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Forum Minireview

Pharmacogenomics of Cardiovascular Pharmacology: Molecular Network Analysis in Pleiotropic Effects of Statin — an Experimental Elucidation of the Pharmacologic Action From Protein-Protein Interaction Analysis

Masayuki Shiota[1,*], Hiromi Kusakabe[1], Yuko Hikita[1], Takafumi Nakao[1], Yasukatsu Izumi[1], and Hiroshi Iwao[1]

[1]*Department of Pharmacology, Osaka City University Medical School, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan*

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Abstract. The ebb and flow of cellular life depends largely on signaling pathways and networks, which are regulated by specific protein-protein interactions. These interactions often involve assembly of large signaling complexes containing many different protein kinases, protein phosphatases, their substrates, and scaffold proteins. Identification of protein complexes is the key to understanding cellular functions. One of the techniques used for the isolation of protein complexes is the affinity purification system. Inhibitors of 3-hydroxyl-3-methyglutaryl coenzyme A (HMG-CoA) reductase (i.e., statins) exert cholesterol-independent vasoprotective effects that are mediated, in part, through the activation of Akt. However, the molecular mechanism remains unknown. To elucidate the molecular mechanisms of the pleioptropic effects of statins, we searched for the binding molecule of Akt1 by using a combined mass spectrometry and affinity purification strategy. By this technique, we identified the protein-protein interactions of 23 proteins from statin-treated rat aortic endothelial cells (rAECs). Our results suggest that this approach is very effective and statin activates many Akt down-stream targets, not only endothelial nitric oxide synthase (eNOS). The methodology presented here would provide a new tool for chemical proteomics in medicinal science.

Keywords: network analysis, interaction, statin, pleiotropic effect, Akt, pharmacogenomics

1. Introduction

Numerous human diseases can be attributed to defects in cellular signal transduction pathways, which are regulated by specific protein-protein interactions. These interactions often involve assembly of large signaling complexes containing many different protein kinases, protein phosphatases, their substrates, and scaffold proteins. Therefore, identification of protein complexes is the key to understanding the physiological functions and molecular mechanisms of disease. A number of ongoing proteomics projects seek to define critical components of signal transduction networks. This type of study enables more intelligent design of therapeutic agents that can specifically correct disease-specific signaling alterations by targeting individual proteins. However, the identification of a protein complex remains a significant challenge because many interactions are transient in nature and the components that form these unstable complexes can be lost during sample preparation. Furthermore, it is also difficult to purify native protein complexes because many of them are expressed at low levels and might not possess a unique physical characteristic that permits their isolation or enrichment from a cell lysate. Therefore, a specific, simple method is necessary to clarify a signal network efficiently. Affinity purification and mass spectrometry (MS)-based protein identification is a powerful and highly sensitive technique for identifying protein-protein interactions and elucidating components of multiprotein complexes. This method can become one of these breakthroughs.

The 3-hydroxyl-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors referred to as statins are

*Corresponding author. sio@med.osaka-cu.ac.jp
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widely prescribed to lower cholesterol level in patients. Numerous studies, however, suggest that statin therapy has additional cardiovascular protective effects that may function independently of their ability to lower serum cholesterol levels (1, 2). These cholesterol-independent or pleiotropic effects of statins involve improving endothelial function, decreasing oxidative stress and inflammation, inhibiting the thrombogenic response, and enhancing the stability of atherosclerotic plaques (3, 4). Recent evidence suggests that most of these effects are mediated by the inhibitory effect of statins on isoprenoid synthesis and by activation of statin on phosphatidylinositol 3-kinase (PI3K)/Akt pathway (5 – 7). Some reports showed that a clinically relevant dose of statins activate Akt (8, 9). However, the molecular mechanism of pleiotropic effects via Akt remains unclear. Akt is critical for the regulation of vascular endothelial functions and maintenance of vascular integrity (10, 11). The Akt-driven signaling network is capable of regulating cellular processes by modulating transcription and translation and by modifying proteins at the post-translational level. Kureishi et al. demonstrated that Akt activation by statins then inhibits apoptosis, acutely increases nitric oxide (NO) production, activates migration, and promotes angiogenesis (12). These effects of statins were prevented by PI3K inhibitors or the dominant negative form of Akt (12). Although many studies have reported that these effects depend on endothelial NO synthase (eNOS) activation via Akt, it is impossible to explain them by only eNOS activation (13, 14). Indeed, each physiological response downstream of Akt appears to be mediated by multiple targets. In fact, a careful search of the literature finds over 100 reported non-redundant Akt substrates, and each signaling pathway is activated (15). Due to the complexity of Akt involvement in various cellular processes, the downstream effectors of Akt that are the most critical to the physiological roles of Akt remain to be determined. It is important to understand the nature and molecular mechanism of statin-induced Akt activation because it may provide novel insights about the regulatory control of endothelial cell function and lead to the identification of new pharmacological targets.

