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

Luciana V. Rossoni[1, 3], Mark Wareing[2], Camilla F. Wenceslau[1], Mahmood Al-Abri [3], Chris Cobb[3], Clare Austin[3*]

[1]Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil; [2]Maternal & Fetal Health Research Centre and [3]Cardiovascular Research Group, The University of Manchester, Manchester, UK

Corresponding author:

Clare Austin*

Cardiovascular Research Group
The University of Manchester, Core Technology Facility
46 Grafton Street, Manchester
M13 9WL
Tel: 0161-275- 1214 Fax: 0171 275 1183
E mail: clare.austin@manchester.ac.uk

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Abstract

Statins can have beneficial cholesterol-independent effects on vascular contractility which may involve increases in the bioavailability of nitric oxide (NO) as a result of phosphorylation of endothelial NO synthase. While this has been attributed to phosphorylation of Akt, studies in cultured cells have shown that statins can phosphorylate AMPK; 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 hours) with simvastatin (0.1 or 1 μM) on contractile responses were examined in the presence and absence of N-Nitro-L-arginine (L-NNA, 10 μM) or mevalonate (1 mM). Effects on endothelial NO synthase (eNOS), phosphorylated eNOS (Ser 1177) and total and phosphorylated Akt and AMPK protein expression were investigated using Western blotting. The effect of AMPK inhibition (Compound C, 10 μM) on eNOS phosphorylation and contractile responses were also studied.

Simvastatin (1 μM, 2 hours) significantly reduced constriction to U46619 and phenylephrine and enhanced dilations to 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 AMPKα phosphorylation but had no effect on Akt protein expression and phosphorylation after 2 hours incubation. Compound C prevented the effects of simvastatin on eNOS phosphorylation and contractility.

Thus, simvastain can acutely modulate resistance arterial contractile function via mechanisms which involve the AMPK/ $p^{Ser1177}$eNOS/ NO-dependent pathway.

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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 reductase and blocking the conversion of HMG-CoA to 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 nitric oxide (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 hours of a single dose of statin in patients with normal serum cholesterol levels [7]. Decreases in NO availability are also noted within hours of simvastatin withdrawal [8]. The bioavailability of NO may be modulated in a variety of different ways but post-translational phosphorylation of endothelial NO synthase (eNOS) 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 protein kinase Akt which can phosphorylate eNOS on Serine 1177 (Ser 1177) 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

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uniform finding [12-13]. AMP-activated protein kinase (AMPK) is another serine/threonine kinase which 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 (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, pravastatin) treatment [19-20] but it is unknown whether this may contribute to the acute effects of statins on arterial reactivity.

The aim of this 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 (200-250g, n= 57). Animals were housed under a 12 h light–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 physiological salt solution (PSS) (mM: NaCl 119, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 2.4, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.07, glucose 6.05, calcium 1.6 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.

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, Denmark) [21]. After mounting, tissues were equilibrated for 30 min in PSS (gassed with 20% O_2 and 5% CO_2 at 37°C) and normalised as previously described [21]. Isometric tension development was continuously recorded (Myodaq data acquisition system). Contractile viability was assessed by two exposures to high K^+ solution (modified PSS containing 120 mM KCl isosmotically substituted for NaCl). Contraction-induced by 120 mM KPSS in the control group was 3.23±0.17 mN/mm (N=31) *vs.* 3.14±0.16 mN/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 acetylcholine (ACh, 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 hours. 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 phenyleprine (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 sodium nitroprusside (SNP, 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 catalyzes 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 hours 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 hours with either N-Nitro-L-arginine (L-NNA, 10 μM) alone or in combination with simvastatin (1 μM). To further investigate the involvement of NO, the influence of simvastatin (1 μM, 2 hours) 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 depolarised rat mesenteric arteries [24].

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The influence of simvastatin (1 μM, 2 hours) 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 hours. 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 hours. Following incubation, arteries were immediately frozen until required. Arteries were then homogenized in lysis buffer containing: RIPA Lysis Buffer (Upstate, USA); protease inhibitor cocktail (1:5,000 dilution; Sigma); sodium fluoride (100 mmol/L); sodium pyrophosphate (10 mmol/L); sodium orthovanadate (100 mmol/L) and PMSF (10 mmol/L). Protein extracts (75 μg) were separated by 7.5% SDS-PAGE and then transferred to polyvinyl difluoride membranes (Amersham, USA) using a Mini Trans-Blot Cell system (Bio-Rad, USA)(overnight, 4^0C) containing transfer buffer (25 mmol/L Tris, 190 mmol/L glycine, 20% methanol and 0.05% SDS) as described previously [27].

