

ORIGINAL ARTICLE

***Bidens pilosa* suppresses interleukin-1 β -induced cyclooxygenase-2 expression through the inhibition of mitogen activated protein kinases phosphorylation in normal human dermal fibroblasts**

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ABSTRACT

Bidens pilosa (*BP*) Linn. var. *radiata* is a plant used in traditional folk medicine. It is clinically effective in various diseases; the pathogenesis of most of these involves cyclooxygenase (COX)-2. To investigate the mechanism on which the clinical effectiveness of *BP* is based, we examined its effects on COX-2 expression and its major product, prostaglandin (PG) E_2 , under conditions of inflammation. We induced inflammation in normal human dermal fibroblasts with interleukin (IL)-1 β and examined the effects of *BP* on COX-2 expression and PGE $_2$ production using Western blotting and competitive enzyme immunoassay, respectively. The functional involvements of mitogen activated protein kinases (MAPK) ERK1/2, p38, and JNK in COX-2 expression were also examined by Western blotting. IL-1 β -induced COX-2 expression was regulated by MAPK pathways, especially by p38. *BP* inhibited the phosphorylation of MAPKs, COX-2 expression, and subsequent PGE $_2$ production. The physiological activities and clinical effectiveness of *BP* observed under diverse conditions may be partly attributable to its ability to inhibit MAPK, mainly p38, activity, COX-2 expression, and subsequent PGE $_2$ production.

Key words: *Bidens pilosa*, cyclooxygenase-2, mitogen activated protein kinases (MAPK), prostaglandin E2 (PGE $_2$).

INTRODUCTION

Bidens pilosa (*BP*), a plant widely found in tropical and subtropical regions, is used in traditional folk medicine.¹ *BP* Linn. var. *radiata*, one variety of *BP*, is native to tropical America and has been introduced to Miyako Island, Okinawa, Japan. Several constituents of *BP*, including flavonoids,^{2–4} polyacetylenes,^{5–8} flavone glycosides,¹ chalcones,⁹ aurones¹⁰ and phenylpropanoids¹⁰ have been isolated. These *BP* extracts exhibit various clinical effects: anti-inflammatory,¹¹ anti-ulcerogenic,¹² hepatoprotective,¹³ anti-hyperglycemic,⁸ diuretic,¹ and anti-platelet-aggregation activities (Iijima, pers. comm. 2003). Inhibitory effects of *BP* on prostaglandin synthesis have also been demonstrated.¹⁴ Recently, beneficial

effects of *BP* on cutaneous disease, livedo reticularis with summer ulceration, have been reported in the Japanese published work by Masuzawa *et al.*¹⁵ We questioned whether there is a common regulator of the physiological functions of *BP*.

Cyclooxygenase (COX) is a physiologically important enzyme that catalyzes the conversion of arachidonic acid to prostaglandin (PG) H_2 , the committed step in the biosynthesis of prostanoids, potent mediators of diverse physiological processes.¹⁶ COX exists in two isoforms, COX-1 and COX-2, each of which is encoded by a unique gene located on a different chromosome.¹⁷ COX-1 is expressed constitutively in most tissues and is involved primarily in cellular homeostasis activities such as gastric cytoprotection, regulation of renal blood flow and

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platelet aggregation. In contrast, COX-2 expression is normally undetectable in most tissues but is highly inducible by a wide variety of extracellular stimuli, indicating that it is involved in the pathogenesis of inflammatory disorders.^{17,18} The association of COX-2 with various disorders for which *BP* is applied has been shown in humans and mice. Basu¹⁹ reported that, in mice, oxidative injury-induced hepatitis was a consequence of COX induction. Others²⁰ found that COX-2 was overexpressed in liver tissue from patients with cirrhosis, suggesting that it may play a role in the pathogenesis of human liver cirrhosis. COX-2 expression in chronic pancreatitis and its correlation with diabetes mellitus have also been reported.²¹ In the kidney, diuretics stimulate COX-2 expression in the macula densa of the juxtaglomerular apparatus and COX-2-selective inhibitor-blunted diuretic-induced salt excretion, suggesting that COX-2 plays a major role in modulating the renal effects of diuretics.^{22,23}

