Biochemical Pharmacology 83 (2012) 1383–1392

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Double-edged sword effect of biochanin to inhibit nuclear factor kappaB: Suppression of serine/threonine and tyrosine kinases

Sunil Kumar Manna *

Laboratory of Immunology, Centre for DNA Fingerprinting & Diagnostics, Nampally, Hyderabad 500 001, India

ARTICLE INFO

Article history:
Received 9 November 2011
Accepted 15 February 2012
Available online 22 February 2012

Keywords:
Protein tyrosine kinase
Ser/Thr kinase
Biochanin
NF-κB
IKK
PTK inhibitors

ABSTRACT

Several protein tyrosine kinase (PTK) inhibitors predominantly isoflavones, such as genistein, erbstatin, quercetin, daidzein, present in red clover, cabbage and alfalfa, show apoptotic effect against cancer cells. In this study I found that biochanin, a methoxy form of genistein, inhibits IL-8-mediated activation of nuclear transcription factor kappaB (NF-κB) and activator protein 1 (AP-1) more potently than genistein as shown in Jurkat T-cell line. Both biochanin and genistein potently inhibited activity of Lck and Syk, but biochanin specifically inhibited activity of IKK. Biochanin inhibited completely NF-κB activation induced by PMA, LPS, pervanadate (PV), or H_2O_2, but only partially that induced by TNFα. Genistein was unable to inhibit IL-8-induced IKK activity, but it blocked PV-induced IKK activity. Biochanin inhibited activation of NF-κB by TRAF6 completely, but by TRAF2 partially. *In silico* data suggested that biochanin interacted strongly with serine/threonine kinase than genistein, though both equally interacted with PTK. The data show that both biochanin and genistein are potent inhibitors of PTK, but biochanin is a potent inhibitor of serine/threonine kinase too. Formononetin, having hydroxyl methoxy group is less potent to inhibit IKK than biochanin. Biochanin inhibits NF-κB activation not only by blocking the upstream IKK, but also PTK that phosphorylate tyrosine residues of IκBα. Thus, the double-edged sword effect of inhibition of NF-κB *via* inhibition of both serine/threonine kinase and PTK by biochanin might show useful therapeutic value against activities of cells that lead to tumorigenesis and inflammation.

1. Introduction

Protein tyrosine kinases (PTKs) play a crucial role in many cell regulatory processes. Functional perturbation of PTKs results in many diseases. PTKs are mostly activated by the growth factors and cytokines through interaction with their specific receptors. Autophosphorylation of these receptors leads to recruitment of several PTKs and thereby activate cell signaling and cell cycle progression [1]. In tumorigenesis several fold activation of these kinases occurs often initiating alarm signal for its regulation. Upon stimulation by cytokines, microbial agents such as bacterial and fungal toxins and viral proteins and DNA, oncogenes, chemotherapeutic agents, and environmental stress, several serine/threonine kinases (Ser/Thr kinases) participate in activation of various transcription factors including nuclear transcription factor kappaB (NF-κB), activator protein 1 (AP-1), cyclic AMP-responsible element binding factor (CREB), *etc.* [2]. The dual kinase inhibitors often help in developing suitable therapeutic molecules against tumorigenesis. Activation of tyrosine and/or Ser/Thr kinases often induces cells for rapid proliferation that lead to tumorigenesis. Interleukin-8 (IL-8), a chemokine and an inducer of angiogenesis, induces cells after interaction with its specific seven transmembrane containing receptors, IL-8Rs [3]. These activate Ser/Thr kinases like mitogen activated protein kinases (MAPKs) through induction of small GTPase and IκBα kinase (IKK) *via* recruitment of TNF receptor associated factor (TRAF)6 [4]. *Clostridium botulinum* C3 transferase (C3-toxin), an inhibitor of Rho-GTPase is shown to inhibit MAPKs [5]. Several inducers like phorbol myristate acetate (PMA), lipopolysaccharide (LPS), TNFα are activators of Ser/Thr kinases and some of them phosphorylate Tyr^{42} of IκBα possibly activating IKK complex [6]. H_2O_2 and pervanadate (PV) activate tyrosine kinases, like Syk [7]. TNFα induces Ser/Thr kinases like MEKK, IKKs, NIK, *etc.* by recruiting TRAF2 and 6 [8]. Epidermal growth factor (EGF) induces cells through binding of EGF receptor, a typical receptor tyrosine kinase (RTK), followed by activating downstream MAPKs [9].

