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PRECLINICAL RESEARCH

Rosuvastatin increases vascular endothelial PPARγ expression and corrects blood pressure variability in obese dyslipidaemic mice

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Aims Statins improve atherosclerotic diseases through cholesterol-reducing effects. Whether the latter exclusively mediate similar benefits, e.g. on hypertension, in the metabolic syndrome is unclear. We examined the effects of rosuvastatin on the components of this syndrome, as reproduced in mice doubly deficient in LDL receptors and leptin (DKO).

Methods and results DKO received rosuvastatin (10 mg/kg/day or 20 mg/kg/day) or saline for 12 weeks. Saline-treated DKO mice had elevated blood pressure (BP) and nitric oxide-sensitive BP variability recorded by telemetry. Compared with saline, rosuvastatin (20 mg/kg/day) had no effect on weight gain and a minor effect on plasma cholesterol. Despite incomplete correction of insulin sensitivity, rosuvastatin fully corrected BP and its variability ($P = 0.01$), in conjunction with upregulation of PPARγ (but not PPARα) in the aortic arch. Rosuvastatin similarly increased PPARγ ($P = 0.002$) and SOD1 ($P = 0.01$) expression in isolated endothelial cells. Both GW9662, a PPARγ-specific antagonist, and siRNA raised against PPARγ abrogated rosuvastatin's effect, which was reproduced in PPARγ- (but not PPARα-) dependent transactivation assays.

Conclusion Beyond partial improvement in insulin sensitivity, rosuvastatin normalized BP homeostasis in obese dyslipidaemic mice independently of changes in body weight or plasma cholesterol. Upregulation of PPARγ and SOD1 in the endothelium may be involved as a unique vasculoprotective effect of statin treatment.

Keywords Statin • Blood pressure • Nitric oxide • Superoxide dismutase • PPARγ

Introduction

Obesity, hypertension, dyslipidaemia, and insulin resistance are clustered in the metabolic syndrome, a predisposing condition for atherosclerotic cardiovascular disease. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) potently reduce cholesterol levels, which largely accounts for the reduction of morbidity and mortality in hypercholesterolaemic patients.[1] Statins also reduce cardiovascular disease risk in patients with the metabolic syndrome,[2] possibly through additional effects on inflammation.[3] Indeed, statins may exert protective effects beyond cholesterol lowering, but their relevance to the clinical benefit of statin treatment is still being debated.

Among the components of the metabolic syndrome, hypertension is commonly preceded by the development of insulin resistance, which has been shown recently to be linked to the production of cellular oxidant radicals.[4] Glitazones, or PPARγ-activating ligands, are widely used as insulin sensitizers and also reduce high blood pressure (BP)[5,6] and vascular oxidative stress.[7] Likewise, several statins have been shown to improve insulin sensitivity in animal models and patients,[3,8] and similarly exhibit anti-inflammatory, as well as BP-reducing effects, which are partly independent of their

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cholesterol-lowering effects.[9,10] Although inhibition of pro-oxidant enzymatic systems, such as NADPH oxidase, has been well documented,[11] additional mechanisms may be at play. In particular, superoxide dismutases (SODs) represent a major antioxidant defence system in the vasculature and the Cu/Zn cytosolic SOD (encoded by *SOD1*), which is largely represented in the endothelium[12] and has been shown to be regulated by PPARs *in vitro*.[13] Whether statins may improve BP homeostasis by regulating the expression of SOD1 in endothelial cells, particularly through PPARγ activation, is however unknown.

To resolve these questions, we studied the effect of rosuvastatin in mice with combined leptin and low-density lipoprotein receptor (LDLR) deficiency that develop all the features of the human metabolic syndrome.[14,15] Their expected resistance to the cholesterol-lowering effect of statins allowed us to examine ancillary effects of rosuvastatin on vascular function independent of decreases in plasma cholesterol. In particular, the variability of BP was analysed by telemetry *in vivo* as a surrogate index of endothelial function and vascular stiffness, both key components of cardiovascular prognosis[16] also known to be profoundly influenced by vascular oxidant status.[17]

Methods

Experimental protocol

DKO mice with both leptin (Ob/Ob) and LDLR deficiency ($LDLR^{-/-}$) were obtained by crossing $LDLR^{-/-}$ and Ob/+ mice as previously described.[15] Experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee. Twelve-week-old mice were injected subcutaneously with rosuvastatin (10 mg/kg/day, $n = 6$ and 20 mg/kg/day, $n = 7$) or vehicle ($n = 9$) for 12 weeks and compared with age-matched (24 weeks) control mice (wild type, WT; C57BL6). Mice were identified by a number and independently assigned to the different experimental groups to avoid selection bias. The results were analysed blindly.

Circadian variation and frequency analysis of blood pressure and heart rate by implanted telemetry

BP signals [and heart rate (HR), derived from pressure waves] from the aortic arch were measured in conscious, unrestrained animals with surgically implanted, miniaturized telemetry devices (Datascience Corp., USA) as described.[18]

Cell culture and transient transfection

Bovine aortic endothelial cells and HEK293 were cultured to confluence in EGM-MV or DMEM containing 10% serum, then serum-starved for 24 h and exposed to the different treatments. Transient transfection of HEK293 was carried out in 24-well plates at 40–50% confluency (see Supplementary material online for details).

mRNA and protein analysis

mRNA expression in the aortic arch and in aortic endothelial cells was measured by reverse transcription (RT)–real-time quantitative polymerase chain reaction (PCR) as described.[15] SOD1 protein expression was measured by western blotting (see Supplementary material online).

Biochemical analysis

Blood was collected from conscious mice by tail bleeding into EDTA tubes after an overnight fast, and biochemical parameters measured as described before[15] (see Supplementary material online). The data reported in *Table 1* were obtained from those mice that underwent the full telemetry protocol.

