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Luteolin sensitizes the antiproliferative effect of interferon α/β by activation of Janus kinase/signal transducer and activator of transcription pathway signaling through protein kinase A-mediated inhibition of protein tyrosine phosphatase SHP-2 in cancer cells

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ABSTRACT

New negative regulators of interferon (IFN) signaling, preferably with tissue specificity, are needed to develop therapeutic means to enhance the efficacy of type I IFNs (IFN-α/β) and reduce their side effects. We conducted cell-based screening for IFN signaling enhancer and discovered that luteolin, a natural flavonoid, sensitized the antiproliferative effect of IFN-α in hepatoma HepG2 cells and cervical carcinoma HeLa cells. Luteolin promoted IFN-β-induced Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway activation by enhancing the phosphorylation of Jak1, Tyk2, and STAT1/2, thereby promoting STAT1 accumulation in the nucleus and endogenous IFN-α-regulated gene expression. Of interest, inhibition of phosphodiesterase (PDE) abolished the effect of IFN-β and luteolin on STAT1 phosphorylation. Luteolin also increased the cAMP-degrading activity of PDE bound with type I interferon receptor 2 (IFNAR2) and decreased the intracellular cAMP level, indicating that luteolin may act on the JAK/STAT pathway via PDE. Protein kinase A (PKA) was found to negatively regulate IFN-β-induced JAK/STAT signaling, and its inhibitory effect was counteracted by luteolin. Pull-down and immunoprecipitation assays revealed that type II PKA interacted with IFNAR2 via the receptor for activated C-kinase 1 (RACK-1), and such interaction was inhibited by luteolin. Src homology domain 2 containing tyrosine phosphatase-2 (SHP-2) was further found to mediate the inhibitory effect of PKA on the JAK/STAT pathway. These data suggest that PKA/PDE-mediated cAMP signaling, integrated by RACK-1 to IFNAR2, may negatively regulate IFN signaling through SHP-2. Inhibition of this signaling may provide a new way to sensitize the efficacy of IFN-α/β.

1. Introduction

Type I interferons (IFN-α/β) are pleiotropic cytokines with broad and potent immunoregulatory and antiproliferative activities that have been applied in a variety of cancers, such as leukemia, Kaposi's sarcoma, and solid tumors. They induce the rapid activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway via binding to the cell-surface IFN receptors (IFNAR1/2), activating JAKs (Jak1 and Tyk2) and subsequently phosphorylating STAT1 and STAT2 proteins, which translocate into the nucleus and recognize a specific ISRE motif in the promoter region of certain genes for transcription [1]. The molecular mechanism of JAK/STAT pathway activation by IFNs has been studied extensively, but the negative regulation of the signaling, which is crucial for maintaining the signaling processes, remains to be fully understood. Several negative regulators of JAKs and STATs participate in the attenuation of JAK/STAT pathway signaling, including Src homology domain 2 containing tyrosine phosphatase-1/2

Abbreviations: luteolin, 3′,4′,5,7-tetrahydroxyflavone; IFN, interferon; JAK, Janus kinase; STAT, signal transducer and activator of transcription; ISRE, interferon α-stimulated response element; PDE, phosphodiesterase; cAMP, cyclic adenosine 3′,5′-monophosphate; IFNAR, type I interferon receptor; PKA, cAMP-dependent protein kinase A; SHP, Src homology domain 2 containing phosphotyrosine phosphatase; PKC, protein kinase C; AKAP, A-kinase anchoring protein; RACK-1, receptor for activated C-kinase 1; MS, multiple sclerosis; IBMX, 3-isobutyl-1-methylxanthine; Na_3O_4, sodium orthovanadate; DMSO, dimethyl sulfoxide; 2′5′-OAS1, 2′5′-oligoadenylate synthetase 1; PKR, interferon-induced, double-stranded RNA-activated protein kinase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; ERK, extracellular signal-regulated kinase; CRE, cAMP responsive element; PKA RIIα, type II PKA regulatory subunit α.

