. In addition, our novel finding that simvastatin inhibits EndMT may have important consequences for the treatment of myocardial fibrosis in both cardiac allografts and nontransplant situations.

Conclusions

Our results demonstrate that donor simvastatin treatment has beneficial molecular and functional effects on microvascular ECs and pericytes and may be used to counteract early microvascular dysfunction and the initiation of fibroproliferative pathways during IRI in cardiac allografts. Our results thus suggest a novel, clinically feasible strategy to protect cardiac allografts.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

Ischemia/reperfusion injury after heart transplantation may result in primary graft dysfunction or initiation of fibroproliferative cascades, leading to the development of cardiac fibrosis, allograft arteriosclerosis, and compromised long-term survival. Vascular dysfunction, including permeability and perfusion disturbances, plays a central role in ischemia/reperfusion injury. Statins are widely used to lower cholesterol levels, but they also have cholesterol-independent pleiotropic effects through Rho GTPase inhibition. We used heterotopic rat heart transplantation models to investigate whether a single dose of simvastatin administered to cardiac allograft donors perorally 2 hours before graft removal protects the cardiac allograft through direct vasculoprotective effects. Donor simvastatin treatment abolished cardiac allograft ischemia/reperfusion injury by preventing the no-reflow phenomenon and reducing vascular permeability, inflammation and cardiomyocyte injury. These early vasculoprotective and cardioprotective effects were mirrored with sustained antiinflammatory, antifibrotic, and antiarteriosclerotic effects in a chronic rejection heart transplantation model. Mechanistic studies indicated that donor simvastatin treatment decreased cardiac allograft microvascular endothelial cell and pericyte RhoA activation, modified the expression of vasculoprotective genes, and improved endothelial barrier function. In contrast to donor simvastatin treatment, recipient simvastatin treatment did not protect against ischemia/reperfusion injury. In vitro studies also showed that simvastatin decreased endothelial-mesenchymal transition, a recently characterized mechanism participating in cardiac fibrosis. Collectively, our results highlight the rapid vasculoprotective effects of simvastatin during ischemia/reperfusion injury and suggest donor simvastatin as a novel, clinically feasible strategy to protect cardiac allografts.
Donor Simvastatin Treatment Abolishes Rat Cardiac Allograft Ischemia/Reperfusion Injury and Chronic Rejection Through Microvascular Protection


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Supplemental Material

Donor simvastatin treatment abolishes rat cardiac allograft ischemia-reperfusion injury and chronic rejection through microvascular protection

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_Tuuminen: Vascular protection by donor simvastatin treatment_

Expanded Methods

Heterotopic Rat Heart Transplantations

Intra-abdominal heterotopic heart transplantations were performed from specific pathogen-free fully MHC-mismatched male Dark Agouti (DA, RT1\textsuperscript{av1}) to male Wistar Furth (WF, RT1\textsubscript{u}) rats (Scanburg, Göteborg, Sweden) weighing 300-350 g. After infusion of 300 IU heparin in 10 ml ice-cold PBS or Plegisol cardioplegia solution (Hospira, Inc., Lake Forest, II; used only in Supplemental Figure 4C experiments) depending on the study model into the inferior vena cava of the heart donor, the vena cava and pulmonary veins were ligated with 6-0 silk and the pulmonary artery and aorta were cut 2 to 3 mm above their origin in the heart. After removal, allografts were left without hypothermic preservation or were preserved either in PBS or in Plegisol cardioplegia solution at +4 °C for 2 or 4 hours depending
on the study model. Cardiac allograft recipients were anesthetized with isoflurane anesthesia (2-5%/l O₂), and received buprenorphine 0.15 mg/kg s.c. (Temgesic 0.3 mg/ml, Schering-Plough, Kenilworth, NJ) for peri- and postoperative analgesia. A midline incision was made, and the aorta and pulmonary artery of the allograft were anastomized to the abdominal aorta and inferior vena cava of the recipient, respectively. Warm ischemia occurring during heart transplantation was standardized to one hour. Permission for animal experimentation was obtained from the State Provincial Office of Southern Finland. The animals received good care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Academy Press (ISBN 0-309-05377-3, revised 1996).

**Drug Administration**

Peroral simvastatin (Merck Research Laboratories, Whitehouse Station, NJ) was given either to the donor as a single dose (5 mg/kg) 2 hours before graft removal, or to the recipient (2 mg/kg) 2 hours before reperfusion and daily thereafter (2 mg/kg/d), or to both. In dose-response analysis (Supplemental Figure 3A) simvastatin 0.5, 2.0 or 5.0 mg/kg was given to the donor 2 hours before graft removal. Simvastatin was diluted in polyethylene glycol (molecular weight 300) (Sigma-Aldrich, St. Louis, MO) to a concentration of 1.5 mg/ml, and the controls received polyethylene glycol vehicle daily p.o. Peroral pravastatin (P4498 pravastatin sodium salt, Sigma-Aldrich) was given to the donor as a single dose (5 mg/kg) 2 hours before graft removal. To prevent severe episodes of acute rejection and to allow the development of an alloimmune response and moderate chronic rejection, allograft recipients with 10-day and 56-day follow-up were given cyclosporine A (CsA, Novartis, Basel, Switzerland) diluted in Intralipid (Fresenius Kabi, Bad Homburg, Germany) 2.0 mg/kg/d s.c. for the first 7 days and 1.0 mg/kg/d thereafter. To inhibit NOS activity, N-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich) was dissolved in drinking water which was changed every other day. Donor animals received the orally active L-NAME in a dose of 50 mg/kg/day for 4 days before allograft removal. To block HO-1 activity, zinc protoporphyrin (ZnPP; Porphyrin Products Inc., Logan, UT) was dissolved in 0.2M NaOH, adjusted to pH of 7.4, and diluted in 0.9% NaCl to a concentration of 1 mg/ml. ZnPP was administered i.p. (20 mg/kg) to donor rats 2
hours before allograft removal. To supplement RhoA GTPase prenylation pathway, geranylgeranyl pyrophosphate (GGPP; Sigma-Aldrich) at a concentration ~1 mg/ml in methanol was administered i.p. (1 mg/kg) to donor rats 2 hours before allograft removal.

**Drug Assay**

After blood samples were collected, they were immediately centrifuged at 2000 rpm for 10 min at +4 °C and stored at -80 °C until further analysis. The blood concentrations of simvastatin lactone and simvastatin β-hydroxy acid were measured by liquid chromatography-ionspray tandem mass spectrometry.¹

**Lipid Analysis**

Serum samples were collected during the harvest at 8 weeks and stored at -20°C until further analysis. The concentration of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were measured using the enzymatic colorimetric test (Roche Diagnostics Cholesterol CHOD-PAP method; Cat. no. 1491458, Basel, Switzerland) and a biochemical analyzer (Hitachi Modular PP-analyzer; Hitachi Ltd., Tokyo, Japan).

**Allograft Survival**

The allograft function was estimated by daily abdominal palpation, and graded according to heart beat from 0 to 4: 0 minimal or no contractility (heart beat <30 bpm); 1, poor; 2, moderate; 3, impaired; and 4 normal. Allografts were removed if the grade fell to 0.

**Histology**

Cardiac fibrosis was determined in a blinded review by two observers from paraformaldehyde fixed paraffin sections stained with Masson’s trichrome and scored semiquantitatively (0 to 3) as follows: 0, no fibrosis; 1, mild fibrosis; 2, moderate fibrosis; and 3, severe myocardial fibrosis. Cardiac allograft vasculopathy was
determined from sections stained with hematoxylin-eosin and Resorcin-Fuchsin for internal elastic lamina using computer-assisted image processing (Zeiss Axiovision 4.4, Carl Zeiss International, Oberkochen, Germany) and measuring the area between the internal elastic lamina and the vessel lumen. The percentage of arterial occlusion was determined as the ratio of neointimal area to internal elastic lamina area.

