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Caffeic acid, a phenolic phytochemical in coffee, directly inhibits Fyn kinase activity and UVB-induced COX-2 expression

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Caffeic acid (3,4-dihydroxycinnamic acid) is a well-known phenolic phytochemical present in many foods, including coffee. Recent studies suggested that caffeic acid exerts anticarcinogenic effects, but little is known about the underlying molecular mechanisms and specific target proteins. In this study, we found that Fyn, one of the members of the non-receptor protein tyrosine kinase family, was required for ultraviolet (UV) B-induced cyclooxygenase-2 (COX-2) expression, and caffeic acid suppressed UVB-induced skin carcinogenesis by directly inhibiting Fyn kinase activity. Caffeic acid more effectively suppressed UVB-induced COX-2 expression and subsequent prostaglandin E_2 production in JB6 P+ mouse skin epidermal (JB6 P+) cells compared with chlorogenic acid (5-*O*-caffeoylquinic acid), an ester of caffeic acid with quinic acid. Data also revealed that caffeic acid more effectively induced the downregulation of COX-2 expression at the transcriptional level mediated through the inhibition of activator protein-1 (AP-1) and nuclear factor-κB transcription activity compared with chlorogenic acid. Fyn kinase activity was suppressed more effectively by caffeic acid than by chlorogenic acid, and downstream mitogen-activated protein kinases (MAPKs) were subsequently blocked. Pharmacological Fyn kinase inhibitor (3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine and leflunomide) data also revealed that Fyn is involved in UVB-induced COX-2 expression mediated through the phosphorylation of MAPKs in JB6 P+ cells. Pull-down assays revealed that caffeic acid directly bound with Fyn and non-competitively with adenosine triphosphate. *In vivo* data from mouse skin also supported the idea that caffeic acid suppressed UVB-induced COX-2 expression by blocking Fyn kinase activity. These results suggested that this compound could act as a potent chemopreventive agent against skin cancer.

Introduction

Skin cancer is one of the most common human cancers with >1 million new cases diagnosed each year, which accounts for ∼40% of all new cancer cases in the USA (1). Solar ultraviolet (UV) radiation has been implicated as a primary cause of skin cancer, and in particular UVB irradiation. UVB not only initiates DNA damage but also causes alterations in signaling molecules involved in tumor promotion, thereby acting as a complete carcinogen. Among the many genes that are abnormally altered by UVB, the *cyclooxygenases-2* (*cox-2*) gene, which is known to be overexpressed in response to UVB in both mouse and human skin, serves as an early skin marker of UVB exposure (2–4). UVB-induced COX-2 expression increases the production of prostaglandin E_2 (PGE_2) by catalyzing the rate-limiting step in the conversion of arachidonic acid into prostaglandins. The UVB-induced production of PGE_2 has been implicated in skin carcinogenesis because increased PGE_2 is associated with the induction of keratinocyte proliferation, angiogenesis and cell migration (5,6). Accumulating evidence suggests that topically applied COX-2 inhibitors such as celecoxib suppress UVB-induced skin inflammation and tumor formation (7). Moreover, COX-2-deficient mice show enhanced UVB-induced epidermal apoptosis (8). In addition, blocking the PGE_2 receptor using PGE_2 antagonists, such as ONO-8713 (9) or PGE_2-receptor-deficient mice (10), decreases UVB-induced skin inflammation and the number of skin tumors. This suggests that agents suppressing UVB-induced COX-2 and PGE_2 production are potential chemopreventive agents against skin cancer.

Fyn is a ubiquitously expressed member of the Src family of non-receptor tyrosine kinases that are involved in transmitting signals from various cell surface receptors to cytoplasmic signal transduction cascades. Fyn comprises an N-terminal region required for plasma membrane binding, two Src homology (SH) domains (SH2 and SH3) involved in protein–protein interactions, a highly conserved catalytic domain including an adenosine triphosphate (ATP)-binding site and a C-terminal tail containing a site that negatively regulates tyrosine phosphorylation (11). Fyn plays a critical role in T cell receptor signaling, brain function and cell adhesion-mediated signaling (12). Fyn is also reportedly involved in skin disorders and mediates 12-*O*-tetradecanoylphorbol-13-acetate-induced keratinocyte differentiation, with Fyn-deficient mice showing skin abnormalities such as epidermal thickness and hyperkeratosis (13,14). Fyn also induces keratinocyte cell–cell adhesion by acting as a downstream mediator of Rho GTPase (15). In addition, accumulating evidence indicates that Fyn has oncogenic potential and is involved in carcinogenesis processes, including skin cancer development. For example, Fyn overexpression induces morphologic transformation and results in anchorage-independent growth of NIH 3T3 murine fibroblasts (16). Fyn is also involved in epidermal growth factor-induced neoplastic transformation of JB6 P+ mouse skin epidermal cells (17). However, the involvement of Fyn in UVB-induced skin cancer remains to be elucidated.

Hydroxycinnamic acids are among the major phenolic compounds derived from fruits, vegetables, grains and coffee. These compounds are commonly found in the esterified form with quinic acid because they are extensively metabolized in the body (18). The major dietary hydroxycinnamic acid is caffeic acid (3,4-dihydroxycinnamic acid, Figure 1A, left), which is found in food mainly as chlorogenic acid (5-*O*-caffeoylquinic acid, Figure 1A, right) because of its conjugation with quinic acid. Chlorogenic acid is an ester formed between caffeic acid and quinic acid and is one of the most widely consumed polyphenols abundant in dietary foods, especially in coffee. Typical consumption of coffee results in the ingestion of 0.5–1 g of chlorogenic acid and 250–500 mg of caffeic acid per day (19). The high dietary content of chlorogenic acid in coffee has resulted in considerable attention being paid to its biological effects. Chlorogenic acid reportedly prevents type 2 diabetes mellitus by inhibiting intestinal glucose absorption (20) and exerts anticarcinogenic effects by attenuating injury from carcinogenic N-nitroso compounds and DNA damage (21–23). However, ∼33% of chlorogenic acid is absorbed in the intact form intestinally, with the remaining 67% being metabolized to

Abbreviations: AP-1, activator protein-1; ATP, adenosine triphosphate; COX-2, cyclooxygenase-2; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEM, minimum essential medium; NF-κB, nuclear factor-κB; PP2, (3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; PGE_2, prostaglandin E_2; PMSF, phenylmethylsulfonyl fluoride; SH, Src homology; UV, ultraviolet.

