Kaohsiung Journal of Medical Sciences (2013) 29, 347–352

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ORIGINAL ARTICLE

Rosuvastatin-regulated post-translational phosphoproteome in human umbilical vein endothelial cells

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Received 11 June 2012; accepted 29 June 2012
Available online 12 January 2013

KEYWORDS
Human umbilical vein endothelial cells;
Phosphorylation;
Pleiotropic effect;
Proteomics;
Rosuvastatin

Abstract Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are widely prescribed as cholesterol-lowering drugs. Statins have recently been found to have pleiotropic effects that are independent of their lipid-lowering properties. Phosphorylation of serine, threonine, and tyrosine residues of functional proteins are considered to be important in the endothelial signaling cascade. In this study, protein phosphorylation status in human umbilical vein endothelial cells (ECs) after rosuvastatin treatment was examined. The proteins were collected from rosuvastatin-treated ECs and then the phosphorylated peptides purified by a Fe^{3+}-immobilized metal-affinity chromatography bead system were examined by liquid chromatography–tandem mass spectrometry analysis. Alterations of the phosphorylation status of proteins were noticed after rosuvastatin treatment. There were 277 and 530 phosphorylated proteins identified from the control and rosuvastatin-treated ECs, respectively. Among those proteins, T78, in addition to S156 of the Ras-GTPase-activating protein, was phosphorylated after rosuvastatin treatment. Rosuvastatin reduced the phosphorylation of Y455 in HSP90 protein. Decreased phosphorylation of T211 with a concurrent increase in the T291 phosphorylation of

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http://dx.doi.org/10.1016/j.kjms.2012.06.002

Akt1 was observed under rosuvastatin treatment. Increased S633 phosphorylation was detected in endothelial nitric oxide synthase. Western blot analysis further showed an earlier and greater S633 phosphorylation than that of S1177 in endothelial nitric oxide synthase after rosuvastatin treatment. Changes in the phosphorylation status of these proteins may alter the protein's function and affect endothelial physiology. The current study provides new insights leading to a better understanding of the pleiotropic effects of statins on the vascular system.

Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly called statins, were first developed to lower total serum cholesterol and to improve the serum lipid profile. The cholesterol-independent pleiotropic effects of statins that have been extensively researched include improved endothelial function, reduced oxidative stress, prevention of platelet aggregation, and stabilization of atherosclerotic plaques [1,2]. Increasing evidence suggests that protein phosphorylation promotes statin-induced pleiotropic effects. Statins induce phosphorylation of Mdm2 and attenuate the p53 response to DNA damage [3]. Moreover, statins phosphorylate the serine (S) at S1177 and S633 of endothelial nitric oxide synthase (eNOS) through the phosphatidylinositol-3-kinase/protein kinase B or AMP-activated protein kinase (AMPK) pathway and thus trigger cardioprotective signaling via nitric oxide [4–6]. Furthermore, statins increased the expression of heme oxygenase-1, which is involved in stress response [7]. Phosphorylation of protein tyrosine phosphatase, which is required for the normal activation of extracellular signal-regulated protein kinases in multiple-receptor tyrosine kinase signaling pathways, is increased after rosuvastatin treatment [8]. This implies that protein phosphorylation is an early response signal that contributes to vascular homeostasis. However, there is currently no known direct link between protein phosphorylation and statin-induced pleiotropic effects.

Currently, six statin compounds are available in the market as cholesterol-lowing drugs; the pharmacokinetics of these compounds differs due to differences in their hydrophobicity. Rosuvastatin exhibits hydrophilic properties and possesses the lowest inhibition coefficient [9]. It exerts protective effects on tissues by suppressing the release of inflammatory mediators such as monocyte chemotactic protein-1 (MCP-1), transforming growth factor-beta 1, interleukin-1 beta, and tumor necrosis factor-alpha [10]. Rosuvastatin also reduced monocyte adhesion, possibly through the decreased expression of intercellular adhesion molecule-1, MCP-1, interleukin-8, interleukin-6, and COX2 in tumor necrosis factor-alpha-treated endothelial cells (ECs) [11]. Rosuvastatin significantly reduced DNA damage by H_2O_2 [12]. Pretreatment of mice with rosuvastatin curbed myocardial necrosis after ischemia and reperfusion [13]. Compared to other statins, rosuvastatin is weakly absorbed by ECs. However, it upregulates eNOS expression and activity via the phosphorylation that provides superior endothelial protection, more than that by other statins [14].