**Immunohistochemistry and Immunofluorescence Stainings**

Cryostat sections were stained using the peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and the reaction was revealed by 3-amino-9-ethylcarbazole (AEC, Vector Laboratories). Counterstaining was performed using Mayer’s hemalum. Immunofluorescent stainings were performed using Alexa 568 red and Alexa 488 green (Promega, Madison, WI) secondary antibodies and DAPI nuclear staining (VECTASHIELD Mounting Medium with DAPI; Vector Laboratories). Antibodies and dilutions used were: CD4 (5 μg/ml, 22021D), CD8 (5 μg/ml, 22071D), ED1 (5 μg/ml, 22451D), CD161a to detect NK cells (5 μg/ml, 555006, BD Pharamingen, Franklin Lakes, NJ); CD42b (GPIb) (NCL-CD42b, dilution 1:100), CD61 (GPIIIa) (NCL-CD61-308, dilution 1:100, Novocastra, Newcastle upon Tyne, United Kingdom); myeloperoxidase to detect neutrophil granulocytes (20μg/ml, ab9535), Tropomyosin (7 μg/ml, ab7785), phospho-Adducin (phospho T445, 10 μg/ml, ab58485), NG2 (15 μg/ml, ab50009, Abcam, Cambridge, United Kingdom); OX-62 (10 μg/ml, MCA 1029G), RECA-1 (50 μg/ml, MCA97, AbD Serotec, Dusseldorf, Germany); HMG-CoA Reductase (10 μg/ml, 07-457, Upstate, Ballerica, MA); α-SMA (1:5000, A2547, Sigma-Aldrich); VCAM (10μg/ml, MMS-141P, Covance, Princeton, New Jersey); S100A4 (FSP-1) (1:100, A5114, DakoCytomation, Glostrup, Denmark); HIF-1α (13 μg/ml, IMG629, Imgenex, San Diego, CA); phospho-Smad2 (Ser465/467, 2.5 μg/ml, AB3849, Millipore, Ballerica, MA) and Prolyl-4-Hydroxylase beta (2 μg/ml, AF 5110-1, Acris antibodies GmbH, Herford, Germany). The number of inflammatory cells and RECA1+ capillaries was determined by counting positive cells and capillaries from four random fields of each quadrant of the cardiac cross section with 40x magnification, and are given as a total for 1 mm².
RNA Isolation and Reverse Transcription

Total RNA was extracted from five random samples per group using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription of mRNA was carried out from 100 ng total RNA using High-RNA-to-cDNA kit (Applied Biosystems Inc., Carlsbad, CA) in a total volume of 20 μl. After completed reverse transcription, 40 μl of PCR-grade water was added to each cDNA sample. Three μl of each sample (corresponding to 5 ng total RNA) were used in each subsequent PCR reaction.

Real-time PCR

Real-time PCR reactions were carried out on a RotorGene-6000 (Corbett Research, Hilden, Germany) using 2X DyNAmo Flash SYBR Green Master mix (Finnzymes, Espoo, Finland). Measurement of the PCR product was performed at the end of each extension period. Amplification specificity was checked using melting curve analysis. The number of mRNA copies of each gene of interest was calculated from a corresponding standard curve using the RotorGene software. The results are given in relation to 18S rRNA molecule numbers. Please see Supplemental Table 2 for primer information.

Transmission Electron Microscopy

For transmission electron microscopy, cardiac mid-axial cross-sectional samples from left ventricle (2 samples) and septum (1 sample) were microdissected, fixed with 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.4, incubated for 2 hours at +25 °C and post-fixed with 1% buffered osmium tetroxide for 1 hour, dehydrated and embedded in epon at room temperature. Sections were post-stained with uranyl acetate and lead and examined with a Jeol EX1200 II transmission electron microscope (Jeol) operating at 60kV. EC-EC junctions were analyzed from 10-15 representative longitudinally cut microvascular vessels per each graft sample using x50,000 magnification and the incidence of EC-EC gaps per graft was quantitated. Images were acquired with an ES500W CCD camera (Gatan Corp., Pleasanton, CA).

Microvascular Leakage and Perfusion
A modified Miles Assay was used to measure extravasation of plasma proteins from the microvasculature into the interstitial space of cardiac allografts. Immediately after reperfusion, recipients were injected i.v. with Evans blue (Sigma-Aldrich) diluted in 0.9% NaCl, at 30 mg/ml concentration, which was allowed to circulate for 30 minutes, whereafter the cardiac allograft coronary network was flushed with 5 ml of 1% PFA in 0.05M citrate buffer, pH 3.5. For quantification of extravasated Evans blue, 100 mg of apical myocardium was dissolved in 500 μl of formamide on a shaker at +60 °C for 24 hours. One hundred μl of formamide containing dissolved Evans blue dye was pipetted to cuvettes and the absorbance was measured by a spectrophotometer at 610 nm wavelength. FITC-labeled *Lycopersicon esculentum* (Tomato) lectin (Vector Laboratories) was used to detect vessels 30 minutes after re-establishment of circulation. Coronary arteries were perfused with 50 μl FITC-labeled Tomato lectin diluted in 150 μl of 0.9% NaCl. The number of FITC+ microvascular vessels from mid-axial cryostat cross sections was analyzed by fluorescence microscopy by counting positive capillaries from four random fields of each quadrant of the cardiac cross section with 40x magnification, and is given as a total for 1 mm².

**Microvascular Interendothelial Gap Detection**

After heart removal, the coronary arteries were perfused with 50 μl of rhodamine-labeled Concavalin A lectin (red) (Vector Laboratories) that binds to the vascular endothelium and was dissolved in 100 μl of NaCl. Thereafter the hearts were predisposed to cold preservation (4 hours), subsequent warm ischemia (1 hour) and then the cardiac allograft coronary network was perfused with 50 μl of biotinylated *Ricinus communis* lectin (Vector Laboratories) that binds to the vascular basement membrane, and flushed with 200 μl 1% PFA in 0.05M citrate buffer, pH 3.5. Cryostat sections were stained with streptavidin and fluorescent-dye conjugate Avidin 350 nm (blue) (Promega Corp.) to detect biotinylated *Ricinus communis* lectin in order to identify endothelial-endothelial barrier disruption.

**Laser Doppler Monitoring**

Cardiac allograft tissue perfusion was analyzed one hour after reperfusion by dynamic
measurement with laser doppler monitor, Transonic BLF21-Series (Transonic Systems Inc., Ithaca, NY). The moving doppler effect was calculated via a 18 gauge (1.2mm) probe head receiving fiber optic light reflected by stationary structures within the tissue as well as by moving particles (red blood cells). A low intensity beam of monochromatic light was emitted to the portion of apical myocardium, to 1mm depth, and the tissue volume under laser doppler monitoring was approximately 1mm$^3$. Flow signal was sampled 200 s$^{-1}$ from transmitted 19200 baud (=pulses per second) and is represented as tissue perfusion units (TPUs) that are relatively proportional to ml/min/100g of tissue.$^2$

**Measurement of Cardiac Troponin T**

The rat serum levels of cTnT were analyzed with the third generation troponin T test (Troponin T STAT, Roche Diagnostics), which shows cross-reactivity of 0.001% with TnT originating in skeletal muscle at a concentration of 2.000 ng/ml. The functional sensitivity is 0.03 μg/l and the lower detection limit 0.01 μg/l. The cTnT was measured by electrochemiluminescence immunoassay (ECLIA) on the Elecsys 2010 immunoassay analyser (Roche Diagnostics).

**Magnetic Resonance Imaging**

MRI studies were performed with a 4.7 T scanner ( PharmaScan, Bruker BioSpin, Billerica, MA) using a 90 mm shielded gradient that is capable of producing maximum gradient amplitude of 300 mT/m with 80-μs rise time. A linear birceage RF coil with an inner diameter of 60 mm was used. After a scout, T2-weighed fast spin echo [rapid acquisition with relaxation enhancement (RARE)] sequence was used (TR/TE$^{eff}$ = 4200/55 ms, rare factor = 8, matrix size = 256 x 256, field-of-view = 30 × 30 mm, 15 slices, slice thickness = 2 mm). The multi-spin multi-echo sequence (MSME), based on CPMG (Carr-Purcell Meiboom-Gill) spin echoes was used for the determination of T2 [repetition time (TR) = 1500 ms, echo time (TE) = 11 - 220 ms and 20 echoes, number of averages = 2, matrix size = 256 x 192, field of-view = 70 x 70 mm, single slice with slice thickness = 2.0 mm, acquisition time = 9 min 36s]. The value of T2 was calculated by fitting the measured intensities to the exponential relaxation curve using the least-squares method.
TGF-β1-induced Endothelial-to-mesenchymal Transition of Human Cardiac Microvascular Endothelial Cells \textit{in vitro}