Statistical analysis

For *in vivo* experiments on BP and BP variability, two different analyses were made. For the assessment of the effect of placebo or two doses of rosuvastatin, the data were analysed by a trend test. To assess whether results after the administration of rosuvastatin were similar to those observed in age-matched C57BL6 mice, we performed one-way ANOVA followed by the Dunnett test. To evaluate the influence of treatment group on the circadian variation, a two-way ANOVA was performed, including an interaction term for treatment by time. All statistical tests were two-sided. For the analysis of the mean 24 h values of haemodynamic parameters, *t*-test was performed. The sample size (i.e. number of animals per group) was decided from our previous experience with highly accurate and reproducible measurements with telemetry.[15] For *in vitro* experiments, we performed one-way ANOVA with *post hoc* analysis using the Bonferroni procedure for selected comparisons as indicated.

Table 1 Blood and metabolic parameters

Parameters	WT ($n = 10$)	DKO placebo ($n = 9$)	DKO rosuvastatin, 10 mg/kg ($n = 6$)	DKO rosuvastatin, 20 mg/kg ($n = 7$)	Test for linear trend
Weight, g	26 ± 4	59 ± 5	63 ± 3	57 ± 4	0.36
Total cholesterol, mg/dL	78 ± 19	570 ± 140	468 ± 323	460 ± 217	0.35
Triglycerides, mg/dL	23 ± 5	467 ± 127	214 ± 194	137 ± 64	0.0001
Glucose, mmol/L	4.3 ± 0.8	9.9 ± 2.2	7.0 ± 1.3	5.0 ± 0.3	0.0001
Insulin, mU/L	130 ± 10	5036 ± 1612	3550 ± 1518	1415 ± 617	0.0001
AUC of GTT	28 ± 5.6	87 ± 4.2	78 ± 4.2	54 ± 75	0.0001

AUC of GTT is the area under the curve in the glucose tolerance test. Data are mean ± SD.

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Results

Weight and metabolic parameters

Compared with placebo, rosuvastatin had no effect on weight gain. The metabolic parameters in the different groups are shown in *Table 1*. All pre-treatment values were identical between placebo- and rosuvastatin-treated animals (data not shown). Rosuvastatin had minimal effects on plasma total cholesterol levels. Conversely, it produced a significant reduction in triglycerides at both doses of the drug. The same doses also decreased glucose and insulin with an improvement in glucose tolerance (*Table 1*).

Rosuvastatin normalized systolic blood pressure and decreased heart rate in LDLR$^{-/-}$/ObOb mice

Figure 1 represents the BP and HR values obtained by telemetry over 24 h in the different groups. Night and day values are also displayed in *Table 2*. Compared with age-matched C57BL6 mice (WT), placebo DKO mice had a significant increase in their mean 24 h systolic BP (SBP, 126.7 ± 2.9 vs. 114.7 ± 2.9 mmHg; $P = 0.0007$), diastolic BP (DBP, 94.7 ± 3.5 vs. 85.8 ± 3.7 mmHg; $P = 0.0008$), and HR (547.8 ± 20.4 vs. 442.6 ± 31.5 b.p.m.; $P = 0.0001$), as well as abolition of their circadian variation of SBP, as measured by two-way ANOVA between WT and placebo (*Figure 1A*; $P = 0.002$). Rosuvastatin (10 and 20 mg/kg/day) decreased mean values of SBP in DKO (111.1 ± 5.6 and 115.3 ± 5.1 for R10 and R20, respectively; $P = 0.002$) and restored the physiological circadian variation of SBP ($P = 0.02$ and $P = 0.01$ for R10 and R20, respectively, vs. placebo).

Rosuvastatin restored the nitric oxide-dependent control of blood pressure variability and its sensitivity to nitric oxide synthase inhibition in LDLR$^{-/-}$/ObOb mice

Spectral analysis of the 24 h SBP recordings was performed, and the variability of SBP (SBPV) in the very low frequency (VLF) band (0.05–0.4 Hz; reflecting neurohumoral control, including nitric oxide, NO) was measured (*Figure 2A*). Compared with WT mice, the SBPV of placebo DKO mice was higher (62.7 ± 1.3 vs. 54.4 ± 2.1; $P = 0.01$), suggesting an altered neurohumoral control of BP. Treatment with both 10 and 20 mg/kg rosuvastatin resulted in a similarly decreased index of SBPV in the VLF domain in DKO mice compared with the placebo group (44.7 ± 1.9 and 44.6 ± 1.0, respectively, $P = 0.01$). The VLF values of both rosuvastatin-treated groups were even lower than in the WT ($P = 0.01$). To verify the involvement of the NO component in the altered control of variability in the VLF, the different groups of mice were subjected to a pharmacological test with an NO synthase (NOS) inhibitor, and the sensitivity of their variability index in the VLF compared (*Figure 2B*). As expected, acute inhibition of NOS upon injection of the NOS inhibitor L-NAME increased the VLF index in WT mice, as previously shown by us.[18] However, this increase was substantially reduced in the