After blockade of nonspecific sites with 5% nonfat dry milk, membranes were incubated overnight at 4°C with the primary antibody against mouse anti-eNOS (1:1,000 dilution; Transduction Laboratories, USA), rabbit anti-peNOS (phospho-eNOS Ser 1177) @ 1:1,000 dilution (Cell Signaling, USA), rabbit anti-Akt1/2/3 @ 1:400 dilution (Santa Cruz Biotechnology, USA), rabbit anti-pAkt1/2/3 (phospho-Akt1/2/3 Ser 473) @ 1:400 dilution (Santa Cruz Biotechnology), rabbit anti-pAkt1/2/3 (phospho-Akt1/2/3 Thr 308) @ 1:500 dilution (Santa Cruz Biotechnology), rabbit anti-AMPKα @ 1:500 dilution (Santa Cruz

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Biotechnology) or rabbit anti-pAMPKα (phospho-AMPKα$_2$ Thr 172) @ 1:1000 dilution (Cell Signaling). After washing, membranes were incubated for 2 hours, at room temperature with anti-mouse IgG antibody (1:1,500 dilution, Bio-Rad) for eNOS and anti-rabbit IgG antibody for peNOS (1:1,500 dilution), Akt-1/2/3, pAkt-1/2/3 (at Ser 473 and Thr 308), AMPKα and pAMPKα (Thr 172) (1:5,000 dilution, Jackson ImmunoResearch, USA). 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 antibody mouse anti-α-actin (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 normalised to the contraction of each tissue to KPSS (120 mM). Dilatory responses to ACh or SNP were normalised to the maintained U46619 or KCl-induced contraction. The maximum effect (E_{max}) and the log of the concentration of agonist that produced half of the E_{max} (log EC_{50}) were calculated using non-linear regression analysis (GraphPad Prism Software).

All values are expressed as mean ± SEM of the number of animals used in each experiment. Results were analysed using two-way ANOVA for comparison between groups or unpaired *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 $P<0.05$.

Drugs and solutions

Unless otherwise stated all drugs were obtained from Sigma (St. Louis, MO), except U46619 that was obtained from Calbiochen (UK). A 10 mM stock solution of U46619 was made by dissolving in a mixture of 100% ethanol and 1 mg kg^{-1} sodium carbonate (1: 2). 10 mM stock solution of mevalonic acid lactone, simvastatin and Compound C were dissolved in DMSO (Merck, UK). All other drugs were dissolved in PSS.

Results

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

Incubation of arteries for 1 hour with either 0.1 or 1 μM simvastatin had no significant effect on U46619-induced contraction (N=5 and N=6, respectively: Figure 1A). Incubation of arteries for 2 hours with 0.1 μM simvastatin was similarly ineffective (Figure 1B). However, 2 hour incubation with 1 μM simvastatin significantly reduced U46619-induced constriction (Figure 1B) but not arterial sensitivity (as evidenced by log EC_{50} values) to U46619 (Table 1). In line with the results obtained with U46619, 2 hour incubation with 1 μM simvastatin also reduced contractile responses to phenylephrine, but had no effect on sensitivity (Emax: Control: 96.9±7.10 (N=4) *vs.* Simvastatin: 68.2±12.57 (N=4) % contraction to KPSS, *t*-Test $P<0.05$; Control -6.28 ± 0.06 *vs* Simvastatin -6.23 ± 0.06, *t*-Test, $P>0.05$).

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

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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 hour incubation with simvastatin (1μM) 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 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 hour co-incubation of L-NNA with 1 μM simvastatin completely blocked the effects of simvastatin on U46619-induced contraction (N=8; Figure 3A, 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 (data not shown).

To further investigate the effects of simvastatin (1 μM for 2 hours) on NO, concentration-response curves to ACh were also constructed in depolarised tissues (80 mM KPSS). ACh-induced relaxation was reduced in depolarized arteries (N=6) when compared to that seen in U46619 pre-constricted arteries (N=12; Figure 3C). Simvastatin (1 μM, 2 hours) significantly enhanced dilations of depolarised vessels to ACh (N=6; Figure 3C).

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Western blotting experiments showed that incubation with simvastatin (1 μM for 2 hours) increased the phosphorylation of eNOS on Ser 1177 (Figure 4A), but did not modify eNOS protein expression (eNOS/ α-actin protein expression: Control: 0.93±0.10 *vs.* Simvastatin: 0.86±0.10 (N=5); *t*-Test, P>0.05). Phosphorylation of Akt 1/2/3 on Ser 473 or on Thr 308 was not significantly modified by simvastatin (Figure 4C and D). Similarly, Akt 1/2/3 protein expression was unaffected (Akt/α-actin protein expression: Control: 0.73±0. 09 *vs.* Simvastatin: 0.86±0.10 (N=8); *t*-Test, P>0.05). However, simvastatin significantly increased phosphorylation of AMPKα on Thr 172 (Figure 4B), without any changes in AMPKα protein expression (AMPKα/ α-actin protein expression: Control: 0.65±0.12 *vs.* Simvastatin: 0.71±0.04 (N=5); *t*-Test, P>0.05). There were no changes in α-actin protein expression between groups (data not shown).

