

Acute Simvastatin Inhibits K_{ATP} Channels of Porcine Coronary Artery Myocytes

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Abstract

Background: Statins (3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors) consumption provides beneficial effects on cardiovascular systems. However, effects of statins on vascular K_{ATP} channel gatings are unknown.

Methods: Pig left anterior descending coronary artery and human left internal mammary artery were isolated and endothelium-denuded for tension measurements and Western immunoblots. Enzymatically-dissociated/cultured arterial myocytes were used for patch-clamp electrophysiological studies and for $[Ca^{2+}]_i$, $[ATP]_i$ and $[glucose]_o$ uptake measurements.

Results: The cromakalim (10 nM to 10 μ M)- and pinacidil (10 nM to 10 μ M)-induced concentration-dependent relaxation of porcine coronary artery was inhibited by simvastatin (3 and 10 μ M). Simvastatin (1, 3 and 10 μ M) suppressed (in okadaic acid (10 nM)-sensitive manner) cromakalim (10 μ M)- and pinacidil (10 μ M)-mediated opening of whole-cell K_{ATP} channels of arterial myocytes. Simvastatin (10 μ M) and AICAR (1 mM) elicited a time-dependent, compound C (1 μ M)-sensitive $[^3H]$ -2-deoxy-glucose uptake and an increase in $[ATP]_i$ levels. A time (2–30 min)- and concentration (0.1–10 μ M)-dependent increase by simvastatin of p-AMPK α -Thr¹⁷² and p-PP2A-Tyr³⁰⁷ expression was observed. The enhanced p-AMPK α -Thr¹⁷² expression was inhibited by compound C, ryanodine (100 μ M) and KN93 (10 μ M). Simvastatin-induced p-PP2A-Tyr³⁰⁷ expression was suppressed by okadaic acid, compound C, ryanodine, KN93, phloridzin (1 mM), ouabain (10 μ M), and in $[glucose]_o$ -free or $[Na^+]_o$ -free conditions.

Conclusions: Simvastatin causes ryanodine-sensitive Ca^{2+} release which is important for AMPK α -Thr¹⁷² phosphorylation via Ca^{2+} /CaMK II. AMPK α -Thr¹⁷² phosphorylation causes $[glucose]_o$ uptake (and an $[ATP]_i$ increase), closure of K_{ATP} channels, and phosphorylation of AMPK α -Thr¹⁷² and PP2A-Tyr³⁰⁷ resulted. Phosphorylation of PP2A-Tyr³⁰⁷ occurs at a site downstream of AMPK α -Thr¹⁷² phosphorylation.

Citation: Seto SW, Au ALS, Poon CCW, Zhang Q, Li RWS, et al. (2013) Acute Simvastatin Inhibits K_{ATP} Channels of Porcine Coronary Artery Myocytes. PLoS ONE 8(6): e66404. doi:10.1371/journal.pone.0066404

Editor: Sudhiranjan Gupta, Texas A & M, Division of Cardiology, United States of America

Received: February 13, 2013; **Accepted:** May 6, 2013; **Published:** June 17, 2013

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Funding: This project is financially supported by University Grant Council (UGC) Earmarked Grants of Hong Kong (Ref. #: 4107/01M; 4166/02M, project code: 2140565) and Direct Grants for Research (The Chinese University of Hong Kong) (Reference no.: 2401149; Project code/ID: 2041231; 2401296). Dr SW Seto, Ms CCW Poon, Ms ALS Au and Ms Q Zhang are recipients of postgraduate studentship of the Department of Pharmacology/School of Biomedical Sciences (The Chinese University of Hong Kong, Hong Kong). Dr SW Seto is supported by the Australia Government National Health and Medical Research Council (NHMRC) Fellowship (1016349) and the National Heart Foundation (NHF) of Australia Fellowship (PF12B6825). Provision of the Student Campus Work Scheme by the Chou's Foundation Fund and the Student Campus Work Scheme (Shaw College, The Chinese University of Hong Kong) is appreciated. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

3-Hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase is a 97-kDa glycoprotein embedded in the endoplasmic

reticulum [1] which is involved in the endogenous cholesterol biosynthesis in mammalian liver and intestine [2]. Pervious study of our group [3] has clearly illustrated the biochemical existence of extra-hepatic HMG-CoA reductase in human and porcine

cardiovascular tissues, suggesting a physiological role of this enzyme in the cardiovascular system. HMG-CoA reductase inhibitors, commonly known as statins, have been shown to be an effective treatment of hypercholesterolemia and cardiovascular diseases via its cholesterol-lowering property and cholesterol-independent effects (pleiotropic effects) [3,4,5,6,7,8].

Regulation of vascular tone relies on complex cellular mechanisms as well as the opening and closing of various ion channels. Previous studies have demonstrated that statins can modify the activities of different ion channels in blood vessels including L-type Ca^{2+} channel and BK_{Ca} channel [3,9,10,11]. In addition to Ca^{2+} channels and BK_{Ca} channels, ATP-sensitive K^+ (K_{ATP}) channels are abundant in vascular tissues and K_{ATP} channels are also important in regulating the vascular tone [12]. In rat isolated aorta, cerivastatin-induced a glibenclamide (a K_{ATP} channel blocker)-sensitive aortic relaxation [13] and pravastatin reduced myocardial infarct size through opening of mitochondrial K_{ATP} channels in rabbit [14]. However, a recent study reported that simvastatin, but not pravastatin, inhibited pinacidil (a K_{ATP} channel opener)-induced relaxation of pig's isolated coronary arteries suggesting that different statins have differential effects on K_{ATP} channels of different cells/tissues [40].

Similar to other ion channels, the opening and closing of K_{ATP} channels are modulated by multiple cell signaling mechanisms, such as phosphorylation by protein kinase A (PKA) [15], protein kinase C (PKC) [16] and cGMP-dependent protein kinase (PKG) [17]. In addition, the intracellular ATP level is an essential determinant of K_{ATP} channel gatings. It is well-known that AMP-activated protein kinase (AMPK) serves as a 'metabolic master regulator' which is sensitive to changes of intracellular AMP/ATP ratio. Activation of AMPK results in suppression of intracellular energy-consuming pathways and generation of ATP i.e. an increase in cellular ATP level. In mouse isolated pancreatic islets, activation of AMPK by AICAR (an AMPK activator) potentiated insulin secretion by inhibiting K_{ATP} channel openings [18]. Moreover, phenformin (another AMPK activator), inhibited K_{ATP} channel openings in mouse aortic smooth muscle cells [19], highlighting the participation of AMPK activity in K_{ATP} channel gatings in VSMC. Unfortunately, in various *ex vivo* studies (multi-cellular preparations), there is no consensus on the vascular effects mediated by AMPK activation as both contraction and relaxation were observed [20,21,22,23,24], and the underlying reason(s) for the discrepancy is unknown. Given the fact that statins promoted phosphorylation of AMPK in human and bovine endothelial cells [25], it is tempting to suggest that activation of AMPK by simvastatin could modulate vascular K_{ATP} channel gatings and vascular reactivity.

Therefore, in this study we hypothesize that acute simvastatin could modulate vascular K_{ATP} channel gatings and the simvastatin-mediated effects involve activation of AMPK signaling pathway. Thus, in this study, experiments were designed to evaluate the effects of acute simvastatin on vascular K_{ATP} channel gatings of pig's coronary artery, and the participation of AMPK activation.

Materials and Methods

Animal and Human Ethics Statements

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996). The protocol was approved by the Animal Ethics Committee of the Chinese University of Hong Kong (Approval Number: 10/003/DRG). Permission prior to the collection of fresh pig's heart for research

purposes was obtained from Sheung Shui Slaughterhouse (Hong Kong).

Fresh human left internal mammary arteries were the leftover obtained from patients with cardiovascular diseases undergoing coronary artery bypass grafting (CABG) procedures, and the use of human tissues for research purposes was approved by the Human Research Ethics Committee of the Chinese University of Hong Kong (CREC Ref. No. 2006.313). Written consents were obtained, prior to surgery, from patients voluntarily involved for the usage of tissues solely for research purposes. Patients had read and understood the patient information document provided, and the aims and methods of this study had been fully explained to them. Patients involved had given written informed consent (as outlined in PLOS consent form) to authors of this manuscript for publication of these data.

Isometric Tension Measurement

Fresh hearts were obtained from pigs (~35 kg) that were slaughtered in the morning of the experiment at a local slaughterhouse. The heart was immediately immersed in an ice-cold physiological salt solution. Segment of the left anterior descending (LAD) coronary artery (tertiary branch, O.D. ~500–800 μ m) was dissected within an hour after the animal was slaughtered.

Fresh human left internal mammary arteries were bathed in an ice-cold physiological salt solution before transported to the laboratory from the operation theatre of the Prince of Wales Hospital (Hong Kong) within an hour. Fat and connective tissues were carefully removed under the dissecting stereo-microscope.

Arterial rings (porcine coronary artery and human internal mammary artery) (endothelium was removed using a blunted watch-maker forceps) were bathed in a 5-ml thermo-regulated wire myograph contained physiological salt solution with the composition (mM): NaCl 118.3, KCl 4.6, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, and glucose 11 (bubbled with 16% O_2 /5% CO_2 balanced with N_2 , pO_2 = ~100 mmHg). Rings (1 mm in length) were equilibrated under resting tension of 1 g [26,27] using two stainless steel wires (diameter ~100 μ m), in the bath solution for 90 min. Resting tension was re-adjusted, if necessary, before commencing the experiments. The reason for choosing these tissues in this study is because previous reports demonstrated the existence of K_{ATP} channels in these vascular tissues [28,29].

Enzymatic Dissociation of Myocytes

Porcine left anterior descending coronary artery myocytes and human left internal mammary artery myocytes were dissociated using collagenase and protease, as reported [3,13] for conventional whole-cell patch-clamp electrophysiology experiments.

Electrophysiological Measurement of K_{ATP} Gatings

Conventional whole-cell, patch-clamp experiments were performed at room temperature (~22°C) using single-cell, voltage-clamp techniques (Axopatch 200B amplifier and Digidata 1200 A/D interface) (Axon Instruments, USA) with recording patch pipettes of 2–4 M Ω (when filled with internal pipette solution). Whole-cell recording configurations were used so as to maintain a "pre-determined concentration" of ATP (i.e. 1 mM) inside all the cells used during the recording of K_{ATP} channels for a fair/accurate comparison of K_{ATP} channel gatings of different cells (pig coronary artery and human internal mammary artery) in response to drug challenges. In addition, this mode of recording offers the convenience of a rapid delivery of drugs (e.g. simvastatin Na^+ , okadaic acid and rottlerin) into the cytosol of cells.