The purpose of this study was to demonstrate the dynamic phosphoproteome in rosuvastatin-treated ECs. Using the immobilized metal-affinity chromatography (IMAC) method to enrich the phosphopeptides from cell lysate [15], we obtained 530 phosphoproteins with high sequence identities. Dynamic changes in phosphorylation status after rosuvastatin treatment were observed in some proteins, including Ras-GTPase-activating protein, heat shock protein 90 (HSP90), Akt1, and eNOS. Western blot confirmed that S633 was phosphorylated earlier and to a greater extent than S1177 in eNOS from ECs after rosuvastatin treatment. The current study provides a global profile of rosuvastatin-induced protein phosphorylation, which provides a basis for further studies into the pleiotropic effects of statins on the vascular system.

Materials and methods

Endothelial cell culture

ECs were isolated from human umbilical cords as described previously [16]. The procedure conformed to the principles outlined in the Declaration of Helsinki for the use of human tissue or subjects. ECs were cultured in M199 medium supplemented with fetal bovine serum (20%), streptomycin (100 μg/mL), and penicillin (100 U/mL). The medium was then replaced with M199 medium containing 2% fetal bovine serum, and the ECs were incubated overnight prior to rosuvastatin (AstraZeneca, Taipei, Taiwan) treatment.

Cell lysis and trypsin digestion

After rosuvastatin treatment, the ECs were washed with cord buffer [NaCl (0.14M), KCl (4 mM), glucose (11 mM), HEPES (10 mM, pH 7.4)] and then lysed with lysis buffer [HEPES (250 mM, pH 7.7), EDTA (1 mM), neocuproine (0.1 mM), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, 0.4%, w/v), protease inhibitor (0.2%, v/v), and Phosphatase arrest (G-Bioscience, MO, USA)]. After centrifugation, detergents and salts in the protein supernatant were removed by spin column (G-Bioscience). The protein concentrations were determined by bicinchoninic acid assay reagent (Thermo Fisher Scientific, IL, USA). Protein lysates (50 μg) were mixed and adjusted to 100 μL with 25 mM trimethylammonium bicarbonate buffer. The samples were then incubated with 1 μg of modified trypsin (Promega, Madison, WI, USA) for 16 hours.

Enrichment of phosphopeptides

An immobilized metal ion affinity chromatography (IMAC) column (5 cm × 500 μm i.d., PEEK tubing, which is an attached component of spin column produced by Qiagen) was packed with nickel nitrilotriacetic acid (Ni-NTA) resin from the spin column (Qiagen, Hilden, Germany). Before use, Ni^{2+} ions were removed with 100 μL of 50 mM EDTA in 1M NaCl, and then incubated with 100 μL of 200 mM $FeCl_3$. The peptide sample was reconstituted in 100 μL loading buffer (6% acetic acid, v/v, pH 3.0) and loaded into the Fe-IMAC column. The unbound peptides were washed out with 100 μL washing solution [acetonitrile (25%, v/v) and acetic acid (6%, v/v; pH 3.5)]. The phosphopeptides were eluted from the IMAC column with 100 μL of 200 mM $NH_4H_2PO_4$ (pH 4.4). The eluted peptide sample was then desalted with a ZipTip (Millipore, Billerica, MA, USA) and dried under a vacuum.