We posited that COX-2 may represent a pivotal molecule in the physiological activities of *BP* and examined the effects of *BP* on COX-2 expression and on PGE₂, the major COX-2 product, under conditions of inflammation. We also investigated the effects of *BP* on the activation of mitogen activated protein kinases (MAPK), ubiquitous kinases involved in various physiological phenomena including COX-2 expression.^{24,25}

In this study, we used normal human dermal fibroblasts (HDF), because we have previously demonstrated that HDF express COX-2 and produce PGE₂ in response to interleukin (IL)-1 β treatment²⁵ and that fibroblasts play an important role in inflammation in various tissues as well as in skin.

MATERIALS AND METHODS

Materials

The *BP* powder produced from the aerial parts of *BP* Linn. var. *radiata* cultivated on Miyako Island was provided by Musashino Research Institute for Immunity (Tokyo, Japan), and purified by aqueous infusion. It was extracted from the dried plant by hot water, filtered and dried. Then the *BP* powder was diluted with phosphate-buffered saline. Cell culture reagents were purchased from Life Technologies (Grand Island, NY, USA), recombinant human IL-1 β

from R&D Systems (Minneapolis, MN, USA), polyclonal antibodies to human COX-1 and COX-2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), antibodies to ERK1/2, p38, and JNK and phosphorylated forms of these kinases from Cell Signaling Technology (Beverly, MA, USA), the inhibitors PD98059 and SB203580 from Promega (Madison, WI, USA), and SP600125 from Biosource (Camarillo, CA, USA).

Cell culture

Normal human dermal fibroblasts were cultured as described previously.²⁶ Briefly, normal human skin samples, minced and placed in sterile 3-cm dishes, were pressed with a coverslip and incubated under sterile conditions at 37°C in a humidified atmosphere containing 5% CO₂ in the presence of 3 ml of complete Eagle's minimum essential medium (EMEM) supplemented with 100 000 IU/l penicillin G, 100 mg/l streptomycin, 1 mg/l Fungizone, 0.1 mmol nonessential amino acids, 292 mg/l glutamine, 50 mg/l ascorbic acid and 10% fetal calf serum (FCS). The medium was changed every 3 days until the cells reached confluence (4–6 weeks), and then they were seeded (10⁵/ml) in 6-cm culture dishes (protein extraction) or 24-well flat-bottom tissue culture plates (PGE₂ measurement) containing the identical medium.

Western blotting

Fibroblasts from normal human skin (10⁵/ml) were seeded in 6-cm tissue culture plates (5 ml/plate), incubated until confluence, and then lysed on ice with lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCL (pH 7.4), 150 mmol NaCl, 1 mmol ethylene diamine tetra acetate (EDTA), 1 mmol sodium orthovanadate, 10 mmol β -glycerol phosphate, 1 mmol phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. The protein concentration in extracts was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Identical amounts (15 μ g) of protein were electrophoresed in a 10% sodium dodecyl sulfate (SDS)/polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% dry milk in Tris-buffered saline containing 0.05% Tween-20 and incubated with primary antibodies for 1 h at room temperature (RT) and then for 30 min at RT

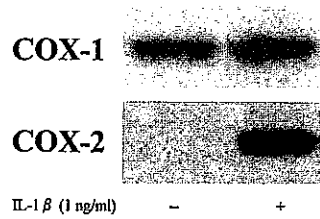


Figure 1. Induction by interleukin (IL)-1 β of cyclooxygenase (COX)-2 protein expression in normal human dermal fibroblasts. Cells (10^5 /ml) seeded in 6-cm tissue culture plates (6 ml/plate) were incubated for 72 h until they reached confluence. The medium was then replaced with fresh Dulbecco's minimum essential medium (DMEM) containing 1% fetal calf serum (FCS) with or without 1 ng/ml IL-1 β . Proteins were extracted after 18 h, and 15 μ g-aliquots were analyzed on Western blots. COX-2 expression, not detected in the absence of IL-1 β treatment, was significantly enhanced by IL-1 β . COX-1, constitutively expressed by the cells, was not increased by IL-1 β . Data are representative of three separate experiments.

with anti-rabbit alkaline phosphatase-linked immunoglobulin. Immunoreactive bands were detected using an ECL kit (Amersham, Arlington Heights, IL, USA).