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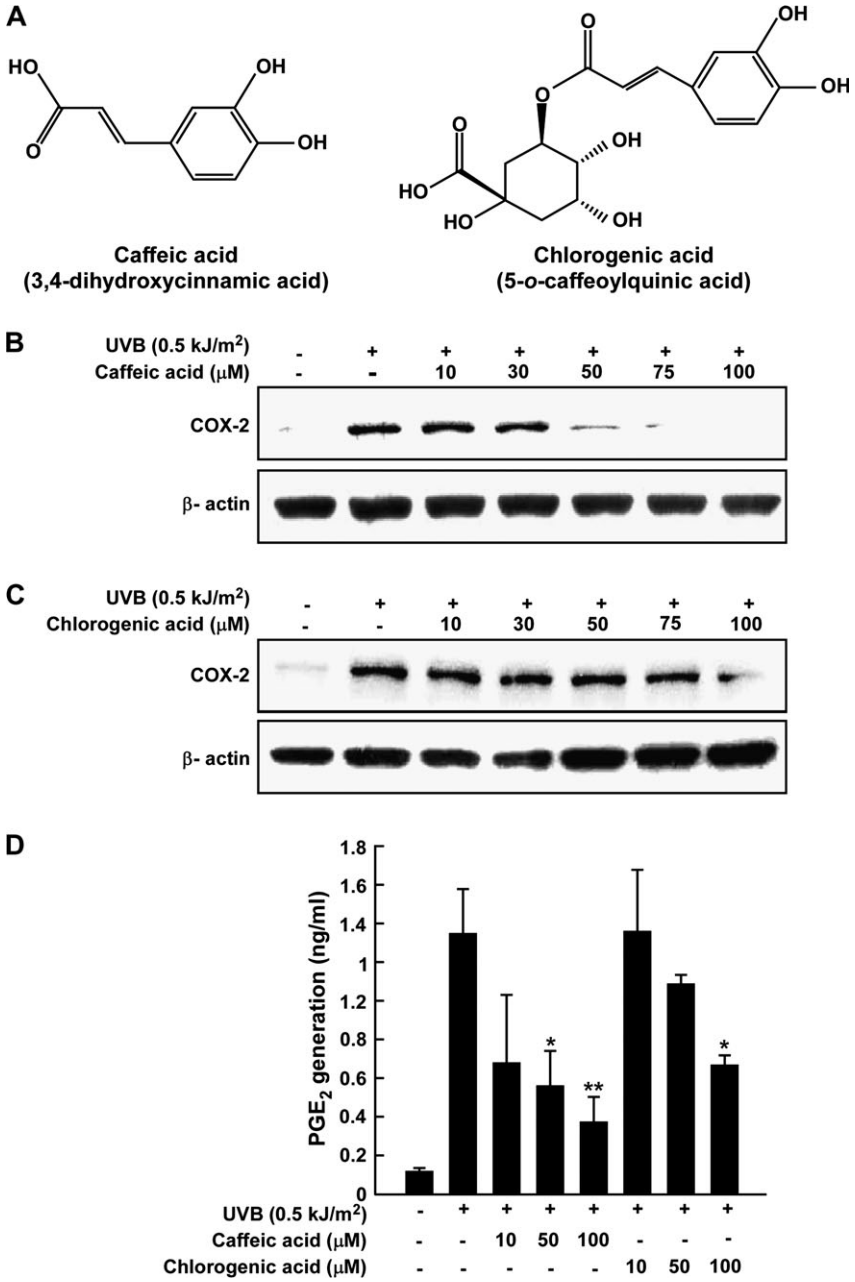

Fig. 1. Effect of caffeic acid or chlorogenic acid on UVB-induced COX-2 expression and PGE_2 production in JB6 P+ cells. (**A**) Chemical structures of caffeic acid (left) and chlorogenic acid (right). (**B** and **C**) UVB-induced COX-2 expression in JB6 P+ cells is inhibited more strongly by caffeic acid than by chlorogenic acid. JB6 P+ cells were treated with caffeic acid or chlorogenic acid at the indicated concentrations (0, 10, 30, 50, 75 or 100 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 4 h later. The cells were disrupted and COX-2 protein level was determined by western blot analysis as described in Materials and Methods. β-Actin was detected to verify equal loading of proteins. Data are representative of two independent experiments that gave similar results. (**D**) UVB-induced PGE_2 production in JB6 P+ cells is inhibited more strongly by caffeic acid than by chlorogenic acid. JB6 P+ cells were treated with caffeic acid or chlorogenic acid at the indicated concentration (0, 10, 50 or 100 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 18 h later. PGE_2 production was measured using a PGE_2 assay kit as described in Materials and Methods. Asterisks indicate significant inhibition of PGE_2 production by caffeic acid or chlorogenic acid compared with the group treated with UVB alone ($^*P < 0.05$ and $^{**}P < 0.01$).

caffeic acid in the colon (20). In contrast, ~95% of caffeic acid is absorbed intestinally. These bioavailability data suggest that the biological effects of chlorogenic acid in the body would manifest after it has been metabolized to caffeic acid, and hence to also study the effects of caffeic acid in the prevention of disease is necessary. Previous studies revealed that caffeic acid exerts protective effects against UVB-induced skin damage by suppressing interleukin-10 and mitogen-activated protein kinase (MAPK) activation in mouse skin (24). However, the underlying molecular mechanisms and targeted proteins involved in the suppression of skin carcinogenesis by caffeic acid are not fully understood.

Here, we report that caffeic acid inhibits UVB-induced COX-2 expression and PGE_2 production by directly targeting Fyn, both in JB6 P+ mouse skin epidermal (JB6 P+) cells and mouse skin *in vivo*. To our knowledge, the present study is the first to suggest that Fyn is required for UVB-induced skin carcinogenesis and that caffeic acid, as an inhibitor of Fyn kinase activity, suppresses skin carcinogenesis more potently than chlorogenic acid.

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Materials and methods

Chemicals

Caffeic acid and chlorogenic acid were purchased from Sigma–Aldrich (St Louis, MO). Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), gentamicin, L-glutamine and penicillin–streptomycin were obtained from GIBCO BRL (Grand Island, NY). U0126, SB203580, SP600125, 3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (PP2) and leflunomide were from Calbiochem (San Diego, CA). The antibody against COX-2 and a PGE_2 enzyme immunoassay kit were obtained from Cayman Chemical (Ann Arbor, MI), and anti-β-actin was purchased from Sigma–Aldrich. Antibodies against phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (Thr202/Tyr204) and total ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated p38 (Tyr180/Tyr182), total p38, phosphorylated c-jun N-terminal kinase (JNK) (Thr183/Tyr185) and total JNK1 were obtained from Cell Signaling Biotechnology (Beverly, MA). The active Fyn protein and anti-Fyn were obtained from Upstate Biotechnology (Lake Placid, NY). CNBr-Sepharose 4B, [γ-^{32}P]ATP and a chemiluminescence detection kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). G418 and the luciferase assay substrate were purchased from Promega (Madison, WI).

Animals

Female ICR mice (5 weeks of age; mean body weight, 25 g) were purchased from the Institute of Laboratory Animal Resources at Seoul National University (Seoul, Korea). Animals were acclimated for 1 week prior to the study and had free access to food and water. The animals were housed in climate-controlled quarters (24°C at 50% humidity) with a 12 h light–12 h dark cycle.

UVB irradiation

A UVB irradiation system was used to stimulate cells in serum-free media. The spectral peak of the UVB source (Bio-Link Crosslinker, Vilber Lourmat, Cedex 1, France) was at 312 nm. The cells were exposed to UVB at 0.5 kJ/m^2 and then cultured for either 15 min or 4 h. ICR mice were exposed to UVB at a dose of 5 kJ/m^2, and then proteins were isolated from the skin 2 or 6 h later.

Cell culture

The JB6 P+ cell line was cultured in monolayers at 37°C in a 5% CO_2 incubator in MEM containing 5% FBS, 2 mM L-glutamine and 25 μg/ml gentamicin. The JB6 P+ cell lines stably transfected with a COX-2, activator protein-1 (AP-1) or nuclear factor-κB (NF-κB) luciferase reporter plasmid were maintained in MEM supplemented with 5% FBS containing 200 μg/ml G418 to exclude non-transfected cells.