Co-incubation of arteries with the AMPK inhibitor Compound C (10 μM) with simvastatin (1 μM, 2 hours) completely blocked the enhanced eNOS phosphorylation at Ser 1177 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 μM) and simvastatin (1 μM), 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 (Emax: 117 ± 8.05% KPSS for control and 82±12.2% KPSS for Compound C) but simvastatin had no additional effect in the presence of Compound C (Emax: 71±8.16% 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 which is associated with phosphroylation of AMPK and not Akt 2 hours 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 responsiveness with enhancement of endothelium-dependent dilation to acetylcholine only evident in tissues constricted by depolarisation but not in those pre-constricted with U466119. In depolarised tissues, in the absence of EDHF action, maximal dilation to acetylcholine 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 depolarised arteries to acetylcholine 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, posttranslational modification of eNOS activity by phosphorylation [30-31, 9-13]. It is well established that phosphorylation of eNOS at Ser 1177 increases its activity [9]. The most widely studied eNOS phosphorylation pathway involves the Ser/Thr protein kinase Akt and it is widely accepted that activation of Akt by phosphorylation (Ser 473) 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 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 Ser 473 or Thr 308 (another possible phosphorylation site) was observed after 2 hours 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.

AMP-activated protein kinase (AMPK) is an upstream kinase which plays a central role in the regulation of energy homeostasis [14]. Activation of AMPK is dependent upon phosphorylation on Thr 172. While it is well established that AMPK activity is regulated by changes in the ratio of AMP:ATP [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 Ser 1177 [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 hours, 1 μM) reduced contraction to both U46619 and phenylephrine via NO-dependent mechanisms which were associated with AMPK and eNOS phosphorylation. Furthermore, inhibition of AMPK with Compound C totally prevented the statin-induced increase in eNOS phosphorylation (at Ser 1177). 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 data suggest that that phosphorylation of AMPK at Thr 172 and

subsequent phosphorylation of eNOS (at Ser 1177) 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 has 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 to 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 upon this pathway. Mevalonate is required for the synthesis of steroids such as cholesterol but also 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 which are associated with an increased phosphorylation of eNOS at Ser 1177 via the AMPK pathway.

Perspectives

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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 which 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 endothelium, 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. Here 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. Whilst 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

Experimental work was performed by L. V. Rossoni, Mark Wareing, Camilla F. Wenceslau, Mahmood Al-Abri and Chris Cobb. The study was directed by Clare Austin.

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Figure legends:

Figure 1: The effect of incubation with simvastatin (0.1 and 1 μM) for 1 (A, C) or 2 (B, D) hours on contractile responses of isolated rat mesenteric small arteries to U46619 and ACh. Contractile responses to U46619 are normalised to the contractile response of each artery to KPSS (120mM). Relaxation responses to ACh are normalised to the maximum contractile response of each artery to U46619. All mean ± SEM. ANOVA 2-way; * P<0.05 *vs.* Control. N = number of animals.

Figure 2: The 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 μM for 2 hours). Contractile responses to U46619 are normalised to the contractile response of each artery to KPSS (120mM). Relaxant responses to ACh are normalised to the maximum contractile response of each artery to U46619. All mean ± SEM. ANOVA 2-way; * P<0.05 *vs.* Control, + P<0.05 *vs.* Mevalonate and # P<0.05 *vs.* Simvastatin. N=number of animals.

Figure 3: The effect of 2 hour incubation with L-NNA (10 μM) 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 μM for 2 hours). C shows a comparison of the effects of 2 hour incubation with 1 μM simvastatin on responses to ACh when pre-constricted with U46619 (10 μM) or KCl (80 mM). Data is shown as mean ± SEM. ANOVA 2-way; * P<0.05 *vs.* Control, + P<0.05 *vs.* L-NNA and # P<0.05 *vs.* arteries pre-constricted with U46619. N= number of animals.