Here, we attempted to perform affinity purification and proteomic analysis to elucidate the role of Akt signaling in the pleiotropic effects of statin. Affinity purification strategies employing epitope tags and physiological conditions have proven to be effective for targeted protein interactions mapping efforts.

2. Methods

The mapping of protein-protein interactions has matured for a few years through significant technology developments and methods standardization. Therefore, the identification of protein interaction partners has become a standard assay in numerous laboratories. In interaction research, several approaches have been developed for purifying and identifying protein complexes, including two-hybrid methods (16), enzyme assays (17), or tandem affinity purification (TAP) of protein complexes followed by mass spectrometric analysis (18, 19). The data from these experiments contain many false positives and false negatives, but these methods provide beneficial information about protein-protein relationships and protein function. These approaches can be applicable to disease-related signaling pathways. Accordingly, to establish a more systematic methodology for these studies, new and efficient proteome technologies are required. In this study, we present an effective method for the identification of multi-protein complexes in rat aortic endothelial cells (rAECs).

We explored the use of strep-tag method for the isolation of Akt1 complexes from rAECs. This protein purification system has previously been shown to allow the rapid, single-step purification of recombinant proteins from mammalian cellular lysates (20 – 22). Strep-tag is an eight amino acid peptide that has strong binding affinity for an engineered streptavidin derivative called strep-Tactin (20, 23). Our method involved the fusion of the strep-tag to Akt1 and introduction of the construct into the rAECs by adenoviral vectors. The cells were maintaining the expression of strep-tagged Akt1 and stimulated by pravastatin or pitavastatin for 10 min because Western blotting showed rapid phosphorylation of Akt1 at S473 and T308 at 10 min, a peak by pravastatin stimulation. Lysates expressing the strep-tagged Akt1 were then passed through a strep-Tactin column and bound proteins were specifically eluted using a derivative of biotin called desthiobiotin under physiological conditions. The purified protein assemblies were separated by denaturing gel electrophoresis, and individual bands were digested by trypsin, analyzed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS), and identified by database search algorithms (24) (Fig. 1)

3. Results and Discussion

Using this technique, the bait protein, Akt, and their 23 interaction partners were systematically purified and identified. Some of these proteins have known cellular functions and they encompass many signaling pathways. These included enzymes, such as protein kinases; translational regulators; cytoskeletal proteins; and hypo-

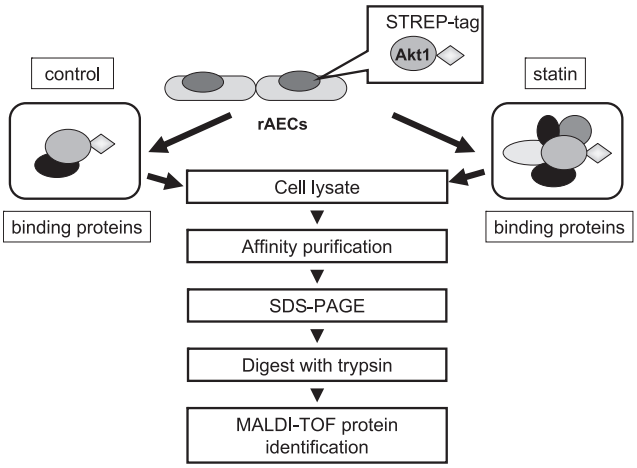

Fig. 1. Schematic overview of the strep-tag purification coupled to MS analysis.

thetical proteins. The identified interactions with Akt involved several known interactions and few previous reported interactions. In addition, we found 14 or 6 candidate proteins that up- or down- regulated the binding after 10 min of statin stimulation, respectively. The wide range of different proteins involved suggests that statin has profound effects on the biomolecular response and physiological consequence of vascular endothelium functions. However it is necessary to validate the specificity of each interaction because some of these results may be false positives. Some examples of the Akt1-associated molecules identified are as follows:

3.1. Hsp90

NO signaling is important in statin-mediated endothelial function (12). A number of studies have revealed that statins may increase eNOS activity via post-translation activation of the PI3K/Akt pathway (8, 12). In the present study, however, we could not detect the binding of eNOS to Akt1 under statin stimulation. This result suggests that eNOS engagements by Akt1 are weak or this is an indirect association. Instead, we identified the molecular chaperone heat-shock protein 90 (Hsp90) as Akt1-binding protein. Brouet et al. reported that statin-induced eNOS activation via Akt was through an interaction with Hsp90 (25). Moreover, Sato et al. reported that Akt interacts with Hsp90 on stimulation and this interaction enhances Akt enzymatic activity (26), suggesting that Hsp90 may serve as a scaffold protein for the efficient phosphorylation of eNOS by Akt (25, 27, 28). Taken together, the interaction between Akt1 and Hsp90 that we observed might be related to control eNOS activation.