Nano-liquid chromatography—tandem mass spectrometry analysis

The purified phosphopeptide samples were reconstituted in sample buffer [formic acid, (0.5%, v/v)] and analyzed by nano-liquid chromatography—tandem mass spectrometry on a Thermo LTQ XL ion trap mass spectrometer (Thermo Fisher Scientific, IL, USA). A split-flow configuration of high-performance liquid chromatography (Agilent1100 HPLC system; Hewlett-Packard, Palo Alto, CA, USA) was used for online sample separation. The sample was injected into a homemade capillary trap column (2 cm × 100 μm i.d.) and packed with Magic C18AQ reversed-phase material (5 μm, 200 Å, Michrom BioResources, CA, USA). The peptides were separated using a 13 cm × 75 μm i.d. capillary column (10 μm electrospray tip, packed with Magic C18AQ; Michrom BioResources), and eluted with a linear gradient of 0—80% buffer A (formic acid, 0.1%, v/v) and buffer B (formic acid, 0.1%, v/v in acetonitrile) for 75 minutes at 300 nL/minute. The mass spectrometer was run in a top five configuration with one mass spectrometry scan followed by five MS/MS scans. Peptide fragmentation by collision-induced dissociation was performed automatically using a dynamic data-dependent method.

Database search

Sequence identification was performed on a Mascot server (in-house, v 2.2, Matrix Science, London, UK). The data sets were searched against the non-redundant protein database of the National Center for Biotechnology Information using the following constraints: trypsic peptides with up to two missed cleavage sites; 2.0 Da mass tolerances for mass spectrometry; and 0.8 Da mass tolerances for MS/MS fragment ions. Modifications specified in the search were differential carbamidomethylation in cysteine, differential oxidation in methionine, and differential phosphorylation in serine, threonine (Thr, T), and tyrosine (Tyr, Y). To evaluate the false discovery rate of identification, the data sets were also searched against a decoy database created by Mascot (false discovery rate <1%). The peptides that contained phosphorylated Ser/Thr/Tyr sites were determined with a mass shift of 80 Da.

Western blot

The phosphorylation on the S633 and S1177 residues in eNOS, which was identified in the mass spectrometric assay, was further verified by Western blot. The blotted membrane was incubated with monoclonal antibody (1:3000; Cell Signaling Technology Inc., Danvers, MA, USA). The hybridized nitrocellulose membrane was developed with SuperSignal West Femto reagent (Thermo Fisher Scientific, IL, USA) and exposed to the radiograph. The film was scanned by a digital scanner (Microtek, International Inc., Taipei, Taiwan), and the relative protein density was calculated by PhotoImpact X3.

Results

Identification of rosuvastatin-induced phosphoproteome

In the present study, the rosuvastatin-induced phosphoproteome was investigated according to the flowchart in Fig. 1. It is notable that we used a polymer-filled spin column to remove inorganic salts and detergents. After trypsin digestion and IMAC enrichment, 277 and 530 phosphorylated proteins were identified from ECs after control and rosuvastatin treatment, respectively. Among those, 76 phosphorylated proteins were simultaneously shown on both treatments (Supplementary Table 1). Those phosphoproteins in each treatment (control/rosuvastatin) were classified according to their physiological functions (Fig. 2): 8.7%/9.6% cytoskeleton proteins; 6.5%/10% nuclear proteins; 8.7%/11.7% transcription factors; 9%/10% HSPs; 24.2%/22.6% proteins involved in energy production; 6.5%/11.9% proteins involved in metabolism; and 36.4%/24.2% others.

Rosuvastatin-induced dynamic shifts of phosphorylated residues on proteins

Further analysis of the MS/MS data revealed changes in phosphorylation sites in the peptide sequences (Fig. 3). An additional phosphorylated T78 was detected in the Ras-GTPase-activating protein after rosuvastatin treatment. Rosuvastatin treatment decreased the Y455 phosphorylation in HSP90 protein. In Akt1, T291 was phosphorylated instead of T211. For eNOS, in addition of the S1177, an increased S633 phosphorylation was observed.

Rosuvastatin induced earlier S633 phosphorylation than S1177 in eNOS

As shown in Fig. 4, the phosphorylation levels of eNOS S633 and S1177 were verified using monoclonal antibodies. After rosuvastatin (10 μM) treatment for 0.5 hours and 24 hours, there was a three-fold and approximately 15-fold increase in the S633 phosphorylation, respectively, when compared to controls were observed. In addition, examination of the phosphorylation status revealed that S633 was phosphorylated earlier than S1177 after rosuvastatin treatment.