TGF-β1 was used to induce EndMT in HMVEC-C at a concentration of 10 ng/ml. Simvastatin was dissolved in EtOH and activated by treatment with NaOH followed by neutralization to pH 7 and concentrations of 0.1, 0.5 and 1.0 μM were used to inhibit EndMT. Antibodies used in \textit{in vitro} studies were CD31 (ab9498, Abcam), ZO-1 (61-7300, Invitrogen, Carlsbad, CA) and Calponin (C2687, Sigma-Aldrich). HMVEC-C and EGM-2 MV growth medium were from Lonza, Basel, Switzerland. The cells used for the experiments were between passages 4-8. For immunofluorescence microscopy the cells were grown on glass coverslips for the indicated times. Coverslips were then washed three times with PBS, and the cells were fixed in ice-cold methanol at -20 °C. After washing three times with PBS, the cells were incubated in Dulbecco's PBS containing 3% BSA to prevent nonspecific binding of the antibodies. The cells were then incubated with the primary antibody in Dulbecco's PBS for 1 hour. The bound antibodies were detected using Alexa Fluor-594 secondary antibodies (Molecular Probes, Invitrogen). The coverslips were finally washed in water, mounted on glass slides using Vectashield anti-fading reagent (Vector Laboratories) and examined under an Axioplan 2 imaging microscope (Zeiss) using a 40x objective. Images were acquired with an AxioCamHRm camera and Axiovision 4.6 software (Zeiss) at the Molecular Imaging Unit of the University of Helsinki. For RNA isolation and real-time RT-PCR analysis, RNeasy mini kit (Qiagen) was used to isolate total cellular RNA. Reverse transcription was carried out with random hexamer primers (Invitrogen) and Superscript III reverse transcriptase (Invitrogen) using 1.0 μg of total RNA according to the manufacturer's instructions. The cDNAs were amplified using TaqMan Assays-on-Demand gene expression products (Applied Biosystems) and GeneAmp 7500 Sequence Detector thermal cycler (Applied Biosystems). Control amplifications directly from RNA were performed in order to rule out DNA contamination. The levels of gene expression were determined using the Ct method and the results are shown as mRNA expression levels normalized to the levels of a gene with a constant expression (TBP, tata binding protein). SDS-PAGE and immunoblotting were performed after the cells were lysed in RIPA buffer. Equal amounts of protein were separated by SDS-PAGE under reducing conditions.
using 4-20% gradient Tris-glycine gels (Lonza). The proteins were transferred to Protran nitrocellulose membranes (Whatman plc., Kent, United Kingdom) using a semi-dry blotting system (BioRad, Hercules, CA).

**Effect of Simvastatin on EC and SMC Gene Expression in vitro**

Human cardiac microvascular endothelial cells (HMVEC-C) were cultured in EGM-2 MV growth medium. The cells used for the experiments were between passages 4-8. Rat coronary artery SMC (kindly provided by Dariusz Leszczynski; Finnish Centre for Radiation and Nuclear Safety, Helsinki, Finland) were cultured in 5% fetal calf serum. HMVEC-C and rat coronary artery SMC were supplemented with activated simvastatin at a concentration of 1.0 μM for 72 hours. RNA isolation and real-time RT-PCR analysis were performed as described above.

**Statistics**

Mann-Whitney U test was used for two-group comparison and Kruskall-Wallis with Dunn test when multiple groups were compared to control. Dunn post hoc test was applied only if Kruskall-Wallis test demonstrated an overall statistically significant difference. For comparison in a longitudinal study, data was analyzed by repeated-measures ANOVA. For survival Kaplan-Meier with Log-rank (Mantel-Cox) was applied. Linear regression analysis was applied to evaluate the relation of different simvastatin concentrations to expression of mesenchymal genes in vitro. Data is given as mean±SEM for longitudinal analysis and for discrete variables and by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) for continuous variables (SPSS Statistics 15.0, SPSS Inc., Somers, NY). The outliers are shown as circles outside the box. P<0.05 was regarded as statistically significant.
Expanded Results

Simvastatin Lactone and β-hydroxy Acid Blood Levels in Rat and Human

Pharmacokinetic analysis with liquid chromatography-ionspray tandem mass spectrometry showed that after single-dose peroral simvastatin 5.0 mg/kg administration, simvastatin lactone was absorbed rapidly within hours (Supplemental Figure 1A). Simvastatin lactone was also absorbed within 2 hours in human organ donors after clinically-approved simvastatin 80 mg administration via nasogastric tube (Supplemental Figure 1B) indicating the applicability of donor simvastatin treatment in a clinical situation. Simvastatin lactone was hydroxylated to its active β-hydroxy acid form and was eliminated much more rapidly in the rat (Supplemental Figure 1A) than in human organ donors (Supplemental Figure 1B).

Simvastatin Treatment Modulates mRNA Levels of Genes Involved in Microvascular Homeostasis Through Endothelial but not of Smooth Muscle Cells

HMVEC-C and rat coronary artery SMC were cultured in vitro with or without simvastatin for 72 hours. Quantitative real time RT-PCR analysis showed that simvastatin resulted in a 50% reduction in HMVEC-C HIF-1α and ET-1 mRNA levels (Supplemental Table 1). In contrast, in similar in vitro experiments with rat coronary artery SMC simvastatin did not alter the mRNA levels of the investigated genes (Supplemental Table 2).

Low Presence of Intravascular Thrombosis After Cardiac Allograft Reperfusion

The no-reflow phenomenon observed after reperfusion of cardiac allografts with 4-hour cold ischemia (Figure 3) could result from intravascular thrombosis. Immunohistochemical platelet stainings showed only a few CD42b+ and CD61+ platelet aggregates in allograft capillaries (Supplemental Figure 2) indicating that wide-spread thrombosis was not the reason for compromised microvascular perfusion.

The Range of Donor Simvastatin Treatment Doses Induces Protection Against Cardiac Allograft IRI
To determine the range of effective donor simvastatin doses and possible dose-response on IRI, we administered simvastatin 0.5, 2 or 5 mg/kg p.o. to donor rats 2 hours before allograft removal (Supplemental Figure 3A). All these simvastatin doses resulted in comparable reduction in serum TnT levels and allograft ED1⁺ macrophage and MPO⁺ neutrophil infiltration (Supplemental Figure 3A) 6 hours after reperfusion of cardiac allografts with 4-hour cold ischemia. A dose-response effect was noted in allograft CD4⁺ and CD8⁺ cell densities with the highest simvastatin 5 mg/kg dose resulting in lowest T cell infiltration (Supplemental Figure 3A).

**Donor Pravastatin Treatment Protects Against Cardiac Allograft IRI**

To determine whether other HMG-CoA reductase inhibitors protect against cardiac allograft IRI, donor rats were treated with pravastatin 5 mg/kg p.o. 2 hours before allograft removal. Donor pravastatin treatment halved serum TnT levels (Supplemental Figure 3B) and reduced allograft ED1⁺ macrophage and MPO⁺ neutrophil infiltration \((P < 0.05;\) Supplemental Figure 3B) 6 hours after reperfusion of cardiac allografts with 4-hour cold ischemia. These results indicate a class-effect for donor statin treatment in cardiac allograft IRI protection.

**Donor Simvastatin Treatment Has Cardioprotective Effects in Experimental Protocols with Concomitant Use of Clinically-relevant Cardioplegia Solution**

Different cardioplegia solutions are used in the clinic to promote cardiac allograft viability and to reduce the early IRI-related complications. As we used heparinized +4 °C PBS for allograft perfusion and preservation so far, we next performed additional experiments using heparinized +4 °C Plegisol cardioplegia solution for allograft perfusion and preservation (Supplemental Figure 3C). In groups with Plegisol cardioplegia solution, concomitant donor simvastatin 5 mg/kg treatment 2 hours before allograft removal decreased allograft MPO⁺ neutrophil infiltration \((P < 0.01;\) Supplemental Figure 3C) 6 hours after reperfusion of cardiac allografts with 4-hour cold ischemia. These results indicate that donor simvastatin treatment effectively protects against IRI also in experimental protocols with concomitant use of a clinically-relevant cardioplegia solution.
Effect of Simvastatin on Cholesterol Levels of Rats with Low-Cholesterol Diet

Serum cholesterol levels were analyzed from cardiac allograft recipients without simvastatin treatment and from recipients with daily simvastatin 2.0 mg/kg p.o. treatment at 56 days. Serum total cholesterol (1.2±0.3 vs. 1.4±0.3; mean±SEM), HDL (0.5±0.2 vs. 0.7±0.2) and LDL fractions (0.5±0.2 vs. 0.4±0.3), and triglyceride levels (0.7±0.1 vs. 0.7±0.2) were low in recipient rats with low-cholesterol diet without simvastatin treatment, and the cholesterol levels were not affected by daily simvastatin treatment.
Supplemental Figure Legends

**Supplemental Figure 1.** Simvastatin is absorbed rapidly after peroral administration in rats and human organ donors. Inactive simvastatin lactone form and its active β-hydroxy acid form were analyzed with liquid chromatography-ionspray tandem mass spectrometry. A, Simvastatin blood levels in rats after peroral simvastatin 5 mg/kg administration. B, Simvastatin blood levels in human organ donors after peroral simvastatin 80 mg administration (B). n=3 per group. Data is given as mean±SEM.