The external bath solution contained (mM): KCl 10, potassium gluconate 135, EGTA 5, glucose 5 and HEPES 10 (pH = 7.4). The internal pipette solution contained (mM): KCl 10, potassium gluconate 133, EGTA 5, glucose 5, K_2ATP 1, NaADP 0.5, $MgCl_2$ 1 and HEPES 10 (pH = 7.4) [15]. The cell was held at 0 mV, and pulse voltages from -100 to +40 mV with a 20-mV increment (with pulse duration of 1 s, stimulated at 0.1 Hz) were applied. Current records were low-pass filtered, digitized and stored on computer hard-disk for later analysis using the Clampfit 9 softwares (Axon Instruments, USA).

Confocal Laser Scanning Microscopy

Porcine coronary artery myocytes were incubated with Fluo-4/AM (5 μM in 0.05% DMSO) (60 min, 37°C) in HEPES buffer (mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10, and HEPES 10 (pH 7.4). After washing, myocytes were imaged using an Eclipse CL Plus Confocal Microscope System (Nikon, Japan) with an excitation at 488 nm and a band-pass filter at 515/530 nm. Fluorescence changes of myocytes (at room temperature) in response to drugs (simvastatin (10 μM) and AICAR (1 mM), with and without ryanodine (100 μM)) were acquired at 15-s intervals. Images were recorded and analyzed by software EZ-C1 3.5 (Nikon, Japan).

Measurement of $[Glucose]_o$ Uptake

$[^3H]$ -2-Deoxy-glucose uptake into porcine coronary artery myocytes was determined using previously described protocols with minor modifications [30]. All experiments were performed in HEPES-buffered Ringer's solution containing (mM): NaCl 135; KCl 5; NaH_2PO_4 3.33; Na_2HPO_4 0.83; $CaCl_2$ 1.0; $MgCl_2$ 1.0 and HEPES 5 (pH 7.4). Confluent monolayer of cultured porcine coronary artery myocytes in 24-well plates were washed three times in Ringer's solution. $[^3H]$ -2-Deoxy-glucose (10 μM , 4 $\mu Ci/ml$) was added to each well and incubated for 30 min (37°C). The plates were then washed three times rapidly with ice-cold phosphate-buffered saline before cells were solubilized in 0.5 ml of Triton X-100 (5% vol./vol.). To determine non-specific uptake of $[^3H]$ -2-deoxy-glucose, cells were incubated in buffer containing $[^3H]$ -2-deoxy-glucose in the presence of cytochalasin B (50 μM) and phloretin (100 μM). The radioactivity was measured using a β -scintillation counter. The protein content was determined spectrophotometrically using the bicinchoninic acid assay (Pierce Biochemicals, USA).

Determination of Cellular ATP Contents

ATP was extracted from cultured porcine coronary artery myocytes (before and after drug treatments) by trichloroacetic acid (final concentration, 0.5% vol./vol.). Trichloroacetic acid in the sample was then neutralized and diluted to a final concentration of 0.1% by adding Tris-acetate buffer (pH 7.75). The ATP content was analyzed using the ATP-dependent luciferin-luciferase bioluminescence assay (ENLITEN[®] ATP assay system, Promega, USA).

Western Immunoblots

Isolated porcine coronary artery and human internal mammary artery (both were endothelium denuded) were homogenized in the presence of protease inhibitors (Roche, USA) to obtain extracts of proteins. Protein concentrations were determined using BCA[™] protein assay kit (Pierce, USA). Samples (40 μg of protein per lane) were loaded onto a 10% SDS-polyacrylamide electrophoresis gel. After electrophoresis (90 V, 120 min), the separated proteins were transferred (12 mA, 45 min) to polyvinylidene difluoride mem-

brane (Bio-Rad, USA). Non-specific sites were blocked with 5% non-fat dry milk (Bio-Rad, USA) for 120 min, and the blots were then incubated with individual type of antibody: anti-HMG CoA reductase, 1:1,000 (Upstate Biotechnology, USA); anti-p-HMG CoA reductase-Ser⁸⁷¹, 1:1,000 (Kinasource, UK); anti-CYP450 3A4, 1:1,000 (Affinity Bioreagent, USA); anti-PP2A, 1:1,000 (Upstate Biotechnology, USA); anti-p-PP2A-Tyr³⁰⁷, 1:1,000 (Upstate Biotechnology, USA); anti-AMPK, 1:1,000 (Upstate Biotechnology, USA), anti-p-AMPK α -Thr¹⁷², 1:1,000 (Upstate Biotechnology, USA); anti-LKB1, 1:1,000 (Upstate Biotechnology, USA) and anti-p-LKB1-Ser⁴²⁸, 1:1,000 (Upstate Biotechnology, USA) overnight at 4°C. Anti-mouse HRP conjugated IgG, 1:1,000 (Bio-Rad, USA) or anti-rabbit HRP conjugated IgG, 1:1,000 (Bio-Rad, USA) was used to detect the binding of the corresponding antibody. Membranes were stripped and re-blotted with anti- β -actin antibody, 1:10,000 (Sigma-Aldrich, USA) to verify an equal loading of protein in each lane. The protein expression was detected with ImmueStar Reagent (Bio-Rad, USA) and quantified using Scion Image analysis programme (Scion Image Ltd., USA).

Chemicals

Simvastatin, cromakalim, glibenclamide, AICAR, compound C, rottlerin, ryanodine, caffeine, U46619, KB R-7953 mesylate, H89 dihydrochloride, okadaic acid, KN93 and KN92 were purchased from Tocris Biosciences (UK). Simvastatin Na⁺ (50 mmol/L) was prepared from simvastatin using NaOH (in ethanol), as suggested by the manufacturer (Tocris Biosciences, UK). Nifedipine, D-mannitol, pinacidil, ouabain, phloridzin, 5-(N-ethyl-N-isopropyl) amiloride (EIPA), N-methyl-D-glucamine chloride and ketoconazole were obtained from Sigma-Aldrich (USA).

Statistical Analysis

All data were obtained from at least 6 independent experiments. Statistical analysis was performed using Student's *t* test or ANOVA (one-way or two-way), where appropriate. A *P* value of <0.05 was considered significant. Data are expressed as mean \pm S.E.M.

Results

Biochemical Existence of HMG-CoA Reductase and the Effects of Simvastatin and Simvastatin Na⁺ on HMG-CoA Reductase Expression

The biochemical existence of HMG CoA reductase was determined in both human isolated left internal mammary artery and porcine isolated coronary artery. Porcine liver served as the positive control. Western blot results confirmed the biochemical existence of HMG-CoA reductase in both human and porcine vascular preparations. Beta actin was used as a loading control (Figure 1A).

We then investigated the effects of simvastatin and simvastatin Na⁺ in inhibiting HMG CoA reductase activity (i.e. phosphorylation of HMG CoA reductase) [29]. Neither simvastatin nor simvastatin Na⁺ (10 μM , incubation \leq 30 min) altered the protein expression of p-HMG-CoA reductase-Ser⁸⁷¹ and HMG-CoA reductase in porcine isolated coronary artery (Figure 1B).

Effects of Simvastatin on K_{ATP} Channel Opener-induced Relaxation

To evaluate the involvement of K_{ATP} channels, effects of simvastatin on K_{ATP} channel opener-mediated vascular relaxation were examined. Cromakalim and pinacidil (both are K_{ATP} channel openers) (10 nM to 10 μM) caused a glibenclamide (1

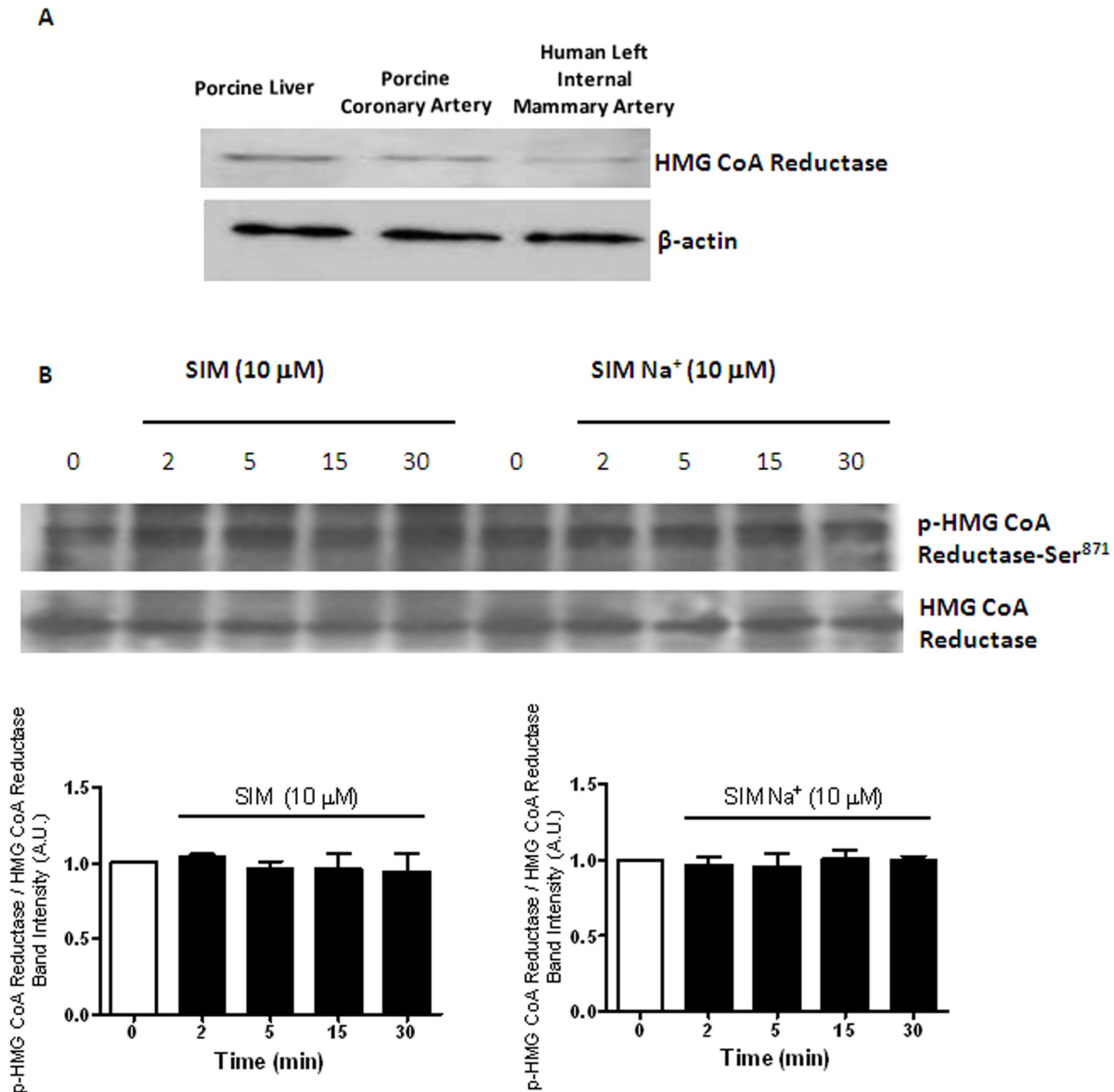


Figure 1. Biochemical existence of HMG-CoA reductase, and the effects of simvastatin and simvastatin Na⁺ on the protein expression of HMG-CoA reductase and p-HMG-CoA reductase. (A) Biochemical existence of HMG-CoA reductase in porcine liver, porcine coronary artery (endothelium-denuded) and human left internal mammary artery (endothelium-denuded). Beta actin was used as loading control. (B) Effects of simvastatin (SIM) (10 μ M) and simvastatin Na⁺ (SIM Na⁺) (10 μ M) (incubation, 2 to 30 min) on the protein expression of p-HMG-CoA reductase-Ser⁸⁷¹ and HMG-CoA reductase of porcine coronary artery. doi:10.1371/journal.pone.0066404.g001

and 3 μ M)-sensitive relaxation of U46619 (10 nM) pre-constricted coronary artery (endothelium-denuded) relaxation in a concentration-dependent manner (data not shown). Glibenclamide alone did not alter the basal tension and U46619-induced contraction. Simvastatin (3 and 10 μ M), but not simvastatin Na⁺ (1, 3 and 10 μ M), significantly attenuated cromakalim- and pinacidil-induced relaxation (Figure 2A and B). Neither simvastatin nor simvastatin Na⁺ altered the basal tension of the preparation.

