

Acute simvastatin increases endothelial nitric oxide synthase phosphorylation via AMP-activated protein kinase and reduces contractility of isolated rat mesenteric resistance arteries

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A B S T R A C T

Statins can have beneficial cholesterol-independent effects on vascular contractility, which may involve increases in the bioavailability of NO (nitric oxide) as a result of phosphorylation of eNOS (endothelial NO synthase). Although this has been attributed to phosphorylation of Akt (also known as protein kinase B), studies in cultured cells have shown that statins can phosphorylate AMPK (AMP-activated protein kinase); it is unknown whether this has functional effects in intact arteries. Thus we investigated the acute effects of simvastatin on resistance arterial contractile function, evaluating the involvement of NO, Akt and AMPK. Isolated rat mesenteric resistance arteries were mounted on a wire myograph. The effects of incubation (1 and 2 h) with simvastatin (0.1 or 1 μ M) on contractile responses were examined in the presence and absence of L-NNA (*N*-nitro-L-arginine; 10 μ M) or mevalonate (1 mM). Effects on eNOS, phospho-eNOS (Ser¹¹⁷⁷), and total and phospho-Akt and -AMPK protein expression were investigated using Western blotting. The effect of AMPK inhibition (compound C, 10 μ M) on eNOS phosphorylation and contractile responses were also studied. Simvastatin (1 μ M, 2 h) significantly reduced constriction to U46619 and phenylephrine and enhanced dilations to ACh (acetylcholine) in depolarized, but not in U46619-pre-constricted arteries. These effects were completely and partially prevented by L-NNA and mevalonate respectively. Simvastatin increased eNOS and AMPK α phosphorylation, but had no effect on Akt protein expression and phosphorylation after 2 h incubation. Compound C prevented the effects of simvastatin on eNOS phosphorylation and contractility. Thus simvastatin can acutely modulate resistance arterial contractile function via mechanisms that involve the AMPK/phospho-eNOS (Ser¹¹⁷⁷)/NO-dependent pathway.

INTRODUCTION

Statins are some of the most commonly prescribed drugs in clinical practice. Statins are widely used

in the treatment of hypercholesterolaemia as they reduce the biosynthesis of cholesterol by inhibiting the enzyme HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase and blocking the conversion of

Key words: acetylcholine, AMP-activated protein kinase, nitric oxide, resistance artery, simvastatin, statin.

Abbreviations: ACh, acetylcholine; AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; eNOS, endothelial NO synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; L-NNA, *N*-nitro-L-arginine; PSS, physiological salt solution; KPSS, potassium PSS; SNP, sodium nitroprusside.

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HMG-CoA into mevalonate. Numerous studies have shown that statin treatment significantly reduces cardiovascular-related morbidity and mortality in patients both with and without coronary artery disease [1,2]; however, it is now apparent that their beneficial effects on the cardiovascular system extend beyond their lipid-lowering actions. These additional effects have led to the recent consideration of statins for the primary prevention of cardiovascular disease in patients without elevated cholesterol [3,4].

The majority of the cholesterol-independent or pleiotropic effects are attributable to effects on endothelial function and, specifically, increases in the bioavailability of endothelium-derived NO [5–7]. NO is essential for the maintenance of cardiovascular health and has numerous beneficial effects including the regulation of arterial tone and thus blood pressure. The pleiotropic effects of statins are rapid with improved large arterial endothelium-dependent dilation being noted within 3 h of a single dose of statin in patients with normal serum cholesterol levels [7]. Decreases in NO availability are also observed within hours of simvastatin withdrawal [8]. The bioavailability of NO may be modulated in a variety of different ways, but post-translational phosphorylation of eNOS (endothelial NO synthase) appears to be particularly important for acute regulation of NOS activity in endothelial cells [9]. The most widely studied phosphorylation pathway involves the serine/threonine Akt (also known as protein kinase B), which can phosphorylate eNOS on Ser¹¹⁷⁷, resulting in activation of the enzyme and increased production of NO [10,11]. Statin modulation of eNOS activity via this pathway has been demonstrated in cultured endothelial cells, although this is not a uniform finding [12,13]. AMPK (AMP-activated protein kinase) is another serine/threonine kinase that has long been known to play a central role in the regulation of energy homeostasis [14]. It is now evident that AMPK may modulate multiple physiological and pathophysiological pathways including enhancement of eNOS activity by phosphorylation [15–17]. Studies in animal models have shown that acute infusion of AICAR (5-amino-4-imidazolecarboxamide riboside; a selective experimental AMPK activator) induces resistance arterial dilation [18]. Given the beneficial effects of AMPK on both metabolism and endothelial function there has been a recent surge of interest in identification of potential clinical activators of the enzyme. Rapid AMPK-dependent activation of eNOS has been demonstrated in both cultured endothelial cells and in mouse aorta following *in vivo* statin (atorvastatin and pravastatin) treatment [19,20], but it is unknown whether this may contribute to the acute effects of statins on arterial reactivity.

The aim of the present study was thus to investigate the acute effects of simvastatin, one of the most widely used and cost-effective statins prescribed in clinical practice, on

resistance arterial function, evaluating the involvement of NO, Akt and AMPK.

MATERIALS AND METHODS

Animals

All studies were performed on tissues isolated from male Wistar rats (200–250 g, $n = 57$). Animals were housed under a 12 h light/12 h dark cycle and had unlimited access to food and water. Rats were killed by stunning and cervical dislocation. The mesenteric vascular bed was removed and placed in ice-cold PSS (physiological salt solution) (119 mM NaCl, 4.7 mM KCl, 2.4 mM MgSO₄ · 7H₂O, 25 mM NaHCO₃, 1.18 mM KH₂PO₄, 0.07 mM EDTA, 6.05 mM glucose and 1.6 mM calcium at pH 7.4). All studies were carried out in accordance with the United States NIH guidelines [Guide for the Care and Use of Laboratory Animals (1985), DHEW Publication No. (NIH) 85-23: Office of Science and Health Reports, DRR/NIH, Bethesda, MD, U.S.A.], the University of Manchester Animal Experimentation Guidelines, and the U.K. Animals (Scientific Procedures) Act 1986.

Drugs and solutions

Unless otherwise stated, all drugs were obtained from Sigma, except U46619 which was obtained from Calbiochem. A 10 mM stock solution of U46619 was made by dissolving in a mixture of 100% ethanol and 1 mg/kg Na₂CO₃ (1:2). 10 mM stock solution of mevalonic acid lactone, simvastatin and compound C were dissolved in DMSO (Merck). All other drugs were dissolved in PSS.