Figure 4: The effects of 2 hour incubation of rat mesenteric arteries with 1 μM simvastatin on phosphorylation of (A) eNOS (Ser 1177) (B), AMPKα (Thr 172) (C), Akt-1/2/3 (Ser 473) and (D) Akt-1/2/3 (Thr 308). Upper panel: representative blot for peNOS (Ser 1177) and eNOS (A), pAMPKα (Thr 172) and AMPKα (B) and pAkt-1/2/3 (Ser 473) (C) or pAkt-1/2/3 (Thr 308) (D) and Akt-1/2/3 protein expression in mesenteric resistance arteries incubated with simvastatin (SIMV) or vehicle (CT). Bottom panel: Densitometric analysis of the ratio for peNOS (Ser 1177) and eNOS (A), pAMPKα (Thr 172) and AMPKα (B), pAkt-1/2/3 (Ser 473) (C) and pAkt-1/2/3 (Thr 308) (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 (Ser 473 or Thr 308) but significantly increased phosphorylation of AMPKα (Thr 172). Data is normalised to the expression of α-actin. Results (means ± SEM). Insert numbers in the bar chart indicate the number of animals used. *t*-Test*;* *P<0.05 *vs.* Control.

Figure 5: A. The effect of the AMPK inhibitor Compound C (10 μM) on enhanced phosphorylation of eNOS at Ser 1177 induced by 2 hour incubation with simvastatin 1 μM. Upper panel: representative blot for peNOS (Ser 1177) 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 peNOS (Ser 1177) and eNOS. Data is normalised to the expression of α-actin. Results (means ± SEM). Insert numbers in the bar chart indicate the number of animals used. ANOVA*;* *P<0.05 *vs.* Control. **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 hours).

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Contractile responses to U46619 are normalised to the contractile response of each artery to KPSS (120mM). Results (mean ± SEM). ANOVA 2-way. N=number of animals.

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Table 1: Changes in the maximal response (R_{max}) and sensitivity (Log EC_{50}) to U46619 in mesenteric resistance arteries incubated for 1 or 2 hours with DMSO (Control) and simvastatin (0.1 or 1 μM).

	R_{max} (% to KPSS)	***Log EC_{50}***
1 hour		
Control	114±18.5	-6.61±0.06 (N=6)
Simvastatin 0.1 μM	104±9.00	-6.71±0.11 (N=5)
Simvastatin 1μM	116±15.0	-6.65±0.09 (N=6)
2 hours		
Control	106±4.24	-6.85±0.11 (N=13)
Simvastatin 0.1 μM	112±6.93	-6.80±0.15 (N=6)
Simvastatin 1μM	52±12.5 *	-6.70±0.04 (N=10)

ANOVA; *P<0.05 *vs.* control.

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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 hours with DMSO (Control) and simvastatin (0.1 or 1 μM).

	ACh		*SNP*	
	R_{max} (%)	**Log EC_{50}**	**R_{max} (%)**	**Log EC_{50}**
1 hour				
Control	71±12.1	-6.51±0.18 (N=6)	79±6.56	-6.74±0.15(N=6)
Simvastatin 0.1 μM	71±12.4	-6.63±0.19 (N=5)	84±7.97	-6.38±0.50(N=5)
Simvastatin 1μM	72±10.3	-6.65±0.16 (N=6)	89±1.37	-6.74±0.12(N=6)
2 hours				
Control	85±4.30	-6.53±0.10 (N=12)	83±4.32	-6.38±0.19(N=13)
Simvastatin 0.1 μM	92±3.07	-6.60±0.17 (N=5)	87±2.71	-6.37±0.12(N=6)
Simvastatin 1μM	79±5.12	-6.69±0.12 (N=10)	83±3.96	-6.65±0.16(N=10)

ANOVA.

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Table 3: Changes in the maximal response (R_{max}) and sensitivity (Log EC_{50}) to U46619 in mesenteric resistance arteries incubated for 2 hours with DMSO (Control), simvastatin 1 μM, L-NNA (10μM) or mevalonate (10mM) in the absence or presence of simvastatin (1 μM).

	R_{max} (% to KPSS)	***Log EC_{50}***
Control	106±4.24	-6.85±0.11 (N=13)
Simvastatin	52±12.5 *	-6.70±0.04 (N=10)
Mevalonate	111±3.01	-6.86±0.13 (N=8)
Mevalonate/ Sinvastatin	83±8.25*$^{+}$	-6.72±0.12 (N=8)
L-NNA	111±6.92	-6.98±0.16 (N=7)
L-NNA/ Simvastatin	103±10.3	-6.98±0.13 (N=8)

ANOVA; *P<0.05 *vs.* control; +P<0.05 *vs.* Simvastatin.

