Journal of Molecular and Cellular Cardiology 80 (2015) 166–174

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Inhibition of the mevalonate pathway ameliorates anoxia-induced down-regulation of FKBP12.6 and intracellular calcium handling dysfunction in H9c2 cells

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ARTICLE INFO

Article history:
Received 21 August 2014
Received in revised form 27 November 2014
Accepted 20 January 2015
Available online 28 January 2015

Keywords:
Mevalonate pathway
Small GTPase
Ryanodine receptor 2
FK506-binding protein 12.6
Calcium handling

ABSTRACT

Statins have beneficial pleiotropic effects beyond lipid lowering on the cardiovascular system. These cardioprotective effects are mediated through inhibition of the intracellular mevalonate pathway, by decreasing isoprenoid intermediate synthesis and the subsequent post-translational modification of small GTPases, such as Ras, Rho, and Rac. Impaired intracellular calcium handling is considered an important pathophysiologic mechanism responsible for cardiac dysfunction. Our study aimed at investigating the influence of mevalonate pathway, including its downstream small GTPases (Ras, RhoA, and Rac1) on anoxia-mediated alterations of calcium handling in H9c2 cardiomyocytes. Cultured H9c2 cardiomyocytes were exposed to acute anoxia after pretreatment with different drugs that specifically antagonize five key components in the mevalonate pathway, including 3-hydroxy-3-methylglutaryl-CoA reductase, farnesyl pyrophosphate synthase, Rho-kinase, Rac1 and Ras farnesyltransferase. Thereafter, we evaluated the effects of the mevalonate pathway on anoxia-induced cell death, expression of the sarcoplasmic reticulum calcium release channel (ryanodine receptor 2) and its regulator FK506-binding protein 12.6, as well as functional calcium release from intracellular calcium stores. Our experiments confirmed the role of prenylated proteins in regulating cardiomyocyte dysfunction, especially via RhoA- and Ras-related signaling pathways. Furthermore, our data demonstrated that inhibition of the mevalonate pathway could ameliorate anoxia-mediated calcium handling dysfunction with the up-regulated expression of FK506-binding protein 12.6 and consequently provided evidence for FK506-binding protein 12.6 as a "stabilizer" of ryanodine receptor 2.

1. Introduction

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, are potent drugs for lowering serum lipid levels. Apart from their lipid-lowering capacity, statins have important lipid-independent "pleiotropic" effects on the cardiovascular system [1]. Clinical studies have provided evidence for the protective effects of statins on myocardial ischemic or non-ischemic injuries [2]. Some research has suggested that statin treatment could improve cardiac function partly through its effects on intracellular calcium handling and excitation-contraction coupling [3–5]. Our previous study also confirmed rosuvastatin's beneficial effect on cardiac dysfunction and found that the molecular mechanism was related to the normalization of sarcoplasmic reticulum calcium ATPase (SERCA) 2a expression, SERCA activity, and phospho-phospholamban at serine-16 levels [6]. However, the more detailed mechanism of statins' effects on cardiac electrophysiology and contractile function is still unclear.

As we know, statins can inhibit the synthesis of intracellular mevalonate: a key upstream precursor of not only cholesterol, but also important isoprenoid intermediates, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). These intermediates play key roles in the post-translational modification of a range of proteins,

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FKBP, FK506-binding protein; FPP, farnesyl pyrophosphate; GAPDH, glyceraldehyde phosphate dehydrogenase; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MTT, methylthiazolyltetrazolium; PKA, cAMP-dependent protein kinase A; PLB, phospholamban; ROCK, Rho-kinase; RT-PCR, reverse transcription polymerase chain reaction; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum Ca2 + ATPase; SR, sarcoplasmic reticulum.

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http://dx.doi.org/10.1016/j.yjmcc.2015.01.010
0022-2828/

including small GTPases (Ras, Rho, Rac) (Fig. 1) [7]. It is now well established that small GTPases are involved in a range of cardiac functions, including heart rhythm, contraction, and pathological hypertrophy. The sustained over-activation of small GTPases or the spatio-temporal dysregulation of corresponding signaling pathways has pathological consequences related to arrhythmia, remodeling, and heart failure [8]. Ras, RhoA, and Rac1, as the three most important small GTPases in cardiomyocytes, have all been implicated in regulating intracellular calcium homeostasis and cardiac contractility, through the modulation of calcium-handling proteins' [Ryanodine receptor (RyR) 2, SERCA2a, and L-type calcium channel] expression or activity [9–11]. Therefore, the inhibition of isoprenoid synthesis and the resulting down-regulation of signaling pathways mediated by small GTPases may be responsible for the beneficial effects of statins. However, previous studies revealed little about the mechanisms driving calcium dyshomeostasis related to mevalonate metabolism. The influence of the mevalonate pathway on calcium handling remains undefined.

The alteration of calcium handling is essential for impaired cardiac function. Sarcoplasmic reticulum (SR) calcium leak due to RyR2 dysfunction contributes to impaired cardiomyocyte calcium handling [12]. Several studies have shown that FK506-binding protein 12.6 (FKBP12.6), a member of the FK506-binding protein family, binds with high affinity to RyR2 and can “stabilize” or reduce SR calcium release [13,14]. Though disputed, the dissociation of FKBP12.6 from RyR2 may be related to enhanced RyR2 open probability, thus leading to defective calcium homeostasis in cardiomyocytes [15].