Western blot analysis

Cells were cultured for 48 h and then incubated in MEM containing 0.1% FBS for an additional 24 h. The cells were then treated with chemicals for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested at various time points. Cell lysates were scraped and treated with lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol and a protease inhibitor cocktail tablet] for 40 min on ice and then centrifuged at 16 000*g* for 10 min. The protein concentration of the supernatant fraction was measured using a dye-binding protein assay kit (Bio-Rad Laboratories) as described in the manufacturer's manual. Lysate protein (40 μg) was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA). After transfer, the membrane was blocked in 5% fat-free dry milk for 1 h and then incubated with the specific primary antibody for 2 h at room temperature. After hybridization with the horseradish peroxidase-conjugated secondary antibody, protein bands were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). For the *in vivo* western blots, respective groups of five ICR mice each received topical application of caffeic acid (0, 40 or 200 nmol) in 200 μl acetone on their shaved backs 1 h before UVB irradiation. To isolate proteins from mouse skin, mice were killed 6 h (COX-2) after UVB treatment and the dorsal skin of each mouse was excised and placed on ice. Any fat was removed and the skin was placed in liquid nitrogen and the skin was immediately pulverized with a mortar and pestle. The pulverized skin was blended on ice with a homogenizer (IKAT10 basic; IKA Laboratory Equipment, Staufen, Germany) and skin lysates were centrifuged at 12 000 r.p.m. for 20 min. After the protein content was determined using the Bio-Rad protein assay kit, 100 μg of mouse skin extract was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

PGE_2 assay

JB6 P+ cells were plated in six-well dishes and grown to 80% confluence in 2 ml of growth medium and then pretreated with caffeic acid or chlorogenic acid for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 18 h later. The amount of PGE_2 released into the medium was measured using the PGE_2 enzyme immunoassay kit.

Luciferase assay for COX-2 promoter activity and AP-1 or NF-κB transactivation

Confluent monolayers of JB6 P+ cells (5×10^3), stably transfected with a COX-2, AP-1 or NF-κB luciferase reporter plasmid, were suspended in 200 μl of 5% FBS–MEM and added to each well of a 96-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. When cells reached 80–90% confluence, they were cultured in 0.1% FBS–MEM for 24 h to reduce background. The cells were then pretreated with chemicals for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 24 h later (i.e. when determining COX-2 activity) or 12 h later (i.e. when determining AP-1 or NF-κB activity). After treatment, cells were disrupted with 100 μl of lysis buffer [0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM DTT and 2 mM EDTA], and luciferase activity was measured using a luminometer (Microlumat Plus LB 96V, Berthold Technologies, Bad Wildbach, Germany).

Fyn kinase assay

Fyn kinase activity was directly determined according to the instructions provided by Upstate Biotechnology. In brief, each reaction contained 6.25 μl of assay buffer [200 mM Tris–HCl (pH 7.5), 0.4 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid and 0.4 mM sodium orthovanadate (Na_3VO_4)] and a magnesium acetate–ATP cocktail buffer [2.5 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (pH 7.4), 50 mM magnesium acetate and 0.5 mM ATP]. The Src substrate peptide was also included at 250 μM with 10 ng of an active Fyn protein. Then 10 μl of diluted [γ-^{32}P]ATP solution was incubated at 30°C for 10 min with the above assay buffer and substrate peptide, and 15 μl aliquots were transferred onto p81 paper and washed three times with 0.75% phosphoric acid for 5 min and once with acetone for 5 min. The radioactive incorporation was measured using a scintillation counter. The effects of caffeic acid (0–100 μM), chlorogenic acid (0–100 μM) and PP2 (1 μM) were evaluated by separately incubating each compound with the reaction mixtures at 30°C for 10 min. Each experiment was performed three times.

Fyn immunoprecipitation and kinase assays in JB6 P+ cells

JB6 P+ cells were cultured to 80% confluence and then incubated with 0.1% FBS–MEM for 24 h at 37°C to reduce background. Cells were treated with various concentrations of caffeic acid (0–100 μM), chlorogenic acid (0–100 μM) or PP2 (10 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested after 15 min. Cells were then disrupted with lysis buffer [20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM ethyleneglycol-bis (aminoethylether)-tetraacetic acid, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mg/ml leupeptin, 1 mM Na_3VO_4 and 1 mM PMSF] and finally centrifuged at 14 000 r.p.m. for 15 min in a microcentrifuge. The lysates containing 500 μg of protein were used for immunoprecipitation with an antibody against Fyn and then incubated at 4°C overnight with Protein A/G Sepharose beads. The beads were washed three times with kinase buffer [200 mM Tris–HCl (pH 7.5), 0.4 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid and 0.4 mM Na_3VO_4] and then resuspended in 6.25 μl of 1× kinase buffer supplemented with 250 μM Src substrate peptide and 10 μl of diluted [γ-^{32}P]ATP solution and incubated for 30 min at 10°C. A 15 μl aliquot was transferred onto p81 paper and washed three times with 0.75% phosphoric acid for 5 min and once with acetone for 5 min. The radioactive incorporation was measured using a scintillation counter. Each experiment was performed three times.

In vivo Fyn immunoprecipitation and kinase assay

Mice were treated with caffeic acid (0, 40 or 200 nmol) topically applied in 200 μl acetone and dorsal skin was prepared 2 h after UVB exposure. Proteins were extracted as described above and centrifuged at 14 000 r.p.m. for 15 min. A mouse skin extract (700 μg) was mixed with protein A/G beads (20 μl) for 1 h at 4°C. The mixture was centrifuged at 12 000 r.p.m. for 1 min at 4°C. The supernatant fraction was added to a Fyn antibody (20 μl) and gently rocked overnight at 4°C. These tubes were centrifuged and washed twice. The pellets were suspended in 6.5 μl of kinase buffer supplemented with 10 μl of diluted [γ-^{32}P]ATP solution and 2.5 μl of Src substrate peptide (250 μM) and incubated for 30 min at 30°C. A 15 μl aliquot was transferred onto P81 paper and washed tree times with 0.75% phosphoric acid for 5 min per wash and one time with acetone for 5 min. Radioactive incorporation was determined using a scintillation counter. Data are presented as the mean ± SD of data points from five mice in each treatment group.

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Direct, cell-based and in vivo pull-down assays

Active Fyn protein (0.2 μg) or a JB6 P+ cellular supernatant fraction (500 μg) was incubated with the caffeic acid–Sepharose 4B, chlorogenic acid–Sepharose 4B or Sepharose 4B only as a control, beads (100 μl, 50% slurry) in a reaction buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 μg/ml bovine serum albumin, 0.02 mM PMSF and 1× protease inhibitor mixture]. After incubation with gentle rocking overnight at 4°C, the beads were washed five times with buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40 and 0.02 mM PMSF], and proteins bound to the beads were analyzed by western blotting. For the *in vivo* pull-down assay, mice received topical application of 200 μl acetone alone or caffeic acid (40 or 200 nmol) in 200 μl acetone on their shaved backs 1 h before UVB irradiation. Dorsal skin was prepared as described above for the *in vivo* western blotting assay, and proteins were extracted as described above for the *in vivo* Fyn immunoprecipitation and kinase assays. Then 500 μg of mouse skin extract was incubated with caffeic acid–Sepharose 4B (or Sepharose 4B alone as a control) beads (100 μl, 50% slurry) in reaction buffer as described for the cell-based pull-down assay. Beads were incubated and washed and proteins bound to the beads were analyzed by western blotting as described above.

ATP and caffeic acid competition assay. Recombinant Fyn (0.2 μg) was incubated with 100 μl of caffeic acid–Sepharose 4B or 100 μl of Sepharose 4B in a reaction buffer (see direct and cell-based pull-down assays) for 12 h at 4°C, and ATP was added at either 10 or 100 μM to a final volume of 500 μl and incubated for 30 min. The samples were washed and proteins were then detected by western blotting.