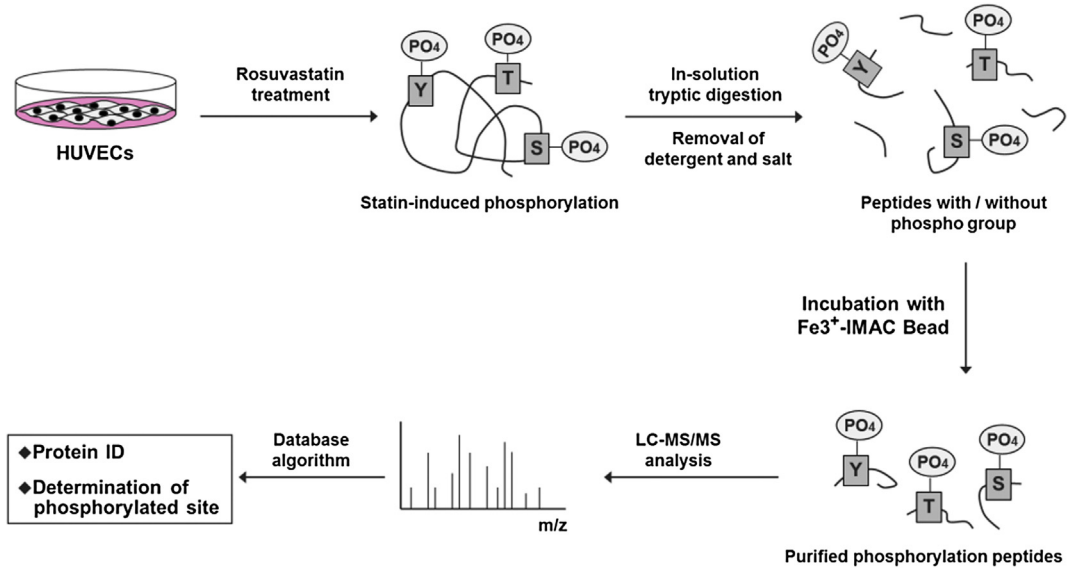

Figure 1. Flowchart of the mass spectrometric technique applied in elucidating the statin-regulated phosphoproteome. Human umbilical vein endothelial cells with either rosuvastatin or control treatment were lysed. The protein lysates were digested by trypsin, and the resulting peptides containing phospho groups (PO4) were conjugated by $Fe3^{+}$-IMAC magnetic beads. The purified phosphorylated peptides were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, and the phosphorylated sites were determined.

Discussion

Statin-induced pleiotropic effects that are independent of their cholesterol-lowering character remain a key issue for clinical and basic research. Ser/Thr/Tyr phosphorylation is regarded as an important posttranslational modification during the signaling cascade [17]. In the present study, we found that rosuvastatin treatment lead to a nearly two-fold increase in the overall phosphorylated proteins in ECs compared to the controls. Moreover, those proteins exhibited different phosphorylation sites. Changes in phosphorylation sites were illustrated in four proteins that were known to be involved in regulating eNOS activity (Fig. 3). In addition to the HMG-CoA reductase inhibition that decreases cholesterol synthesis, statins inhibit the isoprenylation of small GTPases such as Ras, Rho, Rab, and Rap [18]. Ras-GTPase-activating protein is a target for the protein tyrosine kinases of both the receptor and cytoplasmic classes, and may serve to integrate Ras signaling pathways [19]. It is possible that phosphorylation of T78 in the GTPase-activating protein may modulate activation of the Ras signaling pathway, which may contribute to the pleiotropic effects of statins.