**Supplemental Figure 2.** Microvascular platelet aggregates in rat cardiac allografts at 6 hours after reperfusion. Very few CD42b+ (A) or CD61+ (B) platelet aggregates were found in non-treated allografts subjected to 4-hour cold ischemia and 1-hour warm ischemia 6 hours after reperfusion determined by immunohistochemistry. Scale bars = 50 μm.

**Supplemental Figure 3.** Effect of different donor simvastatin treatment doses, donor pravastatin treatment and concomitant use of cardioplegia solution on cardiac allograft IRI. A, Effect of a range of donor simvastatin doses (0.5, 2.0 and 5.0 mg/kg p.o. 2 hours before graft procurement) on cardiomyocyte injury and allograft inflammation 6 hours after reperfusion in allografts with 4-hour cold ischemia. B, Effect of donor pravastatin treatment (5.0 mg/kg p.o. 2 hours before graft procurement) on IRI. C, Effect of PBS and Plegisol cardioplegia solution on the cardiac allograft IRI. n = 6 (A) and 5 (B-C) per group. D, donor treatment; PBS, phosphate buffer solution, PLG, plegisol cardioplegia solution. Kruskall-Wallis with Dunn test was used to compare various donor simvastatin treatment doses to non-treated control (A). Mann-Whitney U test was applied for two-group comparison (B-C). Data is given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) and the outliers are shown as circles outside the box. *P < 0.05, **P < 0.01.

**Supplemental Figure 4.** mRNA levels of genes involved in innate and adaptive
immune responses in rat cardiac allografts 6 hours (A-C) and 10 days after reperfusion (D-F). mRNA expression of genes was measured by real time RT-PCR. The results were normalized to 18S rRNA and are given as the ratio to mRNA expressed in allografts without donor simvastatin treatment and without cold ischemia. n = 6-7 (A-C) and n = 5 (D-F) per group. D, donor; R, recipient; and D/R, donor and recipient simvastatin treatment. Mann-Whitney U test was used to compare prolonged cold ischemia to no cold ischemia. Kruskall-Wallis with Dunn test was used to compare donor, recipient or combined donor and recipient simvastatin to no treatment (A-F). Data is given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) and the outliers are shown as circles outside the box. *P < 0.05, **P < 0.01, ***P < 0.001.

Supplemental Figure 5. Effect of simvastatin on HIF-1α protein expression. A, quantification of allograft HIF-1α immunoreactive cells indicated that simvastatin treatment of donors and both donors and recipients decreases cardiac allograft HIF-1α protein expression 6 hours after reperfusion. B, Photomicrographs of non-treated and simvastatin-treated donors (arrows point to HIF-1α+ cells). n = 6-7 per group. D, donor; R, recipient; and D/R, donor and recipient simvastatin treatment. Mann-Whitney U test was used to compare prolonged cold ischemia to no cold ischemia. Kruskall-Wallis with Dunn test was used to compare donor, recipient or combined donor and recipient simvastatin to no treatment (A). Data is given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers) and the outliers are shown as circles outside the box. **P < 0.01. Scale bar = 75 μm.

Supplemental Figure 6. Allograft infiltrating inflammatory cells at 8 weeks. Representative microphotographs of immunohistochemical stainings of the effect of simvastatin treatment allograft on ED1+ macrophages, MPO+ neutrophils, CD4+ and CD8+ T cells and OX62+ dendritic cells in a chronic rejection model. Please see the details and inflammatory cell quantification in Figure 8. D, donor; R, recipient; and D/R, donor and recipient simvastatin treatment. Scale bar = 50 μm.
References


**Supplemental Table 1.** Relative mRNA levels after simvastatin treatment in EC and SMC *in vitro*.

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HMVEC-C and rat coronary artery SMC were cultured with or without activated simvastatin at a concentration of 1.0 μM for 72 hours. The results were normalized to TBP and are given as a ratio to mRNA expressed in EC and SMC culture without simvastatin treatment. Data is given as mean±SEM. Mann-Whitney U test was used for two-group comparison. p-values were non-significant. Ang, angiopoietin; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; HIF-1α, hypoxia inducible factor-1α; HMVEC-C, human cardiac microvascular endothelial cell; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; SMC, smooth muscle cell; TBP, tata binding protein.
**Supplemental Table 2. RT-PCR primer list.**

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Bold = rat primers; Bold Italic = human primers; fwd = forward sequence; rev = reverse sequence; rev.compl. = reverse complement sequence
Supplement Figure 1

A

Simvastatin blood levels in rat (ng/ml)

- lactone
- β-hydroxy acid

B

Simvastatin blood levels in human (ng/ml)

- lactone
- β-hydroxy acid

Time (h)
Supplement Figure 2

A: CD42b+ vessels

B: CD61+ vessels
**Supplement Figure 3**

A. Effects of Simvastatin on S-TnT levels and inflammatory markers.

B. Effects of Pravastatin on S-TnT levels and inflammatory markers.

C. Effects of Simvastatin on S-TnT levels and inflammatory markers with preservation media.

- **NO TREATMENT**
- **STATIN**

**Key:**
- *P < 0.05
- **P < 0.01
- ***P < 0.001
- ****P < 0.0001
Supplement Figure 5

A

Cold ischemia (h)

Simvastatin

HIF-1α+ cells/mm²

0

1000

2000

3000

**

**

B

NO TREATMENT (4h)

SIMVASTATIN (DONOR)
Donor Simvastatin Treatment Abolishes Rat Cardiac Allograft Ischemia/Reperfusion Injury and Chronic Rejection Through Microvascular Protection

Raimo Tuuminen, MD; Simo Syrjälä, MD; Rainer Krebs, MSc; Mikko A.I. Keränen, MD; Katri Koli, PhD; Usama Abo-Ramadan, PhD; Pertti J. Neuvonen, MD, PhD; Jussi M. Tikkanen, MD, PhD; Antti I. Nykänen, MD, PhD; Karl B. Lemström, MD, PhD

Background—Ischemia/reperfusion injury may have deleterious short- and long-term consequences for cardiac allografts. The underlying mechanisms involve microvascular dysfunction that may culminate in primary graft failure or untreatable chronic rejection.

Methods and Results—Here, we report that rat cardiac allograft ischemia/reperfusion injury resulted in profound microvascular dysfunction that was prevented by donor treatment with peroral single-dose simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase and Rho GT Pase inhibitor, 2 hours before graft procurement. During allograft preservation, donor simvastatin treatment inhibited microvascular endothelial cell and pericyte RhoA/Rho-associated protein kinase activation and endothelial cell--endothelial cell gap formation; decreased intragraft mRNA levels of hypoxia-inducible factor-1α, inducible nitric oxide synthase, and endothelin-1; and increased heme oxygenase-1. Donor, but not recipient, simvastatin treatment prevented ischemia/reperfusion injury--induced vascular leakage, leukocyte infiltration, the no-reflow phenomenon, and myocardial injury. The beneficial effects of simvastatin on vascular stability and the no-reflow phenomenon were abolished by concomitant nitric oxide synthase inhibition with N-nitro-L-arginine methyl ester and RhoA activation by geranylgeranylated pyrophosphate supplementation, respectively. In the chronic rejection model, donor simvastatin treatment inhibited cardiac allograft inflammation, transforming growth factor-β1 signaling, and myocardial fibrosis.

Conclusions—Our results demonstrate that donor simvastatin treatment prevents microvascular endothelial cell and pericyte dysfunction, ischemia/reperfusion injury, and chronic rejection and suggest a novel, clinically feasible strategy to protect cardiac allografts.

Key Words: endothelium • inflammation • ischemia • microcirculation • transplantation

R etoration of compromised blood flow is vital in vascular occlusion, but paradoxically, tissue reperfusion is often accompanied by significant morbidity and mortality. This is especially evident in heart transplantation in which ischemia/reperfusion injury (IRI) may result in primary graft failure and the development of untreatable chronic rejection.1–3 Clinically feasible organ preservation strategies are needed to limit early IRI, to blunt subsequent pathological immunological and tissue remodeling responses, and to prolong cardiac allograft recipient survival.