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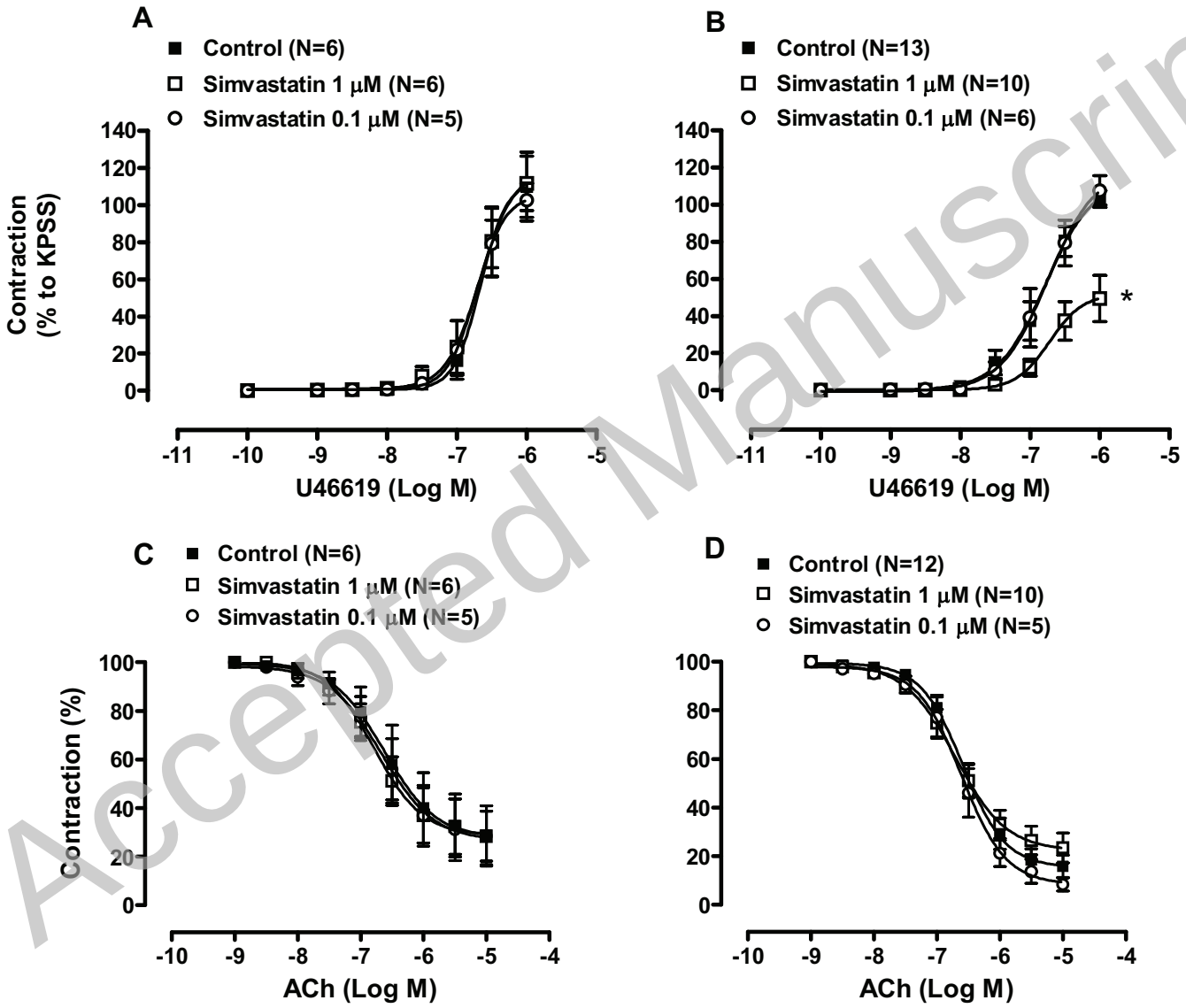

