The Selectivity and Impact of Polyphenols-Protein Kinases Interactions for Chemoprevention

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ABSTRACT

Phosphorylation of target proteins by protein kinases leads to the activation of cellular signaling pathways, which induce a great number of biological processes including cell growth, differentiation, development, and death. Although the activities of protein kinases are stringently regulated in homeostasis system, protein kinases can be deregulated under pathological conditions, leading to perturbation of protein kinase-mediated cell signaling pathways and resulting in various disorders including cancer, diabetes, and inflammation. The data from the Human Genome Project has revealed that 518 protein kinases are encoded in the human genome, thereby creating an enormous repertoire of potential targets for chemoprevention. Polyphenols, as integral constituents of the diet, have been proposed to exert beneficial effects in a multitude of disease states. Recent studies have suggested that the cellular effects of polyphenols are, at least partly, mediated by their interactions with protein kinases central to intracellular signaling cascades. In the present study, we investigated the interaction of polyphenols-protein kinases by pull down assay, affinity assay and computer simulation. The results revealed that some polyphenolic compounds suppressed the phosphorylation of various protein kinases with different affinity and selectivity. Finally, we discussed the impact of these polyphenols-protein kinases interactions on cellular signaling and chemoprevention.

Key words: polyphenol, protein kinase, direct interaction, affinity, docking, chemoprevention

INTRODUCTION

Protein kinase is a type of enzyme that transfers phosphate groups from high-energy donor molecules such as adenosine triphosphate (ATP) to specific substrates. This process is referred to as phosphorylation. According to the catalytic specificity, protein kinases can be subdivided into three categories: Tyrosine (Tyr) kinase, Serine/Threonine (Ser/Thr) kinase, and kinase specific for both Tyr and Ser/Thr. Phosphorylation of these sites in target proteins leads to the activation of signal transduction pathways, which play important roles in a great number of biological processes including cell growth, differentiation, development, and death. The activities of protein kinases are stringently regulated in homeostasis system. However, protein kinases can be deregulated under pathological conditions, leading to perturbation of protein kinase-mediated cell signaling pathways and resulting in various disorders including cancer, diabetes, and inflammation[(1)]. The data from the Human Genome Project has revealed that 518 protein kinases are encoded in the human genome, and each cell will have approximate ~50 - 100 protein kinases[(2)] , thereby creating an enormous repertoire of potential targets for drug discovery[(3)]. Alterations in multiple cellular signaling pathways are frequently found in many cancer cells. It may be the reason why the specific inhibitors that target only one pathway, most often, failed in cancer treatment[(4)]. Thus, compounds that can suppress multiple cellular signaling pathways would have great potential in chemoprevention.

Polyphenolic compounds may be good sources for this since some of them appear to address multiple targets. Polyphenols, as integral constituents of the diet, are one of the biggest families of natural products and have been proposed to exert beneficial effects in a multitude of disease states, including cancer, cardiovascular disease, and neurodegenerative disorders. Classically, many of the biological actions of polyphenols have been attributed to their antioxidant properties, either through their reducing capacities or through their possible influences on intracellular redox status. However, recent studies have speculated that their classical hydrogen-donating antioxidant activity is unlikely to be the sole explanation for cellular effects. Recent studies from our group and other groups have suggested that the cellular effects of polyphenols may be mediated by their interactions with specific proteins central to intracellular signaling cascades[(5)]. In particular, investigations have indicated that polyphenols may interact selectively at different components of a number of protein kinase signaling cascades such as

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phosphoinositide 3-kinase (PI3K), Akt/protein kinase B (Akt/PKB), protein kinase C (PKC), and mitogen-activated protein kinase (MAPKs)[6-8]. Some polyphenols can directly bind these protein kinases and alter their phosphorylation state to regulate multiple cell signaling pathways, and finally affect various cellular functions such as cell defense, neuroprotective, cardioprotective, and cancer chemopreventive actions.