Clinical Perspective on p 143

Endothelial cell (EC) dysfunction is a hallmark of IRI4–6 and the development of cardiac allograft fibrosis and arteriosclerosis, manifestations of chronic rejection,2,3,7 IRI5 and several IRI-related factors such as hypoxia, thrombin, vascular endothelial growth factor, and RhoA GTPase activation are involved in EC-EC gap formation and compromised endothelial stability.6,8–10 Dysfunctional ECs also predispose the patients to thrombosis, leukocyte adhesion, and vasoconstriction and are involved in pathological fibroproliferation.3,7,11 They also directly participate in myocardial fibrosis through endothelial-to-mesenchymal transition (EndMT) in which transforming growth factor (TGF)-β1 induces EC transdifferentiation to fibroblasts.11 Pericytes are perivascular cells that cover endothelial tubes in small arterioles, capillaries, and postcapillary venules.
Pericytes communicate with the underlying ECs, share the same basement membrane, and regulate the microvascular tone\(^\text{12}\) possibly mediated by RhoA GTPase activation.\(^\text{13,14}\) Pericyte dysfunction may result in microvascular hyperten-
sion, pathological angiogenesis, increased capillary permea-
bility,\(^\text{12}\) and the no-reflow phenomenon after IRI.\(^\text{15}\)

Statins are cholesterol-lowering drugs that are widely used in preventing cardiovascular diseases.\(^\text{16}\) They also have im-
portant cholesterol-independent vasculoprotective effects me-
diated largely through the inhibition of Rho GTPases, impor-
tant regulators of cell cytoskeleton and intracellular signaling
pathways.\(^\text{16,17}\) Simvastatin is commonly begun shortly after
heart transplantation because of its beneficial effects against
chronic rejection and on long-term survival.\(^\text{18,19}\) Here, we
investigated whether peroral simvastatin treatment of rat
cardiac allograft donors 2 hours before allograft procurement has vasculoprotective and immunomodulatory effects on IRI
and subsequent pathological tissue remodeling.

Methods

Experimental Design

Cardiac allografts were transplanted from fully major histocom-
patibility complex–mismatched male Dark Agouti (DA; RT1\(^\text{av1}\)) to
male Wistar Furth (RT1\(^\text{n}\)) rats (Scanburg, Stockholm, Sweden).
Allografts were left without hypothermic preservation or were
preserved in PBS at 4°C for 2 to 4 hours, depending on the study
model. Warm ischemia was standardized to 1 hour. Microvascular
RhoA activity and intragraft inflammation were analyzed by immu-
nohistochemistry and immunofluorescence; microvascular interen-
dothelial gaps, by biotinylated Ricinus communis lectin and trans-
mision electron microscopy; microvascular leakage, by a modified
Miles assay; perfusion, by laser Doppler monitoring and intracoro-
nary FITC-labeled Lycopersicon esculentum (Tomato) lectin; myo-
cardial edema, by magnetic resonance imaging; and myocardial
injury, by serum cardiac troponin T. Intragraft mRNA expression
was determined by real-time reverse-transcription polymerase chain
reaction. In the chronic rejection model, fibrosis was analyzed by
Masson trichrome staining, and arteriosclerosis was assessed mor-
phometrically by hematoxylin-eosin and Resorcin-Fuchs staining
for internal elastic lamina. To prevent severe episodes of acute
rejection, allograft recipients were given cyclosporine A (Novartis,
Basel, Switzerland) 2.0 mg · kg\(^{-1}\) · d\(^{-1}\) SC for the first 7 days and
1.0 mg · kg\(^{-1}\) · d\(^{-1}\) thereafter. Sample sizes are given in the figure
legends. The Methods section in the online-only Data Supplement
gives detailed information.

Drug Administration

Peroral simvastatin (MERCK Research Laboratories, Whitehouse Station, NJ) was given to the donor as a single dose (5 mg/kg) 2 hours
before graft removal, to the recipient (2 mg/kg) 2 hours before
reperfusion and daily thereafter (2 mg · kg\(^{-1}\) · d\(^{-1}\)), or to both. The
controls received polyethylene glycol vehicle orally. To inhibit nitric
oxide synthase (NOS) activity, donors received N-nitro-L-arginine
methyl ester (L-NAME; Sigma-Aldrich Co, St. Louis, MO) 50 mg · kg\(^{-1}\) · d\(^{-1}\) for
4 days before allograft removal. To block heme oxygenase-1 (HO-1)
activity, zinc protoporphyrin (Porphyrin Products Inc, Logan, UT) was administered intraperitoneally (20 mg/kg) to
donor rats 2 hours before allograft removal. To supplement RhoA
GTPase prelymphesthesia, geranylgeranyl pyrophosphate
(Sigma-Aldrich) at a concentration of \(1 \mu\)g/mL in methanol was
administered intraperitoneally (1 mg/kg) to donor rats 2 hours before
allograft removal.

Effects of Simvastatin on Transforming Growth Factor-\(\beta\)-Induced Endothelial-to-Mesenchymal Transition In Vitro

Transforming growth factor-\(\beta\) 10 ng/mL was used to induce
EndMT in human cardiac microvascular ECs. Simvastatin was
dissolved in EtOH and activated by NaOH followed by neutraliza-
tion, and concentrations of 0.1, 0.5, and 1.0 \(\mu\)mol/L were used to
inhibit EndMT. Immunohistochemistry identified endothelial CD31
and zonula occludens-1 density. Real-time reverse-transcription
polymerase chain reaction analysis, SDS-PAGE, and immunoblot-
ting were performed to measure the mRNA expression and protein
levels of the mesenchymal markers calponin and \(\alpha\)-smooth muscle
actin (\(\alpha\)-SMA).

Statistics

The Mann-Whitney U test was used for 2-group comparisons, and the
Kruskal-Wallis test with the Dunn test was used when multiple
groups were compared with control. The Dunn post hoc test was
applied only if the Kruskal-Wallis test demonstrated an overall
statistically significant difference. For comparison in a longitudinal
study, data were analyzed by repeated measures ANOVA. For
survival, Kaplan-Meier analysis with log rank (Mantel-Cox) was
applied. Linear regression analysis was applied to evaluate the
relation of different simvastatin concentrations to the expression of
mesenchymal genes in vitro. Data are given as mean ± SEM for
longitudinal analysis and for discrete variables and by box plots
showing the upper extreme (excluding outliers), upper quartile,
median, lower quartile, and lower extreme (excluding outliers) for
continuous variables (SPSS Statistics 15.0, SPSS Inc, Somers, NY).
The outliers are shown as circles outside the box. Values of \(P < 0.05\)
were regarded as statistically significant.

Results

Microvascular Endothelial Cells and Pericytes Express HMG-CoA Reductase in the Rat Heart

To identify the target cells of donor simvastatin treatment, we
performed HMG-CoA reductase immunostainings in normal
DA rat hearts. HMG-CoA reductase immunoreactivity was
found mainly in cardiac vasculature (Figure 1A). Immunoo-
fluorescent double stainings showed that HMG-CoA reduc-
tase was expressed mostly in microvascular rat endothelial
cell antigen-1\(^\text{1}\) (RECA-1\(^\text{1}\)) ECs and in NG2\(^\text{1}\) pericytes but
not in \(\alpha\)-SMA\(^\text{1}\) smooth muscle cells or in tropomyosin\(^\text{1}\)
cardiomyocytes (Figure 1A).

Cold and Warm Ischemia Induce Microvascular Endothelial Cell and Pericyte RhoA/ROCK
Activation and Endothelial Cell–Endothelial Cell Gap Formation

To determine whether cold and warm ischemia lead to RhoA/
ROCK activation, we performed immunostainings of adducin
phosphorylated at Thr445 (p-adducin)\(^\text{20,21}\) in normal DA hearts.
HMG-CoA reductase immunoreactivity was found mainly in cardiac vasculature (Figure 1A). Immunoo-
fluorescent double stainings showed that HMG-CoA reduc-
tase was expressed mostly in microvascular rat endothelial
cell antigen-1\(^\text{1}\) (RECA-1\(^\text{1}\)) ECs and in NG2\(^\text{1}\) pericytes but
not in \(\alpha\)-SMA\(^\text{1}\) smooth muscle cells or in tropomyosin\(^\text{1}\)
cardiomyocytes (Figure 1A).