Molecular modeling

The homology model structure of full-length Fyn was kindly provided by Dr Dubravko Jelic from the GlaxoSmithKline Research Center. Insight II (Accelrys, San Diego, CA) was used for the docking study and structure analysis with the homology model structure of the full-length Fyn.

Statistical analysis

When necessary, data were expressed as mean and standard deviation values, and the Student's *t*-test was used for single statistical comparisons. A probability value of $P < 0.01$ was used as the criterion for statistical significance.

Results

Caffeic acid suppresses UVB-induced COX-2 expression and PGE_2 production in JB6 P+ cells

Because COX-2 activation is known to be an early marker in the development of skin cancer in response to UVB exposure (3), we first examined the effects of caffeic acid or chlorogenic acid on UVB-induced COX-2 expression. Consistent with the results of previous studies, UVB significantly induced COX-2 expression in JB6 P+ cells. In our experimental system, exposure to 0.5 kJ/m^2 UVB was sufficient to induce COX-2 expression in JB6 P+ cells. COX-2 expression was dose dependently inhibited by caffeic acid (Figure 1B) or chlorogenic acid (Figure 1C). The activation of COX-2 by UVB exposure is known to induce PGE_2 production in the skin (10). We also found that the induction of COX-2 upregulation by UVB resulted in an increased PGE_2 production in JB6 P+ cells, and this was significantly inhibited by treatment with either caffeic acid or chlorogenic acid in a dose-dependent manner (Figure 1D). However, caffeic acid was more effective than chlorogenic acid (at the same concentration) at suppressing UVB-induced COX-2 expression and PGE_2 production.

Caffeic acid attenuates UVB-induced COX-2 promoter activity and AP-1 and NF-κB transactivation in JB6 P+ cells

To determine whether the inhibitory effects of caffeic acid and chlorogenic acid on COX-2 expression were mediated by transcriptional regulation, we investigated the effects of caffeic acid or chlorogenic acid on UVB-induced COX-2 promoter activity. Data from the luciferase assay revealed that exposure to 0.5 kJ/m^2 UVB significantly induced COX-2 promoter activity, and the induction was inhibited by caffeic acid or chlorogenic acid in a dose-dependent manner (Figure 2A). Eukaryotic transcription factors such as AP-1 and NF-κB are reportedly involved in UVB-induced COX-2 expression and

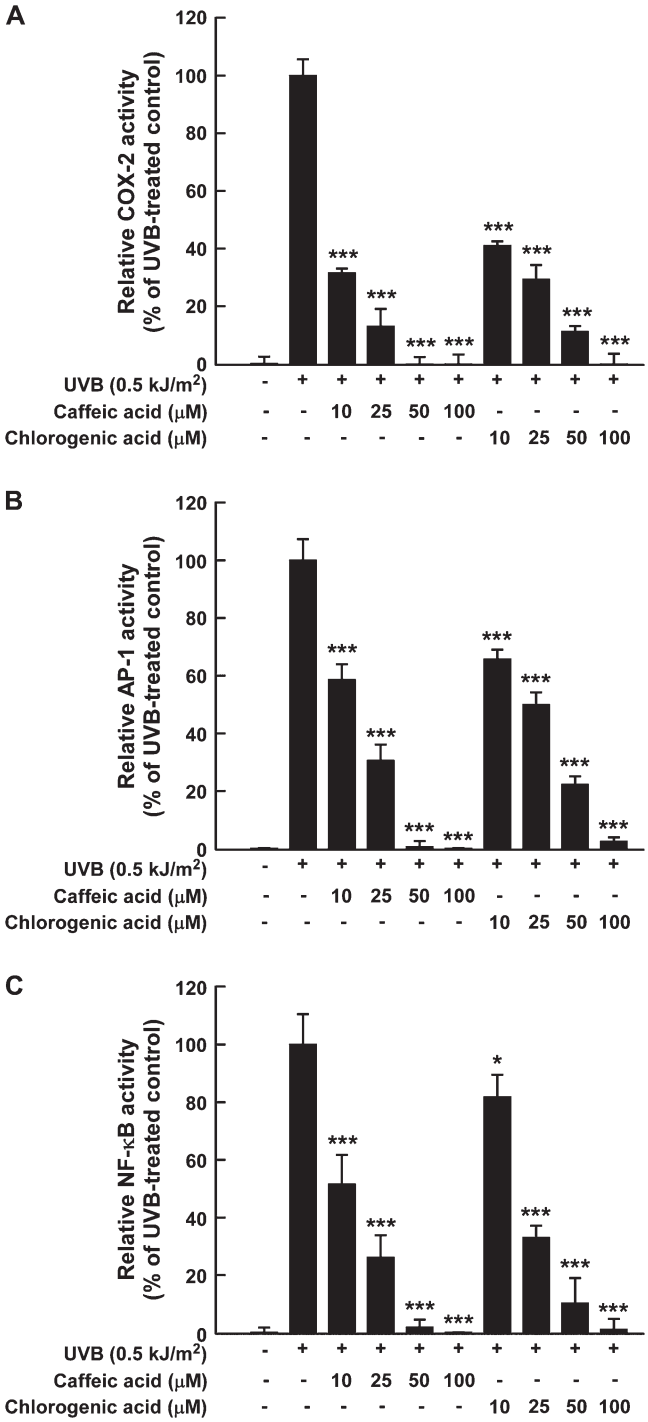

Fig. 2. Effect of caffeic acid or chlorogenic acid on UVB-induced COX-2 promoter activity, and AP-1 or NF-κB transactivation in JB6 P+ cells. Caffeic acid is more effective than chlorogenic acid at suppressing UVB-induced COX-2 promoter activity (**A**), AP-1 (**B**) or NF-κB (**C**) transactivation. JB6 P+ cells, which were stably transfected with COX-2, AP-1 or NF-κB luciferase reporter plasmids, were treated with caffeic acid or chlorogenic acid at the indicated concentration (0, 10, 25, 50 or 100 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 24 h later (i.e. when determining COX-2 activity) or 12 h later (i.e. when determining AP-1 or NF-κB activity). Relative activities were determined using a luciferase assay as described in Materials and Methods. Data are presented as means ± SDs values of the COX-2, AP-1 or NF-κB luciferase activity from three independent experiments. Asterisks indicate significant inhibition of luciferase activity by caffeic acid or chlorogenic acid compared with the group treated with UVB alone ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

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skin carcinogenesis (25). To elucidate the mechanism underlying the transcriptional regulation of the *cox-2* gene, we further measured AP-1 and NF-κB transactivation using JB6 P+ cells stably transfected with an AP-1 or NF-κB luciferase reporter plasmid. Either caffeic acid or chlorogenic acid inhibited UVB-induced transactivation of AP-1 (Figure 2B) and NF-κB (Figure 2C) in a dose-dependent manner, but the inhibitory effect of caffeic acid was greater than that of chlorogenic acid with treatments at the same concentration. Collectively, these results indicated that the suppression of UVB-induced COX-2 expression at the transcriptional level by the inhibition of AP-1 and NF-κB activities was stronger for caffeic acid than for chlorogenic acid.

UVB-induced phosphorylation of JNK, p38 and ERK in JB6 P+ cells is inhibited more strongly by caffeic acid than by chlorogenic acid

UVB-induced AP-1 and NF-κB activations are mediated by the activation of MAPK-signaling pathways including ERK, JNK and p38 (26). To elucidate the manner by which caffeic acid or chlorogenic acid modulate AP-1 and NF-κB activities, we examined the effects of each substance on UVB-induced phosphorylation of MAPKs. Exposing JB6 P+ cells to 0.5 kJ/m^2 UVB markedly induced the phosphorylation of JNK, p38 and ERK, and this induction was suppressed by caffeic acid or chlorogenic acid (Figure 3A). Similar to the other observations, this inhibitory effect was stronger for caffeic acid than for chlorogenic acid.