Figure 1

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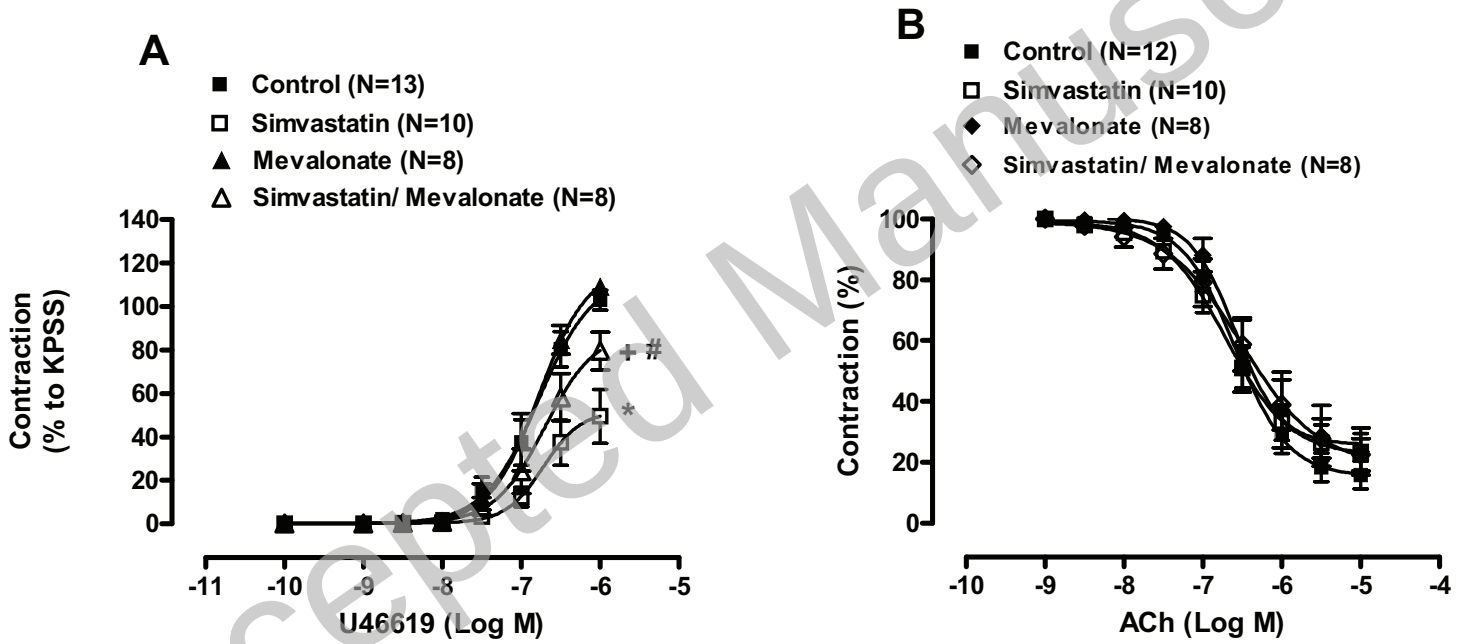

Figure 2

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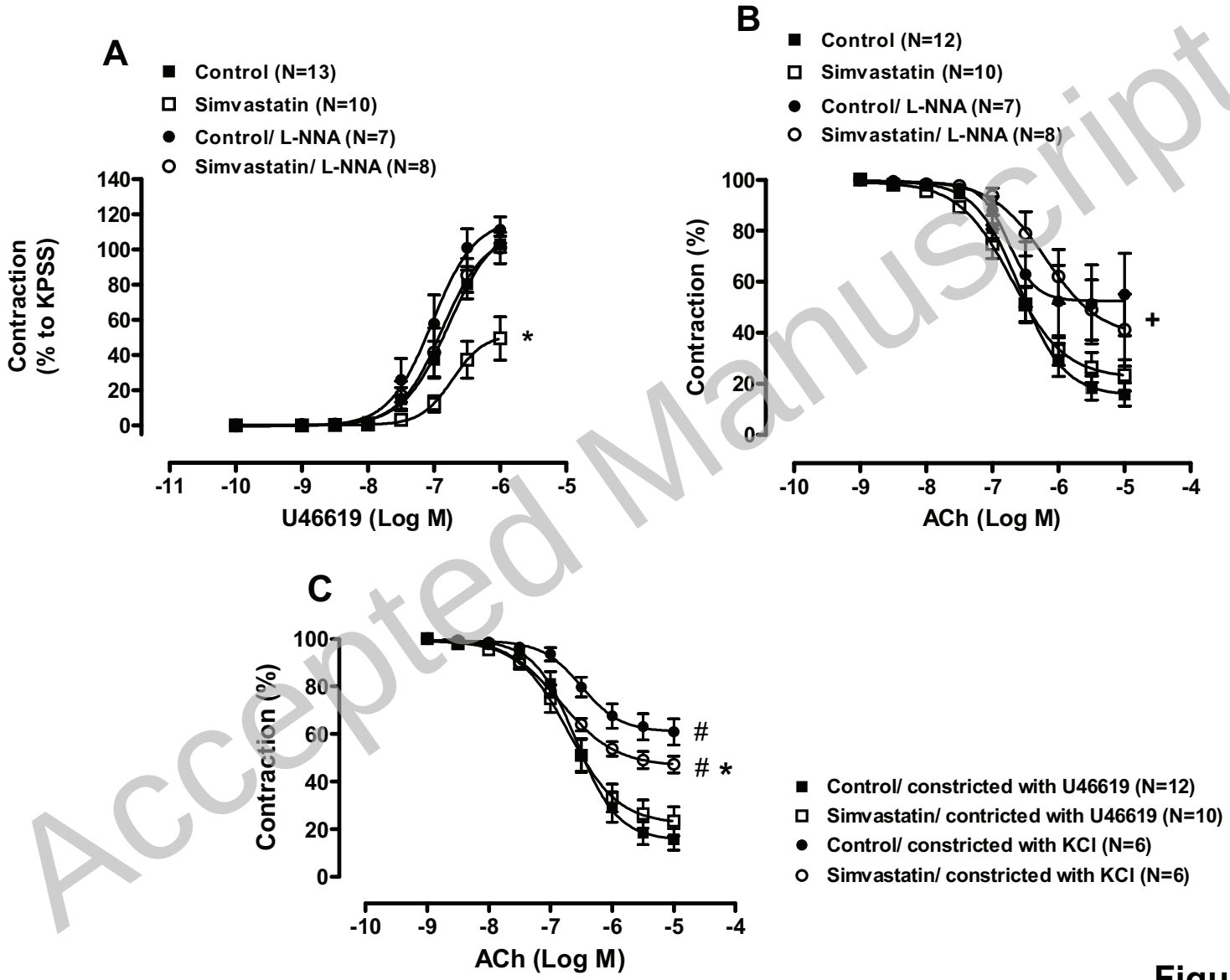