Transmission electron microscopy analysis indicated that
combined 4-hour cold and 1-hour warm ischemia markedly
increased the incidence of microvascular EC-EC gaps
\(\text{(P < 0.01; Figure 1E). The presence of microvascular EC-EC}
\text{gaps during cold and warm ischemia was confirmed by}
\text{fluorescence microscopy of grafts that received intracoronary}
\text{Downloaded from http://circ.ahajournals.org/ on November 13, 2011 by IMED Korea}
Figure 1. Donor simvastatin treatment prevents microvascular RhoA/ROCK activation and endothelial cell (EC)-EC gap formation in the rat heart subjected to 4-hour cold and 1-hour warm ischemia. A, Dark Agouti rat heart microvascular HMG-CoAR reductase (HMG-CoAR) immunoreactivity colocalized with rat endothelial cell antigen-1 (RECA-1) ECs and NG2 pericytes but not with \( \alpha \)-smooth muscle actin–positive (SMA) smooth muscle cells, or tropomyosin–cardiomyocytes. B through D, Immunoreactivity for adducin phosphorylated at Thr445 (p-adducin) as an in situ indicator of RhoA/ROCK activation. E, Microvascular EC-EC gaps (arrows) determined by transmission electron microscopy of left ventricular longitudinally cut microvascular vessels. F, Microvascular EC-EC gaps confirmed by intracoronary perfusion with EC-binding rhodamine-labeled Concanavalin A (red) lectin and basement membrane-binding biotinylated Ricinus communis lectin (blue, arrows). Negative controls are shown in the insets (A and D). n=5 to 6 per group. The Kruskal-Wallis test with the Dunn test was used to compare the effect of ischemia and no ischemia (B through E). The Mann-Whitney U test was used to compare donor simvastatin treatment and no treatment with the same ischemia time (B through E). Data are given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers), and the outliers are shown as circles outside the box (B through E). Rbc indicates red blood cell. Arrows indicate EC-EC junctions. Scale bars=50 \( \mu \)m (A through D and F) and 100 nm (E). *P<0.05, **P<0.01, ***P<0.001.
perfusion of endothelium-binding rhodamine-labeled Conca
vain A lectin and basement membrane-binding biotin-
labeled R. communis lectin22 (Figure 1F).

**Donor Simvastatin Treatment Prevents Microvascular RhoA/ROCK Activation and Endothelial Cell-Endothelial Cell Gap Formation During Cold and Warm Ischemia**

Next, we investigated whether peroral donor treatment with simvastatin 5 mg/kg 2 hours before graft procurement inhibits graft RhoA/ROCK activation and EC-EC gap formation. Simvastatin was absorbed and metabolized to its active form in hours in rats (simvastatin 5 mg/kg; Figure IA in the online-only Data Supplement) and in human organ donors (simvastatin 80 mg; Figure 1B in the online-only Data Supplement). Donor simvastatin treatment decreased p-adducin immunoreactivity in capillaries (P < 0.05; Figure 1B), postcapillary venules, and arterioles (P < 0.05; Figure 1C), as well as in the media of arteries (P < 0.05; Figure 1D), and decreased the formation of EC-EC gaps (P < 0.001; Figure 1E and 1F), indicating decreased RhoA/ROCK activation and enhanced endothelial stability.

**Donor Simvastatin Treatment Decreases Hypoxia-Inducible Factor-1α, Inducible Nitric Oxide Synthase, and Endothelin-1 and Increases Heme Oxygenase-1 mRNA During Cold and Warm Ischemia**

Real-time reverse-transcription polymerase chain reaction analysis showed that 4-hour cold and 1-hour warm ischemia significantly decreased inducible NOS (iNOS) (P < 0.01; Figure 2A), vascular endothelial growth factor (P < 0.001; Figure 2A), and HO-1 (P < 0.05; Figure 2B) mRNA levels, whereas it increased heat shock protein 27 mRNA levels (P < 0.05; Figure 2B) in the rat heart. Donor simvastatin treatment significantly decreased mRNA levels of hypoxia-inducible factor-1α (HIF-1α; P < 0.05; Figure 2A), its downstream INOS (P < 0.05; Figure 2A), and vasoconstrictive endothelin-1 (P < 0.05; Figure 2B) and increased the expression of protective HO-1 (P < 0.05; Figure 2B). Simvastatin treatment resulted in a 50% reduction in EC HIF-1α and endothelin-1 mRNA levels in vitro (Table I in the online-only Data Supplement).

**Cold and Warm Ischemia Induce Vascular Leakage, the No-Reflow Phenomenon, and Myocardial Edema During Reperfusion**

To determine the effect of allograft reperfusion on microvascular permeability and perfusion, DA hearts with or without 4-hour cold ischemia were transplanted to Wistar Furth recipients using a standardized 1-hour warm ischemia. In a vascular permeability assay, 4-hour cold ischemia significantly increased Evans blue dye extravasation 30 minutes after reperfusion (P < 0.001; Figure 3A). This was accompanied by a prominent no-reflow phenomenon with diminished numbers of perfused capillaries determined by intracoronary injection of endothelium-binding FITC-labeled L. esculentum lectin 30 minutes after reperfusion (P < 0.01; Figure 3B). The loss of microvascular perfusion was not due to widespread intravascular thrombosis (Figure II in the online-only Data Supplement).

Furthermore, serial laser Doppler measurements from the apex of normal DA hearts and cardiac allografts with 4-hour cold ischemia (Figure 3C) showed a prominently diminished microvascular perfusion early after cardiac allograft reperfusion (15 mL·min⁻¹·100 g⁻¹ tissue 1 to 30 minutes after reperfusion) that gradually increased (30 mL·min⁻¹·100 g⁻¹ tissue 1 hour after reperfusion) toward normal DA heart values (53 ± 0.7 mL·min⁻¹·100 g⁻¹ tissue), indicating that IRI results in impaired microvascular perfusion early after reperfusion that gradually resolves over time.

To assess myocardial edema, we used cardiac allograft magnetic resonance imaging to analyze myocardial transverse relaxation time (T2) value 4 hours after reperfusion. T2 >40 milliseconds is considered an indicator of edema.3 The T2 median value of normal DA hearts was 32 milliseconds (Figure 3D, dashed line), whereas that of cardiac allografts with 4-hour cold ischemia was 55 milliseconds, indicating myocardial edema (Figure 3D).

**Donor Simvastatin Treatment Prevents Vascular Leakage, the No-Reflow Phenomenon, and Myocardial Edema in During Reperfusion**

To investigate the effect of donor simvastatin treatment on microvascular dysfunction, donor DA rats were given simva-
statin 5 mg/kg via a nasogastric tube 2 hours before allograft removal. Donor simvastatin treatment prevented Evans blue dye extravasation (P<0.001; Figure 3A), maintained normal myocardial capillary perfusion 30 minutes after reperfusion (P<0.01; Figure 3B), resulted in rapid restoration of microvascular perfusion to normal levels as early as 1 minute after reperfusion (P<0.001; Figure 3C), and led to normal myocardial T2 values 4 hours after reperfusion (P<0.001; Figure 3D). These results indicate that donor simvastatin treatment offers immediate and profound microvascular protection after cardiac allograft reperfusion.

**Donor But Not Recipient Simvastatin Treatment Prevents Ischemia/Reperfusion Injury**

We next treated the donor (5 mg/kg), the recipient (2 mg/kg), or both with simvastatin. Six hours after reperfusion, the 4-hour cold ischemia increased serum cardiac troponin T levels (P<0.01; Figure 4A) and the number of allograft-infiltrating ED-1+ macrophages (P<0.05; Figure 4B) and myeloperoxidase+ neutrophils (P<0.01; Figure 4B) compared with allografts without 4-hour cold ischemia. Donor simvastatin treatment and combined donor and recipient simvastatin treatment decreased serum cardiac troponin T levels (P<0.05, P<0.01; Figure 4A) and the number of allograft-infiltrating ED1+ macrophages (P<0.01, P<0.05; Figure 4B) and myeloperoxidase+ neutrophils (P<0.05, P<0.01; Figure 4B). A range of donor simvastatin treatment doses (0.5, 2.0, and 5.0 mg/kg) and donor pravastatin 5.0 mg/kg treatment inhibited IRI (Figure IIIA and IIIB in the online-only Data Supplement). In contrast, recipient simvastatin treatment alone did not have significant effects on serum cardiac troponin T levels (Figure 4A) or on inflammation (Figure 4B).

Donor and combined donor and recipient simvastatin treatment significantly increased allograft heat shock protein...
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Benificial Effects of Donor Simvastatin Treatment on Microvasculature Stability and Perfusion Are Mediated Through Nitric Oxide and RhoA During Ischemia/Reperfusion Injury

We next investigated whether endothelial NOS, HO-1, and RhoA activation through prenylation mediated the protective effects of simvastatin. Inhibition of NOS by donor L-NAME administration reversed the antipermeability (P<0.01; Figure 5A) and antiinflammatory (P<0.05; Figure 5D and 5E) effects achieved with donor simvastatin treatment. Inhibition of HO-1 by donor zinc protoporphyrin administration did not result in significant changes in permeability, inflammation, or serum troponin T levels (Figure 5A through 5D). Supplementation of RhoA GTPase prenylation pathway by concomitant geranylgeranyl pyrophosphate reversed the beneficial effects of donor simvastatin treatment on capillary perfusion (P<0.05; Figure 5B and 5F). These results indicate that NO and the RhoA prenylation pathway participate in microvascular stability and perfusion after donor simvastatin treatment, respectively.