In this study, author reports the results in the interaction of protein kinases-polyphenols, special in the direct binding and molecular modeling of protein kinases-polyphenols. Moreover, the binding sites, selectivity, and the impact on cellular signaling and chemoprevention of polyphenol-protein kinase interactions are discussed.

MATERIALS AND METHODS

I. *Pull Down Assay*

Myricetin-Sepharose 4B beads were prepared as described previously[9]. Briefly, polyphenols (3 mg) was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B beads (25 mg) in a coupling buffer[17] overnight at 4°C according to the manufacturer's instructions. The mixture was washed in 5 volume of coupling buffer and then centrifuged at 1,000 rpm for 3 min at 4°C. The precipitate was resuspended in 5 volume of 0.1 M Tris-HCl buffer (pH 8.0) with 2 h rotation at room temperature (RT). After washing three times with 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl, the mixture was further washed with 0.1 M Tris-HCl (pH 8.0) buffer containing 0.5 M NaCl. The cell lysates (500 μg for *ex vivo* assay) or recombinant active protein kinase (100 ng for *in vitro assay*) were incubated at 4°C overnight with Sepharose 4B beads or Sepharose 4B-polyphenol-coupled beads (100 μL, 50% slurry) in a reaction buffer[17]. The beads were then washed 5 times with a washing buffer[17]. The proteins were applied to SDS-PAGE and then detected by immunoblotting.

II. *Affinity Analysis of Polyphenol-Protein Kinase Interaction*

Affinity of polyphenol to protein kinase was measured by Quartz-crystal microbalance (QCM)[9]. Recombinant protein kinase (100 ng) was immobilized into a QCM electrode plate for 1 h at RT. After washing 5 times with a binding buffer[11], the electrode plate was immersed in the analysis chamber with binding buffer at 25°C. Polyphenol was injected stepwise into the analysis chamber (0.1 - 12.6 μM). The binding affinity was indicated by frequency changes of QCM, and disassociation constant (K_d) was calculated by AFFINIX Q User Software (AQUA; Initium).

III. *Western Blotting*

Immunoblotting was performed as described previously[9]. The cells were lysed in a modified RIPA buffer. The lysed cells were homogenized in an ultrasonicator for 15 s twice on ice and then centrifuged at 14,000 g for 15 min at 4°C. The protein concentration of the supernatant was determined using dye-binding protein assay kit (Bio-Rad) according to the manufacturer's manual. Approximate 10 - 30 μg proteins were run on SDS-PAGE and then transferred to PVDF membrane (Amersham Biosciences). The blotted membrane was incubated with a specific primary antibody for 2 h at RT, and then incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by Immobilon Western System (MILLIPORE), and further quantified by Lumi Vision Imager software (TAITEC Co.).

IV. *Molecular Modeling of Polyphenol-Protein Kinase Docking*

Computer modeling of polyphenolic compound to protein kinase was performed using Molecular Operating Environment™ software (MOE, Version 2008.10, Chemical Computing Group Inc.). The 3D structure of protein kinase was obtained from Protein Data Base (PDB, http://www.rcsb.org/pdb/) and crystallization water and other molecules involved in protein kinase structure were deleted. Hydrogen atoms were first added, and forcefield (MMFF94x) atomic charges were assigned. Docking of myricetin to Akt1 was done using MOE- ASEDock 2005 software[10].

RESULTS AND DISCUSSION

I. *A Polyphenolic Compound Can Target Multiple Protein Kinases*

We chose myricetin, a typical polyphenol existing in many fruits and vegetables, to investigate the direct targets of protein kinase. Binding assay revealed that myricetin bound to Akt directly by competing with ATP. Molecular modeling further suggested that myricetin easily docks to the ATP-binding site of Akt with hydrogen bonds. On the other hand, myricetin also could directly bind to JAK1/STAT3 molecules. Affinity data further demonstrated that myricetin had a higher affinity for JAK1 than STAT3. Other groups reported that myricetin further bound to MEK1[11], PI3K[12], Fyn[13], and MKK4 [14]. These data shown that one polyphenol can target several protein kinases. The number and substitution of hydroxyl groups on the B-ring and the degree of unsaturation of the C2 - C3 bond of polyphenol appear important determinants of this particular bioactivity[15]. The potential of polyphenol binding to protein kinases will depend on both the structure of polyphenol (e.g. the number and position of hydroxyl group on the B-ring, and the degree of unsaturation of the C2 - C3 bond of flavonoids) and the properties of different protein kinase (e.g. selectivity of ATP-pocket, the architecture in the adjacent to the

ATP-binding site).