Okadaic acid (a potent PP2A inhibitor) was used to elucidate the involvement of PP2A in simvastatin-suppressed cromakalim- and pinacidil-induced relaxation. Okadaic acid (10 nM) eradicated simvastatin (10 μ M)-induced inhibition of cromakalim- and pinacidil-induced relaxation (n = 6) (Figure 2C and D). Okadaic

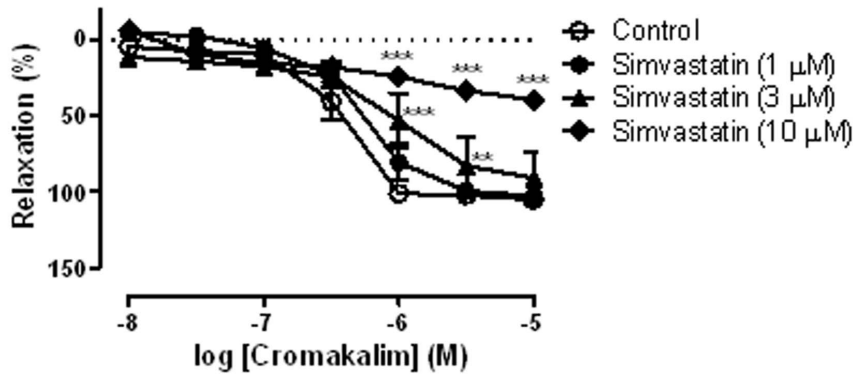
acid (10 nM) alone did not modify cromakalim-induced relaxation.

Effects Simvastatin on K_{ATP} Openings

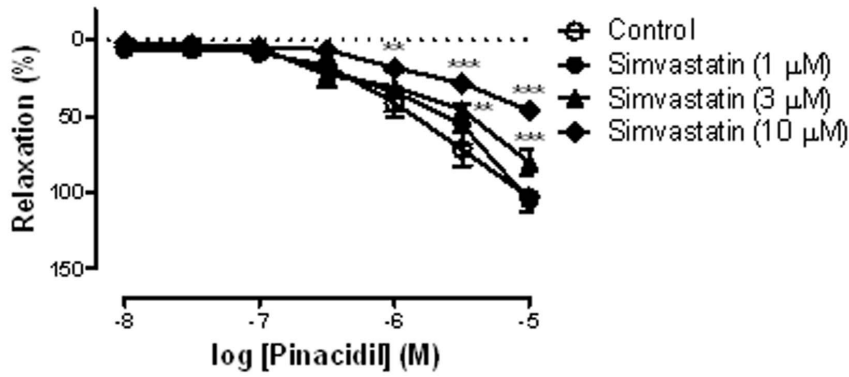
In order to get a better understanding on the modulation of K_{ATP} channels gatings by simvastatin, experiments were performed in single vascular myocytes. Cromakalim (10 μ M) (Figure 3A) and pinacidil (10 μ M) (data not shown) significantly enhanced the recorded outward K^+ current amplitude which is inhibited by glibenclamide (a K_{ATP} channel blocker), indicating that the recorded K^+ current is the genuine K_{ATP} current.

In human internal mammary artery myocytes, neither simvastatin (1, 3 and 10 μ M) nor simvastatin Na⁺ (1, 3 and 10 μ M) altered the basal K_{ATP} channel gatings (data not shown).

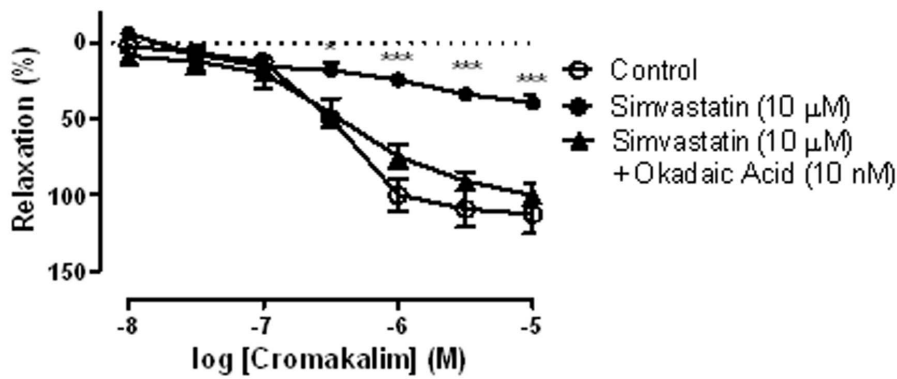
A



B



C



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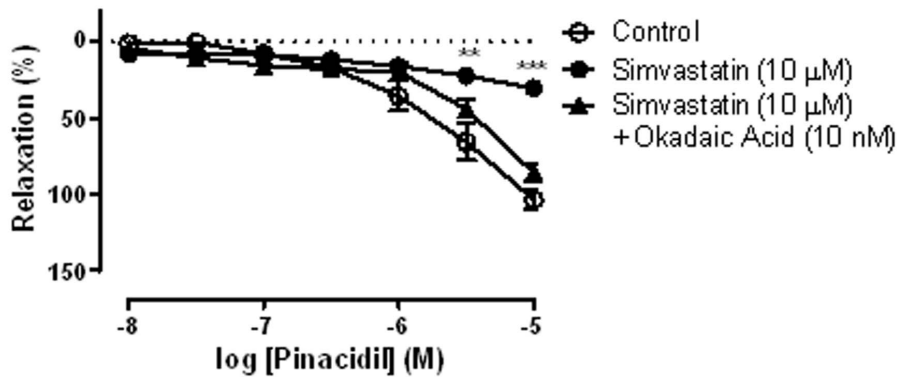


Figure 2. Effects of simvastatin on K_{ATP} channel openers-induced vasorelaxation. (A) Effect of simvastatin (1, 3 and 10 μ M) ($n=6$ to 8) on cromakalim-induced relaxation of U46619 (10 nM) pre-contracted porcine coronary artery (endothelium-denuded). (B) Effect of simvastatin (1, 3 and 10 μ M) ($n=6$ to 8) on pinacidil-induced relaxation of U46619 (10 nM) pre-contracted porcine coronary artery (endothelium-denuded). (C) Effect of okadaic acid (10 nM) ($n=6$ to 8) on simvastatin-inhibited cromakalim-induced relaxation of U46619 (10 nM) pre-contracted porcine coronary artery (endothelium-denuded). (D) Effect of okadaic acid (10 nM) ($n=6$ to 8) on simvastatin-inhibited pinacidil-induced relaxation of U46619 (10 nM) pre-contracted porcine coronary artery (endothelium-denuded). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared to controls. doi:10.1371/journal.pone.0066404.g002

Interestingly, simvastatin caused a concentration-dependent inhibition of cromakalim (10 μ M)-induced K_{ATP} channel opening, with no apparent recovery after washout (Figure 3B). However, simvastatin Na^+ (10 μ M, applied either in external bath solution or included in the pipette solution) did not alter cromakalim (10 μ M)-induced K_{ATP} opening (data not shown).

Due to the irregular/limited supply of human left internal mammary artery for research purposes, the following experiments were performed using porcine coronary artery myocytes. All drugs/inhibitors were tested against both cromakalim- and pinacidil-induced K_{ATP} opening, however only representative figures of drug modulation of cromakalim-mediated responses were illustrated in the Figures.

Okadaic acid (a potent PP2A inhibitor) was used to examine the involvement of PP2A in simvastatin-mediated suppression of cromakalim- and pinacidil-induced K_{ATP} opening. Okadaic acid (10 nM) did not alter the basal K_{ATP} openings, and cromakalim (10 μ M)- and pinacidil (10 μ M)-induced K_{ATP} openings. However, okadaic acid (10 nM, in the pipette solution) significantly attenuated simvastatin (10 μ M)-mediated suppression of cromakalim (10 μ M)- and pinacidil (10 μ M)-induced K_{ATP} openings (Figure 3C). In contrast, okadaic acid failed to alter glibenclamide (3 μ M)-mediated inhibition of cromakalim- and pinacidil-induced K_{ATP} openings (Figure 3C).

The involvement of AMPK on cromakalim- and pinacidil-induced K_{ATP} channel openings was examined. Similar to simvastatin (10 μ M), AICAR (1 mM, an AMPK activator) attenuated cromakalim- and pinacidil-induced K_{ATP} channel openings (Figure 3D). However, AICAR (1 mM) did not alter the basal K_{ATP} amplitude (data not shown).

Effects of Simvastatin on AMPK and PP2A Phosphorylation

To strengthen our hypothesis on the participation of AMPK activation on mediating simvastatin-induced responses, we evaluated AMPK activity using Western blots. Activity of AMPK is represented by p-AMPK α -Thr¹⁷²/total AMPK, as previously demonstrated [31]. AICAR (1 mM) and simvastatin (10 μ M) caused a time-dependent (2–30 min) increase of AMPK activation (i.e. increased p-AMPK α -Thr¹⁷² expression) (Figure 4A and B). These responses were sensitive to Compound C (an AMPK inhibitor) (1 μ M; 30 min) (Figure 4C). Okadaic acid (10 nM, 30 min) (Figure 4D) did not alter simvastatin (10 μ M)-mediated increase of AMPK activity.

The role of PP2A activation (represented by p-PP2A-Tyr³⁰⁷/total PP2A), as previously reported [32], in response to simvastatin and AICAR challenges was evaluated using okadaic acid and Compound C. Simvastatin (10 μ M) and AICAR (1 mM) elicited a time-dependent (2 to 30 min) increase in p-PP2A-Tyr³⁰⁷/total PP2A (i.e. a decreased PP2A activity) (Figure 5A and B). Simvastatin (10 μ M)- and AICAR (1 mM)-induced decrease of PP2A activity was eradicated by okadaic acid (10 nM, 30 min) and Compound C (1 μ M, 30 min) (Figure 5C and D).