Prostaglandin E₂ measurement

Fibroblasts (10^5 /ml) were seeded in 24-well tissue culture plates (0.8 ml/well) and incubated for 24 h until they reached confluence. The growth medium was then replaced with fresh DMEM containing 1% FCS and 1 ng/ml IL-1 β with or without appropriate concentrations of BP. Culture media were collected after 24 h and analyzed for PGE₂ production using the Prostaglandin E₂ EIA Kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturer's instructions.

RESULTS

IL-1 β induces the expression of COX-2, but not COX-1, in normal human dermal fibroblasts

We used Western blots to examine the expression of COX-1 and COX-2 in normal human dermal fibroblasts grown in the presence or absence of IL-1 β . No COX-2 protein expression was detected in IL-1 β -untreated cells. However, fibroblasts treated for 18 h with 1 ng/ml IL-1 β expressed COX-2 at significant levels. COX-1 protein was constitutively

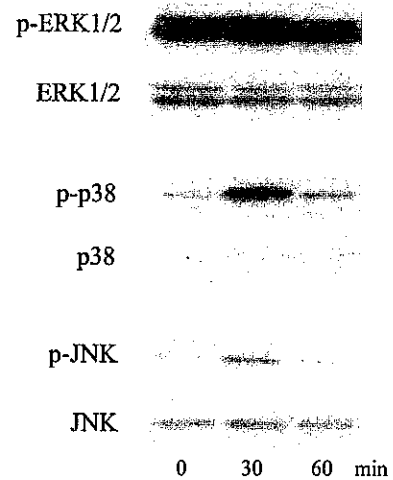


Figure 2. Phosphorylation of mitogen activated protein kinases (MAPK) by IL-1 β in normal human dermal fibroblasts. Cells (10^5 /ml) seeded in 6-cm tissue culture plates (6 ml/plate) were incubated for 72 h until confluence. The medium was then replaced with fresh DMEM containing 1% FCS with or without 1 ng/ml IL-1 β . Proteins were extracted at the indicated times and 15- μ g aliquots were analyzed by Western blotting. Although transient phosphorylation of MAPK was induced by 30-min IL-1 β treatment, it declined thereafter. There was marked activation of p38 and JNK; ERK1/2-activation was moderate. Data are representative of three separate experiments.

expressed by these cells but was not increased by IL-1 β (Fig. 1). These results indicate that inflammation was induced by IL-1 β treatment.

IL-1 β induces MAPK phosphorylation

As the MAPK pathway is reportedly involved in COX-2 expression,^{24,25} we examined the functional contribution of MAPK signaling in IL-1 β -induced COX-2 expression. First, we examined the activation of three MAPK, ERK1/2, p38, and JNK in response to IL-1 β treatment by detecting their phosphorylated forms by Western blotting. The phosphorylation of MAPK was transiently induced by 30-min exposure to IL-1 β and declined thereafter (Fig. 2). MAPK p38 was phosphorylated most remarkably. While JNK was activated clearly, the activation of ERK1/2 was moderate. These results confirm that inflammatory cytokines such as IL-1 β activate p38 and JNK, but ERK1/2 is preferentially activated by mitogens of the receptor tyrosine kinase family.²⁷