Myography

Third-/fourth-order mesenteric arteries were dissected out, cleared of fat and connective tissue, cut into small segments (2–4 mm) and mounted in the chamber of a wire myograph (Danish Myo Technology A/S) [21]. After mounting, tissues were equilibrated for 30 min in PSS (gassed with 20% O₂ and 5% CO₂ at 37°C) and normalized as previously described [21]. Isometric tension development was continuously recorded (Myo-DAQ data acquisition system). Contractile viability was assessed by two exposures to high K⁺ solution (modified PSS containing 120 mM KCl iso-osmotically substituted for NaCl). Contraction induced by 120 mM KPSS (potassium PSS; identical with PSS, except with sodium replaced by potassium on an equimolar basis) in the control group was 3.23 ± 0.17 mM/mm ($n = 31$) compared with 3.14 ± 0.16 mM/mm ($n = 33$) in the group that was subsequently exposed to simvastatin ($P > 0.05$). Functional endothelial integrity was assessed by determining relaxation to the endothelium-dependent dilator ACh (acetylcholine; 10 μM) in thromboxane mimetic 9,11-dideoxy-11α,9α-epoxy methanoprostaglandin (U46619,

1 μM) pre-contracted rings; only the rings that relaxed more than 80% to 10 μM ACh were included.

Cumulative concentration–response curves were constructed to U46619 (0.1 nM–1 μM) following incubation with either 0.1 μM or 1 μM simvastatin for 1 or 2 h. These concentrations are similar to plasma concentrations in patients treated with therapeutic doses of statins [22,23]. The influence of simvastatin on contractile responses to the α -adrenergic agonist phenylephrine (0.1 nM–1 μM) was also investigated. Time-control experiments were performed in parallel. Cumulative concentration–response curves to ACh (1 nM–10 μM) and the endothelium-independent dilator SNP (sodium nitroprusside; 1 nM–100 μM) were also constructed in arteries pre-constricted with U46619. In the absence of ACh or SNP, U46619 produced sustained contractions.

HMG-CoA reductase catalyses the production of mevalonate from HMG-CoA. To determine whether the acute effects of simvastatin were dependent on the mevalonate pathway, arteries were incubated with mevalonate (1 mM) for 2 h in both the absence and presence of 1 μM simvastatin. Post-incubation, concentration–response curves to U46619 and ACh were constructed (see above).

To determine the role of NO in the acute effects of simvastatin on contractile function, responses to U46619 and to ACh were examined after incubation for 2 h with either L-NNA (*N*-nitro-L-arginine; 10 μM) alone or in combination with simvastatin (1 μM). To further investigate the involvement of NO, the influence of simvastatin (1 μM , 2 h) on responses to ACh was investigated in arteries pre-constricted by 80 mM KPSS. We have previously shown that relaxation to carbachol is completely blocked by L-NNA in depolarized rat mesenteric arteries [24].

The influence of simvastatin (1 μM , 2 h) on contractile responses to U46619 was also examined in the presence of the AMPK inhibitor compound C {6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo-[1,5-a]pyrimidine} (10 μM) [19].

Western blot analysis

Isolated resistance mesenteric arteries were incubated in PSS either in the presence or absence of simvastatin (1 μM) for 2 h. In some experiments, arteries were also incubated with the AMPK inhibitor compound C (10 μM) [21] both in the presence and absence of simvastatin (1 μM) for 2 h. Following incubation, arteries were immediately frozen until required. Arteries were then homogenized in lysis buffer containing [RIPA lysis buffer (Upstate) containing protease inhibitor cocktail (1:5000 dilution; Sigma), 100 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium orthovanadate and 10 mmol/l PMSF]. Protein extracts (75 μg) were separated by SDS/PAGE (7.5% gel) and then transferred on to PVDF membranes

(Amersham) using a Mini Trans-Blot Cell system (Bio-Rad) (overnight, 40°C) containing transfer buffer (25 mmol/l Tris, 190 mmol/l glycine, 20% methanol and 0.05% SDS) as described previously [25].

After blockade of non-specific sites with 5% non-fat dried milk, membranes were incubated overnight at 4°C with the primary antibody against mouse anti-eNOS (1:1000 dilution; Transduction Laboratories), rabbit anti-phospho-eNOS (Ser¹¹⁷⁷) (1:1000 dilution; Cell Signaling), rabbit anti-Akt-1/-2/-3 (1:400 dilution; Santa Cruz Biotechnology), rabbit anti-phospho-Akt-1/-2/-3 (Ser⁴⁷³) (1:400 dilution; Santa Cruz Biotechnology), rabbit anti-phospho-Akt-1/-2/-3 (Thr³⁰⁸) (1:500 dilution; Santa Cruz Biotechnology), rabbit anti-AMPK α (1:500 dilution; Santa Cruz Biotechnology) or rabbit anti-phospho-AMPK α (Thr¹⁷²) (1:1000 dilution; Cell Signaling). After washing, membranes were incubated for 2 h at room temperature with an anti-(mouse IgG) antibody (1:1500 dilution; Bio-Rad) for eNOS and an anti-(rabbit IgG) antibody for phospho-eNOS (1:1500 dilution), Akt-1/-2/-3, phospho-Akt-1/-2/-3 (at Ser⁴⁷³ and Thr³⁰⁸), AMPK α and phospho-AMPK α (Thr¹⁷²) (1:5000 dilution; Jackson ImmunoResearch). Membranes were thoroughly washed, and immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECLPlus; Amersham) and autoradiography (Hyperfilm ECL; Amersham). The same membrane was used to determine α -actin protein expression as an internal control using a monoclonal mouse anti- α -actin antibody (1:30 000 dilution; Sigma).

Immunoblot signals were quantified using Scion Image. All densitometric raw data values were normalized to α -actin expression in the same sample run on the same gel/membrane. This permitted the ratio of the amounts of each protein of interest, relative to α -actin, to be calculated and directly compared under each experimental condition.

Data analysis and statistical procedures

U46619 or phenylephrine-induced contractions were normalized to the contraction of each tissue to KPSS (120 mM). Dilatory responses to ACh or SNP were normalized to the maintained U46619 or KCl-induced contraction. The maximum effect (E_{max}) and the logarithm of the concentration of agonist that produced half of the E_{max} (log EC₅₀) were calculated using non-linear regression analysis (GraphPad Prism Software).