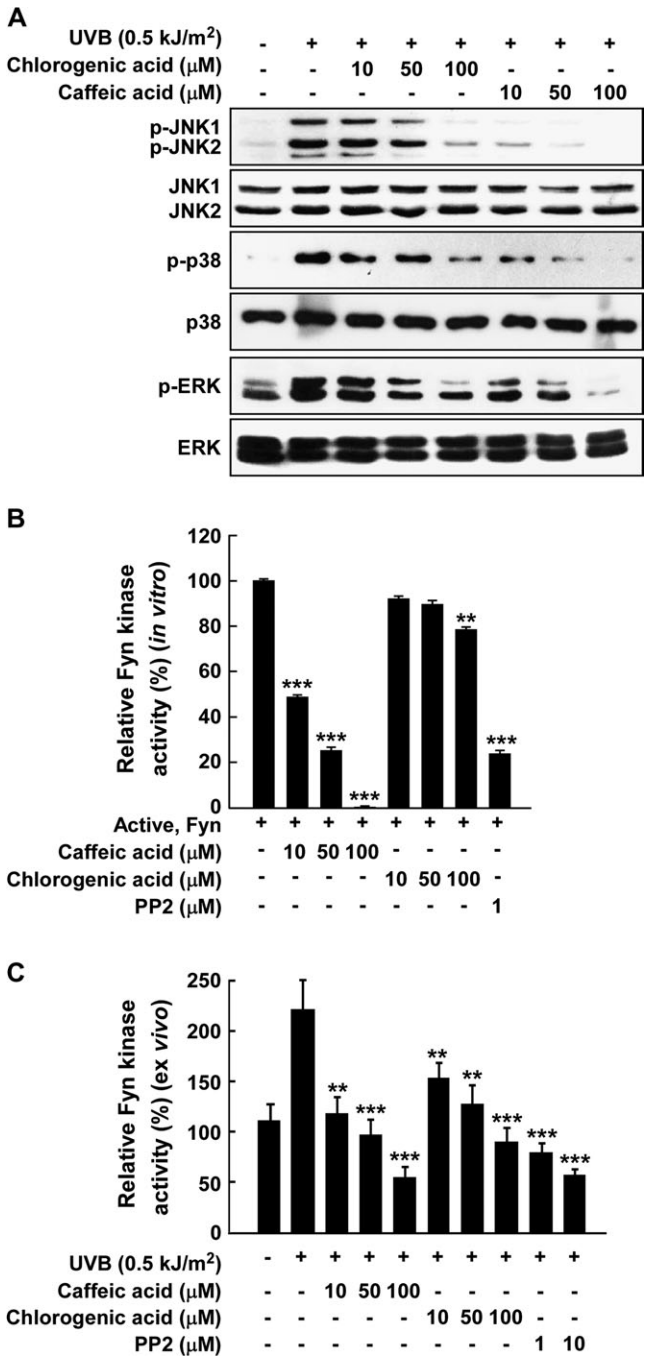

Fig. 3. Effect of caffeic acid or chlorogenic acid on UVB-induced phosphorylation of MAPKs and on the activation of Fyn. (**A**) UVB-induced phosphorylation of JNK, p38 MAPK or ERK is inhibited more strongly by caffeic acid than by chlorogenic acid at the same relative concentration. JB6 P+ cells were treated with caffeic acid or chlorogenic acid at the indicated concentration (0, 10, 50 or 100 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 15 min later. The cells were disrupted and the levels of phosphorylated and total JNK, p38 and ERK proteins were measured by western blot as described in Materials and Methods using specific antibodies against each protein. Data are representative of two independent experiments that gave similar results. (**B**) Caffeic acid is more effective than chlorogenic acid at directly suppressing Fyn kinase activity. A direct Fyn kinase assay was performed as described in Materials and Methods, and the effect of caffeic acid or chlorogenic acid is expressed as the percent inhibition relative to the activity of the Fyn protein alone. (**C**) Caffeic acid is more effective than chlorogenic acid at suppressing UVB-induced Fyn kinase activity. JB6 P+ cells were treated with caffeic acid or chlorogenic acid at the indicated concentration (0, 10, 50 or 100 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 15 min later. The cells were disrupted, and Fyn kinase activity was measured as described in Materials and Methods. The effect of caffeic acid or chlorogenic acid is expressed as the percent inhibition relative to the group treated with UVB alone. Data in (B) and (C) are shown as means ± SDs values as determined from three independent experiments. Asterisks indicate significant inhibition of Fyn kinase activity by caffeic acid or chlorogenic acid compared with the active Fyn only group (A) or the group treated with UVB (B) only ($^{**}P < 0.01$ and $^{***}P < 0.001$).

Caffeic acid is more effective than chlorogenic acid at inhibiting Fyn kinase activity

Src family kinases including Fyn transmit extracellular signals in response to various stimuli, such as growth factors and oxidative stresses, to intracellular signaling proteins (27–29). To elucidate the mechanism by which caffeic acid and chlorogenic acid inhibit UVB-induced MAPK activation, we determined the effects of each compound on the activation of Fyn as a possible upstream kinase of MAPKs. The results of a direct Fyn kinase assay indicated that caffeic acid strongly suppressed Fyn kinase activity in a dose-dependent manner, whereas chlorogenic acid slightly suppressed this activity and only when applied at a concentration of 100 μM (Figure 3B). Similar to the result of the direct kinase assay, the inhibition of UVB-induced Fyn kinase activity in JB6 P+ cells was stronger for caffeic acid (Figure 3C). Additionally, to confirm whether caffeic acid can affect the other kinases, we also examined the effects of caffeic acid on the kinase activities of ERK2, JNK1 and p38, but caffeic acid had no effect on these kinase activities (data not shown). Collectively, these results indicated that the strong downregulation of UVB-induced COX-2 signaling by caffeic acid was attributable to the suppression of Fyn kinase activity and the subsequent inhibition of downstream effectors such as MAPKs, AP-1 and NF-κB.

Fyn is involved in UVB-induced COX-2 upregulation by modulating the activation of MAPK pathways

To confirm whether the inhibition of Fyn kinase activity suppresses UVB-induced COX-2 upregulation in JB6 P+ cells, we examined the role of Fyn as an upstream kinase modulating COX-2 expression and promoter activity. Treatment with PP2 or leflunomide, which are commercial pharmacological inhibitors of Fyn kinase activity, suppressed UVB-induced COX-2 expression and promoter activity (Figure 4A and B) and UVB-induced phosphorylation of JNK, p38 and ERK (Figure 4C). These results indicated that Fyn is required for