Figure 3

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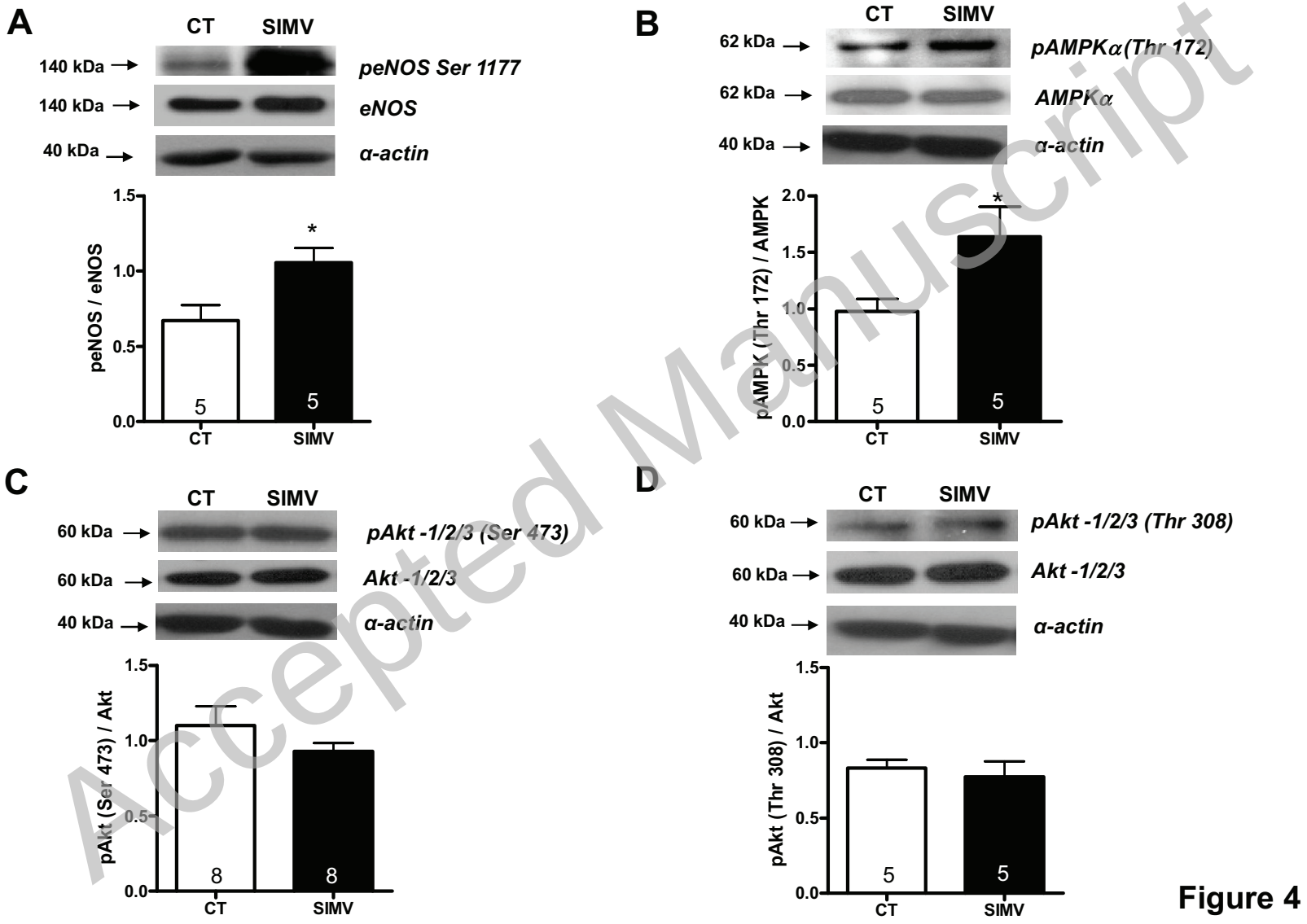