All values are expressed as means \pm S.E.M. for the number of animals used in each experiment. Results were analysed using a two-way ANOVA for comparison between groups or an unpaired Student's *t* test when appropriate. When ANOVA showed a significant treatment effect, Bonferroni's post-hoc test was used to compare individual means. Differences were considered statistically significant at $P < 0.05$.

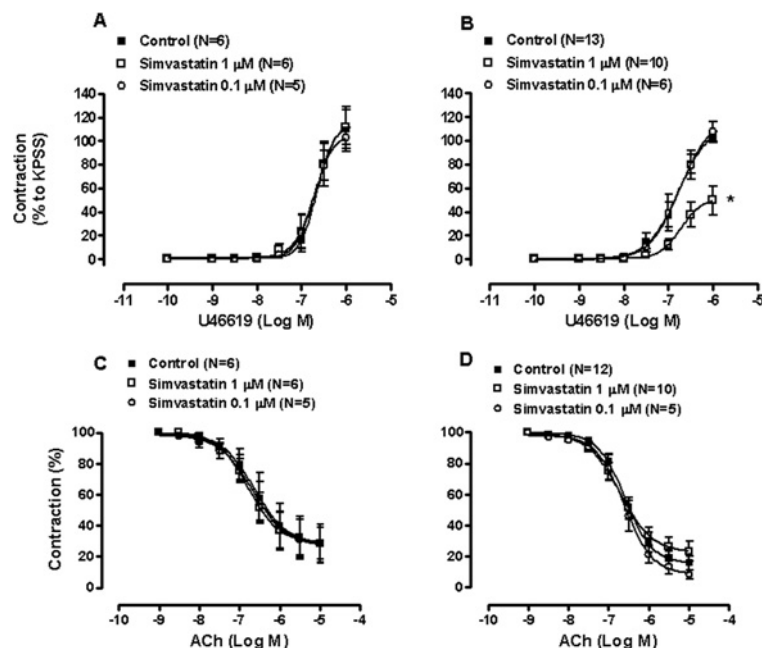


Figure 1 Effect of incubation with simvastatin (0.1 and 1 μ M) for 1 (A and C) or 2 (B and D) h on contractile responses of isolated rat mesenteric small arteries to U46619 and ACh

Contractile responses to U46619 are normalized to the contractile response of each artery to KPSS (120 mM). Relaxation responses to ACh are normalized to the maximum contractile response of each artery to U46619. Values are means \pm S.E.M. (N = number of animals). * P < 0.05 compared with control, using a two-way ANOVA.

RESULTS

Effects of acute incubation with simvastatin on contractile responses of isolated rat mesenteric arteries

Incubation of arteries for 1 h with either 0.1 or 1 μ M simvastatin had no significant effect on U46619-induced contraction ($n=5$ and 6 respectively; Figure 1A). Incubation of arteries for 2 h with 0.1 μ M simvastatin was similarly ineffective (Figure 1B). However, 2 h incubation with 1 μ M simvastatin significantly reduced U46619-induced constriction (Figure 1B), but not arterial sensitivity (as evidenced by log EC₅₀ values) to U46619 (Table 1). In line with the results obtained with U46619, 2 h incubation with 1 μ M simvastatin also reduced contractile responses to phenylephrine, but had no effect on sensitivity [E_{\max} , 96.9 \pm 7.10% contraction to KPSS ($n=4$) for Control compared with 68.2 \pm 12.57% contraction to KPSS for Simvastatin ($n=4$) (P < 0.05, using a Student's t test); -6.28 ± 0.06 for control compared with -6.23 ± 0.06 for Simvastatin; (P > 0.05, using a Student's t test)].

Incubation with simvastatin (0.1 or 1 μ M; 1 or 2 h) had no significant effect on dilations to ACh or SNP in arteries pre-constricted with U46619 (Figures 1C and 1D, and Table 2).

Table 1 Changes in the maximal response (R_{\max}) and sensitivity (log EC₅₀) to U46619 in mesenteric resistance arteries incubated for 1 or 2 h with DMSO (control) and simvastatin (0.1 or 1 μ M)

* P < 0.05 compared with control (ANOVA)

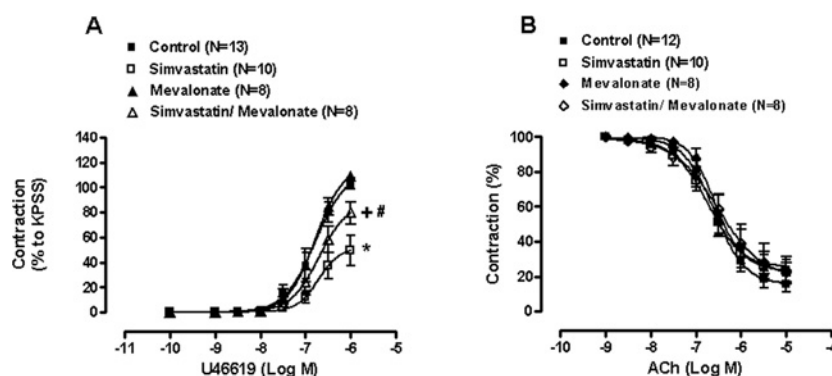
Treatment	R_{\max} (% to KPSS)	log EC ₅₀
1 h		
Control	114 \pm 18.5	-6.61 ± 0.06 ($n=6$)
Simvastatin 0.1 μ M	104 \pm 9.00	-6.71 ± 0.11 ($n=5$)
Simvastatin 1 μ M	116 \pm 15.0	-6.65 ± 0.09 ($n=6$)
2 h		
Control	106 \pm 4.24	-6.85 ± 0.11 ($n=13$)
Simvastatin 0.1 μ M	112 \pm 6.93	-6.80 ± 0.15 ($n=6$)
Simvastatin 1 μ M	52 \pm 12.5*	-6.70 ± 0.04 ($n=10$)

Effects of mevalonate

Incubation with mevalonate (1 mM) alone had no effect on contractions to U46619 ($n=8$) (Figure 2A). However, the reduction in contraction to U46619 observed after 2 h incubation with simvastatin (1 μ M) was partially restored following concomitant incubation with mevalonate plus simvastatin ($n=8$) (Figure 2A). Arterial sensitivity to U46619 was unaffected (Table 3). Mevalonate had no

Table 2 Changes in the maximal response (R_{max}) and sensitivity ($\log EC_{50}$) to ACh and SNP in mesenteric resistance arteries incubated for 1 or 2 h with DMSO (control) and simvastatin (0.1 or 1 μ M)