Donor Simvastatin Treatment Has Sustained Antiinflammatory Effects in Immunosuppressed Rat Cardiac Allografts

We next investigated the effect of donor, recipient, and both donor and recipient simvastatin treatment on cardiac allograft inflammation 10 days after transplantation in cyclosporine A–immunosuppressed recipients. In the re-

Figure 4. Donor (D), but not recipient (R), simvastatin treatment prevents ischemia-reperfusion injury in cardiac allografts 6 hours after reperfusion. A, Serum cardiac troponin T (s-TnT) levels (dashed line shows TnT levels in nontransplanted Dark Agouti hearts). B, The number of allograft-infiltrating inflammatory cells by immunohistochemistry. C and D, Allograft mRNA expression by real time reverse-transcription polymerase chain reaction. The results were normalized to 18S rRNA and are presented as the ratio to mRNA expressed in allografts without donor simvastatin treatment and without cold ischemia. n=6 to 10 (A and B) and n=5 (C and D) per group. The Mann-Whitney U test was used to compare the effect of ischemia with no ischemia. The Kruskal-Wallis test with the Dunn test was used to compare simvastatin treatment of donors, recipients, or both donors and recipients and no treatment with the same ischemia time (A through C). Data are given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers), and the outliers are shown as circles outside the box (A through D). Ang indicates angiopoietin; HIF-1α, hypoxia-inducible factor-1α; eNOS, endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; TGF-β1, transforming growth factor-β1; ET-1, endothelin-1; HO-1, heme oxygenase-1; and HSP, heat shock protein. *P<0.05, **P<0.01.
Recipient simvastatin groups, simvastatin was given before the operation and daily thereafter. Donor simvastatin treatment and combined donor and recipient simvastatin treatment decreased the number of vascular cell adhesion molecule-1–positive postcapillary venules \( (P < 0.01; \text{Figure 6A}) \) and the number of graft-infiltrating ED1+ macrophages, CD4+ T cells, and OX62+ dendritic cells \( (P < 0.05; \text{Figure 6B}) \) in cardiac allografts. These results show that inhibition of IRI by single-dose donor simvastatin treatment has sustained antiinflammatory effects.

**Figure 5.** The beneficial effects of donor (D) simvastatin treatment on microvascular permeability and perfusion are mediated through nitric oxide (NO) and inhibition of the RhoA GTPase pathway, respectively. Donor Dark Agouti rats received simvastatin and either the NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME) or the heme oxygenase-1 inhibitor zinc protoporphyrin (ZnPP) or geranylgeranyl pyrophosphate (GGPP) supplementation before allograft removal. A, Extravasated Evans blue dye 30 minutes after reperfusion. B and F, The density of perfused vessels 30 minutes after reperfusion. C, Serum troponin T (s-TnT) levels 6 hours after reperfusion. n=6 to 7 per group. The Kruskal-Wallis test with the Dunn test was used to compare the effect of L-NAME, ZnPP, or GGPP with donor simvastatin treatment (A through D). Data are given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers), and the outliers are shown as circles outside the box (A through D). Scale bars=100 \( \mu \text{m} \). MPO indicates myeloperoxidase. \* \( P < 0.05 \), \** \( P < 0.01 \), \*** \( P < 0.001 \).
Endothelial Cells Transition of Human Cardiac Microvascular

We next investigated whether simvastatin interferes with allograft TGF-β signaling play a pathological role in myocardial fibrosis, cardiac allografts were stained with fibroblast marker prolly-4-hydroxylase and phosphorylated Smad2 (p-Smad2), which indicates TGF-β1 activation. At 10 days after transplantation, numerous prolly-4-hydroxylase⁺ fibroblasts (Figure 7A) and p-Smad2⁺ cells (Figure 7B) were present in the border zone of fibrotic areas in allografts. Treatment of donors and both donors and recipients decreased the number of allograft prolly-4-hydroxylase⁺ fibroblasts (P<0.05, P<0.01; Figure 7A). Donor simvastatin treatment significantly decreased the number of allograft p-Smad2⁺ cells (P<0.01; Figure 7B). These results indicate that donor simvastatin treatment has sustained effects on allograft TGF-β1 signaling and initiation of fibrotic pathways.

Simvastatin Decreases Transforming Growth Factor-β1-Induced Endothelial-to-Mesenchymal Transition of Human Cardiac Microvascular Endothelial Cells

We next investigated whether simvastatin interferes with TGF-β1-induced EndMT by stimulating cultured human cardiac microvascular ECs with TGF-β1 in the presence of different concentrations of simvastatin. Indeed, TGF-β1 induced a clear morphological change characterized by human cardiac microvascular EC filopodia formation and loss of cell-to-cell contact at 3 days. In addition, the tight junction protein zonula occludens-1 was clearly lost from EC surfaces (Figure 7C). In addition, TGF-β1 markedly increased mRNA levels of mesenchymal genes calponin and α-SMA (Figure 7D), as well as calponin protein levels, at 6 days (Figure 7E), indicating EndMT.

Simvastatin inhibited human cardiac microvascular EC filopodia formation, cell detachment, and relocation of zonula occludens-1 induced by TGF-β1 (Figure 7C), and a concentration-dependent reduction was noted in the human cardiac microvascular EC calponin (R²=0.751, P=0.005; Figure 7D) and α-SMA (R²=0.623, P=0.020; Figure 7D) mRNA levels and calponin protein levels (Figure 7E). These findings indicate that inhibition of EndMT may directly contribute to the antifibrotic effects of simvastatin.

Treatment of Both Donor and Recipient With Simvastatin Has Beneficial Effects on Long-Term Survival and the Development of Cardiac Allograft Fibrosis and Arteriosclerosis

Next, we investigated the long-term effects of simvastatin treatment on cardiac allograft inflammation, survival, capillary density, fibrosis, and arteriosclerosis using a chronic rejection model with cyclosporine A background immunosuppression. Because our preliminary studies indicated that the 4-hour cold ischemia resulted in poor 56-day allograft survival (~30%), the grafts were subjected to 2-hour cold ischemia.

At 56 days, the 2-hour cold ischemia increased allograft CD4⁺ T-cell density (P<0.05; Figure 8A), reduced cardiac allograft survival from 86% (54±2 days) to 44% (47±4 days; Figure 8B), decreased myocardial RECA-1⁺ capillary density (P<0.05; Figure 8C), and enhanced cardiac allograft arteriosclerosis (P<0.05; Figure 8G) compared with allografts without the 2-hour cold ischemia.
Figure 7. Simvastatin treatment decreases cardiac allograft fibroproliferative activity and transforming growth factor-β1 (TGF-β1) signaling at 10 days and TGF-β1-induced endothelial-to-mesenchymal transition in vitro. A. The number of cardiac allograft fibroblast marker prolyl-4-hydroxylase immunoreactive cells. B. The activation of TGF-β1 pathway analyzed by phosphorylated Smad2 immunoreactivity. Pathological fibroproliferation was profound in perifibrotic areas of nontreated cardiac allografts (arrows, inset). C. Human cardiac microvascular endothelial cells (ECs) were stimulated with TGF-β1 (10 ng/mL) in the presence of different concentrations of simvastatin. Simvastatin reversed the TGF-β1–induced morphological changes in filopodia formation and loss of cell-to-cell contact and prevented the loss of tight junction associated protein zonula occludens-1 (ZO-1) from the EC surface at 3 days. D, mRNA expression levels of the mesenchymal marker proteins calponin and α-smooth muscle actin (α-SMA) and (E) protein levels of calponin were inhibited by simvastatin in a concentration-dependent manner. n=5 per group (A and B). Arrow indicates perifibrotic borderline. The Mann-Whitney U test was used to compare the effects of ischemia and no ischemia. The Kruskal-Wallis test with the Dunn test was used to compare simvastatin treatment of donors (D), recipients (R), or both donors and recipients and no treatment with the same ischemia time (A and B). Linear regression analysis was applied to evaluate the relation of different simvastatin concentrations to expression of mesenchymal genes in TGF-β1–stimulated cells (D). Data are given by box plots showing the upper extreme (excluding outliers), upper quartile, median, lower quartile, and lower extreme (excluding outliers), and the outliers are shown as circles outside the box (A and B). Dashed line shows expression in cells without TGF-β1 (D). Scale bars=50 μm. *P<0.05, **P<0.01.
Donor and combined donor and recipient simvastatin treatment decreased the number of allograft-infiltrating ED1+ macrophages, myeloperoxidase+ neutrophils, and CD4+ T cells (P<0.05; Figure 8A and Figure VI in the online-only Data Supplement) and reduced myocardial fibrosis (P<0.05; Figure 8E and 8F). Treatment of both donors and recipients with simvastatin also significantly prolonged cardiac allograft survival (P<0.05; Figure 8B), increased RECA-1+ capillary density (P<0.01; Figure 8C and 8D), and reduced cardiac allograft arteriosclerosis (P<0.05; Figure 8G and 8H). Our findings thus indicate that combining the beneficial early effects of donor simvastatin treatment with daily recipient simvastatin treatment gives the best long-term protection for cardiac allografts.