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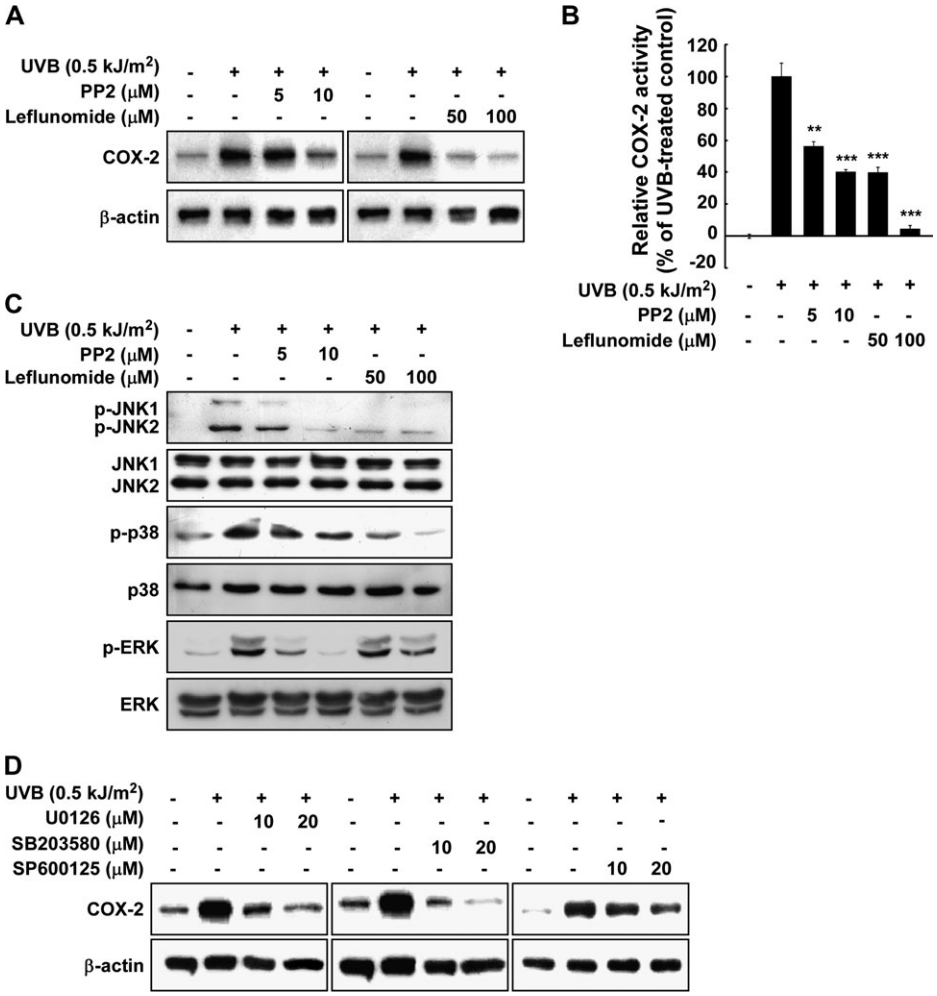

Fig. 4. Involvement of Fyn as an upstream kinase of MAPKs in mediating UVB-induced COX-2 upregulation. (**A**) PP2 or leflunomide inhibits UVB-induced COX-2 expression in JB6 P+ cells. JB6 P+ cells were treated with PP2 (0, 5 or 10 μM) or leflunomide (0, 50 or 100 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 4 h later. The cells were disrupted and the level of the COX-2 protein was determined by western blot analysis as described in Materials and Methods. (**B**) PP2 or leflunomide suppresses UVB-induced COX-2 promoter activity in JB6 P+ cells. JB6 P+ cells stably transfected with a COX-2 luciferase reporter plasmid were treated with PP2 (0, 5 or 10 μM) or leflunomide (0, 50 or 100 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 24 h later. Relative activity was measured using a luciferase assay as described in Materials and Methods. Data were presented as means ± SDs values of the COX-2 luciferase activity from three independent experiments. Asterisks indicate significant inhibition of luciferase activity by PP2 or leflunomide compared with the group treated with UVB only ($^{**}P < 0.01$ and $^{***}P < 0.001$). (**C**) PP2 or leflunomide suppresses UVB-induced phosphorylation of JNK, p38 and ERK in JB6 P+ cells. JB6 P+ cells were treated with PP2 (0, 5 or 10 μM) or leflunomide (0, 50 or 100 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 15 min later. The cells were lysed and then the levels of phosphorylated and total JNK, p38 and ERK proteins were determined by western blot as described in Materials and Methods using specific antibodies against each protein. (**D**) U0126, SB203580 or SP600125 inhibits UVB-induced COX-2 expression in JB6 P+ cells. JB6 P+ cells were treated with U0126, SB203580 or SP600125 at the indicated concentrations (0, 10 or 20 μM) for 1 h before being exposed to 0.5 kJ/m^2 UVB and harvested 4 h later. The cells were disrupted, and the level of the COX-2 protein was determined by western blot analysis as described in Materials and Methods. Data in (A), (C) and (D) are representative of two independent experiments that gave similar results.

the UVB-induced COX-2 upregulation and acts by modulating MAPK pathways in JB6 P+ cells. To further determine whether the activation of MAPK pathways increases COX-2 expression in JB6 P+ cells exposed to UVB, the cells were treated with pharmacological MAPK inhibitors before being exposed to UVB. U0126 (a MEK inhibitor), SB203580 (a p38 inhibitor) or SP600125 (a JNK inhibitor) significantly suppressed UVB-induced COX-2 expression in JB6 P+ cells (Figure 4D), which indicated that MAPKs are involved in UVB-induced COX-2 expression.

Caffeic acid binds with Fyn non-competitively with ATP

To elucidate the mechanism by which caffeic acid and chlorogenic acid modulate Fyn kinase activity, we determined whether each compound binds directly with Fyn. Pull-down assays revealed that the active Fyn protein directly bound with caffeic acid-conjugated Sepharose 4B beads (Figure 5A, lane 3), but not with unconjugated Sepharose 4B beads (Figure 5A, lane 2). The input lane (Figure 5A, lane 1) revealed that only 50 ng of an active Fyn protein was loaded as a marker, verifying that the detected band represented the Fyn protein. In contrast, chlorogenic acid bound only weakly with Fyn (Figure 5A, lane 4). Cell-based pull-down assays also revealed that the binding with UVB-induced Fyn in JB6 P+ cells was stronger for caffeic acid than for chlorogenic acid (Figure 5B). Furthermore, the ability of caffeic acid to bind with Fyn did not vary with the level of ATP (Figure 5C) implying that caffeic acid inhibits Fyn non-competitively with ATP.

Caffeic acid suppresses UVB-induced COX-2 expression through the inhibition of Fyn kinase activity in vivo

We next investigated the effect of caffeic acid on UVB-induced COX-2 expression and Fyn kinase activation using mouse skin. Western blot analysis revealed that topical pretreatment with caffeic acid

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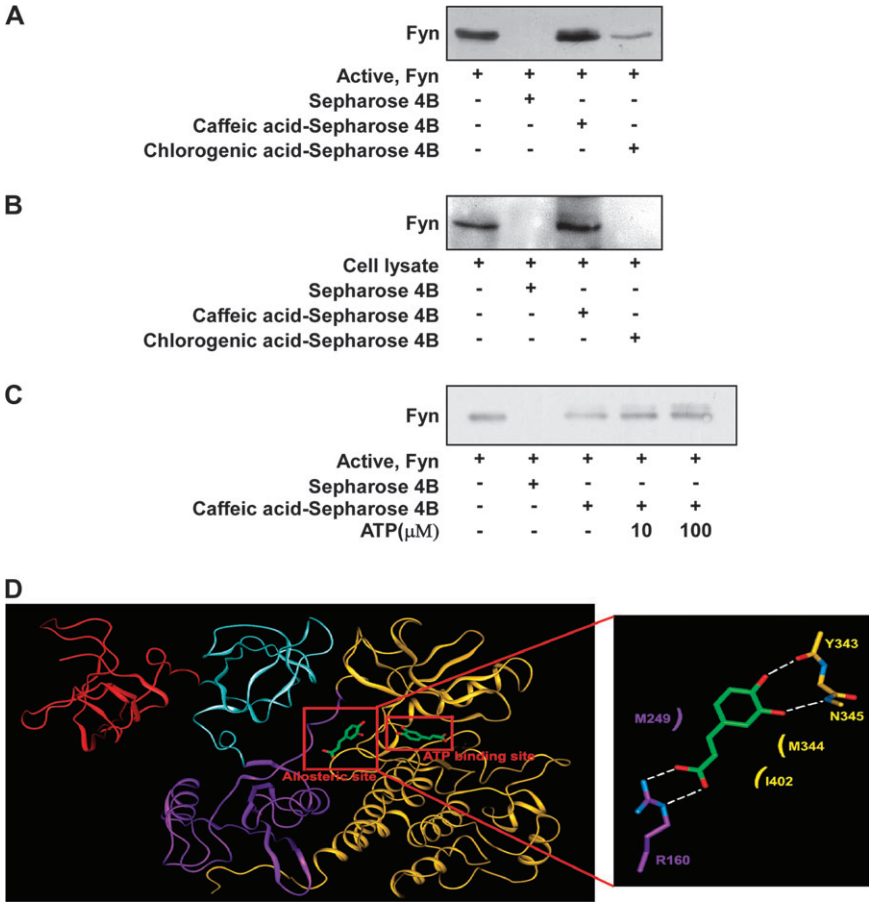