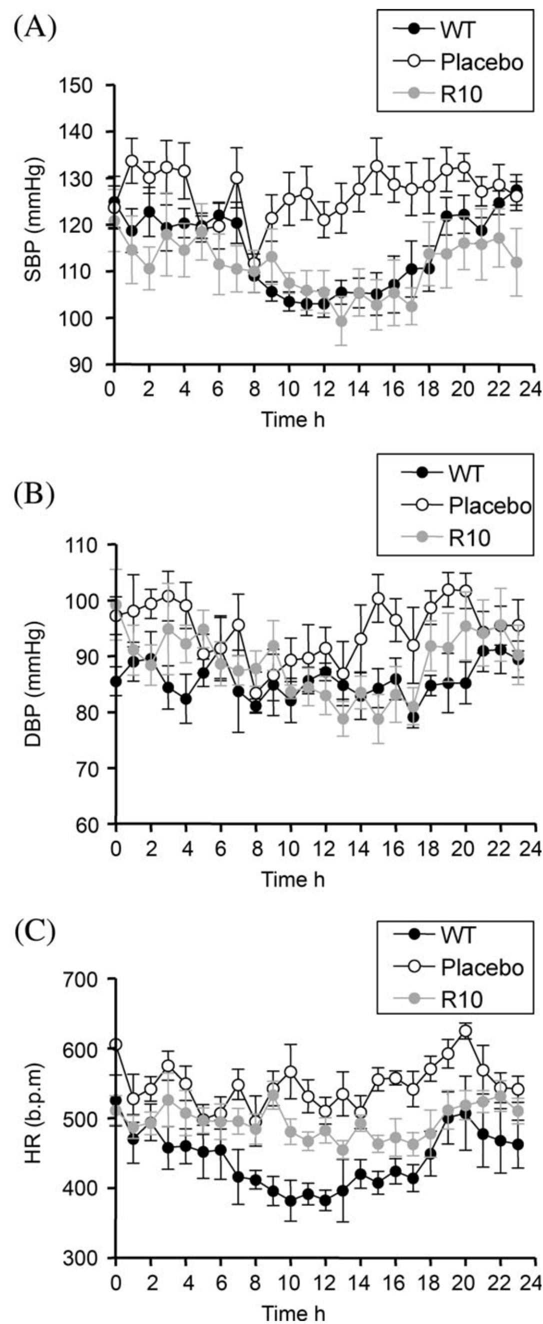

Figure 1 Rosuvastatin corrects circadian variation of blood pressure and heart rate in low-density lipoprotein receptor/ObOb-deficient mice. Circadian variation of blood pressure and heart rate in wild type mice ($n = 10$) and DKO mice treated with placebo for 12 weeks (Placebo, $n = 9$) and DKO mice treated for 12 weeks with 10 mg/kg of rosuvastatin (R10, $n = 6$). Time course of systolic blood pressure (*A*), diastolic blood pressure (*B*), and heart rate (*C*) over 24 h. Shaded areas on the *x*-axis represent dark cycles (activity period in mice). Mean values (±SEM) of systolic blood pressure, diastolic blood pressure, and heart rate were calculated for each 60 min sequence of recording during the 24 h period

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Table 2 Mean, night and day values of systolic blood pressure, diastolic blood pressure, and heart rate in wild type (C57BL/6), DKO mice treated with placebo and rosuvastatin 10 mg/kg/day and 20 mg/kg/day

	WT (n = 10)	DKO placebo (n = 9)	DKO rosuvastatin 10 mg/kg (n = 6)	DKO rosuvastatin 20 mg/kg (n = 7)
SBP (mmHg)				
Mean (24 h)	114.7 ± 2.9	126.7 ± 2.9	111.1 ± 5.6	115.3 ± 5.1
Night	121.8 ± 2.8	127.9 ± 4.9	115.0 ± 3.3	117.8 ± 6.1
Day	107.5 ± 5.2	125.6 ± 5.6	107.1 ± 4.7	108.5 ± 3.7
P-value night vs. day	0.0001	0.29	0.0001	0.0002
DBP (mmHg)				
Mean (24 h)	85.8 ± 3.7	94.7 ± 3.5	88.7 ± 2.7	87.3 ± 3.0
Night	87.5 ± 3.1	96.6 ± 3.5	92.7 ± 3.6	94.9 ± 4.6
Day	84.0 ± 2.3	92.5 ± 5.8	85.0 ± 4.8	86.9 ± 3.1
P-value night vs. day	0.005	0.05	0.0002	0.0001
HR (b.p.m.)				
Mean (24 h)	442.6 ± 31.5	547.8 ± 20.4	495.4 ± 22.7	514.6 ± 12.3
Night	470.5 ± 28.5	553.1 ± 36.8	508.5 ± 14.7	520.9 ± 15.0
Day	414.6 ± 33.1	542.5 ± 28.4	482.4 ± 22.2	506.8 ± 9.7
P-value night vs. day	0.0002	0.44	0.003	0.01

Data are mean ± SD.

placebo DKO mice, suggesting a reduced NO-dependent buffering of their SBPV. Importantly, the L-NAME-sensitivity of the VLF index was restored after treatment with rosuvastatin (at both doses) (*Figure 2B*). These results suggest that the decreased SBP buffering capacity in DKO mice is indeed NO-dependent and that the beneficial effect of rosuvastatin involves restored production (and/or bioavailability) of NO.

Rosuvastatin restored the sympathetic and parasympathetic control of blood pressure and heart rate variability in DKO ($LDLR^{-/-}$/ObOb) mice

Notably, rosuvastatin also partly normalized the higher SBPV in the low frequency domain (LF, 0.4–1.5 Hz; reflective of the sympathetic tone; $P = 0.03$, *Figure 3A*), which was compatible with the decrease in HR as illustrated in *Figure 1C*; moreover, the adrenergic tone was assessed from the HR response after an acute challenge with propranolol. There was a more pronounced decrease in HR in DKO placebo compared with WT mice (−148.9 ± 18.3 vs. −63.5 ± 15.1 b.p.m.; $P = 0.008$), which was compatible with an increased basal sympathetic tone in DKO mice. This response was reduced after rosuvastatin treatment (R20: −97.5 ± 9.9 b.p.m.; $P = 0.03$). Rosuvastatin also partly reversed the decreased variability of HR in the high frequency (HF, 1.5–5.0 Hz; reflective of parasympathetic tone; $P = 0.04$; *Figure 3B*), which suggests a restoration of vagal tone, as assessed from the HR response to an acute challenge with atropine. Atropine induced less elevation in HR in DKO placebo compared with WT mice (61.3 ± 23.9 vs. 141.8 ± 12.0 b.p.m.; $P = 0.04$), confirming the diminished basal control of HR in DKO mice. This reduced control of HR by the parasympathetic nervous system was partly restored after rosuvastatin treatment (R20: 126.2 ± 9.1 b.p.m.; $P = 0.04$).