The present study was designed to investigate the influence of the mevalonate pathway including its downstream small GTPases (Ras, RhoA, and Rac1) on anoxia-mediated intracellular calcium handling dysfunction and to determine whether FKBP12.6 plays a cardio-protective role in the regulation of RyR2 function. We utilized multiple pharmacologic compounds including inhibitors of HMG-CoA reductase, FPP synthase, Ras farnesyltransferase, Rho-kinase (ROCK), and Rac1 (Fig. 1), and evaluate the effects of the mevalonate pathway on anoxia-induced cell death, as well as the alterative expression and function of RyR2 in H9c2 cardiomyocytes. The underlying molecular mechanisms will be discussed.

2. Materials and methods

2.1. Cell culture

H9c2 cardiac cells (American Type Culture Collection, Rockville, MD), as a generous gift from Prof. Qiang Xia (Zhejiang University, China), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM of glutamine, 100 μg/mL of penicillin, 100 μg/mL of streptomycin, and 1 mM of pyruvate in humidified air (5% CO_2) at 37 °C. These cells are primitive, undifferentiated myoblasts of cardiac lineage derived from embryonic rat ventricular tissue [16]. Although H9c2 cells are no longer able to beat, they still show many similarities to primary cardiomyocytes, including the expression of calcium channels (L-type calcium channels and RyRs) that are involved in the depolarization and caffeine-induced calcium responses [17–19]. For anoxic conditions, cells were transferred to a glucose-free DMEM buffer that had been pre-equilibrated with 95% N_2 and 5% CO_2 for at least 20 min. Then, the cells were placed in an anaerobic incubator (at 37 °C) containing 95% N_2 and 5% CO_2 for 2 h. At the same time, incubations under normoxic conditions were carried out.

2.2. Drugs and chemicals

The HMG-CoA reductase inhibitor rosuvastatin (AstraZeneca Pharmaceutical Co., Sweden) and FPP synthase inhibitor alendronate (Merck Sharp & Dohme Co., USA) were dissolved in water and sterilized by filtration. ROCK inhibitor fasudil hydrochloride was obtained from Chase Sun Pharmaceutical Co., Ltd. (Tianjin, China). Manumycin A, which is a Ras farnesyltransferase inhibitor that effectively suppresses Ras biological functions, was purchased from Sigma-Aldrich (Dorset, UK) and dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Dorset, UK) before dilution in DMEM so that the final concentration of DMSO would not exceed 0.1%. NSC23766 was obtained from Tocris Bioscience (Bristol, UK) and dissolved in water. As a selective Rac1 activation inhibitor, NSC23766 prevents Rac1 activation by Rac-specific

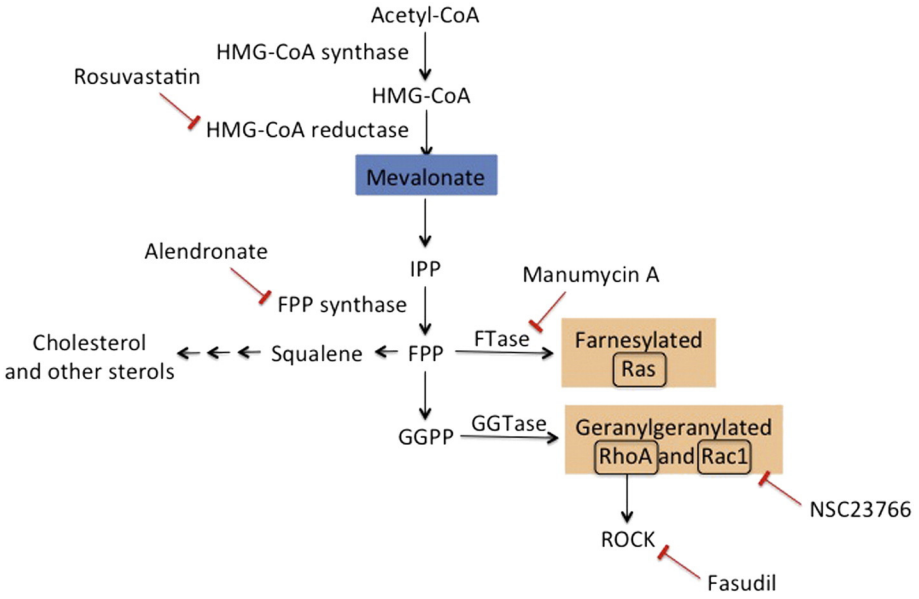

Fig. 1. The mevalonate pathway and effect of different inhibitors. Rosuvastatin blocks the conversion of HMG-CoA to mevalonate by suppressing HMG-CoA reductase and thereby inhibits Rac1/RhoA geranylgeranylation and Ras farnesylation. Alendronate inhibits the FPP synthase and interferes with the isoprenoid intermediates, FPP and GGPP biosynthesis downstream. Manumycin A is a Ras farnesyltransferase inhibitor. Fasudil inhibits the RhoA-dependent signaling pathway by targeting ROCK, the major downstream effector of RhoA. NSC23766 prevents Rac1 activation by Rac-specific guanine nucleotide exchange factors TrioN and Tiam1 without affecting RhoA activation. Abbreviations: Acetyl-CoA, acetoacetyl coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IPP, isopentenyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate. ROCK, Rho-kinase.