Fig. 5. Direct binding of caffeic acid with Fyn. (**A**) Caffeic acid, but not chlorogenic acid, binds directly with the Fyn protein. The Fyn–caffeic acid and Fyn–chlorogenic acid binding were confirmed by immunoblotting using an antibody against Fyn: lane 1 (input control)—Fyn protein standard; lane 2 (control)—Sepharose 4B was used to pull down Fyn as described in Materials and Methods and lanes 3 and 4—Fyn was pulled down using caffeic acid–Sepharose 4B or chlorogenic acid–Sepharose 4B beads as described in Materials and Methods, respectively. (**B**) Caffeic acid, but not chlorogenic acid, specifically binds with the UVB-activated Fyn protein. The Fyn–caffeic acid or Fyn–chlorogenic acid binding in UVB-exposed JB6 P+ cells was confirmed by immunoblotting using an antibody against Fyn: lane 1 (input control)—whole-cell lysates from JB6 P+ cells; lane 2 (control)—lysates of JB6 P+ cells were precipitated with Sepharose 4B beads as described in Materials and Methods and lanes 3 and 4—whole-cell lysates from JB6 P+ cells were precipitated by caffeic acid–Sepharose 4B or chlorogenic acid–Sepharose 4B beads as described in Materials and Methods, respectively. (**C**) Caffeic acid binds to Fyn non-competitively with ATP. Active Fyn (0.2 μg) was incubated with ATP at the indicated concentration (0, 10 or 100 μM) and 100 μl of caffeic acid–Sepharose 4B or 100 μl of Sepharose 4B (as a negative control) in a reaction buffer to a final volume of 500 μl. The pulled-down proteins were detected by western blot analysis as described in Materials and Methods: lane 1 (input control)—Fyn protein standard; lane 2 is the negative control, which indicates that Fyn does not bind with Sepharose 4B and lane 3 is the positive control, which indicates that Fyn binds with caffeic acid–Sepharose 4B. Data presented in (A), (B) and (C) are representative of three independent experiments that gave similar results. (**D**) Hypothetical model of the Fyn kinase domain in complex with caffeic acid or chlorogenic acid. Putative caffeic acid-binding sites in the homology model structure of Fyn. Different parts of Fyn are presented in different colors: the N-terminal domain in red, SH3 in cyan, SH2 in violet and the kinase domain in yellow. Caffeic acid (atomic color) binds to both the ATP-binding site in the kinase domain of Fyn and the putative allosteric site between the SH2 domain and the kinase domain. In the close-up view of the inhibitor interaction in the allosteric site, the hydrogen bonds and salt bridge are depicted as dashed lines and the hydrophobic contacts by small curves.

suppressed UVB-induced COX-2 upregulation in mouse dorsal skin (Figure 6A). We also found that caffeic acid inhibited UVB-induced Fyn kinase activity through its direct binding with Fyn in mouse dorsal skin extracts (Figure 6B and C).

Discussion

Caffeic acid is one of the major metabolites produced by the hydrolyzation of chlorogenic acid, a major phenolic phytochemical in various foods, including coffee. Because a large amount of chlorogenic acid is absorbed in the metabolized form, considerable attention has been focused on the biological effects of metabolites such as caffeic acid in order to evaluate possible *in vivo* effects of chlorogenic acid-containing diets. Previous studies have shown that chlorogenic acid or caffeic acid inhibits skin tumor promotion induced by 12-*O*-tetradecanoylphorbol-13-acetate in mouse skin (30). Chlorogenic acid reportedly suppresses the effects of high-dose UVB-induced activation of MAPKs, AP-1 and NF-κB by inducing cellular antioxidant systems such as Nrf2 signaling (31). Also, caffeic acid has been implicated as a protective agent against UVB-induced skin damage (24,32). Although accumulating evidence suggests that caffeic acid and chlorogenic acid have the potential to inhibit skin cancer development, their actual effects and the molecular mechanisms involved in the modulation of COX-2, which is a biomarker of UVB-induced skin damage, have remained unclear. The present study clearly showed that caffeic acid inhibits UVB-induced COX-2 upregulation and PGE_2 production in JB6 P+ cells more strongly than does chlorogenic acid.

The expression of COX-2 is primarily regulated by eukaryotic transcription factors such as NF-κB or AP-1, inhibition of AP-1 and/or NF-κB might lead to the suppression of cell transformation through the blocking of COX-2 expression (33–35). Stimulation of cells with various tumor promoters results in the activation of AP-1

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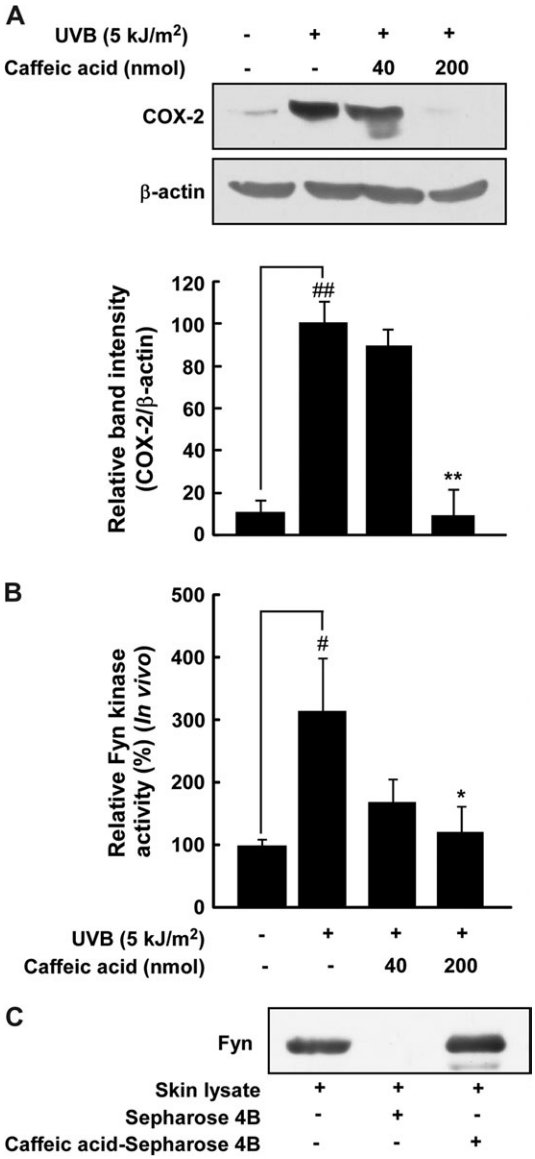