Rosuvastatin increased PPARγ and SOD1 expression in aortic tissue and endothelial cells

Because of the involvement of the transcription factors PPARs in the control of insulin sensitivity and inflammation, which may determine the development of endothelial dysfunction, the expression of PPARs was examined in extracts of the aortic arch by RT–PCR. Our previous study had shown a decrease of aortic PPARγ and α expression in DKO vs. WT mice.[15] Here, rosuvastatin restored PPARγ expression compared with placebo DKO (R10 and R20: 100%; $P = 0.007$) (*Figure 4A*), but had no significant effect on PPARα expression (R10: 0.62 ± 0.29; R20: 0.49 ± 0.20 vs. placebo: 0.36 ± 0.15; $P = 0.09$ and 0.23, respectively). Rosuvastatin also increased SOD1 expression in aortic tissue.[19]

To ascertain a specific effect on the endothelium, we next examined the effect of rosuvastatin on PPARγ expression in cultured endothelial cells. After 24 h of incubation with rosuvastatin at 10^{-5} mol/L, expression of PPARγ mRNA was significantly increased (70% vs. control; $P = 0.002$) (*Figure 4B*).

We then measured the expression of Cu/Zn SOD (SOD1) as a target gene for PPAR-mediated transcriptional control that may account for the restored NO-dependent endothelial function, as observed *in vivo*. Under the same conditions as earlier, rosuvastatin produced a significant increase in SOD1 mRNA expression (43%; $P = 0.001$) compared with control (*Figure 4C*). This was confirmed with dose-dependent increases in SOD1 protein levels (*Figure 4D*).

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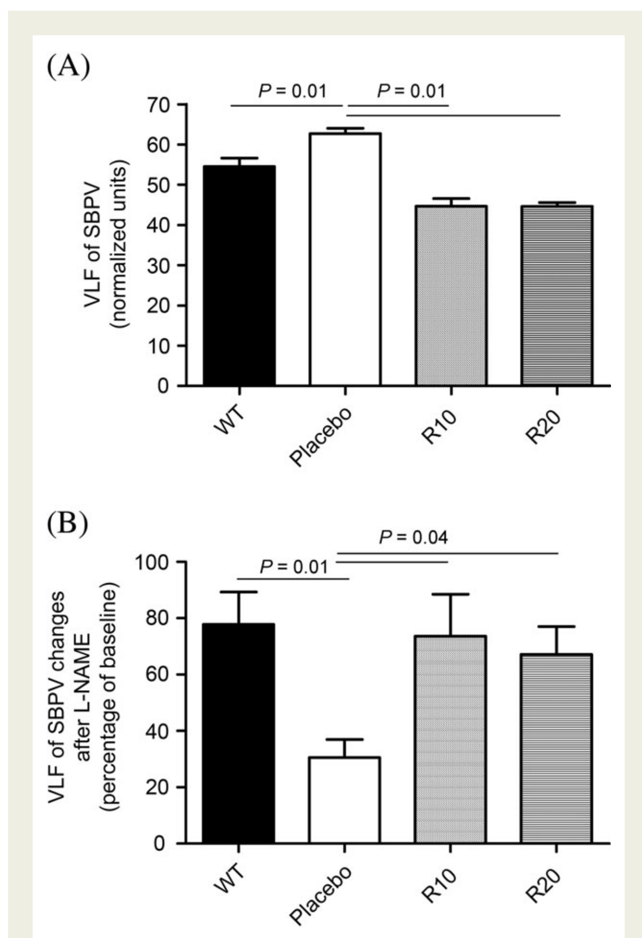

Figure 2 Rosuvastatin corrects nitric oxide-dependent systolic blood pressure variability in low-density lipoprotein receptor/ObOb-deficient mice. Spectral analysis of systolic blood pressure variability in DKO mice treated with placebo (Placebo, $n = 9$) and DKO mice treated for 12 weeks with 10 mg/kg of rosuvastatin (R10, $n = 6$) or 20 mg/kg (R20, $n = 7$) compared with control C57BL6 (WT, $n = 10$). After normalization to whole power spectra, area under the curve for the variability of systolic blood pressure was calculated for each group. Results are presented for specific frequency bands, i.e. very low frequency of systolic blood pressure variability (0.05–0.4 Hz, reflecting neurohumoral control) (*A*) and its increase after acute nitric oxide synthase inhibition using intraperitoneal injection of L-NAME (30 mg/kg) (*B*)