Treatment	ACh		SNP	
	R_{max} (%)	$\log EC_{50}$	R_{max} (%)	$\log EC_{50}$
1 h				
Control	71 \pm 12.1	- 6.51 \pm 0.18 (<i>n</i> = 6)	79 \pm 6.56	- 6.74 \pm 0.15 (<i>n</i> = 6)
Simvastatin 0.1 μ M	71 \pm 12.4	- 6.63 \pm 0.19 (<i>n</i> = 5)	84 \pm 7.97	- 6.38 \pm 0.50 (<i>n</i> = 5)
Simvastatin 1 μ M	72 \pm 10.3	- 6.65 \pm 0.16 (<i>n</i> = 6)	89 \pm 1.37	- 6.74 \pm 0.12 (<i>n</i> = 6)
2 h				
Control	85 \pm 4.30	- 6.53 \pm 0.10 (<i>n</i> = 12)	83 \pm 4.32	- 6.38 \pm 0.19 (<i>n</i> = 13)
Simvastatin 0.1 μ M	92 \pm 3.07	- 6.60 \pm 0.17 (<i>n</i> = 5)	87 \pm 2.71	- 6.37 \pm 0.12 (<i>n</i> = 6)
Simvastatin 1 μ M	79 \pm 5.12	- 6.69 \pm 0.12 (<i>n</i> = 10)	83 \pm 3.96	- 6.65 \pm 0.16 (<i>n</i> = 10)

**Figure 2** Effect of incubation with mevalonate (1 mM) on responses to (A) U46619 and (B) ACh (in tissues pre-constricted with U46619) in both the presence and absence of simvastatin (1 μ M for 2 h)

Contractile responses to U46619 are normalized to the contractile response of each artery to KPSS (120 mM). Relaxant responses to ACh are normalized to the maximum contractile response of each artery to U46619. Values are means \pm S.E.M. (*N* = number of animals). ANOVA two-way; **P* < 0.05 compared with control; + *P* < 0.05 compared with mevalonate; and # *P* < 0.05 compared with simvastatin, using a two-way ANOVA.

Table 3 Changes in the maximal response (R_{max}) and sensitivity ($\log EC_{50}$) to U46619 in mesenteric resistance arteries incubated for 2 h with DMSO (control), simvastatin 1 μ M, L-NNA (10 μ M) or mevalonate (10 mM) in the absence or presence of simvastatin (1 μ M)

**P* < 0.05 compared with control; + *P* < 0.05 compared with simvastatin (ANOVA).

Treatment	R_{max} (% to KPSS)	$\log EC_{50}$
Control	106 \pm 4.24	- 6.85 \pm 0.11 (<i>n</i> = 13)
Simvastatin	52 \pm 12.5*	- 6.70 \pm 0.04 (<i>n</i> = 10)
Mevalonate	111 \pm 3.01	- 6.86 \pm 0.13 (<i>n</i> = 8)
Mevalonate/simvastatin	83 \pm 8.25* +	- 6.72 \pm 0.12 (<i>n</i> = 8)
L-NNA	111 \pm 6.92	- 6.98 \pm 0.16 (<i>n</i> = 7)
L-NNA/simvastatin	103 \pm 10.3	- 6.98 \pm 0.13 (<i>n</i> = 8)

effect on responses to ACh either in the presence or absence of simvastatin (Figure 2B).

Involvement of NO, Akt and AMPK

Incubation with L-NNA had no significant effect on contraction to U46619 (*n* = 7) (Figure 3A). However, 2 h co-incubation of L-NNA with 1 μ M simvastatin completely blocked the effects of simvastatin on U46619-induced contraction (*n* = 8; Figure 3A and Table 3). Arterial sensitivity to U46619 did not change among groups (Table 3). The dilatory response to ACh in both the presence (*n* = 8) and absence (*n* = 7) of simvastatin was reduced by L-NNA, although this only reached a level of significance in the presence of the statin (Figure 3B). Arterial sensitivity to ACh was unaffected (results not shown).

To investigate further the effects of simvastatin (1 μ M for 2 h) on NO, concentration–response curves to ACh were also constructed in depolarized tissues (80 mM KPSS). ACh-induced relaxation was reduced in depolarized arteries (*n* = 6) when compared with that seen in U46619 pre-constricted arteries (*n* = 12; Figure 3C). Simvastatin (1 μ M, 2 h) significantly

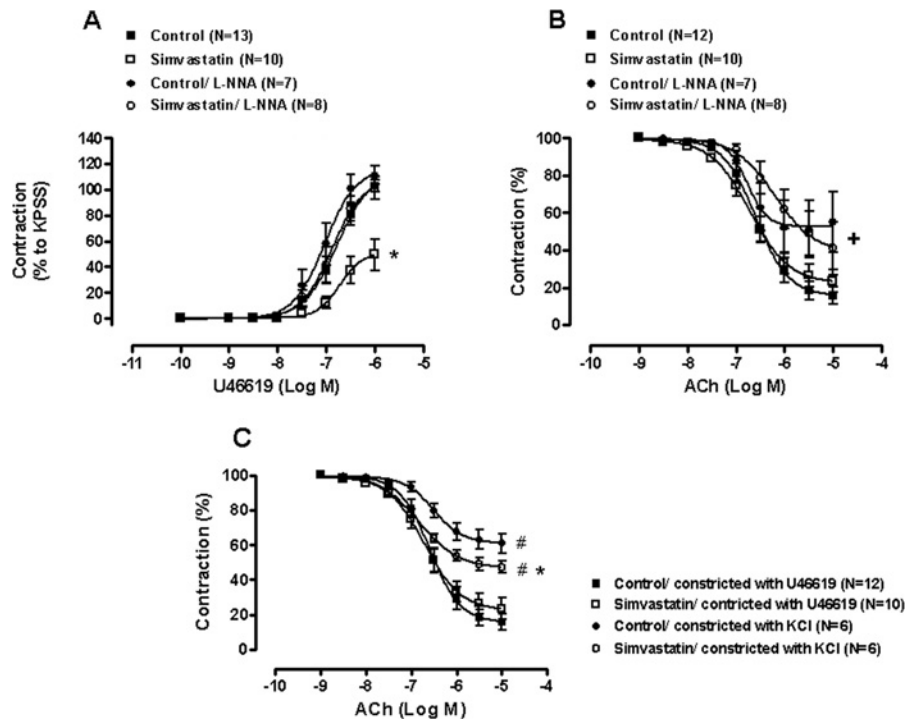


Figure 3 Involvement of NO in acute functional effects of simvastatin in isolated rat mesenteric arteries

The effect of 2 h incubation with L-NNA (10 μ M) on responses of isolated rat mesenteric small arteries to (A) U46619 and (B) ACh (in tissues pre-constricted with U46619) in both the presence and absence of simvastatin (1 μ M for 2 h). (C) Comparison of the effects of 2 h incubation with 1 μ M simvastatin on responses to ACh when pre-constricted with U46619 (10 μ M) or KCl (80 mM). Values are means \pm S.E.M. (N = number of animals). * P < 0.05 compared with Control; + P < 0.05 compared with L-NNA; and # P < 0.05 compared with arteries pre-constricted with U46619, using a two-way ANOVA.

enhanced dilations of depolarized vessels to ACh (n = 6; Figure 3C).