Figure 4

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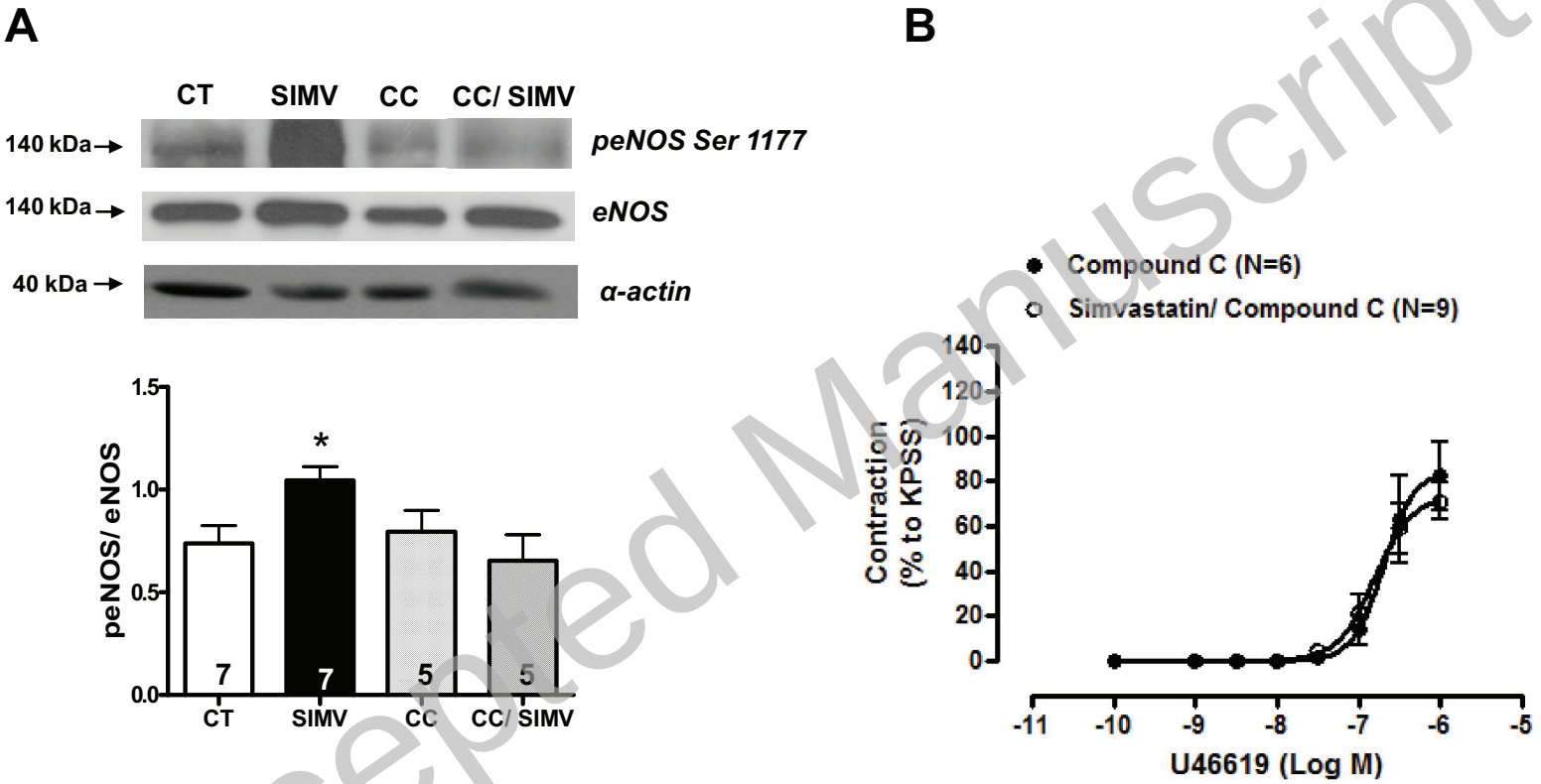

Figure 5