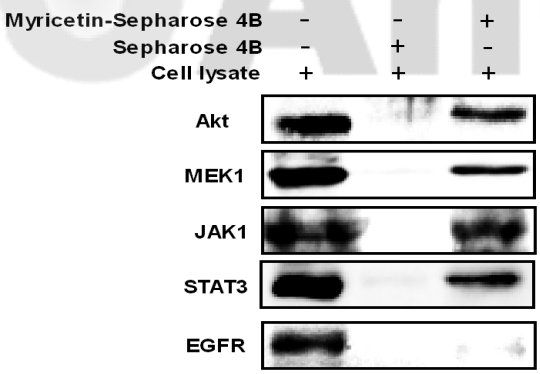

Figure 1. Myricetin directly binds to Akt, MEK1, JAK1 and STAT3, but not EGFR. Whole cell lysate was precipitated with Sepharose 4B beads or Sepharose 4B-myricetin-coupled beads as described in Materials and methods. Whole cell lysate (input control, lane 1), precipitate with Sepharose 4B beads (negative control, lane 2) or Sepharose 4B-myricetin-coupled beads were applied to SDS-PAGE, and detected with their antibodies, respectively, after transferring to membrane.

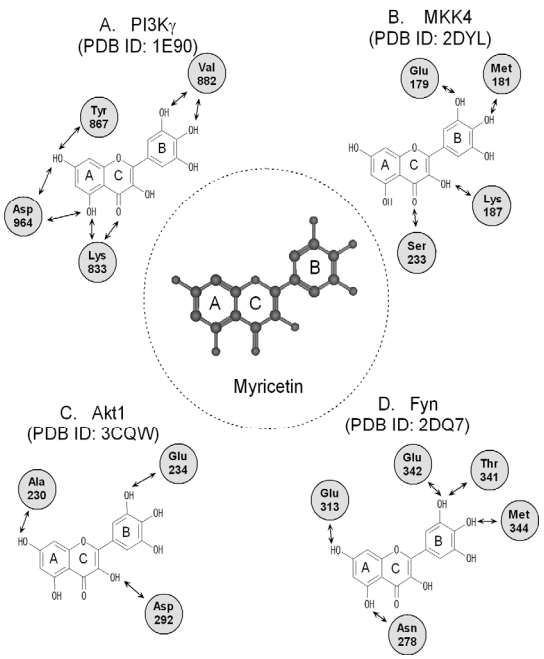

Figure 2. Myricetin binds the ATP-pocket of multiple protein kinases. The chemical structure of myricetin is showing in the middle. (A) PI3Kγ (PDB ID : 2DQ7) [12]. (B) MKK4 (PDB ID : 2DYL) [14]. (C) Akt (PDB ID : 3CQW) [10]. (D) Fyn (PDB ID : 2DQ7) [13]. Hydrogen bonds between myricetin and protein kinase are indicated by arrows.

II. *A Protein Kinase Can be Targeted by a Variety of Polyphenolic Compounds*

MEK1/2 is a critical kinase in MAPK signaling pathway. It is found that this protein kinase, at least, can be directly bound with delphinidin, quercetin, and equol. As shown in Figure 3C, Equol, a metabolite of daidzein, is docked to a pocket separate from but adjacent to the ATP-binding site similar to PD318088 (ATP noncompetitive inhibitor of MEK1) as observed in the crystal structure of the MEK1-PD318088 complex I[16] (Figure 3A). The hydroxyl group at the 7 position of equol can make a hydrogen bond with the backbone carbonyl group of Val127 in the ATP noncompetitive binding site.