Nitric oxide, a gaseous molecule, significantly affects vascular tone. It is considered to be significantly involved in mediating the pleiotropic effects of statins [2,20]. In this

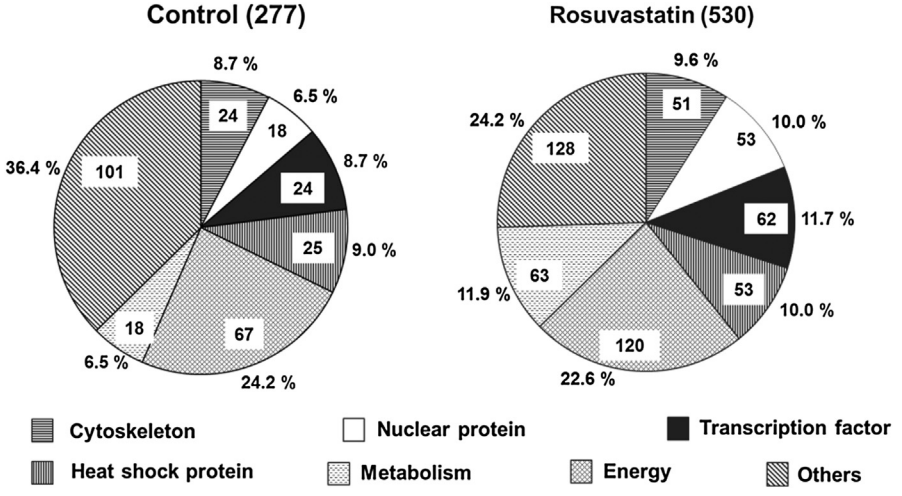

Figure 2. Functional classification of the rosuvastatin-regulated phosphoproteome. The identified phosphorylated proteins were categorized into seven groups according to the annotated functions by Ingenuity Pathway Analysis. The total number and the percentage of each protein are indicated.

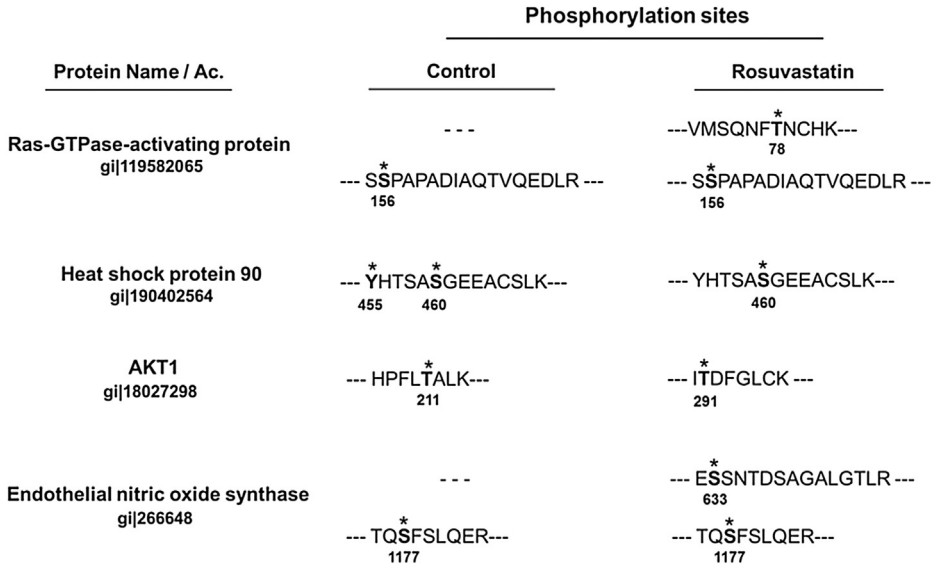

Figure 3. Rosuvastatin regulates dynamic phosphorylation on protein residues. Following a Mascot in-house software algorithm, four proteins were shown to have dynamic variations of phosphorylated peptides on proteins. The asterisk indicates the locations of phosphorylated Ser/Thr/Tyr residues. Ac represents the accession number in the National Center for Biotechnology Information database.

study, we show the phosphorylation of HSPs and Akt, two signaling proteins involved in nitric oxide generation, are modulated by posttranslational modification. With rosuvastatin treatment, the number of phosphorylated HSPs increased from 25 to 53 (Fig. 2). Among these HSPs, HSP90 is known to enhance eNOS activity [21]. In this study, we observed a decrease of Y455 phosphorylation in HSP90. Whether this decreased phosphorylation affects its stimulation effect on eNOS remains unclear. Akt is a part of the PI3K-Akt-eNOS signaling cascade and has shown a dynamic phosphorylation response to rosuvastatin treatment. It is well known that Akt is activated by phosphorylation at S473, and this is essential for the subsequent activation of eNOS by phosphorylation at S1177 [22]. Another report showed that treatment with pitavastatin induced Akt phosphorylation at S473 [4]. Although phosphorylation at S473 was not shown in the present MS/MS data, phosphorylation of T291 instead of T211 of Akt was observed after rosuvastatin treatment. It is unclear whether this changed pattern of phosphorylation can lead to alterations in the enzymatic activities of Akt.