Role(s) of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ in Mediating Effects of Simvastatin on AMPK and PP2A Activities

Ca^{2+} ions are important in mediating various cellular signaling cascades. The significance of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ in mediating simvastatin-induced responses was therefore evaluated. Simvastatin (10 μ M) caused an increase in $[Ca^{2+}]_i$ level and contraction of single myocytes (data not shown). In myocytes challenged with ryanodine (100 μ M), there was a transient increase in $[Ca^{2+}]_i$, (plus contraction of single myocytes) and the subsequent application of simvastatin (10 μ M) (Figure 6A and B) failed to alter $[Ca^{2+}]_i$ levels.

To elucidate the role of changes in $[Ca^{2+}]_i$ in mediating simvastatin-induced AMPK activation as shown above, effects of ryanodine, $[Ca^{2+}]_o$ -free solution, KB R-7953 and nifedipine were examined. Ryanodine (100 μ M, 30 min pre-treatment) abolished simvastatin (10 μ M)-, but not AICAR (1 mM)-, induced AMPK activation (Figure 6C). Caffeine (1 mM) caused a time-dependent (2 to 30 min) (ryanodine-sensitive) AMPK activation (Figure 6D). Ryanodine, on its own, did not alter AMPK activation.

Ryanodine pre-treatment (100 μ M) abolished simvastatin (10 μ M)-, AICAR (1 mM) and caffeine (1 mM)-induced changes of p-PP2A-Tyr³⁰⁷/total PP2A (Figure 6E). Moreover, effects of simvastatin (10 μ M)- and caffeine (1 mM)-, but not AICAR (1 mM)-, on PP2A activities were abolished by KN 93 (10 μ M) (Figure 6F), but not by KN 92 (10 μ M) (data not shown). In addition, effects of simvastatin (10 μ M) and AICAR (1 mM) on AMPK and PP2A activities were not modified by $[Ca^{2+}]_o$ -free solution (with EGTA, 2 mM), KB R-7953 (10 μ M)- or nifedipine (10 μ M)-containing solutions (data not shown).

Effects Simvastatin and AICAR on $[Glucose]_o$ Uptake

In order to establish the role of $[glucose]_o$, effects of simvastatin and AICAR on $[glucose]_o$ uptake was determined. Simvastatin (10 μ M) and AICAR (1 mM) caused a significant increase in [³H]-2-deoxy-glucose uptake into coronary artery myocytes, and the “enhanced” $[glucose]_o$ uptake was eradicated by Compound C (10 μ M) (Figure 7A).

The Role(s) of $[Glucose]_o$ and $[Na^+]_o$ in Mediating Simvastatin Effects on AMPK and PP2A Activities

After the confirmation of the essential role of $[glucose]_o$ as mentioned above, identification of the $[glucose]_o$ uptake transporter involved was performed. $[Glucose]_o$ -free solution (osmotic balanced with D-mannitol), phloridzin (1 mM, a Na^+ /glucose cotransporter-1 (SGLT-1) blocker), ouabain (10 μ M, a Na^+ / K^+ ATPase inhibitor), 5-(N-ethyl-N-isopropyl) amiloride (EIPA, 10 μ M) (a Na^+ / H^+ exchanger-1 blocker) and $[Na^+]_o$ -free (replaced with N-methyl-D-glucamine) solution failed to alter simvastatin (10 μ M)- and AICAR (1 mM)-mediated increase of p-AMPK α -Thr¹⁷² expression (data not shown). In contrast, simvastatin (10 μ M)- and AICAR (1 mM)-mediated increase of p-PP2A-Tyr³⁰⁷/total PP2A (i.e. PP2A inhibition) was eradicated in $[glucose]_o$ -free (Figure 7B) or $[Na^+]_o$ -free conditions (Figure 7C), and with phloridzin (1 mM)- (Figure 7D) or ouabain (10 μ M)-containing solutions (Figure 7E). However, EIPA (10 μ M) did not

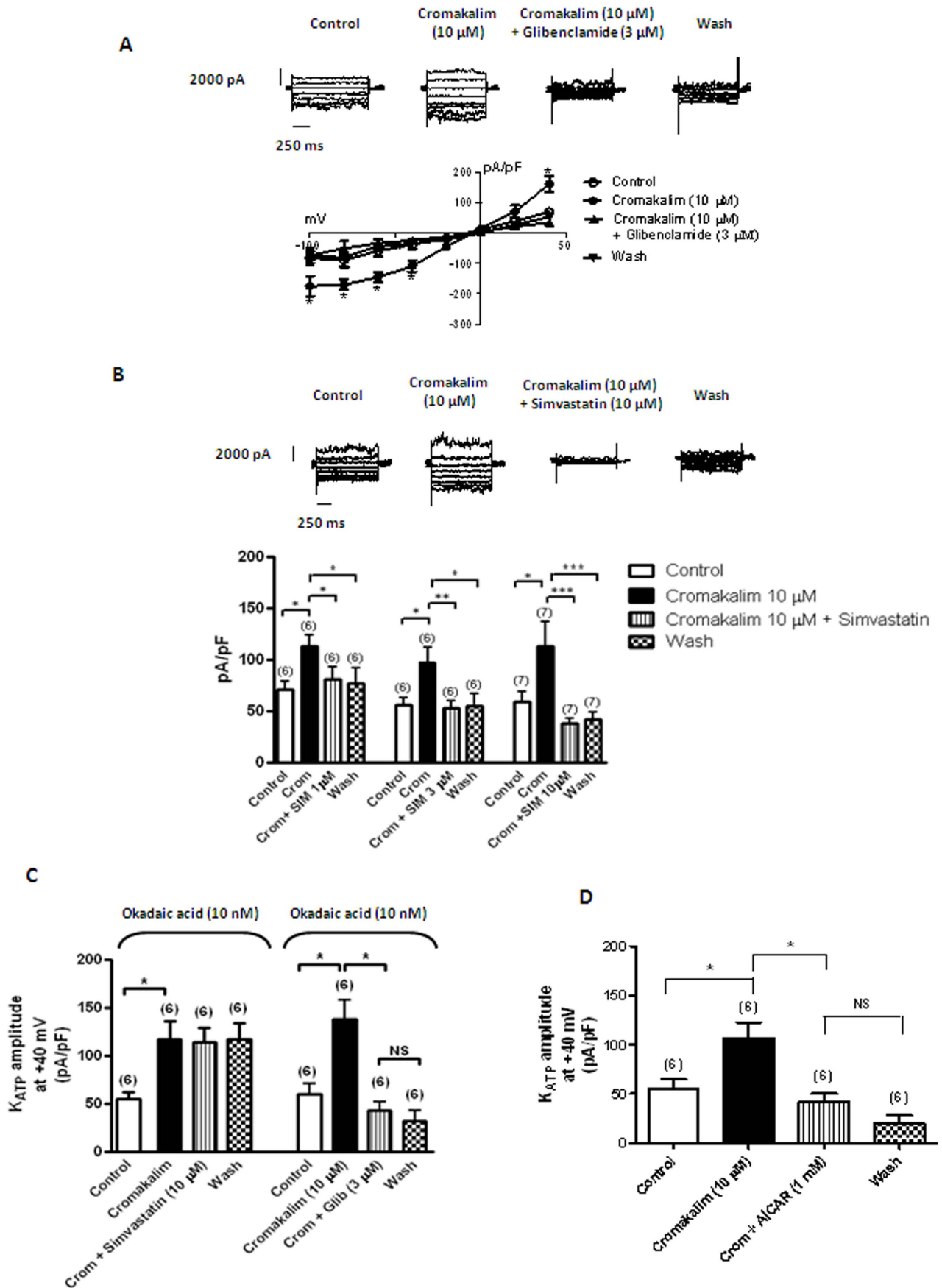
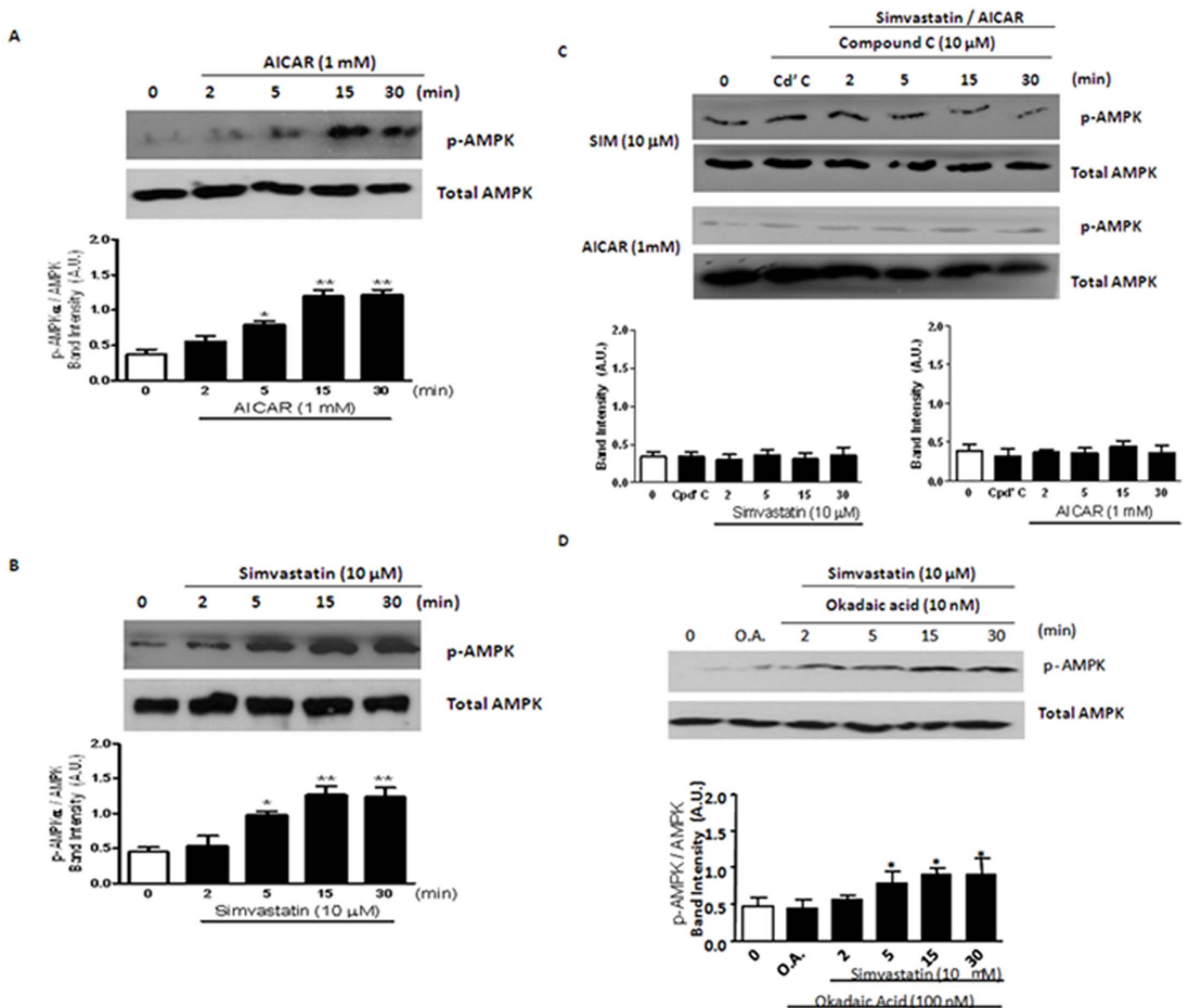