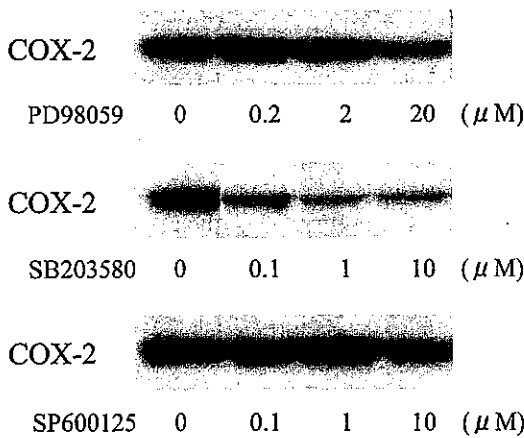


Figure 3. Inhibition of IL-1 β -induced COX-2 expression by MAPK inhibitors in normal human dermal fibroblasts. Cells (10⁵/ml) seeded in 6-cm tissue culture plates (6 ml/plate) were incubated for 72 h until confluence. The medium was replaced with fresh DMEM containing 1% FCS and 1 ng/ml IL-1 β and the cells were incubated in the presence or absence of MAPK inhibitors (PD98059 for ERK1/2, SB203580 for p38, and SP600125 for JNK) at the indicated concentrations. Proteins were extracted after 18 h and 15- μ g aliquots were analyzed by Western blotting. All MAPK inhibitors suppressed COX-2 expression; SB203580 showed the most prominent inhibitory effect. Data are representative of three separate experiments.

MAPK inhibition reduces IL-1 β -induced COX-2 expression

Next, we examined whether selective MAPK inhibitors, PD98059 for ERK1/2, SB203580 for p38, and SP600125 for JNK, suppress IL-1 β -induced COX-2 expression. The concentrations we used were 0.1- and 10-fold the 50% inhibitory concentration (IC₅₀) as well as the IC₅₀. All the MAPK inhibitors suppressed COX-2 expression, and SB203580 showed the strongest inhibitory effect (Fig. 3). These results demonstrate that IL-1 β -induced COX-2 expression is regulated by a MAPK pathway, especially by p38 kinase.

BP suppresses IL-1 β -induced MAPK phosphorylation

To investigate whether *BP* affects MAPK pathways, we examined its effects on the activation of MAPK. When *BP* (1 mg/ml) was added 15 min before the 30-min IL-1 β treatment, the phosphorylation of p38 and JNK and, to a lesser degree of ERK1/2, was suppressed (Fig. 4).

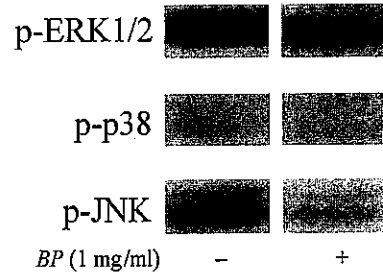


Figure 4. Inhibition of IL-1 β -induced MAPK phosphorylation by *Bidens pilosa* (*BP*) in normal human dermal fibroblasts. Cells (10⁵/ml) seeded in 6-cm tissue culture plates (6 ml/plate) were incubated for 72 h until confluence. The medium was then replaced with fresh DMEM containing 1% FCS with or without 1 mg/ml *BP* and IL-1 β was added after 15 min. Proteins were extracted at the indicated times after IL-1 β treatment, and 15- μ g aliquots were analyzed by Western blotting. Exposure (30 min) to IL-1 β suppressed the phosphorylation of p38 and JNK, and less strongly, of ERK1/2. Data are representative of three separate experiments.

BP suppresses IL-1 β -induced COX-2 expression

Because IL-1 β -induced COX-2 expression is regulated through MAPK pathways and *BP* suppressed IL-1 β -induced MAPK phosphorylation, we examined the effect of *BP* on IL-1 β -induced COX-2 expression. As expected, the IL-1 β -induced COX-2 expression was inhibited by *BP* in a dose-dependent manner. COX-1 expression was not altered by *BP* in the absence or presence of IL-1 β (Fig. 5).