**Discussion**

Similar to the response-to-injury hypothesis on atherosclerosis,27 our present findings highlight the importance of early injury to cardiac allografts in the development of subsequent pathological responses. A clinically relevant 4-hour cold ischemia followed by 1-hour warm ischemia increased allograft cardiomyocyte injury and inflammation 6 hours after reperfusion of cardiac allografts. This early injury had sustained effects on the development of chronic rejection, as...
shown in previous experimental transplantation studies. Interestingly, cold and warm ischemia resulted in cardiac allograft microvascular RhoA/ROCK activation and formation of EC-EC gap and led to profound microvascular permeability and the no-reflow phenomenon after reestablishment of circulation. Thus, our findings support the critical role of microvascular injury and dysfunction in cardiac allograft IRI and in the development of chronic rejection.

It is important that administration of a single dose of simvastatin to cardiac allograft donors prevented IRI and had beneficial long-term consequences (main findings of the study are summarized in the Table). The rapid effects of peroral administration of simvastatin to the allograft donor only 2 hours before graft removal may have important implications because this time frame fits the window of organ donor treatment in clinical transplantation and makes bench-to-bedside studies feasible. The short-term effects of statins are supported by previous clinical observations on the beneficial effects of peroral statin administration hours before percutaneous coronary intervention, although most studies reporting IRI protection are performed with long-term statin administration for days, weeks, or months before IRI.

A multitude of evidence in our study indicates that the protective effects of donor simvastatin treatment on microvasculature were cholesterol-independent pleiotropic effects. First, the cholesterol levels of rats were low with a low-cholesterol diet, and the levels were not affected by simvastatin treatment (see Results in the online-only Data Supplement). Second, HMG-CoA reductase expression was detected mainly in cardiac microvasculature. Third, donor, but not recipient, simvastatin treatment effectively blunted IRI. Fourth, donor simvastatin treatment resulted in beneficial microvascular effects in that it stabilized the endothelium during allograft preservation and reversed the profound microvascular EC permeability and the no-reflow phenomenon after reperfusion. Fifth, simvastatin prevented microvascular RhoA/ROCK activation, the pathway considered central in the pleiotropic effects of statins in both experimental and clinical studies.

In addition to microvascular ECs, the surrounding pericytes have an important regulatory role in physiological and pathological microvascular functions. Our present results suggest that simvastatin controls vascular permeability and the no-reflow phenomenon in cardiac allograft IRI by regulating microvascular EC integrity and pericyte contraction, respectively. Inhibition of NOS with L-NAME reversed the beneficial effect of donor simvastatin treatment on vascular permeability but had no effect on capillary perfusion. This supports the role of simvastatin and endothelial NOS in the regulation of endothelial integrity and indicates that the no-reflow phenomenon in our study was not due to edema-induced capillary collapse. In addition, the lack of intravascular thrombosis in the cardiac allografts suggests that there must be additional mechanisms behind the observed myocardial perfusion defects. Interestingly, HMG-CoA reductase was expressed in microvascular pericytes, and supplementation of the geranylgeranyl pyrophosphate pathway, which is important for RhoA activation, recapitulated the no-reflow phenomenon. Previous studies show that sustained microvascular pericyte contraction impairs capillary reflow after cerebral ischemia and that RhoA modulates the contractile phenotype of pericytes. Thus, our results present the novel possibility that statins control the no-reflow phenomenon through regulation of pericyte contraction. Importantly, we also found that donor simvastatin treatment increased cardiac allograft HO-1 and heat shock protein 27 expression, which may also participate in the protective effects of statins in IRI.

Interestingly, we found that simvastatin decreased the mRNA levels of HIF-1α in rat hearts. Previously, statins have been shown to decrease HIF-1α DNA binding in ECs and smooth muscle cells and HIF-1α immunoreactivity in pig arteries. However, to the best of our knowledge, this is the first study to show that simvastatin decreases HIF-1α mRNA.

### Table: Summary of the Main Findings of the Study

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<td>Preservation ex vivo</td>
<td>4-h cold + 1-h warm ischemia</td>
<td>Decreased microvascular RhoA activation, EC-EC gap formation, HIF-1α, iNOS, and ET-1 mRNA expression and increased HO-1 mRNA expression in preserved hearts</td>
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<tr>
<td>Reperfusion injury</td>
<td>30 min to 6 h after reperfusion</td>
<td>Decreased microvascular leakage and leukocyte infiltration (mediated by NOS), increased tissue perfusion (mediated by RhoA), and decreased myocardial injury (possibly mediated by HO-1 and HSP27) in cardiac allografts</td>
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<td>Alloimmune response</td>
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<td>In vitro</td>
<td>3 and 6 d after stimulation</td>
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RhoA indicates ras homolog gene family member A; EC, endothelial cell; HIF-1α, hypoxia-inducible factor-1α; iNOS, inducible nitric oxide synthase; ET-1, endothelin-1; HO-1, heme oxygenase-1; HSP27, heat shock protein 27; VCAM-1, vascular cell adhesion molecule-1; TGF-β1, transforming growth factor-β1; EndMT, endothelial-to-mesenchymal transition; and HMVEC, human cardiac microvascular endothelial cell.
Mechanistically, these effects may be attributed to RhoA inhibition by simvastatin because RhoA regulates HIF-1α mRNA expression in renal cell carcinoma and trophoblast cells.

Recent observations show that ECs contribute directly to myocardial fibrosis in a process of EndMT. A similar phenomenon, epithelial-to-mesenchymal transition, occurs in kidney fibrosis; it involves TGF-β1. RhoA, hypoxia, and HIF-1α, and is inhibited by statins. Here, we found that donor simvastatin treatment reduced cardiac allograft TGF-β1 activation and fibrosis and that simvastatin also inhibited TGF-β1-induced EndMT in vitro. Our results thus support the findings that cardiac allograft EC dysfunction may directly participate in myocardial fibrosis through EndMT. In addition, our novel finding that simvastatin inhibits EndMT may have important consequences for the treatment of myocardial fibrosis in both cardiac allografts and nontransplant situations.

Conclusions
Our results demonstrate that donor simvastatin treatment has beneficial molecular and functional effects on microvascular ECs and pericytes and may be used to counteract early microvascular dysfunction and the initiation of fibroproliferative pathways during IRI in cardiac allografts. Our results thus suggest a novel, clinically feasible strategy to protect cardiac allografts.

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Disclosures
None.

References
Ischemia/reperfusion injury after heart transplantation may result in primary graft dysfunction or initiation of fibroproliferative cascades, leading to the development of cardiac fibrosis, allograft arteriosclerosis, and compromised long-term survival. Vascular dysfunction, including permeability and perfusion disturbances, plays a central role in ischemia/reperfusion injury. Statins are widely used to lower cholesterol levels, but they also have cholesterol-independent pleiotropic effects through Rho GTPase inhibition. We used heterotopic rat heart transplantation models to investigate whether a single dose of simvastatin administered to cardiac allograft donors perorally 2 hours before graft removal protects the cardiac allograft through direct vasculoprotective effects. Donor simvastatin treatment abolished cardiac allograft ischemia/reperfusion injury by preventing RhoA activation and myosin regulatory light chain phosphorylation. 


**CLINICAL PERSPECTIVE**

**Ischemia/reperfusion injury after heart transplantation may result in primary graft dysfunction or initiation of fibroproliferative cascades, leading to the development of cardiac fibrosis, allograft arteriosclerosis, and compromised long-term survival. Vascular dysfunction, including permeability and perfusion disturbances, plays a central role in ischemia/reperfusion injury. Statins are widely used to lower cholesterol levels, but they also have cholesterol-independent pleiotropic effects through Rho GTPase inhibition. We used heterotopic rat heart transplantation models to investigate whether a single dose of simvastatin administered to cardiac allograft donors perorally 2 hours before graft removal protects the cardiac allograft through direct vasculoprotective effects. Donor simvastatin treatment abolished cardiac allograft ischemia/reperfusion injury by preventing RhoA activation and myosin regulatory light chain phosphorylation.**


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