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(SHP-1/2), suppressor of cytokine signaling family members, protein inhibitor of activated STAT family members, and the ubiquitin/26S proteasome pathway [2]. Type I IFNs have been used to treat a variety of cancers and viral diseases, but associated side effects such as neuropsychiatric and hematologic toxicities and drug tolerance severely limit their clinical uses [3]. The severity of many of these side effects appears to be directly related to the dose and duration of IFN therapy. Thus, understanding the negative regulation mechanisms of the JAK/STAT pathway will greatly facilitate the development of new therapeutic adjuvants that allow decreases in IFN dosage and relief from its side effects.

Cyclic adenosine 3′,5′-monophosphate(cAMP) acts as an intracellular messenger for numerous hormones and neurotransmitters, regulating a wide range of important biological processes through cAMP-dependent protein kinases such as protein kinase A (PKA) [4]. The amplitude and duration of cAMP signaling depend on the activity of cAMP-degrading phosphodiesterases (PDEs) [5]. Of the 11 families of PDEs identified thus far, 8 (PDE1–4, 7, 8, 10, and 11) have specific or dual-specific activity to degrade cAMP, and most individual cells express multiple PDE variants, with each isoform showing a unique combination of subcellular localization and regulatory mechanisms. Spatial confinement of PKA and PDE to specific subcellular structures is mediated by A-kinase anchoring proteins (AKAPs), a large and diverse family of scaffolding proteins with more than 50 members identified thus far, which orchestrate PKA/PDE-regulated cAMP signaling with other signaling components such as phosphatases in a compartmentalized manner [6]. Although the cAMP signaling and the JAK/STAT pathway are generally activated signaling pathways in response to extracellular hormones or cytokines, the cross-talk between them is still unclear. cAMP-activated PKA reportedly inhibits the tyrosine phosphorylation of IFNARs, JAKs, and STATs [7–9], and down-modulation of cAMP pathway signaling is proposed as a survival response to the apoptosis induced by IFN-α [10]. However, the mechanism through which PKA regulates IFN signaling is still unclear. Receptor for activated C-kinase 1 (RACK-1), a protein kinase C (PKC)-anchoring protein, physically interacts with the type I interferon receptor 2 (IFNAR2) and is required for recruitment and activation of STAT1 [11,12], but its role in assembling the cross-talk between cAMP signaling and IFN signaling is unknown. Thus, elucidating the mechanism of cAMP signaling in the regulation of the JAK/STAT pathway will provide new insights on the complexity of the negative regulation of IFN signaling and inform new therapeutic means with tissue or cell-type specificity to obviate the side effect of IFNs.

The rich sources of flavonoids contained in vegetables and fruits play important roles in reducing the risk of many chronic diseases [13]. However, their influence on the JAK/STAT pathway, the major anticancer and antiviral systems in cells, is rarely studied. Luteolin, 3′,4′,5,7-tetrahydroxyflavone, is a flavonoid that is widely distributed in the plant kingdom with multiple pharmacological activities [14]. Accumulating evidence suggests that luteolin could be developed as an anticancer agent to sensitize tumor cells for apoptosis by suppressing cell survival pathways such as nuclear factor kappa B, X-linked inhibitor of apoptosis protein, STAT3, and PKCε [15–18]. Luteolin is also proposed as a promising adjuvant for multiple sclerosis (MS) therapy owing to its additive effects with IFN-β in modulating, via an unknown mechanism, the immune responses of peripheral blood mononuclear cells isolated from MS patients [19]. We show herein that luteolin sensitizes the antiproliferative effect of IFN-α/β on various human cancer cells by enhancing the activation of JAK/STAT signaling. Interestingly, such potentiation is achieved via decreases in intracellular cAMP level through activation of IFNAR2-bound PDE activity and subsequent PKA inhibition. Furthermore, we show that PKA interacts with IFNAR2 through RACK-1 and targets SHP-2 to exert its regulatory role in JAK/STAT pathway signaling. Data from this study thus reveal a novel signal complex consisting of PKA, PDE, and SHP-2 localized in the cytoplasmic region of IFNAR2 that fine-tunes cAMP regulation of IFN signaling and further advances our understanding of the molecular mechanism involved in the anticancer activity of luteolin.