Abbreviations: AP-1, activator protein 1; C3-toxin, *Clostridium botulinum* C3 transferase; CE, cytoplasmic extracts; Cox2, cyclooxygenase 2; ICAM, intercellular adhesion molecule; NF-κB, nuclear transcription factor kappaB; IL, interleukin; IκB, inhibitory subunit of NF-κB; IκBα-DN, IκBα dominant negative; IKK, IκB kinase; Lck, lymphocyte-specific PTK (protein tyrosine kinase); LPS, lipopolysaccharide; NE, nuclear extracts; PV, pervanadate; RMSD, root mean square deviation; Syk, spleen tyrosine kinase; TNFα, tumor necrosis factor alpha; TRAF, TNF receptor-associated factor.

* Tel.: +91 40 24749412; fax: +91 40 24749448.
E-mail address: manna@cdfd.org.in.

0006-2952/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2012.02.011

Phytoestrogens possess anti-inflammatory, anti-allergic, anti-oxidant, anti-thrombotic, anti-neoplastic, and hepatoprotective activities [10]. Red clover dietary supplements contain varying ratios of the four isoflavones commonly found in legume-based diets, namely, daidzein, genistein, formononetin, and biochanin. Several phytoestrogens are isoflavonoids and are inhibitors of PTK. Genistein is a trihydroxyisoflavone that inhibits PTK by competing with ATP [11]. Like genistein, biochanin is known to inhibit PTK and to interfere cell growth [12,13]. Biochanin is a dihydroxy methoxyisoflavone. Erbstatin, a dihydroxymethylcinnamate, inhibits PTK by competing with ATP and peptide substrate at the catalytic site [14]. Daidzein, a dihydroxyisoflavone and a structural analog of genistein, lacks PTK inhibitory activity. Quercetin, a pentahydroxyisoflavone inhibits PTK at lesser extent. Formononetin, a hydroxymethoxyisoflavone in food materials, is absorbed as daidzein in the gut and shows PTK inhibitory activity. Formononetin is also increased the allergic responses by increasing the amount of IL-4 [15] and induces cell death [16]. Several isoflavonoids, including biochanin, have been shown to inhibit growth and induce apoptosis in bladder, prostate, and breast cancer cells [17–19].

This study examined the ability of these isoflavones found in red clover to inhibit inflammatory and proliferative activities of cells. Isoflavones have shown to reduce the synthesis of prostaglandin E2 and/or thromboxane B2 by inhibiting cyclooxygenase 2 (cox2) [20]. However, the mechanism of anti-proliferation and anti-inflammatory activity of biochanin has not been understood. Nuclear transcription factor kappaB is a rate-limiting transcription factor underlying both inflammatory and proliferative activities of cells. NF-κB activation depends upon the translocation of homo- or hetero-dimers of Rel family proteins from cytoplasm to nucleus. Dimer of Rel family proteins are often arrested in the cytoplasm by inhibitory subunit of kappaB (IκB) family proteins. Upon stimulation, IκB family proteins are phosphorylated followed by ubiquitinated and degraded. The IκB family proteins are phosphorylated by IκBα kinases, predominantly a Ser/Thr kinase. The Tyr^{42} residue of IκBα is also phosphorylated by unknown tyrosine kinase and these are activated by H_2O_2 and PV [6]. We have shown previously that biochanin exhibits anti-proliferative, antiinflammatory activities by inhibiting NF-κB [21]. The present study shows that biochanin is a potent inhibitor of not only PTK, like other isoflavones, but also inhibits Ser/Thr kinases especially IKK complex. First time, I am providing data that methoxy genistein is a potent inhibitor of Ser/Thr kinase. Thus, biochanin is a dual kinase inhibitor and showed double-edged sword effect to inhibit NF-κB and may be use as a potent anti-inflammatory and/or anti-tumor agent.