Figure 3. Effects of simvastatin on K_{ATP} channel openings. (A) Effects of cromakalim (Crom., 10 μ M) on whole-cell K_{ATP} channel openings of single human internal mammary artery myocytes in the presence of glibenclamide (Glib., 3 μ M) ($n=5$ to 6). (B) Effects of cromakalim (Crom., 10 μ M) on whole-cell K_{ATP} channel openings of single human internal mammary artery myocytes with and without simvastatin (1, 3 and 10 μ M). (C) Effects of simvastatin (10 μ M) and glibenclamide (Glib., 3 μ M) on cromakalim (Crom., 10 μ M)-induced whole-cell K_{ATP} channel openings of single porcine artery myocytes (in the presence of okadaic acid, 10 nM). Number of cells studied is indicated in parenthesis. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared to controls. (D) Effects of cromakalim (Crom., 10 μ M) on whole-cell K_{ATP} channel openings of single porcine coronary artery myocytes in the presence of AICAR (1 mM). Number of cells studied is indicated in parenthesis. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared to controls. doi:10.1371/journal.pone.0066404.g003

modify simvastatin (10 μ M)- and AICAR (1 mM)-mediated changes of p-PP2A-Tyr³⁰⁷/total PP2A (data not shown).

Effects of Simvastatin on $[ATP]_i$ Levels and LKB1 Activation

To confirm the generation of $[ATP]_i$ after $[glucose]_o$ uptake induced by simvastatin, cellular ATP level was estimated in response to drug challenges. AICAR caused a Compound C (10 μ M)-sensitive increase in cellular ATP level of the arterial



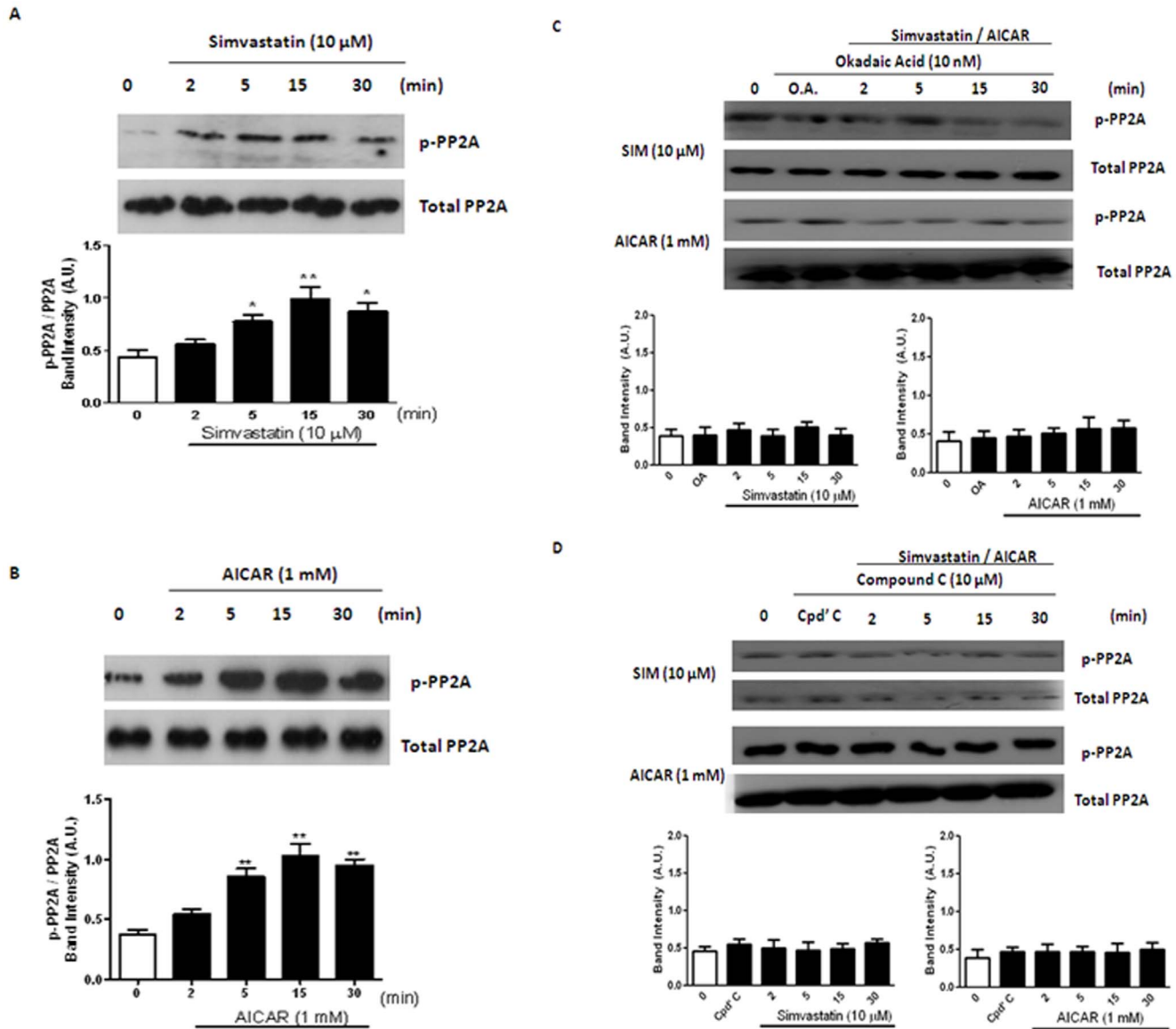


Figure 5. Effects of simvastatin on PP2A activation in porcine coronary artery. (A) Effects of simvastatin (10 μ M) on the protein expression of p-PP2A/total PP2A in porcine coronary artery. * P <0.05 and ** P <0.01 compared to controls (i.e. time 0). (B) Effects of AICAR (1 mM) on the protein expression of p-PP2A/total PP2A in porcine coronary artery. * P <0.05 and ** P <0.01 compared to controls (i.e. time 0). (C) Effect of okadaic acid (O.A., 10 nM) on simvastatin- and AICAR-induced protein expression of p-PP2A/total PP2A in porcine coronary artery. * P <0.05 and ** P <0.01 compared to controls (i.e. time 0). (D) Effect of compound C (10 μ M) on simvastatin- and AICAR protein expression of p-PP2A/total PP2A in porcine coronary artery. * P <0.05 and ** P <0.01 compared to controls (i.e. time 0). doi:10.1371/journal.pone.0066404.g005

myocytes (Figure 8A) and a time-dependent (2 to 30 min) increase in LKB1 activity (i.e. an increase in p-LKB1-Ser⁴²⁸/total LKB1 [33]) (Figure 8B). However, simvastatin increased intracellular ATP level of the arterial myocytes with no apparent change in p-LKB1/total LKB1 (Figure 8C).

Participation of Cytochrome P450 3A4

To elucidate the importance of cytochrome P450 (CYP450)-mediated drug metabolism in mediating simvastatin-induced responses, effects of CYP450 3A4 inhibitor was examined. The biochemical existence of CYP450 3A4 protein was confirmed in porcine coronary artery and human internal mammary artery (Figure 9A). Porcine liver served as the positive control. Ketoconazole (10 μ M, a selective CYP450 3A4 inhibitor) failed

to modify simvastatin (10 μ M)-induced changes of AMPK and PP2A activities (Figure 9B and C).

Discussion

In this study, acute simvastatin (membrane permeable) suppressed cromakalim- and pinacidil-induced relaxation of U46619 pre-constricted (endothelium-denuded) arteries with no effects on basal tension. In single myocytes of porcine coronary artery and human left internal mammary artery, simvastatin and AICAR inhibited cromakalim- and pinacidil-evoked K_{ATP} channel openings with no apparent effect on basal K_{ATP} channel gatings. Thus, a prerequisite opening of K_{ATP} channels (by two structurally different K_{ATP} channel openers cromakalim and