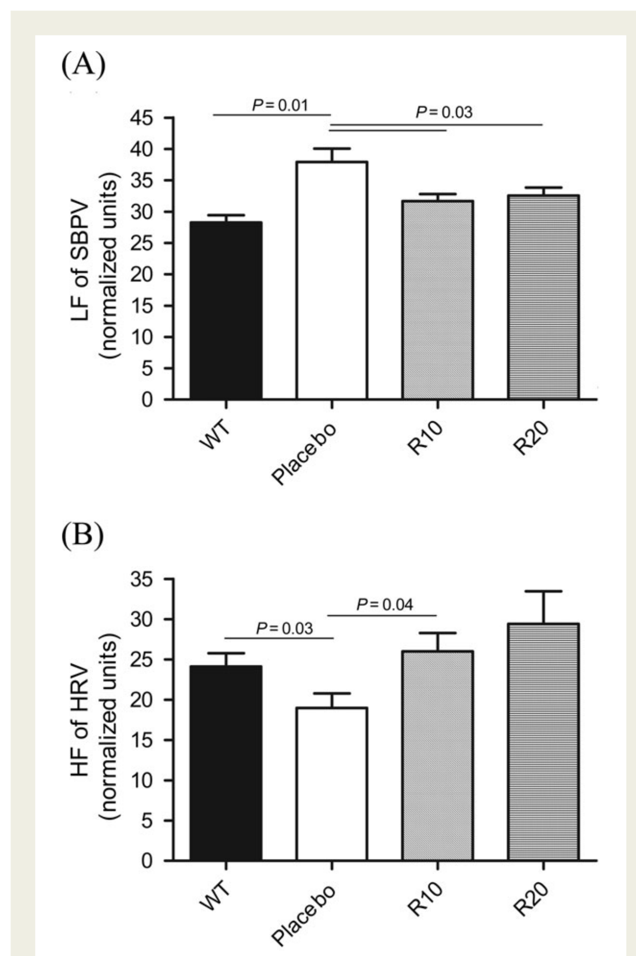

Figure 3 Rosuvastatin corrects autonomic control of blood pressure and heart rate in low-density lipoprotein receptor/ObOb-deficient mice. Spectral analysis of systolic blood pressure variability and heart rate variability in DKO mice treated with placebo (Placebo, $n = 9$) and DKO mice treated for 12 weeks with 10 mg/kg of rosuvastatin (R10, $n = 6$) or 20 mg/kg (R20, $n = 7$) compared with control C57BL6 (WT, $n = 10$). After normalization to whole power spectra, area under the curve for the variability of systolic blood pressure and heart rate was calculated for each group. Results are presented for specific frequency bands, i.e. LF of systolic blood pressure variability (0.4–1.5 Hz, reflective of the adrenergic tone) (*A*) and high frequency of variability (1.5–5 Hz, reflective of parasympathetic tone) (*B*)

To verify that the increase in SOD1 expression was a result of PPARγ transactivation, we also treated cells with GW9662, a PPARγ antagonist.[20] As shown in *Figure 4C*, GW9662 alone had no significant effect on basal levels of SOD1. However, GW9662 abrogated the upregulation of SOD1 in response to rosuvastatin, supporting the causality of PPARγ activation on this effect.

We next assessed the effects of the PPARγ agonist pioglitazone and the PPARα agonist WY14643 on PPARγ and SOD1 expression in endothelial cells. Pioglitazone induced a significant increase in PPARγ expression (73%, $P = 0.04$, again inhibited by GW9662, $P = 0.001$), whereas WY14643 had no effect ($P = 0.25$). However, as shown in *Figure 5A*, both pioglitazone and WY14643 induced an increase in SOD1 expression (although more modest with the PPARα agonist). GW9662, again, selectively inhibited the effect of pioglitazone only.

To gain further proof of the involvement of PPARγ in rosuvastatin-induced SOD1 upregulation, we used two different siRNAs targeting PPARγ. Both siRNAs decreased PPARγ expression (by 66 and 49%, respectively; $P = 0.001$) and abrogated the increase in PPARγ ($P = 0.09$ and 0.21, respectively) as well as SOD1 expression by rosuvastatin (*Figure 5B*).

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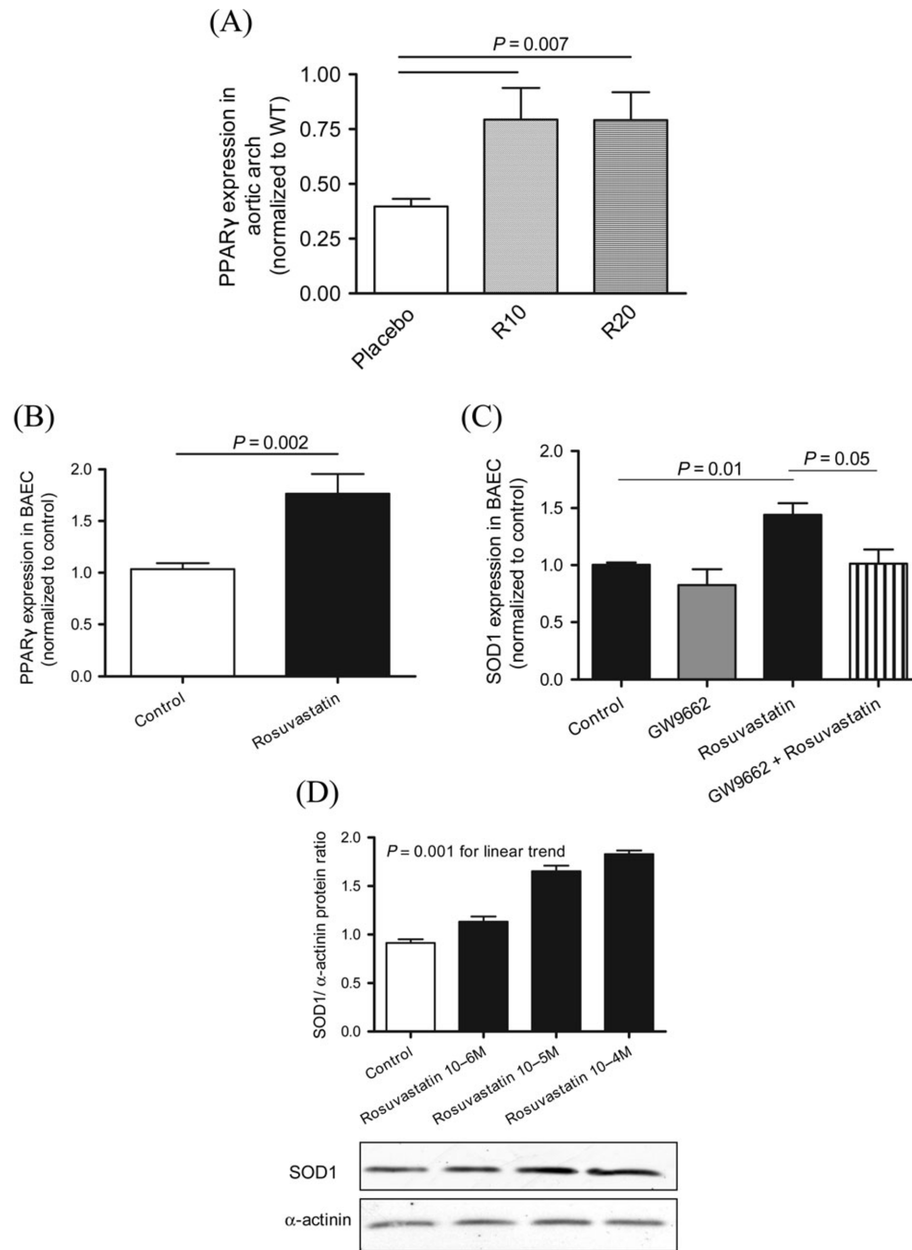