2. Materials and methods

2.1. Materials

Biochanin, quercetin, genistein, formononetin, daidzein, quercetin, phorbol myristate acetate, doxorubicin, lipopolysaccharide, MTT, DMSO, H_2O_2, sodium orthovanadate, and anti-tubulin antibody were obtained from Sigma–Aldrich Chemicals (St Louis, MO, USA). Erbstatin was obtained from LC Laboratories (Woburn, MA, USA). Penicillin, streptomycin, neomycin, RPMI 1640, DMEM medium, and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY, USA). Antibodies against ICAM1, cox2, IκBα, p65, p50, CRM1, IKKα, IKKβ, Lck, Syk, and phospho-Syk and gel shift oligonucleotides for NF-κB and AP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant IL-8, EGF, and TNFα was purchased from Peprotech (Rocky Hill, NJ, USA). *Clostridium botulinum* toxin (C3-toxin) was kindly provided by Prof. G.S. Chhatwal (German Center for Biotechnology, Braunschweig, Germany). Plasmid constructs for *TRAF2*, *TRAF6*, *p65*, *IκBα-DN*, *Cox2-luciferase*, and *NF-κB-luciferase* were obtained from Prof. Bharat B. Aggarwal (MD Anderson Cancer Center, Houston, USA).

PV was prepared freshly for treatment of cells as described before [6]. Briefly, 20 μl of 1 M sodium orthovanadate was taken in 270 μl phosphate-buffered saline. Ten microliters of 33% H_2O_2 was added to the mixture and reaction was continued for 5 min at room temperature. The pH of the solution was neutralized by adding 1N HCl and excess H_2O_2 was deactivated with catalase (60 μg). The concentration of PV generated is denoted by the vanadate concentration taken in the reaction mixture. The vehicle control was used the mixture without sodium orthovanadate for treatment.

2.2. Cell line

The cell line used in this study Jurkat (human T-cells) was obtained from American Type culture collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were free from mycoplasma, as detected by Gen-Probe mycoplasma rapid detection kit (Fisher Scientific, Pittsburgh, PA, USA).

2.3. NF-κB and AP-1 DNA binding assay

DNA binding of AP-1 and NF-κB was determined by gel shift assay [22]. Briefly, the cells were subjected to different treatments as described and cytoplasmic and nuclear extracts were prepared. Nuclear extract (8 μg protein) was incubated with ^{32}P-end-labeled double-stranded NF-κB oligonucleotide of HIV-LTR, 5′-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3′ for 30 min at 37 °C, and the DNA–protein complex was separated from free oligonucleotides on 6.6% native PAGE. AP-1 DNA binding was assayed similarly using specific double-stranded labeled oligonucleotides. Visualization of radioactive bands was done in a fluorescent image analyzer FLA-3000 (Fuji, Japan).

2.4. NF-κB-, AP-1-, and cox2-dependent reporter gene transcription assay

The amount of different transcription factor-dependent reporter gene, luciferase expression was carried out as described previously [23]. Jurkat cells were transfected with *NF-κB-luciferase*, *AP-1-luciferase*, or *Cox2-lucifearse* (0.5 μg) and GFP (0.5 μg) constructs. After 3 h of transfection cells were washed and cultured for 12 h. GFP positive cells were counted (35–40% for different combinations). Cells, after different treatments were extracted with lysis buffer (part of Luciferase assay kit from Promega) and luciferase activity was determined and indicated as fold activation considering vector-transfected value as 1 fold.

2.5. IKK and Lck activities assay

The IKK and Lck activities were assayed by a method described previously [23,24]. Briefly, IKK or Lck complex from whole-cell extract (300 μg) was precipitated with anti-IKKα and -IKKβ antibodies (1 μg each) or anti-Lck antibody (1 μg), followed by incubation with protein A/G-Sepharose beads (Pierce, Rockford, IL, USA). After 2 h incubation, the beads were washed with lysis buffer and then assayed for IKK by using 2 μg of substrate GST-IκBα (aa1–aa54) or Lck activity was assayed by autophosphorylation of Lck using assay buffer with ^{32}P-γATP.

2.6. Study of molecular docking

2.6.1. Proteins setup for docking

Experimental structures of serine/threonine kinase (PDB ID: 2QON) [25] and tyrosine kinase (PDB ID: 3GQL) [26] were retrieved