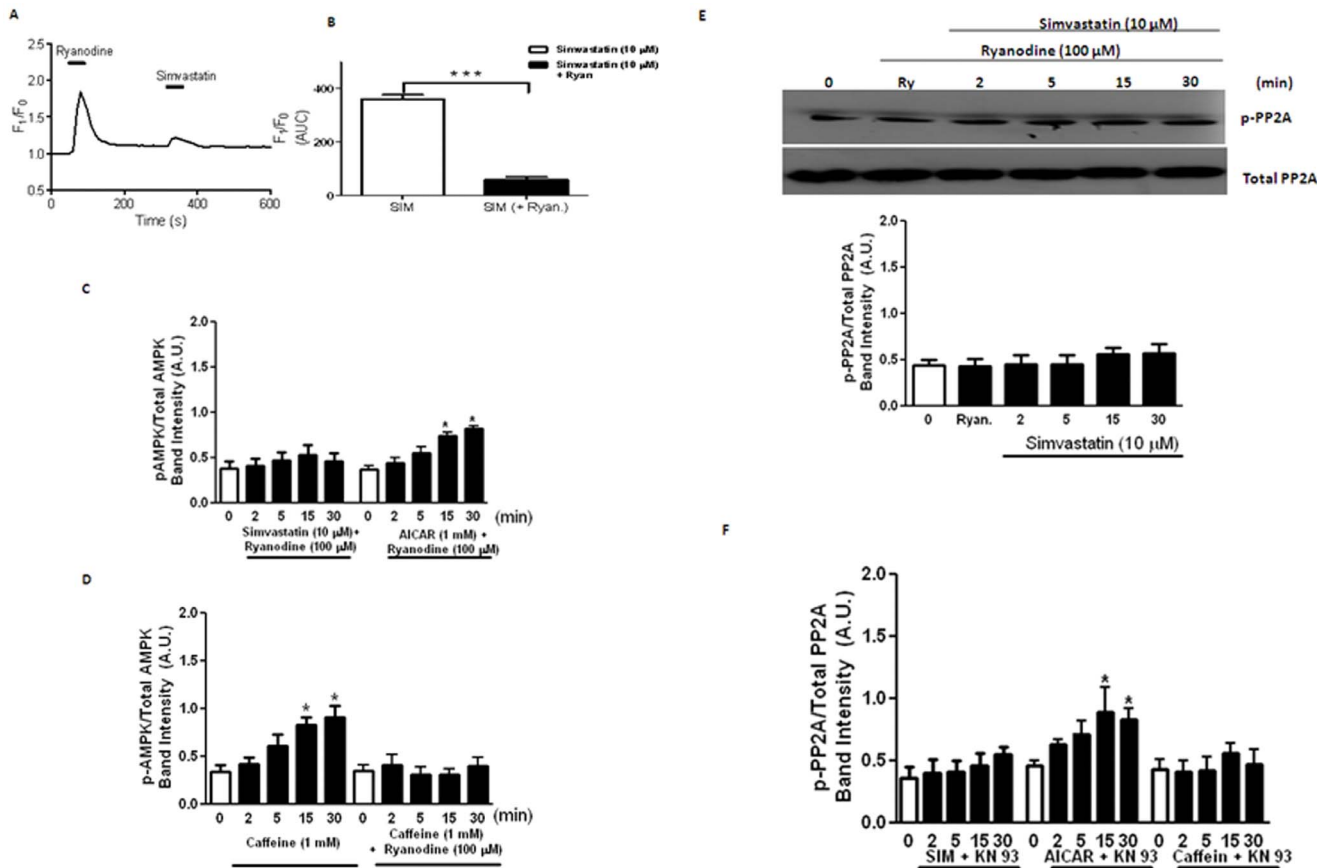


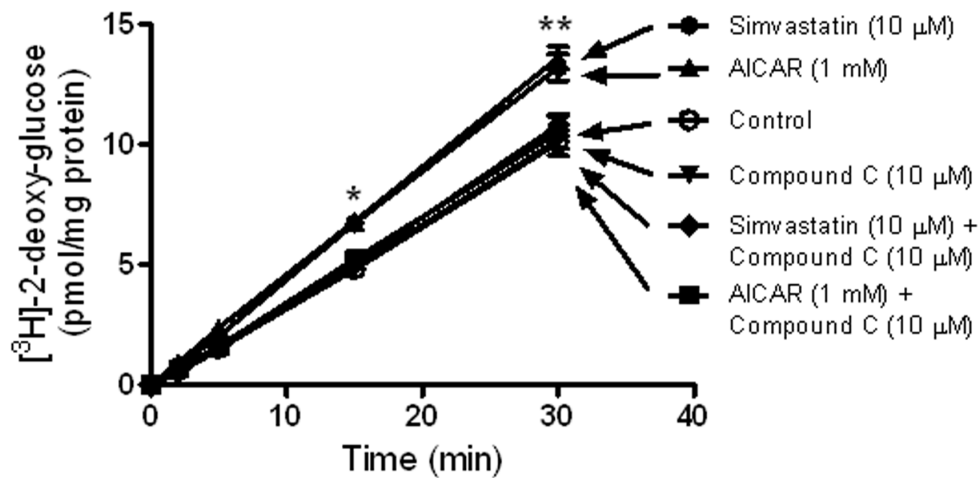
Figure 6. Role(s) of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ in mediating the effects of simvastatin on AMPK and PP2A activities. (A) Effects of simvastatin (10 μ M), with and without ryanodine (100 μ M) pre-treatment, on $[Ca^{2+}]_i$ changes (F_1/F_0) of porcine coronary artery myocytes, estimated using Fluo-4/AM with confocal laser scanning microscope. (B) Summary of $[Ca^{2+}]_i$ changes in response to simvastatin (10 μ M) before and after ryanodine (100 μ M) challenges. Results are expressed (Area Under Curve, AUC) as mean \pm SEM of 13–15 cells (** $P < 0.001$). (C) Summary of the effects of ryanodine (100 μ M) on simvastatin (10 μ M)- or AICAR (1 mM)-induced protein expression of p-AMPK/total AMPK in porcine coronary artery. * $P < 0.05$ and ** $P < 0.01$ compared to controls (i.e. time 0). (D) Summary of the effects of caffeine (1 mM) on the protein expression of p-AMPK/total AMPK in porcine coronary artery, with and without ryanodine (100 μ M). * $P < 0.05$ and ** $P < 0.01$ compared to controls (i.e. time 0). (E) Effect of ryanodine (100 μ M) on simvastatin (10 μ M)-induced protein expression of p-PP2A/total PP2A in porcine coronary artery. * $P < 0.05$ and ** $P < 0.01$ compared to controls (i.e. time 0). (F) Summary of the effect of simvastatin (10 μ M), AICAR (1 mM) and caffeine (1 mM) on the protein expression of p-PP2A/total PP2A, with and without KN93 (10 μ M) in porcine coronary artery. * $P < 0.05$ and ** $P < 0.01$ compared to controls (i.e. time 0). doi:10.1371/journal.pone.0066404.g006

pinacidil) is necessary for simvastatin and AICAR to demonstrate K_{ATP} channel blocking properties. However, simvastatin Na^+ (membrane impermeable) did not alter the cromakalim-/pinacidil-induced relaxation and the K_{ATP} channel openings. Therefore, these results suggest that the lipophilic property of simvastatin is essential [3,34].

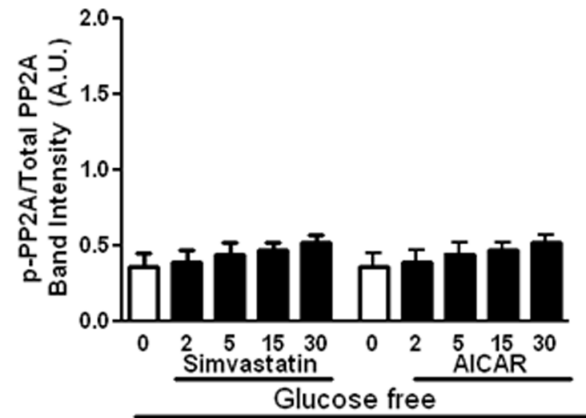
Acute application of HMG-CoA reductase inhibitors (pravastatin, atorvastatin and cerivastatin) elicited an endothelium-dependent relaxation of pre-constricted rat isolated aorta, and cerivastatin-induced relaxation was attenuated by glibenclamide and ouabain [13]. Activation of cardiac K_{ATP} channels by statins has been reported [14,35,36]. However, a recent study [37] and our current study demonstrated that simvastatin inhibits pinacidil-induced relaxation in porcine isolated coronary artery. Furthermore, our result illustrate that simvastatin consistently suppressed, instead of enhanced, cromakalim- and pinacidil-induced K_{ATP} channel openings of arterial myocytes of pig coronary artery and human left internal mammary artery. Taken together, these results clearly illustrate that simvastatin could alter vasodilatation via the inhibition of K_{ATP} channels.

AMPK (formerly termed HMG-CoA reductase kinase) is activated by an increase in $[AMP/ATP]_i$ ratio and a rise in $[Ca^{2+}]_i$ which signals an increase in energy demands [38,39]. Once activated, AMPK decreases ATP consumption and/or stimulates ATP production (e.g. via oxidative phosphorylation) and the $[ATP]_i$ level is thus restored [40]. In human umbilical vein endothelial cells (HUVECs), atorvastatin activated AMPK [25] whereas in mouse pancreatic islets β -cells, AICAR (an AMPK activator) inhibited K_{ATP} openings [41]. Acute application of simvastatin significantly suppressed vasoconstriction of rat mesenteric resistance arteries via an AMPK α -phosphorylation-dependent mechanism [42]. In addition, AICAR activates AMPK via an increased phosphorylation, and phosphorylation (i.e. inactivation) of a known target for AMPK i.e. HMG-CoA reductase occurred [18,43,44]. However, in our study neither simvastatin nor simvastatin Na^+ (incubation ≤ 30 min) altered the expression of HMG-CoA reductase and p-HMG-CoA reductase-Ser⁸⁷¹ (the inactivated isoform of HMG-CoA reductase) suggesting that AICAR and simvastatin are acting through different cellular mechanisms (see below). In rat's liver, AMPK is associated with HMG-CoA reductase [45]. In our study, simvastatin and AICAR

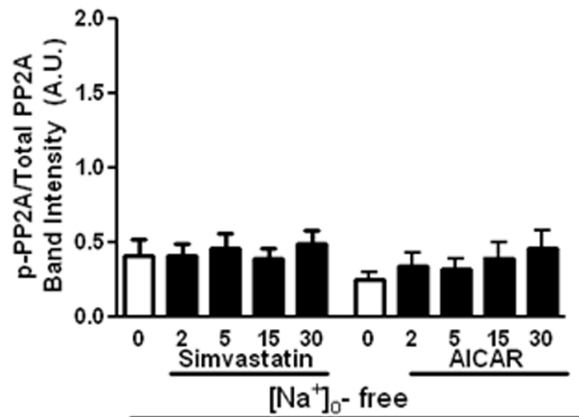
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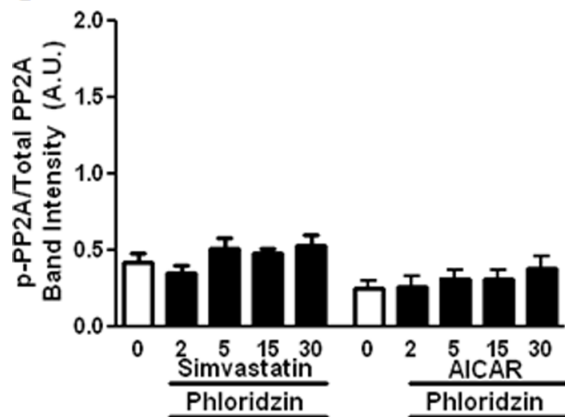
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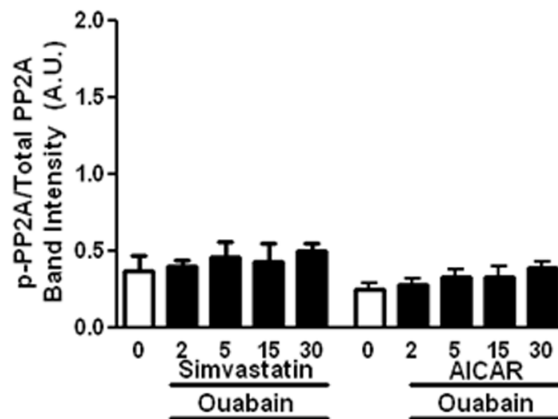
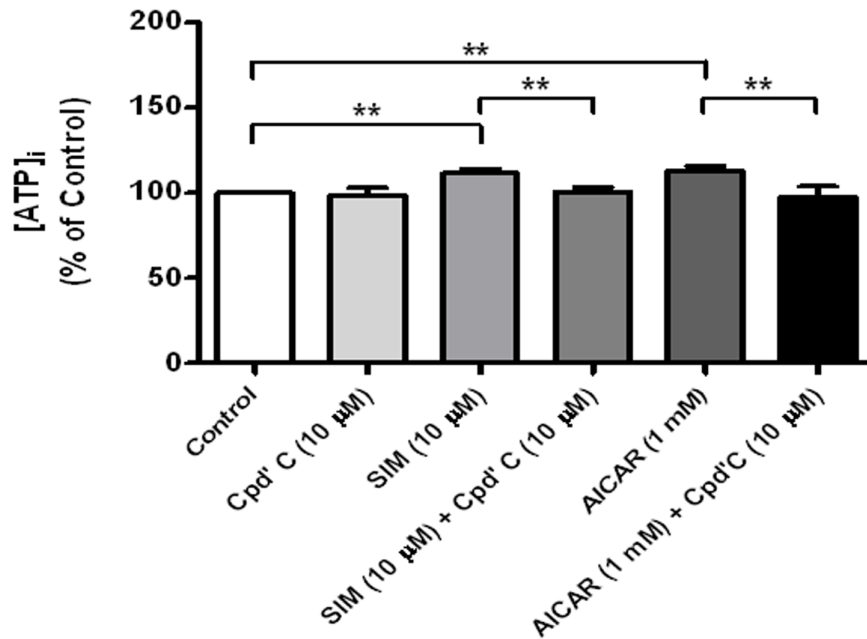
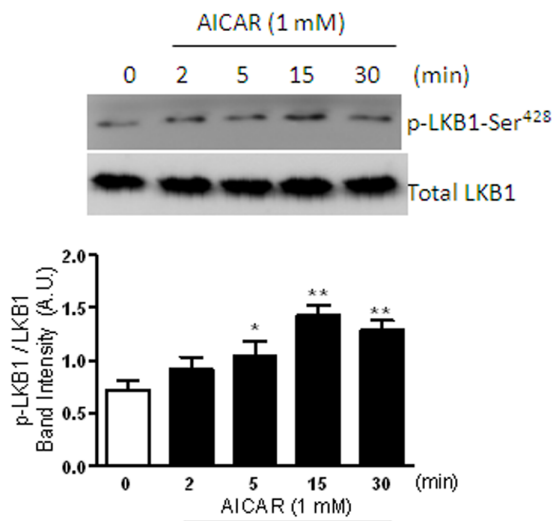