3.2. mTOR/S6K

We found an association between Akt1 and the mammalian target of rapamycin (mTOR), which is already identified as substrate of Akt. Furthermore, we validated the specificity of this interaction by immunoprecipitation with endogenous proteins. The serine /threonine kinase mTOR is one of the most important downstream targets of PI3K/Akt. mTOR signaling has been demonstrated to be involved in the control of cell growth and proliferation (29). S6K plays a role in regulating the translation machinery and is controlled by mTOR, an immediate downstream effector of Akt (30). We analyzed activation of S6K to validate the mTOR/S6K pathway. Western blot analysis showed that pravastatin induced the phosphorylation of S6K on T389, thereby leading to enzyme activation. Pretreatment of cells with LY294002, a PI3K inhibitor, abolished the phosphorylation of Akt and S6K, whereas pretreatment with rapamycin, an mTOR inhibitor, inhibited only S6K phosphorylation. These data indicated that pravastatin activated the PI3K/Akt /mTOR/S6K pathway in this sequential manner in rAECs. Furthermore, rapamycin depressed pravastatin-induced rAEC proliferation. Nevertheless, the mTOR pathway contributed little to in vitro tube formation.

3.3. Cytoskeletal proteins

We identified several cytoskeletal proteins, non-muscle alpha-actinin 1, vimentin, and beta-actin, as Akt1 binding proteins. Furthermore, we confirmed endogenous binding of some of these proteins by immunoprecipitation. Confocal microscopic analysis also revealed that endogenous Akt rapidly translocated to perikaryon and cytoplasm from the nucleus by pravastatin stimulation. Akt translocation to the cytoplasm by statin stimulation seemed to be related to the interaction of Akt1 with the cytoskeletal proteins. Skaletz-Rorowski et al. has shown that Akt rapidly translocated to discrete sites at membrane ruffles in the simvastatin-treated endothelial cells (31). Their results agree with our results from mass spectrometric analysis, validating the confocal microscopic analysis. Cellular movement requires the reorganization of the actin cytoskeleton and distinct patterns of actin reorganization. This is required as cells establish a leading edge and then generate contractile force to migrate forward (32). It has been shown that Akt transiently localized to the leading edge membrane of migratory cells in a PI3K-dependent manner (33, 34). Recently, Enomoto et al. identified an Akt substrate, designated Girdin/APE, which is an actin binding protein (35). Akt phosphorylated Girdin accumulates at the leading edge of migrating cells. Thus, Akt seems to be important for regulation

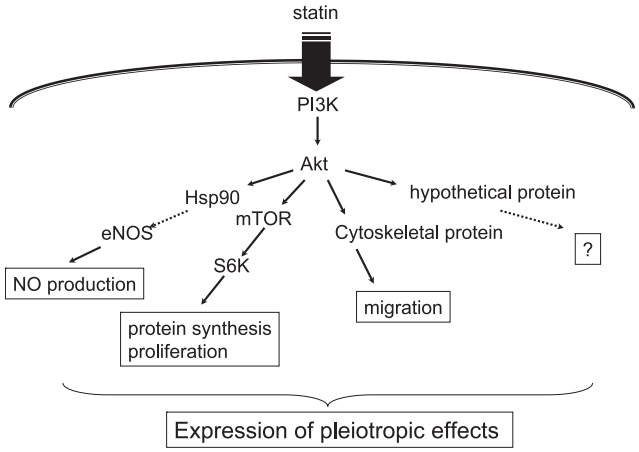

Fig. 2. Schematic representation of the proposed molecular mechanisms activated by statins in endothelial cells and responsible for pleiotropic effects.

of cell migration via actin reorganization (36) and through phosphorylation of the cytoskeletal protein Girdin. These data suggest that thanslocation of Akt1 and binding with cytoskeletal proteins of Akt1 participate in migration.

Taken together, statin induced activation of the PI3K/Akt pathway was shown to active many effectors and play an important role in mediating NO production, proliferation, and migrations in ECs (10, 37) (Fig. 2).

4. Conclusions

Identification of protein complexes is the key to understanding cellular functions including the transduction of inter- and/or intra-cellular signals and the regulation of gene expression. Aberrant protein-protein interactions are implicated in a number of diseases; therefore the study of protein complexes is the object of significant research in pharmacology. Furthermore, the information gained from protein interaction studies will ultimately lead to the discovery of novel pathway associations and therapeutic targets.

In this study, we have introduced a method for identifying protein-protein interactions using affinity purification and MS-based protein identification. We demonstrated that the strep method is very effective for purifying and identification of associated protein complexes. We tried to clarify the molecular mechanisms of the pleiotropic effects of statin with this method. Furthermore, we have identified many known interactions and several unreported proteins and hypothetical proteins to be Akt-binding proteins. As a result, it was revealed that statins activate not only the eNOS pathway but also many other pathways via Akt. Thus, the clarification of mechanisms for the pleiotropic effects of statins may lead to a better understanding of EC functions and provide avenues for the development of novel therapeutic interventions.

The simplicity and convenience of the single-step strep method, as well as its reliability and efficacy, would also make it useful for high-throughput applications both in academic research and in industrial drug development projects. The methodology presented here provides a new tool for proteomics in post-genomic medicinal science.

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