BP suppresses IL-1 β -induced PGE₂ production

We tested the effect of *BP* on the production of PGE₂, a major COX-2 product. The production of PGE₂ was significantly enhanced by IL-1 β and reduced, in a dose-dependent manner, by *BP* (Fig. 6). The amount of PGE₂ produced by IL-1 β -treated fibroblasts correlated well with the expression level of COX-2, as shown in Figure 5.

DISCUSSION

We demonstrated that IL-1 β -induced COX-2 expression was regulated by MAPK pathways and that *BP* inhibited the phosphorylation of MAPK, COX-2 expression, and subsequent PGE₂ production. The MAPK family consists of three subgroups, ERK1/2, p38 and JNK, cell ubiquitous serine/threonine kinases

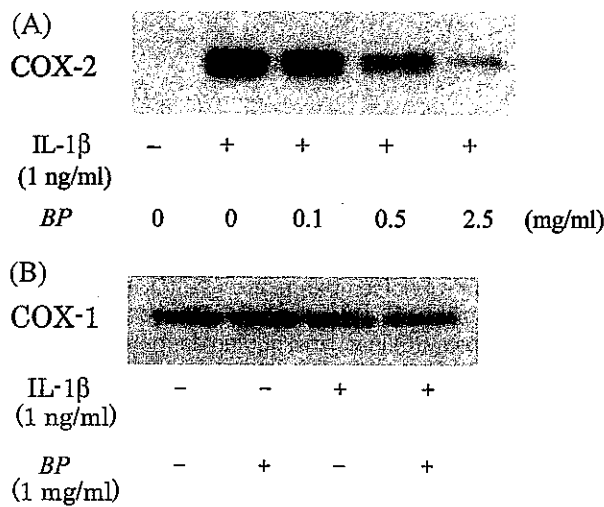


Figure 5. (A) Inhibition of IL-1 β -induced COX-2 expression by BP in normal human dermal fibroblasts. Cells (10^5 /ml) seeded in 6-cm tissue culture plates (6 ml/plate) were incubated for 72 h until confluence. The medium was then replaced with fresh DMEM containing 1% FCS with or without 1 ng/ml IL-1 β . The cells were incubated for 18 h in the presence or absence of BP at the indicated concentrations, proteins were extracted, and 15- μ g aliquots were analyzed by Western blotting. IL-1 β -induced COX-2 expression was inhibited by BP in a dose-dependent manner. Data are representative of three separate experiments. (B) COX-1 expression was not altered by BP in the absence or presence of IL-1 β .

that are activated by various extracellular signals and involved in a wide variety of physiological phenomena such as cell proliferation and differentiation, apoptosis and gene expression.^{27,28} MAPK is reported to regulate COX-2 expression,^{24,25} and COX-2 is functionally active in various organ systems and disease states.^{19-23,29} Thus, the physiological activities and clinical effectiveness of BP in diverse conditions may be partly attributable to its ability to inhibit MAPK activity and COX-2 expression. In the present study, while the activation of ERK1/2 by IL-1 β was moderate, the inhibitory effect of the ERK1/2 inhibitor on IL-1 β -induced COX-2 expression was apparent. These results indicate that ERK1/2 pathway contributes to COX-2 induction possibly by constitutively phosphorylated ERK1/2 as shown in Figure 2. Although BP suppressed the phosphorylation of JNK most profoundly, the inhibitory effect of the JNK inhibitor on IL-1 β -induced COX-2 expression was only modest, indicating that JNK