Figure 4 Rosuvastatin increases PPARγ and SOD1 expressions in aortic tissue and aortic endothelial cells. The expression of PPARγ was measured in aortic tissue extracts and compared with placebo ($n = 9$) and rosuvastatin-treated animals (R10, $n = 6$; R20, $n = 7$). Results are normalized to the levels in wild type (C57Bl6) animals (*A*). Bovine aortic endothelial cells were cultured and incubated for a period of 24 h with vehicle ($n = 12$), 10^{-5} mol/L of rosuvastatin ($n = 11$) (*B*), 5×10^{-6} mol/L of GW9662 (PPARγ antagonist, $n = 6$), and 10^{-5} mol/L of rosuvastatin + 5×10^{-6} mol/L of GW9662 ($n = 6$) (*C*). mRNA levels, as measured by real-time polymerase chain reaction (see Methods for technical details) for PPARγ (*B*) and SOD1 (*C*). Results are expressed as a percentage of the control (vehicle) level. Dose-dependent effect of rosuvastatin on SOD1 protein level in bovine aortic endothelial cells ($n = 6$), compared with α-actinin as loading control (*D*)

Rosuvastatin increased PPARγ-dependent transactivation of PPAR-responsive genes

Finally, we analysed the effect of rosuvastatin in HEK293 cells transiently transfected with a luciferase reporter construct containing three copies of the PPRE site of the human apolipoprotein A-II promoter flanking the thymidine kinase promoter (PPRE3-TK-Luc). Co-transfection of plasmids encoding PPARγ or PPARα (at low or high amounts) induced promoter activity, which was robustly enhanced by pioglitazone (PPARγ synthetic agonist) and WY14643 (PPARα synthetic agonist), respectively (Supplementary material online, *Figure S1*). Cell treatment with rosuvastatin

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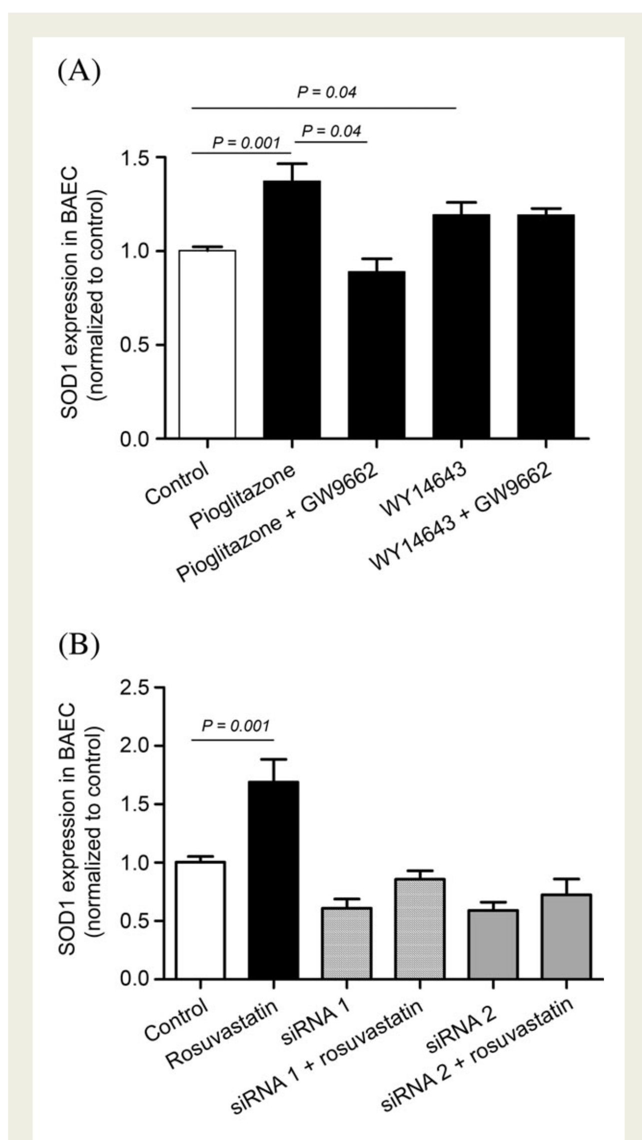