Western blotting experiments showed that incubation with simvastatin (1 μ M for 2 h) increased the phosphorylation of eNOS on Ser¹¹⁷⁷ (Figure 4A), but did not modify eNOS protein expression [eNOS/ α -actin protein expression, 0.93 ± 0.10 in control compared with 0.86 ± 0.10 with Simvastatin (n = 5); P > 0.05, using a Student's t test]. Phosphorylation of Akt-1/-2/-3 on Ser⁴⁷³ or on Thr³⁰⁸ was not significantly modified by simvastatin (Figures 4C and 4D). Similarly, Akt-1/-2/-3 protein expression was unaffected [Akt/ α -actin protein expression, 0.73 ± 0.09 in Control compared with 0.86 ± 0.10 with Simvastatin (n = 8); P > 0.05, using a Student's t test]. However, simvastatin significantly increased phosphorylation of AMPK α on Thr¹⁷² (Figure 4B), without any changes in AMPK α protein expression [AMPK α / α -actin protein expression, 0.65 ± 0.12 in Control compared with 0.71 ± 0.04 with Simvastatin (n = 5); P > 0.05, using a Student's t test]. There were no changes in α -actin protein expression between groups (results not shown).

Co-incubation of arteries with the AMPK inhibitor compound C (10 μ M) with simvastatin (1 μ M, 2 h) completely blocked the enhanced eNOS phosphorylation at Ser¹¹⁷⁷ seen with simvastatin alone (Figure 5A).

Similar effects were seen with the vascular reactivity experiments; in the presence of the AMPK inhibitor compound C (10 μ M) and simvastatin (1 μ M), U46619-induced contractions were similar to those seen with compound C alone (Figure 5B). Compound C alone did have some effect on contractility to U46619 (E_{max} , 117 ± 8.05 % of contraction to KPSS for control and 82 ± 12.2 % of contraction to KPSS for compound C), but simvastatin had no additional effect in the presence of compound C (E_{max} , 71 ± 8.16 % contraction to KPSS).

DISCUSSION

In the present study, we have clearly demonstrated that short-term incubation with simvastatin can directly modulate the contractility of isolated rat mesenteric small arteries via a NO-dependent mechanism that is associated with phosphorylation of AMPK and not Akt 2 h after incubation. Simvastatin had no further effect on contractility when AMPK was inhibited with compound C. Although the emphasis of most previous studies has been improvement of endothelial function, in the present study endothelial function was not compromised. Here the predominant effect of simvastatin was a NO-dependent reduction in contractile

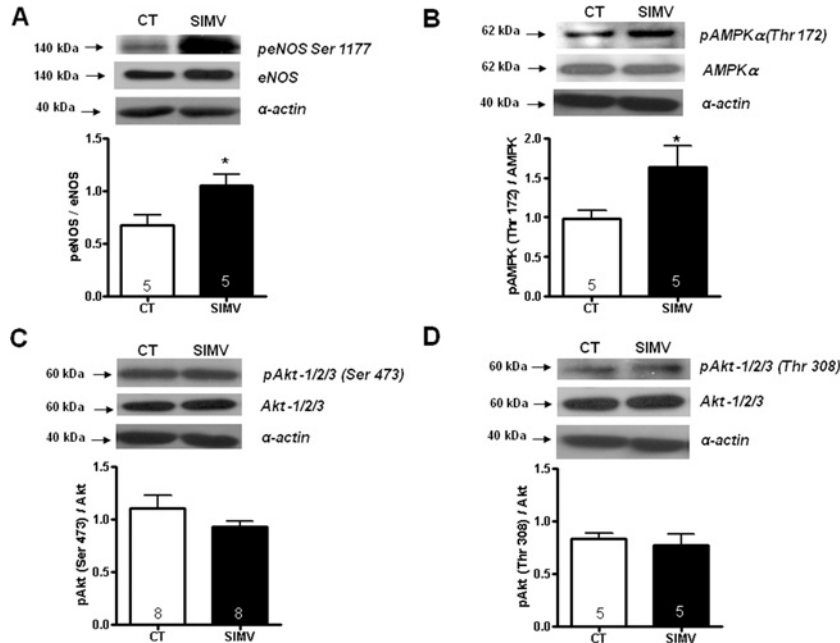


Figure 4 Effects of 2 h incubation of rat mesenteric arteries with 1 μM simvastatin (SIMV) on phosphorylation of (A) eNOS (Ser¹¹⁷⁷) (B), AMPK α (Thr¹⁷²) (C), Akt-1/-2/-3 (Ser⁴⁷³) and (D) Akt-1/-2/-3 (Thr³⁰⁸)

Upper panel: representative blot for phospho-eNOS (Ser¹¹⁷⁷) and eNOS (A), phospho-AMPK α (Thr¹⁷²) and AMPK α (B), phospho-Akt-1/-2/-3 (Ser⁴⁷³) (C) or phospho-Akt-1/-2/-3 (Thr³⁰⁸) (D) and Akt-1/-2/-3 protein expression in mesenteric resistance arteries incubated with simvastatin (SIMV) or vehicle (CT). Lower panel: densitometric analysis of the ratio for phospho-eNOS (Ser¹¹⁷⁷) and eNOS (A), phospho-AMPK α (Thr¹⁷²) and AMPK α (B), phospho-Akt-1/-2/-3 (Ser⁴⁷³) (C) and phospho-Akt-1/-2/-3 (Thr³⁰⁸) (D) and Akt-1/-2/-3 protein expression. Phosphorylation of eNOS was significantly increased in the presence of simvastatin. Simvastatin had no effect on Akt phosphorylation at either site (Ser⁴⁷³ or Thr³⁰⁸), but significantly increased phosphorylation of AMPK α (Thr¹⁷²). Results are normalized to the expression of α -actin. Values are means \pm S.E.M. (number of animals used is indicated on the bars). * $P < 0.05$ compared with control using a Student's t test. CT, control.