Figure 7. Effects of simvastatin and AICAR on $[Glucose]_o$ uptake and the role(s) of $[glucose]_o$ and $[Na^+]_o$ in mediating simvastatin effects on AMPK and PP2A activities. (A) Effects of simvastatin (10 μM) and AICAR (1 mM) on [³H]-2-deoxy-glucose uptake, with and without compound C (10 μM), of porcine coronary artery myocytes (n=6 for each treatment). * $P < 0.05$ and ** $P < 0.01$ compared to controls. Summary of the effect of simvastatin and AICAR on the protein expression of p-PP2A/total PP2A in (B) $[glucose]_o$ -free, (C) $[Na^+]_o$ -free, (D) with phloridzin (1 mM) and (E) with ouabain (10 μM) in porcine coronary artery. * $P < 0.05$ and ** $P < 0.01$ compared to controls (i.e. time 0). doi:10.1371/journal.pone.0066404.g007

A



B



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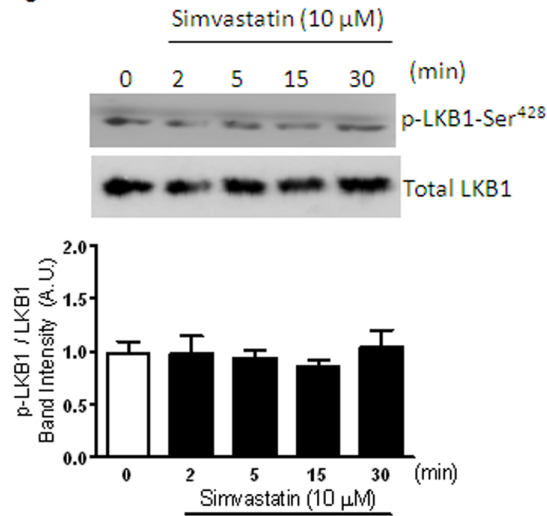


Figure 8. Effects of simvastatin on $[ATP]_i$ levels and LKB1 activation. (A) Summary of the effects of simvastatin (10 μ M) and AICAR (1 mM) on $[ATP]_i$ level with and without compound C (10 μ M) of porcine coronary artery myocytes ($n=6$ for each treatment). * $P<0.05$ and ** $P<0.01$ compared to controls. (B) Effect of AICAR (1 mM, $n=4$) on the protein expression of p-LKB1/total LKB1 in porcine coronary artery. * $P<0.05$ and ** $P<0.01$ compared to controls (i.e. time 0). (C) Effect of simvastatin (10 μ M, $n=4$) on the protein expression of p-LKB1/total LKB1 in porcine coronary artery. * $P<0.05$ and ** $P<0.01$ compared to controls (i.e. time 0). doi:10.1371/journal.pone.0066404.g008

consistently increased p-AMPK α -Thr¹⁷² expression (i.e. activation) [44], and suppressed cromakalim- and pinacidil-induced K_{ATP} channel openings. Taken together, our results illustrate that acute simvastatin inhibits vascular K_{ATP} channel openings probably via AMPK α -Thr¹⁷² phosphorylation (i.e. activation).

Okadaic acid (a potent PP2A inhibitor), but not rottlerin (a PKC- δ inhibitor) or H89 (a PKA inhibitor), reversed the inhibitory effects of simvastatin on cromakalim- and pinacidil-mediated

K_{ATP} channel openings strengthen our conclusion on the participation of PP2A. In contrast to cultured bovine aortic endothelial cells (BAECs) [46], simvastatin- and AICAR-mediated increase in PP2A-Tyr³⁰⁷ phosphorylation was abolished by Compound C (an AMPK inhibitor) illustrating that PP2A is phosphorylated (i.e. inactivated) in an ‘‘AMPK α -dependent’’ manner, and PP2A phosphorylation occurs at a site downstream of AMPK α activation/phosphorylation. In addition, AMPK can

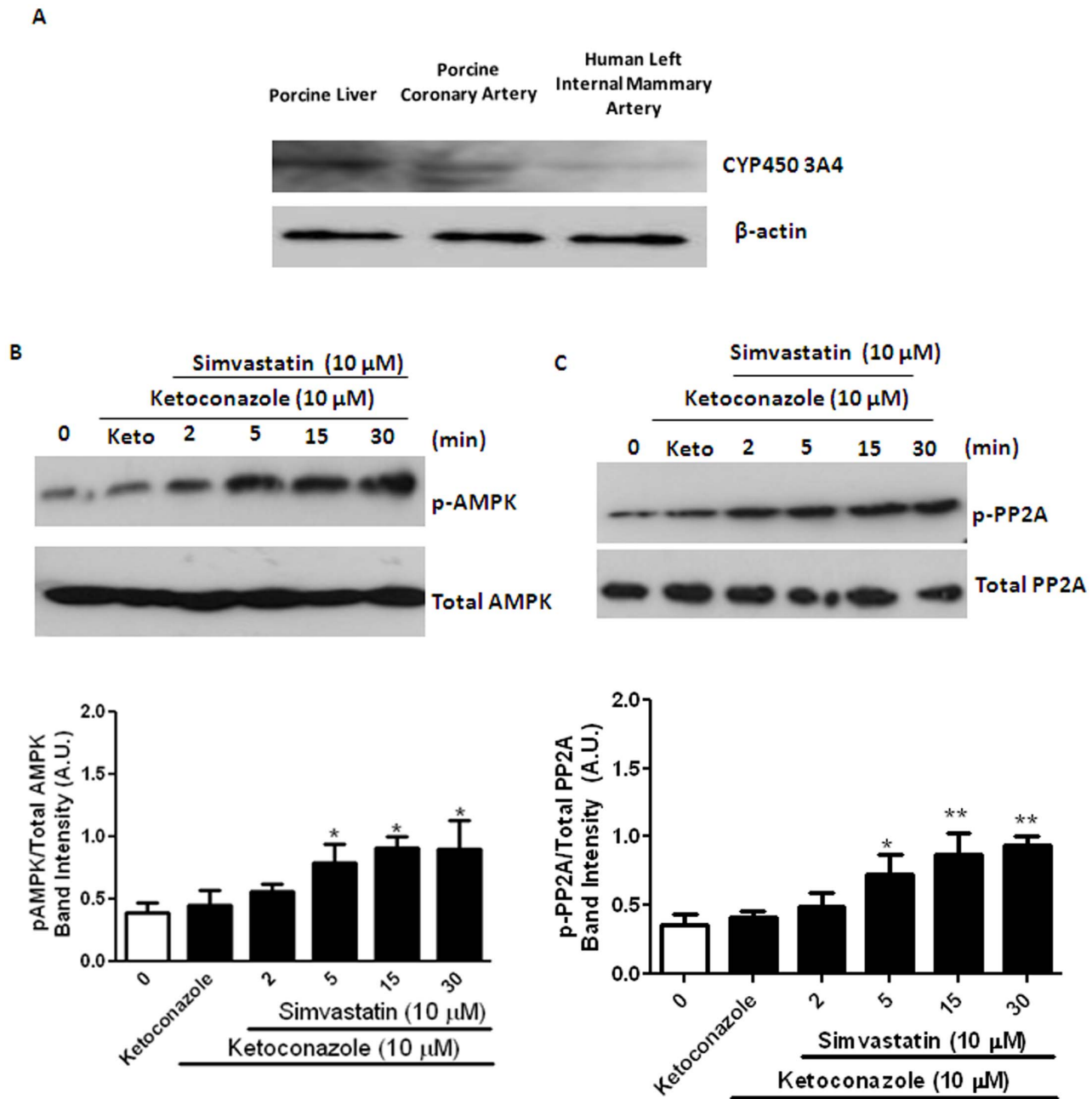


Figure 9. Participation of cytochrome P450 3A4. (A) Biochemical existence of cytochrome 450 (CYP450 3A4) in porcine liver, porcine coronary artery (endothelium denuded) and human left internal mammary artery (endothelium denuded). Beta actin was used as loading control. (B) Effects of simvastatin on the protein expression of p-AMPK/total AMPK, with ketoconazole (Keto, 10 μ M, n=4), in porcine coronary artery (endothelium denuded). * P <0.05 and ** P <0.01 compared to controls (i.e. time 0). (C) Effects of simvastatin on the protein expression of p-PP2A/total PP2A, with ketoconazole (Keto, 10 μ M, n=4), in porcine coronary artery (endothelium denuded). * P <0.05 and ** P <0.01 compared to controls (i.e. time 0). doi:10.1371/journal.pone.0066404.g009

be activated via the LKB1 (the upstream serine/threonine kinase of AMPK) cascade [47]. In cultured BAECs, simvastatin (10 μ M) increased AMPK α -Thr¹⁷² phosphorylation via LKB1-Ser⁴²⁸ phosphorylation [5,8]. However, in our study, simvastatin (unlike AICAR) did not cause LKB1-Ser⁴²⁸ phosphorylation of porcine coronary artery suggesting that simvastatin-induced AMPK α -Thr¹⁷² phosphorylation is mediated via a LKB1-Ser⁴²⁸ phosphorylation-independent pathway.