Figure 5 Pioglitazone increases SOD1 expression and PPARγ-targeted siRNA transfection abolishes the upregulation of SOD1 in response to rosuvastatin in aortic endothelial cells. Graphs illustrate mRNA levels, as measured by real-time polymerase chain reaction (see Methods for technical details) for SOD1. (*A*) Bovine aortic endothelial cells were cultured and incubated for a period of 24 h with vehicle ($n = 14$), 10^{-5} mol/L of pioglitazone (PPARγ agonist, $n = 9$) alone or with 5×10^{-6} mol/L of GW9662 (PPARγ antagonist, $n = 6$), 10^{-5} mol/L of WY14643 (PPARα agonist, $n = 6$) alone or with 5×10^{-6} mol/L of GW9662 ($n = 4$). (*B*) Bovine aortic endothelial cells were cultured and transfected with two different siRNAs targeting PPARγ. Cells were then incubated for a period of 24 h with vehicle ($n = 12$ for control, $n = 7$ for siRNA-1, $n = 7$ for siRNA-2) or 10^{-5} mol/L of rosuvastatin ($n = 9$ for control, $n = 9$ for siRNA-1, $n = 9$ for siRNA-2). Results are expressed as a percentage of the control (vehicle) level

enhanced PPARγ-dependent luciferase activity ($P = 0.04$) and had a marginal effect on PPARα transactivation ($P = 0.13$). PPARγ transactivation was again blocked by GW9662 (*Figure 6*).

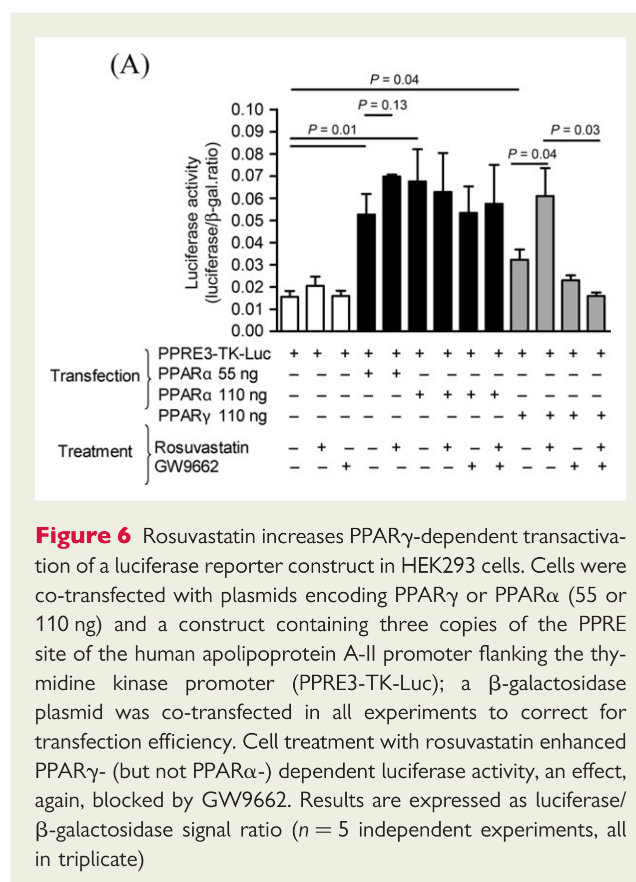

Figure 6 Rosuvastatin increases PPARγ-dependent transactivation of a luciferase reporter construct in HEK293 cells. Cells were co-transfected with plasmids encoding PPARγ or PPARα (55 or 110 ng) and a construct containing three copies of the PPRE site of the human apolipoprotein A-II promoter flanking the thymidine kinase promoter (PPRE3-TK-Luc); a β-galactosidase plasmid was co-transfected in all experiments to correct for transfection efficiency. Cell treatment with rosuvastatin enhanced PPARγ- (but not PPARα-) dependent luciferase activity, an effect, again, blocked by GW9662. Results are expressed as luciferase/β-galactosidase signal ratio ($n = 5$ independent experiments, all in triplicate)

Discussion

The main findings of our study are that in a mouse model of the human metabolic syndrome, rosuvastatin (i) reduced plasma triglycerides and improved insulin sensitivity, with only a marginal effect on total cholesterol; (ii) corrected high BP and NO-dependent SBPV; (iii) increased PPARγ expression in the aortic arch *in vivo* and in cultured endothelial cells *in vitro*, as well as endothelial SOD1, an antioxidant, PPAR-responsive gene; (iv) enhanced PPARγ- (but not PPARα-) transactivation of a PPAR-responsive reporter gene.

Our analysis showed that the development of high BP was paralleled with increases in triglycerides, glucose, and insulin in DKO mice, consistent with the proposed pathophysiological importance of insulin resistance for the development of hypertension in the metabolic syndrome.[6,21] The relationship between insulin resistance and abnormal vascular reactivity has been demonstrated in a variety of clinical conditions, and a reduction in systemic insulin resistance is accompanied by improved endothelial function and vice versa.[4,22] Accordingly, the dose-dependent effect of rosuvastatin on systemic insulin resistance may be part of the explanation for the beneficial effect on BP homeostasis in our model.

However, an important observation in this study is that, even at the lower dose, rosuvastatin completely corrected SBP and SBPV despite incomplete correction of insulin resistance. This suggests that additional mechanisms must be at play, perhaps through direct effects on molecular targets in the central nervous system or the vascular wall.[9] Indeed, our observation of rosuvastatin's

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effects on LF and HF variability (*Figure* 3), reflecting a restoration of the sympathovagal balance, suggests central effects on specific brain nuclei, possibly through inhibition of Rho/Rho-kinase.[23] In addition, our frequency analysis of BP tracings indicates an effect on SBPV in the VLF domain, an index of humoral control of vessel tone, including by the vascular relaxant NO.[24] We and others have previously validated this parameter as reflecting the 'buffering' capacity of NO on SBP in the mouse,[18,24] i.e. decreased production/activity of NO results in increased SBPV, as observed in the untreated DKO mice. Regardless of the dose used, rosuvastatin decreased SBPV. That this effect involved a restoration of NO is confirmed by the comparative sensitivity to acute inhibition of NOS with L-NAME (*Figure 2B*).[18] Rosuvastatin's effect on variability is unlikely to be only the consequence of SBP normalization, because variability was lowered to levels below those observed in wild-type animals (*Figure 2A*) at similar-day SBP levels (*Figure 1A* and *Table* 2). Therefore, a direct effect on vascular NO is more likely to be the cause, rather than the consequence of BP correction.