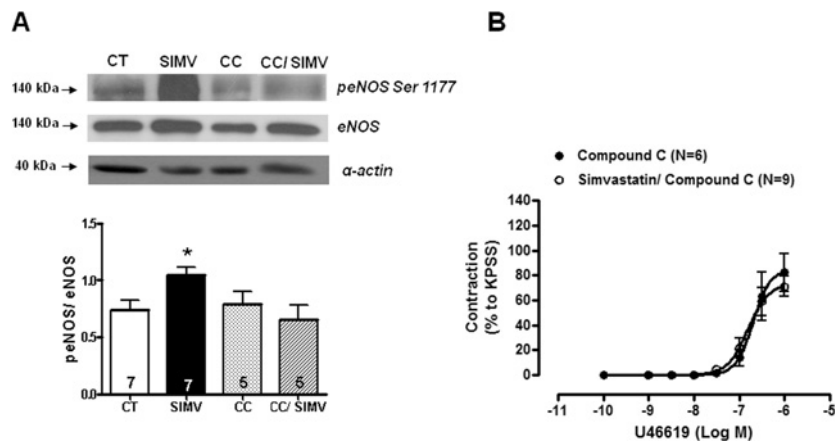


Figure 5 Effects of the AMPK inhibitor compound C on responses to acute simvastatin (SIMV)

(A) The effect of the AMPK inhibitor compound C (10 μM) on enhanced phosphorylation of eNOS at Ser¹¹⁷⁷ induced by 2 h incubation with simvastatin 1 μM . Upper panel: representative blot for phospho-eNOS (Ser¹¹⁷⁷) and eNOS protein expression in mesenteric resistance arteries incubated with vehicle (CT), simvastatin (SIMV), compound C (CC) or compound C plus simvastatin (CC/SIMV). Bottom panel: densitometric analysis of the ratio for phospho-eNOS (Ser¹¹⁷⁷) and eNOS. Results are normalized to the expression of α -actin. Values are means \pm S.E.M. (number of animals used is indicated on the bars). * $P < 0.05$ compared with control using a Student's t test. (B) The effect of incubation with compound C (10 μM) on responses to U46619 in the presence and absence of simvastatin (1 μM for 2 h). Contractile responses to U46619 are normalized to the contractile response of each artery to KPSS (120 mM). Values are means \pm S.E.M. (N = number of animals).

responsiveness with enhancement of endothelium-dependent dilation to ACh only evident in tissues constricted by depolarization but not in those pre-constricted with U46619. In depolarized tissues, in the absence of EDHF (endothelium-derived hyperpolarizing factor) action, maximal dilation to ACh was less than that seen in U46619-pre-constricted tissues; as such there is the capacity for enhancement of dilation. We have previously shown that dilation of depolarized arteries to ACh is entirely due to the release of NO [24] supporting the notion that acute simvastatin enhanced NO bioavailability.

Although it has been known for some time that statins may directly modulate NO bioavailability and NO-dependent vascular function, the mechanisms underlying these effects appear multiple and may depend on various factors, including the type, dose and duration of statin exposure. These mechanisms may include changes in the expression of eNOS mRNA and protein [26–28], reductions in NO breakdown [29] and, of particular importance in endothelial cells, post-translational modification of eNOS activity by phosphorylation [9–13,30,31]. It is well established that phosphorylation of eNOS at Ser¹¹⁷⁷ increases its activity [9]. The most widely studied eNOS phosphorylation pathway involves the serine/threonine Akt and it is widely accepted that activation of Akt by phosphorylation (Ser⁴⁷³) may also modulate vascular contractility in an eNOS-dependent manner [10,32,33]. Previous studies have shown that treatment of cultured endothelial cells with simvastatin causes rapid phosphorylation of both Akt and eNOS increasing the activity of the latter [11–13]. Such effects are thought to contribute to the acute effects of cerivastatin on endothelial function in isolated rat aorta [34]. However, in the present study, no Akt phosphorylation at either Ser⁴⁷³ or Thr³⁰⁸ (another possible phosphorylation site) was observed after 2 h incubation. We report for the first time in intact arteries that the acute effects of simvastatin on resistance arterial contractility may involve an AMPK-dependent pathway.

AMPK is an upstream kinase that plays a central role in the regulation of energy homeostasis [14]. Activation of AMPK is dependent on phosphorylation of Thr¹⁷². Although it is well established that AMPK activity is regulated by changes in the AMP/ATP ratio [35], it is now clear that AMPK may also be activated by AMP-independent pathways [36]. Activated AMPK may have multiple actions including the activation of eNOS via phosphorylation at Ser¹¹⁷⁷ [16,17]. This has functional consequences as it has been shown that the selective pharmacological AMPK activator AICAR induces endothelium-dependent dilation of resistance arteries [18]. Previous studies in cultured endothelial cells have shown that atorvastatin can acutely increase the phosphorylation of AMPK and subsequently eNOS

phosphorylation [19,37]. Chronic treatment of mice with simvastatin has also been shown to increase aortic AMPK phosphorylation, but it is unknown whether this was associated with changes in eNOS phosphorylation or alteration of vascular function [38]. In the present study, we have shown that acute incubation of isolated mesenteric arteries with simvastatin (2 h, 1 μ M) reduced contraction to both U46619 and phenylephrine via NO-dependent mechanisms that were associated with AMPK and eNOS phosphorylation. Furthermore, inhibition of AMPK with compound C totally prevented the statin-induced increase in eNOS phosphorylation (at Ser¹¹⁷⁷). Although we acknowledge that compound C did itself reduce contraction to U46619, in the presence of compound C, simvastatin had no additional effect on arterial contractility. Taken together our results suggest that that phosphorylation of AMPK at Thr¹⁷² and subsequent phosphorylation of eNOS (at Ser¹¹⁷⁷) is a fundamental mechanism underlying the pleiotropic effects of the statin on resistance arterial contractility. Whether further control may be afforded by AMPK phosphorylation of eNOS at alternative sites is yet to be determined [19,37].

The mechanism by which simvastatin enhances AMPK phosphorylation is unclear. Simvastatin inhibits HMG-CoA reductase which converts HMG-CoA into mevalonic acid. In the present study, the functional effects of simvastatin were partially inhibited by mevalonate suggesting that the effects we observed were partially dependent on this pathway. Mevalonate is required not only for the synthesis of steroids such as cholesterol, but also for the production of a number of other compounds including isoprenoids, ubiquinone and dolichols. Isoprenoids are important regulators of a variety of small molecules, including GTPases Rho, Rac1 and Ras [39], and have previously been implicated in the effects of statins on eNOS expression and stability (see [40]). Previous studies in cultured endothelial cells have demonstrated simvastatin may increase GTP-bound Rac1 and increase AMPK phosphorylation [38]. Clearly such a mechanism may contribute to the effects observed in the present study, although further work is required to clarify the involvement of mevalonate in the acute effects of simvastatin on simvastatin-stimulated AMPK and eNOS phosphorylation and upstream signalling pathways involved.