Apart from LKB1, AMPK is activated/phosphorylated by Ca²⁺/calmodulin-dependent kinase kinase (CaMKK) [48]. In our

study, caffeine- and simvastatin-induced AMPK α -Thr¹⁷² phosphorylation was abolished by KN93 (a selective Ca²⁺/calmodulin-dependent protein kinase II (CaMK II) inhibitor) illustrating the participation of Ca²⁺/CaMK II. In contrast, AICAR-induced AMPK α -Thr¹⁷² phosphorylation was not affected by KN93 suggesting that AICAR-mediated AMPK α -Thr¹⁷² phosphorylation is CaMK II-independent [48]. In fact, our results illustrate that AICAR increased p-LKB1-Ser⁴²⁸ expression suggesting that AICAR phosphorylates AMPK α -Thr¹⁷² via the LKB1 pathway [49].

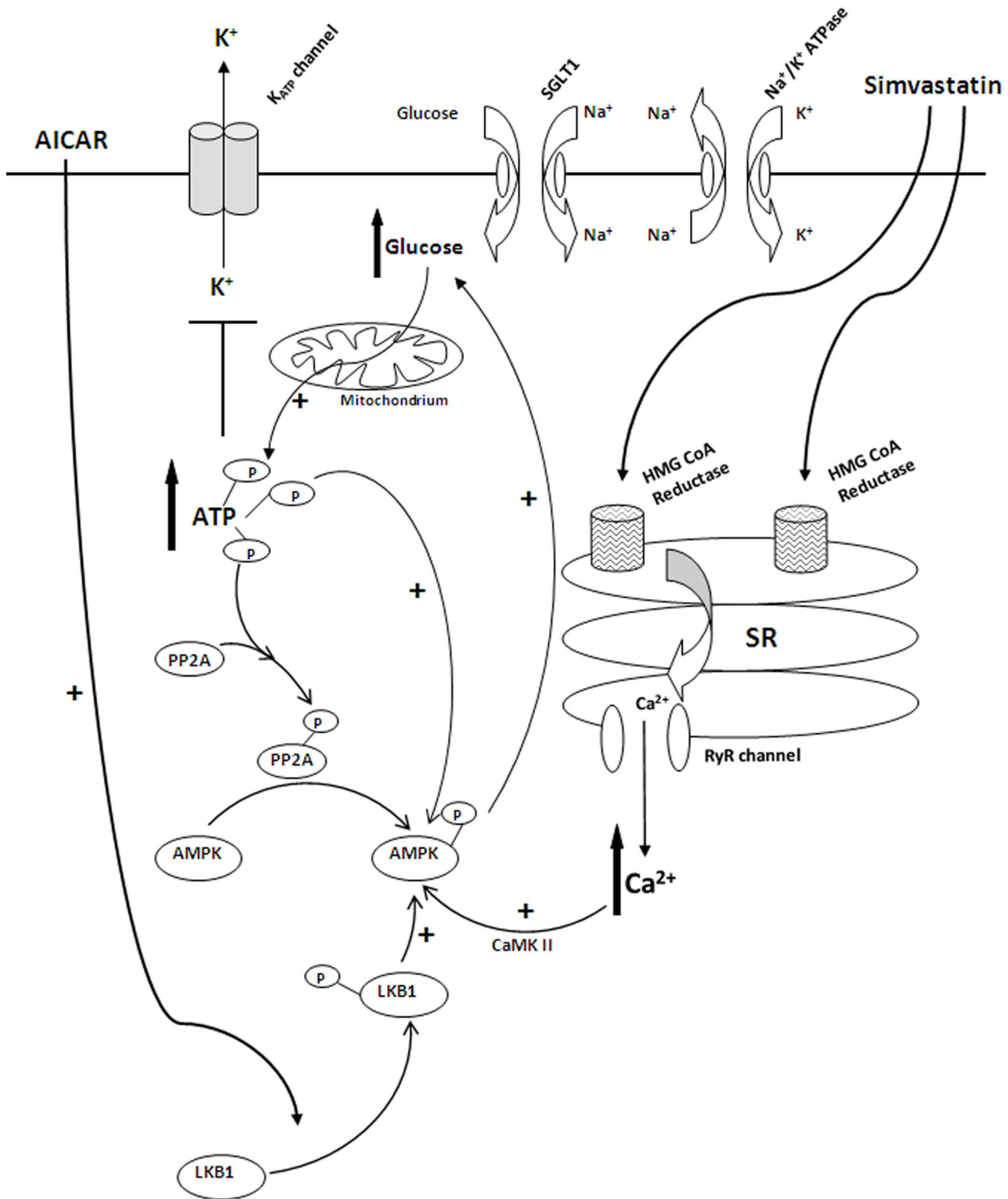


Figure 10. Proposed mechanisms for acute simvastatin-induced closure of K_{ATP} channels of vascular myocytes. Simvastatin (lipophilic) crosses the plasma membrane and reaches the sarcoplasmic reticulum (SR) of vascular myocytes. Binding of simvastatin to SR leads to the release of ryanodine (Ryr)-sensitive Ca^{2+} into the cytosol. Elevation of Ca^{2+} activates CaMK II which leads to the subsequent activation (phosphorylation) of AMPK α . Phosphorylation of AMPK α -Thr¹⁷² causes [glucose]_o uptake with the participation of SGLT1 and Na^+/K^+ ATPase. Increase in cytosolic [glucose] leads to an elevation of ATP levels via oxidative phosphorylation. Elevation of [ATP]_i serves two purposes: (1) closure of vascular K_{ATP} channels, (2) providing phosphate groups for cellular proteins (e.g. PP2A and AMPK) phosphorylation. Phosphorylation of PP2A occurs downstream of AMPK phosphorylation. PP2A phosphorylation results in PP2A inactivation which “releases” AMPK and thus phosphorylation of AMPK α -Thr¹⁷² resulted. AICAR produces similar effects as simvastatin except the initial step involves LKB1-Ser⁴²⁸ phosphorylation.
doi:10.1371/journal.pone.0066404.g010

Simvastatin inhibited Ca^{2+} release from intracellular stores of smooth muscle cells [50,51]. As mentioned above, caffeine- and simvastatin-induced AMPK α -Thr¹⁷² phosphorylation involved CaMK II activation which is a Ca^{2+} -dependent process. Similar to rat aorta [52] and cultured BAECs [53], in our study simvastatin caused a ryanodine-sensitive $[Ca^{2+}]_i$ increase in porcine coronary artery myocytes. Apart from Ca^{2+} release from intracellular stores, the $[Ca^{2+}]_i$ level is also modified by $[Ca^{2+}]_o$ influx [54]. However, our results reveal that $[Ca^{2+}]_o$ -free conditions did not modify simvastatin-induced p-AMPK α -Thr¹⁷² expression. Neither nifedipine (a L-type Ca^{2+} channel blocker) nor KB R-7953 (a reverse-mode Na^+/Ca^{2+} exchanger blocker) altered simvastatin-induced AMPK α -Thr¹⁷² phosphorylation. Taken together, the increased p-AMPK α -Thr¹⁷² expression is solely dependent on ryanodine-sensitive $[Ca^{2+}]_i$ release which is probably related to the distinct physiological location of HMG-CoA reductase (i.e. sarco/endoplasmic reticulum) [1].

Activation of AMPK causes $[glucose]_o$ uptake [55], and in yeast AMPK activation is regulated by PP2A in a $[glucose]_o$ -dependent manner [56]. Although simvastatin and AICAR caused a Compound C-sensitive increase in [³H]-2-deoxy-glucose uptake into the myocytes, simvastatin- and AICAR-induced p-AMPK α -Thr¹⁷² expression was $[glucose]_o$ -/ $[Na^+]_o$ -independent. Hence, ryanodine-sensitive Ca^{2+} release and activation of CaMK II, but not $[glucose]_o$ uptake, are essential initial steps necessary for AMPK α -Thr¹⁷² phosphorylation upon simvastatin challenge. Nonetheless, our results demonstrate that simvastatin and AICAR caused an increase in $[ATP]_i$ levels. The elevated $[ATP]_i$ contributed to the closure of vascular K_{ATP} channels [57] as well as providing phosphate groups necessary for protein (e.g. PP2A-Tyr³⁰⁷) phosphorylation (see below).

AMPK is regulated negatively by serine/threonine phosphatase(s) e.g. PP2A [46], and in rat's liver HMG-CoA reductase is associated with PP2A [45]. Our results clearly illustrate that simvastatin-induced PP2A-Tyr³⁰⁷ phosphorylation (i.e. inactivation) [58] requires $[glucose]_o$. In addition, Na^+/K^+ -ATPase provides the favorable trans-cellular Na^+ gradient for $[glucose]_o$ uptake via Na^+ -glucose co-transporter (SGLT-1) [59]. Phloridzin (a SGLT-1 inhibitor), $[Na^+]_o$ depletion or ouabain suppressed PP2A-Tyr³⁰⁷, but not AMPK α -Thr¹⁷², phosphorylation illustrating

the obligatory role of $[glucose]_o$ uptake via SGLT-1 [60] with the participation of Na^+/K^+ -ATPase for PP2A-Tyr³⁰⁷ phosphorylation.

The biochemical existence of cytochrome P450 (CYP450) 3A4 was demonstrated in porcine coronary artery and human left internal mammary artery, and the possible local enzymatic conversion of simvastatin into simvastatin Na^+ by CYP450 3A4 [61] was considered. However, ketoconazole (a selective CYP450 3A4 inhibitor) failed to modify simvastatin-induced increase in p-AMPK α -Thr¹⁷² and p-PP2A-Tyr³⁰⁷ expression refuted the possibility of local bio-transformation of simvastatin. Thus, our results strengthen the conclusion on the involvement of simvastatin, but not simvastatin Na^+ , in inhibiting vascular K_{ATP} channel openings.

In conclusion, our results demonstrate that acute simvastatin caused phosphorylation of PP2A-Tyr³⁰⁷ and AMPK α -Thr¹⁷², but not HMG-CoA reductase-Ser⁸⁷¹, of porcine coronary artery. Ryanodine-sensitive Ca^{2+} stores, but not $[Ca^{2+}]_o$ entry, play an obligatory role in simvastatin-elicited $[Ca^{2+}]_i$ increase and initiated the activation of Ca^{2+} /CaMK II cascade which are essential for the subsequent AMPK α -Thr¹⁷² phosphorylation (activation). Activation of AMPK α leads to $[glucose]_o$ uptake (and $[ATP]_i$ elevation resulted) with the participation of SGLT-1 and Na^+/K^+ ATPase. An increase in $[ATP]_i$ levels not only closed the K_{ATP} openers-induced channels openings but also provided the necessary phosphate groups for protein phosphorylation. Phosphorylation (inactivation) of PP2A-Tyr³⁰⁷ probably occurs at a site downstream of AMPK α -Thr¹⁷² phosphorylation (Figure 10).

Acknowledgments

Proofreading of the manuscript by Dr HY Lam is acknowledged.

Author Contributions

Conceived and designed the experiments: SWS JHKY SMYL SWC GPHL YWK. Performed the experiments: SWS ALSA CCWP QZ RWSL. Analyzed the data: SWS ALSA CCWP QZ RWSL MPMH. Contributed reagents/materials/analysis tools: JHKY SKK SMN SW HPH. Wrote the paper: SWS YWK.

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