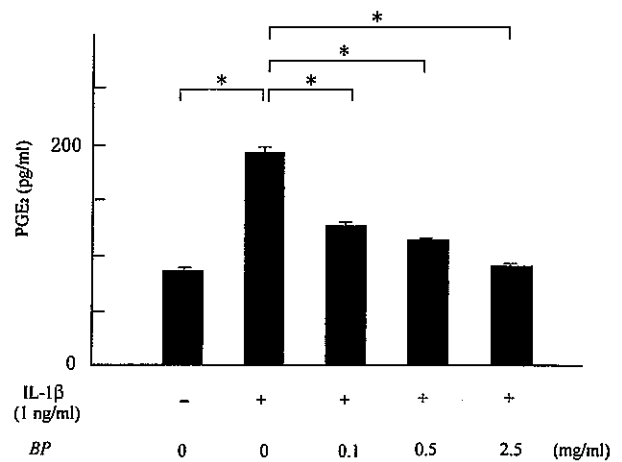


Figure 6. Inhibition of IL-1 β -induced PGE₂ production by BP in normal human dermal fibroblasts. Cells (10^5 /ml) seeded in 96-well tissue culture plates (0.5 ml/well) were incubated for 48 h until confluence. The medium was replaced with fresh DMEM containing 1% FCS and 1 ng/ml IL-1 β with or without BP at the indicated concentrations, collected after 24 h, and analyzed for PGE₂ production using the Prostaglandin E₂ EIA Kit (Cayman Chemicals, Ann Arbor, MI, USA). PGE₂ production was significantly induced by IL-1 β and dose-dependently reduced by BP. Data shown are mean \pm SE of triplicate determinations and representative of three separate experiments. *Significantly different at $P < 0.05$.

is involved in COX-2 induction to a less extent. According to the remarkable phosphorylation of p38 by IL-1 β and the strongest inhibitory effect of the p38 inhibitor, the main target of BP in IL-1 β -induced COX-2 expression might be p38 MAPK.

The constituents of BP are structurally classified as coffee tannins, flavonoids and polyacetylenes.³⁰ Of these, coffee tannins and flavonoids have been shown to have antioxidant activity in scavenging superoxide, hydrogen peroxide and oxygen-free radicals that underlie the development of various diseases.^{30,31} Because oxidative stress has been implicated in a broad range of disease states including inflammatory diseases,^{32,33} ischemia and reperfusion injury,³⁴ diabetes,³⁵ neurodegenerative diseases,³⁶ cancer³⁷ and other diseases,³⁸ the antioxidant activity of BP may exert beneficial effects in clinical situations.

COX is the key enzyme required for PG biosynthesis, a two-step process that depends on the release of arachidonic acid (AA) into the cytoplasm from

membrane phospholipids by phospholipase A₂ and the subsequent conversion by COX of AA to PG. COX is a bi-functional enzyme with cyclooxygenase activity that converts AA to PGG₂ and peroxidase activity that generates PGH₂, a direct precursor of PGE₂, from PGG₂. In addition to its role in PG synthesis, the peroxidase activity of COX leads to superoxide production and subsequent alterations in the intracellular redox status, which is associated with the perturbation of cellular homeostasis, cell growth and transformation.^{39,40} COX-2 is known to regulate cell proliferation and differentiation, and tumorigenesis⁴¹⁻⁴⁴ and COX-2 expression has been reported to be increased in disease states including carcinoma of various organs.^{45,46} The cellular redox status regulates COX-2 expression. IL-1- and lipopolysaccharide (LPS)-induced COX-2 expression is decreased in antioxidant-treated rat mesangial cells and alveolar macrophages, respectively.^{47,48} The enhanced expression of COX-2 by human colorectal cancer cells is decreased by antioxidants, pyrrolidinedithiocarbamate (PDTC), *N*-acetylcysteine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, and U74006,³⁹ and PDTC decreased COX-2 expression by human skin squamous carcinoma cells.⁴⁹ Because of its antioxidant activity, *BP* might be able to suppress IL-1 β -induced COX-2 expression. We also demonstrated that *BP* reduced the production of PGE₂, a major product of COX-2. This observation further supports the putative clinical effects of *BP*, because PGE₂ is a potent mediator of pathophysiological processes.¹⁶

The antioxidant properties that result in the inhibition of COX-2 expression under conditions of inflammation lead us to suspect that *BP* may be of dietary value. Studies are underway to further elucidate the constituents of *BP* and their potential pharmacokinetic and clinical utility.

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