In summary, we have shown that simvastatin acutely directly modulates resistance arterial contractile function in healthy animals via NO-dependent mechanisms that are associated with an increased phosphorylation of eNOS at Ser¹¹⁷⁷ via the AMPK pathway.

Perspectives

The role of AMPK in the maintenance and restoration of metabolic homeostasis has led to considerable interest in it as a potential target for the treatment of

metabolic disorders, including obesity, Type II diabetes and the metabolic syndrome [41,42]. These disorders are associated with endothelial dysfunction and indeed cardiovascular disease is responsible for considerable morbidity and mortality in these patients [43]. The identification of safe and effective compounds that activate AMPK may represent an effective strategy to improve both metabolic homeostasis and endothelial function in cardiometabolic disorders [14]. In support of this notion the pharmacological activators of AMPK A769662 and AICAR have been shown to elicit beneficial effects on metabolism and endothelial function, but problems of oral bioavailability and short half-life limit their clinical use [18,44]. There is a continued search for identification of safe and effective modulators of AMPK. In the present study, we show that, in addition to those benefits on endothelial function associated with a reduction in cholesterol, simvastatin can acutely modulate AMPK via phosphorylation of eNOS. Further studies are clearly required to fully understand the pleiotropic effects of statins on eNOS, to investigate the relative importance of this mechanism with other statins and the time- and dose-dependence of these effects. Although the effects of statins on NO bioavailability are multiple, the results of the present study do show that simvastatin, which is a safe and cost-effective medication, can modulate AMPK- and eNOS-dependent arterial function even in the absence of endothelial dysfunction. Such effects may represent a new strategy for use of statins in both treatment and primary prevention of cardiometabolic disorders.

AUTHOR CONTRIBUTION

Luciana Rossoni, Mark Wareing, Camilla Wenceslau, Mahmood Al-Abri and Christopher Cobb performed the experimental work; and Clare Austin directed the study.

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REFERENCES

- Scandinavian Simvastatin Survival Study Investigators (1994) Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the scandinavian simvastatin survival study. (45). *Lancet* **344**, 1383–1389
- Heart Protection Study Collaborative Group (2002) MRC/BHF heart protection study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* **360**, 7–22
- Tziomalos, K., Athyros, V. G., Karagiannis, A. and Mikhailidis, D. P. (2009) JUPITER: major implications for vascular risk assessment. *Curr. Med. Res. Opin.* **25**, 133–137
- Mora, S. and Ridker, P. M. (2006) Justification for the use of statins in primary prevention: an intervention trial evaluating rosuvastatin (JUPITER): can C-reactive protein be used to target statin therapy in primary prevention? *Am. J. Cardiol.* **97**, 33A–41A
- Anderson, T. J., Meredith, I. T., Yeung, A. C., Frei, B., Selwyn, A. P. and Ganz, P. (1995) The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion. *N. Engl. J. Med.* **332**, 488–493
- O'Driscoll, G., Green, D. and Taylor, R. R. (1997) Simvastatin, an HMG-coenzyme A reductase inhibitor, improves endothelial function within 1 month. *Circulation* **95**, 1126–1131
- Omori, H., Nagashima, H., Tsurumi, Y., Takagi, A., Ishizuka, N., Hagiwara, N., Kawana, M. and Kasanuki, H. (2002) Direct *in vivo* evidence of a vascular statin: a single dose of cerivastatin rapidly increases vascular endothelial responsiveness in healthy normocholesterolaemic subjects. *Br. J. Clin. Pharmacol.* **54**, 395–399
- Chen, H., Ren, J. Y., Xing, Y., Zhang, W. L., Liu, X., Wu, P., Wang, R. J. and Luo, Y. (2009) Short-term withdrawal of simvastatin induces endothelial dysfunction in patients with coronary artery disease: a dose-response effect dependent on endothelial nitric oxide synthase. *Int. J. Cardiol.* **131**, 313–320
- Sessa, W. C. (2004) eNOS at a glance. *J. Cell Sci.* **117**, 2427–2429
- Fulton, D., Gratton, J.-P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A. and Sessa, W. C. (2000) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597–601
- Sugawara, T., Ayer, R., Jadhav, V., Chen, W., Tsubokawa, T. and Zhang, J. H. (2008) Simvastatin attenuation of cerebral vasospasm after subarachnoid hemorrhage in rats via increased phosphorylation of Akt and endothelial nitric oxide synthase. *J. Neurosci. Res.* **86**, 3635–3643
- Harris, M. B., Blackstone, M. A., Sood, S. G., Li, C., Goolsby, J. M., Venema, V. J., Kemp, B. E. and Venema, R. C. (2004) Acute activation and phosphorylation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *Am. J. Physiol. Heart Circ. Physiol.* **287**, H560–H566
- Wang, J., Xu, Z., Kitajima, I. and Wang, Z. (2008) Effects of different statins on endothelial nitric oxide synthase and Akt phosphorylation in endothelial cells. *Int. J. Cardiol.* **127**, 33–39
- Wong, A. K.F., Howie, J., Petrie, J. R. and Lang, C. C. (2009) AMP-activated protein kinase pathway: a potential therapeutic target in cardiometabolic disease. *Clin. Sci.* **116**, 607–620
- Chen, Z. P., Mitchelhill, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., Witters, L. A., Power, D. A., Ortiz de Montellano, P. R. and Kemp, B. E. (1999) AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett.* **1443**, 285–289
- Chen, H., Montagnani, M., Funshashi, T., Shimomura, I. and Quon, M. J. (2003) Adiponectin stimulates production of nitric oxide in vascular endothelial cells. *J. Biol. Chem.* **278**, 45021–45026
- Morrow, V. A., Foufelle, F., Connell, J. M., Petrie, J. R., Gould, G. W. and Salt, I. P. (2003) Direct activation of AMP-activated protein kinase stimulates nitric oxide synthesis in human aortic endothelial cells. *J. Biol. Chem.* **278**, 31629–31639
- Bradley, E. A., Eringa, E. C., Stehouwer, C. D., Korstjens, I., van Nieuw Amerongen, G. P., Musters, R., Sipkema, P., Clark, M. G. and Rattigan, S. (2010) Activation of AMP-activated protein kinase by 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside in the muscle microcirculation increases nitric oxide synthesis and microvascular perfusion. *Arterioscler. Thromb. Vasc. Biol.* **30**, 1137–1142