Quercetin[17] is also reported to dock to the pocket separate from but adjacent to the ATP-binding site of MEK1 (Figure 3B). The hydroxyl group at the 7 position can make a hydrogen bond with the backbone carbonyl group of Val127 in the ATP noncompetitive binding site. The C-ring interacts with the residues in the activation loop of the inactive MEK1. Val211 and Leu215 form *van der Waals* interactions with the C-ring of quercetin. The hydroxyl group at the 3' position of the C-ring can make a critical hydrogen bond with the backbone amide group of Ser212. These interactions of quercetin with the activation loop would lock MEK1 into a catalytically inactive species by stabilizing the inactive conformation of the activation loop.

Delphinidin, a typical aglycone of anthocyanins, is reported to dock to the pocket separate from but adjacent to the ATP-binding site of MEK1[18]. The interactions by the C-ring moiety are critical for the holding of the activation loop of the inactive MEK1 (Figure 3C).

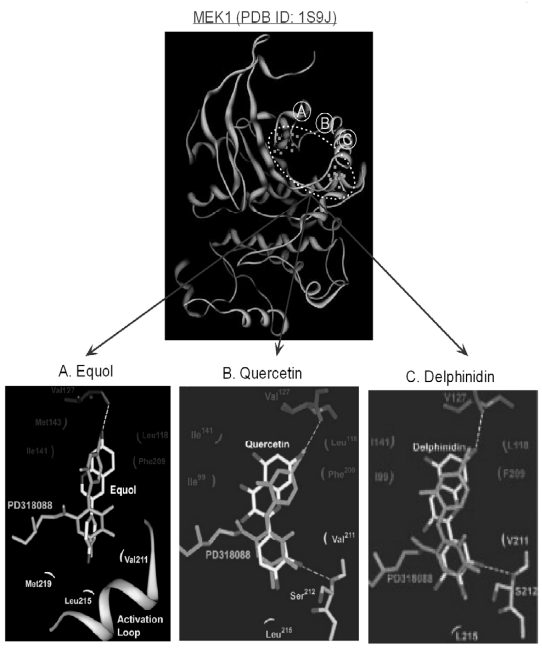

Figure 3. Molecular modeling of equol (A) [16], quercetin (B) [17], and delphinidin (C) [18] binding to MEK1. (Top) The tube model of MEK1 structure (PDB ID : 1S9J)).

III. *Selectivity vs. Multiple Targets*

Most diseases involve multiple molecular abnormalities with more than one dysfunctional proteins although a highly selective drug can target a single molecule responsible for the etiology of a disease. Therefore,

drugs whose efficacy is based on rebalancing the several proteins that contribute to the pathogenesis and progression of a disease may be ideal[4].

The lack of selectivity of protein kinase inhibitor can sometimes be advantageous since anticancer drugs that act on multiple tyrosine kinases are thought to be more effective than those that are specific in their mode of action[19]. Flavonoids that can suppress these multiple pathways would have great potential in cancer chemoprevention and cancer treatment. On the other hand, cross-reactivity with unrelated ATP-binding proteins is also a frequent problem, resulting in undesirable side effects that cause many protein kinase inhibitors to fail in either preclinical or clinical development[20]. Therefore, multiple targets and side effect possibility of flavonoids as protein kinase inhibitors are required to be clarified in further.

The evidence indicates that most of the plant-based agents used in traditional Ayurvedic and Chinese medicine contain a variety of polyphenols, and do indeed suppress multiple pathways[4]. Importantly, all of these signaling pathways have been found malfunctioning in cancer cells, resulting in cancer cell proliferation and inhibition of apoptosis. Therefore, it is important to design a strategy that could simultaneously target multiple cellular signaling pathways so that cancer cells could get killed effectively. Therefore, targeting multiple signaling by natural compounds has opened a new avenue for cancer chemoprevention and therapy.

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