Several mechanisms may account for such direct vascular effects. In addition to upregulation of endothelial NO synthase (eNOS) expression or activity,[25,26] rosuvastatin may restore vascular NO signalling by increasing NO bioavailability (for a review, see Pelat and Balligand[9]). A prominent factor influencing the latter is the prevailing oxidative stress in the vascular wall. PPARγ exerts well-established anti-inflammatory effects through both transcriptional regulation and trans-repressional effects on key pro-inflammatory and pro-oxidant signalling pathways, such as NF-kappaB.[27,28] Accordingly, rosuvastatin upregulated PPARγ mRNA expression in the aortic arch of our telemetered mice, as well as in isolated endothelial cells (*Figure 4A* and *B*), which are known to express this PPAR isoform.[13] Statins can activate the transcription of PPARγ through a SREBP response element in the PPARγ promoter.[29] However, this does not necessarily translate into increased PPARγ protein abundance (not directly measured here), which was previously shown to be reduced by ligand binding as a result of receptor degradation.[30] This does not exclude activation of PPARγ transcriptional activity by the statin, as illustrated in our transactivation assay. Of note, the expression and activity of endothelial PPARγ have been causally linked with BP regulation independently of changes in systemic insulin sensitivity.[31] Our observations *in vivo* now provide an additional mechanism for the correction of endothelial dysfunction by rosuvastatin beyond its effects on systemic insulin resistance. The fact that, compared with glitazones,[32] rosuvastatin has a more prominent BP-lowering effect suggests that statins probably activate additional mechanisms beyond PPARγ activation.

Among PPAR-regulated genes with known antioxidant effects, we found the copper–zinc-containing SOD (Cu/Zn SOD or SOD1) to be upregulated in isolated endothelial cells (*Figure 4*). This cytosolic enzyme represents the predominant SOD in the vasculature and is abundantly expressed in the endothelium.[12] The *SOD1* gene promoter contains a PPAR-response element that mediates its induction by PPARs and may contribute to the antioxidant effects of PPARγ agonists in the endothelium.[13] Accordingly, both GW9662, a PPARγ-specific antagonist, and cell transfection with siRNA raised against PPARγ abrogated the upregulation of SOD1 in response to rosuvastatin, demonstrating the causal involvement of PPARγ in the upregulation of SOD1 (*Figures 4C* and *5B*). Furthermore, in transactivation assays using co-transfection of plasmids encoding PPARγ or PPARα with a luciferase reporter construct under the control of PPAR-responsive elements, cell treatment with rosuvastatin enhanced PPARγ- (but not PPARα-) dependent luciferase activity, an effect, again, selectively blocked by GW9662. The ensuing protection of NO from scavenging by superoxide anions likely participates in the restoration of NO-dependent endothelial function and BP regulation. SOD1 also protects against oxidant-mediated vascular smooth muscle hyperplasia and hypertrophy, two key pathogenic factors for vascular stiffness that are directly correlated with increased SBPV[17] and were recently shown to be attenuated by pitavastatin in hypercholesterolaemic rabbits.[33] Finally, insulin resistance has recently been shown to be mechanistically linked with the generation of cellular ROS,[4] so rosuvastatin's effect on PPARγ and SOD1 may provide a unifying link for the restoration of both NO-dependent endothelial BP control and endothelial insulin sensitivity.

Previous studies indicate that statins activate PPARα through a molecular mechanism implicating the geranylgeranyl-pyrophosphate pathway and prenylation of Rho family proteins.[34] Moreover, it has been shown that the anti-inflammatory effect of simvastatin occurs via PPARα by a mechanism involving inhibition of PKCα inactivation of PPARα transrepression activity in murine macrophages and neutrophils.[35] As SOD1 was also upregulated by a PPARα agonist in our endothelial cells, rosuvastatin may have exerted some of its effects through PPARα as well; however, rosuvastatin had no effect on PPARα expression in aortic tissue and marginally affected PPARα-mediated transactivation, suggesting a more prominent effect through PPARγ with this statin.

Clinical significance

A recent analysis comparing the effect of high vs. low dose of statin treatment in patients with the metabolic syndrome and stable coronary disease showed a benefit of the higher dose irrespective of the presence of glycaemic abnormalities,[2] suggesting this effect to be at least in part independent of improvements in glucose homeostasis, as in our mouse model. Contrary to this clinical study, where most of the benefit was attributed to incremental cholesterol lowering, rosuvastatin corrected haemodynamics in the absence of major changes in plasma cholesterol in our obese, LDLR-deficient mice, lending support for direct vascular effects of the drug, as suggested for statins in acute coronary syndromes.[36] Our demonstration of endothelial PPARγ and SOD1 upregulation with rosuvastatin, resulting in decreased oxidative stress and improved endothelial dysfunction, adds to the expected benefits in high-risk patients, particularly through the decrease in vascular stiffness and SBPV, both independent predictors of cardiovascular morbidity and mortality.[16,17]

In conclusion, despite incomplete correction of insulin sensitivity, rosuvastatin normalized BP regulation in DKO mice in the absence of major change in plasma cholesterol. The increase in PPARγ expression and activity as well as of SOD1 (a PPAR-responsive gene) observed *in vivo* in aorta and *in vitro* in endothelial

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cells may represent a unique mechanism of vascular protection and BP correction by statin treatment.

Supplementary material

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

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