- 19 Sun, W., Lee, T.-S., Zhu, M., Gu, C., Wang, Y., Zhu, Y. and Shyy, J. Y. (2006) Statins activate AMP-activated protein kinase *in vitro* and *in vivo*. *Circulation* **114**, 2655–2662
- 20 Izumi, Y., Shiota, M., Kusakabe, H., Hikita, Y., Nakao, T. and Nakamura, Y. (2009) Pravastatin accelerates ischemia-induced angiogenesis through AMP-activated protein kinase. *Hypertens. Res.* **32**, 675–678
- 21 Mulvany, M. and Halpern, W. (1977) Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.* **41**, 19–26
- 22 Lilja, J. J., Kivisto, K. T. and Neuvonen, P. J. (1998) Grapefruit juice-simvastatin interaction: effect of serum concentrations of simvastatin, simvastatin acid and HMG-CoA reductase inhibitors. *Clin. Pharmacol. Ther.* **64**, 477–483
- 23 Kantola, T., Kivisto, K. T. and Neuvonen, P. J. (1998) Erythromycin and verapamil considerably increase serum simvastatin and simvastatin acid concentrations. *Clin. Pharmacol. Ther.* **64**, 177–182
- 24 Shaw, L., Taggart, M. J. and Austin, C. (2000) Mechanisms of 17 β -oestradiol induced vasodilatation in isolated pressurised rat small arteries. *Br. J. Pharmacol.* **129**, 555–565
- 25 Xavier, F. E., Davel, A. P., Fukuda, L. E. and Rossoni, L. V. (2009) Chronic ouabain treatment exacerbates blood pressure elevation in spontaneously hypertensive rats: the role of vascular mechanisms. *J. Hypertens.* **27**, 1233–1242
- 26 Laufs, U. and Liao, J. K. (1998) Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J. Biol. Chem.* **272**, 24266–24271
- 27 Laufs, U., La Fata, V., Plutzky, J. and Liao, J. K. (1998) Upregulation of endothelial nitric oxide synthase by HMG-CoA reductase inhibitors. *Circulation* **97**, 1129–1135
- 28 Kalinowski, L., Dobrucki, I. T. and Malinski, T. (2002) Cerivastatin potentiates nitric oxide release and eNOS expression through inhibition of isoprenoids synthesis. *J. Physiol. Pharmacol.* **53**, 585–595
- 29 Suzumura, K., Yashuhara, M., Tanaka, K., Odawara, A., Narita, H. and Suzuki, T. (1999) An *in vitro* study of the hydroxyl radical scavenging property of fluvastatin, and HMG-CoA reductase inhibitor. *Chem. Pharm. Bull.* **47**, 1010–1012
- 30 Lane, P. and Gros, S. S. (2002) Disabling a C-terminal autoinhibitory control element in endothelial nitric-oxide synthase by phosphorylation provides a molecular explanation for activation of vascular NO synthesis by diverse physiological stimuli. *J. Biol. Chem.* **277**, 19087–19094
- 31 Wu, H., Lu, D., Jiang, H., Xiong, Y., Qu, C. and Li, B. (2008) Increase in phosphorylation of Akt and its downstream signalling targets and suppression of apoptosis by simvastatin after traumatic brain injury. *J. Neurosci.* **109**, 691–698
- 32 Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. and Zeiher, A. M. (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**, 601–605
- 33 Luo, Z., Fujio, Y., Kureishi, Y., Rudic, R. D., Damerie, G., Fulton, D., Sessa, W. C. and Walsh, K. (2000) Acute modulation of endothelial Akt/PKB activity alters nitric oxide-dependent vasomotor activity *in vivo*. *J. Clin. Invest.* **106**, 493–499
- 34 Mukai, Y., Shimokawa, H., Matoba, T., Hiroki, J., Kunihiro, I., Fujiki, T. and Takeshita, A. (2003) Acute vasodilator effects of HMG-CoA reductase inhibitors: involvement of PI3-kinase/Akt pathway and Kv channels. *J. Cardiovasc. Pharmacol.* **42**, 118–124
- 35 Hardie, D. G. and Carling, D. (1997) The AMP-activated protein kinase—fuel gauge of the mammalian cell? *Eur. J. Biochem.* **246**, 259–273
- 36 Hawley, S. A., Davison, M., Woods, A., Davies, S. P., Beri, R. K., Carling, D. and Hardie, D. G. (1996) Characterization of the AMP-activated protein kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J. Biol. Chem.* **271**, 27879–27887
- 37 Chen, Z., Peng, I.-C., Sun, W., Su, M.-I., Hsu, P. H., Fu, Y., Zhu, Y., De Fea, K., Pan, S., Tsai, M. D. and Shyy, J. Y. (2009) AMP-activated protein kinase functionally phosphorylates endothelial nitric oxide synthase Ser⁶³³. *Circ. Res.* **104**, 496–505
- 38 Kuo, R., Sartoretto, J. and Michel, T. (2009) Regulation of Rac1 by simvastatin in endothelial cells: differential roles of AMP-activated protein kinase and calmodulin-dependent kinase kinase- β . *J. Biol. Chem.* **284**, 14734–14743
- 39 Caey, P. J. (1995) Mechanisms of protein prenylation and role in G protein function. *Biochem. Soc. Trans.* **23**, 161–166
- 40 Rikitake, Y. and Liao, J. K. (2005) Rho GTPases, statins and nitric oxide. *Circ. Res.* **97**, 1232–1235
- 41 Viollet, B., Mounier, R., Leclerc, J., Yazigi, A., Foretz, M. and Andreelli, F. (2007) Targeting AMP-activated protein kinase as a novel therapeutic approach for the treatment of metabolic disorders. *Diabetes Metab.* **33**, 395–402
- 42 Towler, M. C. and Hardie, D. G. (2007) AMP-activated protein kinase in metabolic control and insulin signalling. *Circ. Res.* **100**, 328–341
- 43 Haffner, S. M., Lehto, S., Rönnemaa, T., Pyörälä, K. and Laakso, M. (1998) Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N. Engl. J. Med.* **339**, 229–234
- 44 Cool, B., Zinker, B., Chiou, W., Kifle, L., Cao, N., Perham, M., Dickinson, R., Adler, A., Gagne, G., Iyengar, R. et al. (2006) Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab.* **3**, 403–416

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