2. Materials and methods

2.1. Reagents and plasmids

pCMV-SHP-2 plasmid was from Fisher Scientific (Pittsburgh, PA). pCRE-luc plasmid was purchased from Stratagene (La Jolla, CA). pSV-β-galactosidase control vector was purchased from Promega (Madison, WI). IFN-α (recombinant human IFN-α2a) or IFN-β (recombinant human IFN-β1b) was purchased from ProSpec-Tany Techno Gene Ltd (Shanghai, China). The lyophilized protein was reconstituted in sterile water with carrier protein (0.1% bovine serum albumin) at a concentration of 100 μg/mL and stored at −80 °C. Lipofectamine 2000 reagent was from Invitrogen (Carlsbad, CA). JAK inhibitor 1 was purchased from Calbiochem (Gibbstown, NJ). Luteolin, 3-isobutyl-1-methylxanthine (IBMX), 8-Br-cAMP, Rp-cAMPS, forskolin, H-89, staurosporine, PD98059, LY294002, and sodium orthovanadate (Na_3VO_4) were purchased from Sigma-Aldrich (Shanghai, China). Bortezomib was purchased from Millennium Pharmaceuticals (Cambridge, MA). The compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma) and stored in small aliquots at −20 °C. Anti-phospho-Jak1, anti-phospho-Tyk2, anti-phospho-STAT1, anti-phospho-STAT2, anti-STAT1 and anti-STAT2 were obtained from Cell Signaling Technology (Danvers, MA). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), anti-RACK-1, anti-type II PKA regulatory subunit α (PKA RIIα), anti-glutathione *S*-transferase (GST), anti-histone H3, and anti-SHP2 were purchased from Epitomics (Burlingame, CA). Anti-PDE4D antibody was purchased from Millipore (Billerica, MA). Recombinant expressed PKA RIIα was purchased from ProSpec (Protein-Specialists, East Brunswick, NJ).

2.2. Cell lines and cultures

The HepG2-ISRE-Luc2 cell line was established and maintained as previously reported (20). Human liver cell line HepG2 and human cervical cancer cells HeLa were from American Type Culture Collection (Manassas, VA); human embryonic kidney HEK293A cells were purchased from Invitrogen. HeLa and HEK293A cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) with 10% fetal bovine serum (Invitrogen); HepG2 cells were maintained in Roswell Park Memorial Institute 1640 medium (Invitrogen) with 10% fetal bovine serum.

2.3. Screening and luciferase reporter assay

On the day of assay, HepG2-ISRE-Luc2 cells were seeded at 1×10^4 cells/well in 96-well plates and incubated in a cell incubator overnight. The cells were then pretreated with the test compounds (10 μg/mL) for 2 h before 200 U/mL IFN-α was added for another 24 h. The luciferase activity of the total cell lysate was measured using a Luciferase Reporter Assay System (Promega) according to manufacturer's instructions.

2.4. Cell viability assay

HepG2 and HeLa cells were plated at 0.5×10^4 cells/well in 96-well plates with 100 μL medium. Cultured HeLa and HepG2 cells were then treated with various concentrations of luteolin or a combination of luteolin with IFN. After 72 h, 10 μL Alamar Blue reagent (SunBio Medical Biotechnology, Shanghai, China) was added to the medium and incubated for another 2–4 h until the blue color changed to pink. The relative fluorescence intensity in each well was measured using a Varioskan® Flash (Thermo Scientific, Waltham, MA).

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