Phosphorylation of either S1177 or S633 significantly activates eNOS and increases NO production [5,23]. From the mass spectrometry data in this study, we further verified the phosphorylation of both activation sites and showed that S633 exhibited an earlier response to rosuvastatin treatment than that by S1177. Furthermore, the increase in eNOS phosphorylation was sustained for at least 24 hours. With rosuvastatin treatment, S633 appears to be a major phosphorylation site for nitric oxide production. It has been suggested that S633 phosphorylation is more potent than S1177 phosphorylation in augmenting eNOS-derived nitric oxide production. Both Akt and AMPK can phosphorylate S633 in eNOS when ECs are subjected to

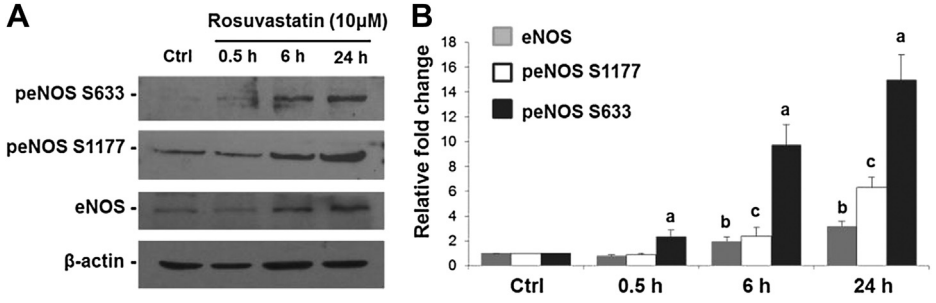

Figure 4. Rosuvastatin enhanced phosphorylation of eNOS S635 and S1177. (A) Protein lysates (40 μg) extracted from ECs treated with rosuvastatin (10 μM) for 0.5, 6.0, and 24.0 hours were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and subjected to Western blotting with antibodies against eNOS (1:3000), peNOS S1177 (1:3000), peNOS S633 (1:500), and β-actin (1:5000). (B) The relative fold changes are shown by mean ± standard error as compared to control treatments from three replications. The changes were individually compared to the control by Fisher's least significant difference for significant ($p < 0.05$) differences, and indicated as a, b, and c.

shear stress [23,24]. AMPK phosphorylation of S633 in eNOS is required to prime SIRT1-induced deacetylation of eNOS to promote nitric oxide production, indicating an atheroprotective flow via AMPK and SIRT1 to increase nitric oxide bioavailability in the endothelium [25]. Further investigation of the interrelationship between the AMPK pathway and phosphatidylinositol-3-kinase/protein kinase signaling to eNOS activation in rosuvastatin-mediated cardioprotection is necessary.

The pharmacology of statins has been under comprehensive investigation because of its pleiotropic effects which contribute to improved vascular functions during diabetes-induced complications [2]. In this study, with the help of advanced proteomic techniques, we have examined global phosphoproteome and our study provides a basis for further phosphorylation profiling that may contribute to the understanding of the pleiotropic effects of statins.

Acknowledgments

This work was supported in part by grants from the National Science Council (NSC 98-2752-B-001-001 and NSC 100-2320-B-037-001) and projects from Kaohsiung Medical School (KMU-Q100003) and Chi-Mei Medical Center and Kaohsiung Medical University Research Foundation (100CM-KMU-01). We are grateful to the core facility laboratory of the Institute of Biomedical Sciences, Academia Sinica, and the Center for Resources, Research and Development of Kaohsiung Medical School for mass spectrometric analysis. We also thank AstraZeneca, Taiwan, for providing the rosuvastatin powder.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.kjms.2012.06.002

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