EXPERIMENTAL CELL RESEARCH 318 (2012) 1673–1684

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Research Article

Atorvastatin preserves the integrity of endothelial adherens junctions by inhibiting vascular endothelial cadherin tyrosine phosphorylation

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ARTICLE INFORMATION

Article Chronology:
Received 27 January 2012
Received in revised form
3 May 2012
Accepted 6 May 2012
Available online 30 May 2012

Keywords:
VE-cadherin
Adherens junction
Atorvastatin
RhoA
HRas
Statins

ABSTRACT

Vascular endothelial cadherin (VE-cad) tyrosine (Tyr) phosphorylation has been implicated in the disruption of adherens junctions (AJs) induced by inflammatory reactions. The impacts of statins on integrity of AJs and VE-cad Tyr phosphorylation have not been explored. The effects of atorvastatin on IL-1β and monocyte-induced VE-cad Tyr phosphorylation in human umbilical vein endothelial cells (ECs) were studied. In ECs treated with interleukin (IL)-1β for 30 min, VE-cad Tyr phosphorylation, dissociation of the VE-cad/β-catenin complex and transendothelial migration (TEM) of monocytes were increased. These processes were mediated by activation of HRas and RhoA that leads to phosphorylation of myosin light chain (MLC). Atorvastatin inhibited IL-1β-induced Tyr phosphorylation of VE-cad by inhibiting RhoA and by dephosphorylating MLC. The attenuating effect of atorvastatin on VE-cad Tyr phosphorylation was reversed when RhoA was activated or MLC phosphatase was inhibited. Furthermore, inhibiting farnesyl transferase or geranylgeranyl transferase reproduced the inhibitory effects of atorvastatin on VE-cad Tyr phosphorylation. In addition, atorvastatin inhibited monocyte-induced VE-cad Tyr phosphorylation in ECs and attenuated IL-1β-induced TEM of monocytes. Our study introduces a novel pleiotropic effect of atorvastatin and suggests that statins protect the integrity of AJs in ECs by inhibiting RhoA-mediated Tyr phosphorylation of VE-cad.

Introduction

Atherogenesis is a vascular inflammatory process characterized by the enhanced recruitment of leukocytes to dysfunctional endothelium [1]. Adherens junctions (AJs) are the main structures that mediate intercellular adhesion between endothelial cells (ECs) and together with tight junctions, play an important role in regulating paracellular macromolecular permeability.

Nonstandard abbreviations: VE-cad, vascular endothelial cadherin; Tyr, tyrosine; ECs, endothelial cells; AJs, adherens junctions; IL-1β, interleukin-1β; MLC, myosin light chain; TEM, transendothelial migration; Pyk2, proline-rich tyrosine kinase 2; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate

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http://dx.doi.org/10.1016/j.yexcr.2012.05.009

In the formation of AJs, vascular endothelial cadherin (VE-cad) dimers within the plasma membrane of one cell interact with VE-cad dimers of an adjacent cell [2]. In the cytoplasm, the highly conserved cytoplasmic domain of VE-cad binds to p120-catenin and either β-catenin or γ-catenin (plakoglobin) [3]. These, in turn, bind α-catenin, which links VE-cad to the actin cytoskeleton [4]. VE-cad tyrosine (Tyr) phosphorylation has been implicated in the dissociation of AJs of ECs and in the increased transendothelial migration (TEM) of leukocytes [5]. During in vitro monocyte and neutrophil diapedesis, there is a sequential loss of AJ components at the site of diapedesis while adjacent AJs remain intact [6,7]. In vascular ECs, the actin–myosin interaction is regulated by the phosphorylation status of myosin light chain (MLC). Both the Tyr phosphorylation of VE-cad and actin reorganization mediated by MLC phosphorylation regulate the barrier function of the endothelium [5,8] but the relationship between these regulatory mechanisms remained unclear. Recently, we revealed a link between MLC phosphorylation and Tyr phosphorylation of VE-cad [9]. We showed that the attachment of monocytes to ECs induces Tyr phosphorylation of VE-cad, which is mediated by the sequential activation of the HRas/Raf/MEK/ERK signaling cascade, MLC phosphorylation, and Src and proline-rich tyrosine kinase 2 (Pyk2) Tyr kinases [9].

Inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme-A reductase (statins) are widely used to treat high cholesterol. The beneficial effects of statins stem from their ability to reduce cholesterol biosynthesis, but, because they are also potent inhibitors of mevalonate, which governs diverse cell signaling pathways, statins may exhibit pleiotropic effects. Statins inhibit prenylation, which is a covalent posttranslational modification involving the addition of either farnesyl (15-carbon) or geranylgeranyl (20-carbon) lipid side chains to proteins anchored to the inner membrane leaflet, including Ras, and RhoA GTPase. Farnesyl pyrophosphate (FPP) represents a branch point in the cholesterol metabolic pathway, not only serving as a precursor of cholesterol, but as a precursor of geranylgeranyl pyrophosphate (GGPP), which is required for the posttranslational lipidation and membrane localization of Rho family GTPases, such as RhoA and Rac. FPP is also required for the lipidation and membrane localization of Ras [10]. The effects of statins on the stability of AJs have not been determined. We hypothesized that statins confer ECs barrier protection by inhibiting cytokine and leukocyte-induced VE-cad Tyr phosphorylation and disruption of AJs. Interleukin-1 (IL-1) is a major pro-inflammatory cytokine that plays a crucial role in pathophysiology of inflammatory diseases [11]. It is well-established that treatment of ECs with IL-1 or TNF-α for 4 hours or longer enhances transendothelial migration of leukocyte [12]. This effect is likely due to increase in adhesion of leukocyte to endothelial cells mediated by cytokine-induced over expression of adhesion molecules on ECs. However, it has been demonstrated that treatment of endothelial cells with IL-1 and TNF-α for as short as 1 h increases TEM of leukocytes, an effect which was not dependent on the leukocyte adhesion [13]. The underlying molecular mechanism that explains how IL-1 enhances TEM of leukocyte in such a short time (1 h) has not been determined. To assess the impacts of atorvastatin on the stability of AJs, we studied the mechanisms underlying IL-1β-induced disruption of AJ integrity and how statins modulate these mechanisms. In the present study, we show for the first time that statins protect ECs from IL-1β and monocyte-induced disruption of AJs by inhibiting VE-cad Tyr phosphorylation through inhibition of RhoA and dephosphorylation of MLC.

Materials and methods

Reagents

Phospho-specific and nonphospho-specific antibodies against Src (pY416), Pyk2 (pY402), β-catenin, MYPT and ERK1/2 were obtained from Abcam (Cambridge, MA, USA). Phospho-specific antibodies and nonphospho-specific antibodies against VE-cadherin (pY658, Y731) and monoclonal 4G10 anti-phosphotyrosine Ab were obtained from Invitrogen (Camarillo, CA). Monoclonal phospho-antibody against MLC, specific antibody against MLC, farnesyl pyrophosphate and geranylgeranyl pyrophosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant interleukin-1β (IL)-1, monocyte chemoattractant protein (MCP), Tumor necrosis factor (TNF-α), Lipopolysaccharides (LPS), geranylgeranyltransferase I inhibitor (GTI-298) and farnesyl transferase inhibitor (FTI-277) were purchased from Calbiochem (La Jolla, CA, USA). Atorvastatin and rosuvastatin were obtained from LKT Laboratories (St. Paul, MN). Premade recombinant Ras V12 (constitutively active [CA]), Ras N17 (dominant negative [DN]), RhoA N19 (DN), RhoA L63 (CA), ERK2 (DN), null control and GFP adenoviruses and ViraDuctin adenovirus transduction reagents were purchased from Cell Biolabs (San Diego, CA, USA). The nonphosphorylatable mutant construct for MLC in which Thr18 and Ser19 were replaced with alanines (AA-MLC) was a generous gift from Dr. Andreas Kapus, University of Toronto, Toronto, Canada [14]. Small-interfering RNA (siRNA) and TaqMan primers and probes for MYPT, Src and Pyk2 (PTK2B) were purchased from Applied Biosystems (Foster City, CA).

Cells

Human umbilical vein endothelial cells (HUVECs) and human acute monocytic leukemia (THP-1) cells were purchased from American Type Culture Collection (Manassas, VA, USA). HUVECs were grown in Lonza's EGM-2 medium on collagen-coated (20 μg/ml) tissue culture dishes. HUVECs from fewer than 4 generations were used for all experiments. THP-1 cells were maintained in RPMI 1640 medium with 10% heat-inactivated FCS.

Western blotting

HUVECs were grown to confluence in 35 mmol/l dishes or 6-well plates. Cells were extracted in radioimmunoprecipitation assay (RIPA) buffer, which contained 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholate, 1% NP-40, 10 mmol/l sodium phosphate, 150 mmol/l NaCl, 2 mmol/l EDTA, 50 mmol/l NaF, 5 mmol/l sodium pyrophosphate, 0.1 mmol/l sodium vanadate, 2 mmol/l PMSF, 0.1 mg/ml leupeptin, and 100 KIU/ml aprotinin. Samples were loaded onto an SDS-polyacrylamide electrophoresis (PAGE) gel and run at 150 V for 1 h. The proteins were then transferred onto nitrocellulose paper at 300 mA for 1.5 h, followed by Western blot analysis. Blots were blocked with 5% dry milk in 0.1% Tween 20 in phosphate-buffered saline (PBS) for 1 h at room temperature. The primary antibodies were used at a dilution of 1:500–1:1000. All antibodies were added for 1 h at room temperature or overnight at 4 °C. After washing, the appropriate secondary antibodies (Pierce) were added at a dilution of 1:10000 for 1 h at room temperature. After extensive washing,