Fig. 6. Effects of caffeic acid on UVB-induced COX-2 expression and Fyn kinase activity in mouse dorsal skin. (**A**) Caffeic acid inhibits UVB-induced COX-2 expression in mouse skin extracts. The levels of COX-2 and β-actin were determined by western blot analysis using specific antibodies against the corresponding COX-2 and β-actin proteins. Each band was quantified by densitometry. Results are shown as means ± SD ($n = 5$). The pound symbols (##) indicate a significant difference ($P < 0.01$) between the control group and the group exposed to UVB only, and the asterisks (**) indicate a significant difference at $P < 0.01$ between groups treated with caffeic acid and irradiated with UVB and the group exposed to UVB alone. (**B**) Caffeic acid inhibits UVB-induced Fyn kinase activity in mouse skin extracts. For the Fyn kinase activity assay, dorsal skin protein lysates were prepared from the epidermis, and the assays were carried out as described in Materials and Methods. Each band was quantified by densitometry. Results are shown as means ± standard errors ($n = 5$). The pound sign (#) indicates a significant difference ($P < 0.05$) between the control group and the group exposed to UVB (5 kJ/m^2) only; the asterisk (*) indicates a significant difference ($P < 0.05$) between groups treated with UVB and caffeic acid and the group exposed to UVB alone. (**C**) Caffeic acid specifically binds with Fyn in mouse skin extracts. The Fyn–caffeic acid binding *in vivo* was confirmed by immunoblotting using an antibody against Fyn; lane 1 (input control)—mouse dorsal skin lysate; lane 2 (control)—a lysate of mouse dorsal skin precipitated with Sepharose 4B beads as described in Materials and Methods; lane 3—mouse dorsal skin lysate precipitated by caffeic acid–Sepharose 4B affinity beads. Each experiment was performed three times.

and/or NF-κB by a series of upstream kinases, including MAPKs. In particular, considerable attention has focused on the involvement of AP-1 and NF-κB in tumor promoter-induced JB6 P+ cell transformation, and the inhibition of AP-1 and NF-κB activity by theaflavin or epigallocatechin gallate has been found to reduce UVB-induced skin carcinogenesis (36,37). TAM67, a dominant-negative *c-Jun* mutant transgene, was also found to suppress UVB-induced AP-1 activation and subsequently inhibit skin tumor development (38). These results imply that JNK, an upstream regulator of c-Jun, plays a role in UVB-induced skin carcinogenesis. JNK reportedly plays a critical role in transmitting responses to UV irradiation, and exposure to UV impairs apoptosis in JNK-deficient murine embryonic fibroblasts (29,39,40). Other studies have found that ERK and p38 are also involved in UVB-induced carcinogenesis. In JB6 cells, ERK activation is required for the induction of AP-1 via cross talk with the ERK pathway after exposure to UV (41), and p38 is reportedly involved in UVB-induced COX-2 expression in HaCaT human keratinocyte cells (42). We found that caffeic acid inhibited UVB-induced COX-2 upregulation at the transcriptional level by suppressing COX-2 promoter activity, which resulted from the inhibition of AP-1 and NF-κB transactivation. This transcriptional regulation of COX-2 by caffeic acid was mediated by blocking JNK, p38 and ERK phosphorylation. These results indicated that caffeic acid is effective at suppressing the UVB-activated-signaling pathway.

Accumulating evidence indicates that inhibition of MAPK phosphorylation and subsequent blocking of AP-1 and NF-κB activity lead to a reduction of neoplastic transformation. For example, cyanidin-3-glucoside inhibits UVB-induced COX-2 expression in JB6 P+ cells by downregulating ERK, p38 and JNK phosphorylation (43). Strawberry and apple-peel extracts also suppress UVB-induced JB6 cell transformation by blocking ERK and JNK phosphorylation (26,44). However, these studies focused on the antioxidant activities of each naturally occurring chemical in reducing UVB-induced oxidative stress, and the possibility of an upstream mediator of MAPK activation has not been investigated. Previous studies showed that the earliest UV response is the activation of Fyn kinase activity, which subsequently transmits a UV-related signal to the nucleus by activating the Ras/Raf pathway (45). Therefore, we investigated whether Fyn is involved in UVB signaling related to COX-2 expression in JB6 P+ cells as an upstream regulator of MAPK pathways. In this study, the use of pharmacological Fyn inhibitors (PP2 or leflunomide) confirmed that Fyn kinase activity is required for the induction of UVB-induced COX-2 expression by acting as an upstream regulator of MAPKs. Furthermore, caffeic acid directly inhibited Fyn kinase activity as well as UVB-induced Fyn kinase activity in JB6 P+ cells, and this inhibition was much more potent than that exerted by chlorogenic acid. On the other hand, caffeic acid did not affect ERK2, JNK1 or p38 kinase activities *in vitro* (data not shown). Together, these results indicated that the direct inhibition of Fyn kinase activity contributes to the inhibitory effect of caffeic acid on UVB-induced COX-2 expression by blocking MAPK phosphorylation.

Considerable attention has recently focused on the possibility that polyphenols act as kinase inhibitors, contributing to their biological effects in preventing disease. Polyphenols are small molecules with structures similar to those of other pharmacological kinase inhibitors, and they can bind with specific kinases to suppress their activities. For example, equol (a metabolite of soy isoflavone daidzein) and quercetin (a major flavonol in red wine) reportedly bind with MEK1 and subsequently inhibit its activity (46,47). A few studies have determined whether natural compounds can act as Fyn inhibitors. Rosmarinic acid and epigallocatechin 3-gallate, which both have a higher affinity to the non-ATP-binding site of Fyn than to the ATP-binding pocket, were reported to be possible inhibitors of Fyn (17,48). Similarly, the present study showed that caffeic acid binds with Fyn non-competitively with ATP.

To investigate the molecular basis of Fyn inhibition by caffeic acid or chlorogenic acid, we carried out a modeling study using the homology model structure of the full-length Fyn. Fyn comprises four domains: an N-terminal membrane-anchoring domain, a SH3

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domain, a SH2 domain and a catalytic kinase domain. The kinase domain of the enzyme consists of an N-lobe and a C-lobe. The N- and C-lobes are linked through a loop, which is referred to as the 'hinge region', and the ATP-binding site is flanked by these two lobes. The backbone of this loop interacts with the adenine moiety of ATP by hydrogen bonding. We docked caffeic acid to the ATP-binding site and the putative allosteric site, where rosmarinic acid binds in the homology model structure of Fyn (48). Considering the experimental result showing that caffeic acid binds to Fyn non-competitively with ATP, we suggest that the major binding site of caffeic acid would be the putative allosteric site and not the ATP-binding site. Because of the structural similarity between caffeic acid and rosmarinic acid, caffeic acid can be easily docked to the putative allosteric site in a manner similar to rosmarinic acid. Caffeic acid can make a salt bridge with Arg160 of the SH2 domain and forms hydrogen bonds with the backbone atoms of Tyr343 and Asn345 in the hinge region of the kinase domain (Figure 6D). In addition, some hydrophobic interaction with Met249, I402 and M344 would also be possible. The interaction between the SH2 domain and caffeic acid could have some influence on protein–protein interaction through the SH2 domain. The interaction with the hinge region could also induce some structural distortion of the ATP-binding site, and thus reduce the binding affinity of ATP and the catalytic activity of the kinase domain allosterically. On the other hand, chlorogenic acid cannot be docked to the site due to its additional quinic acid moiety and a greater structural change of Fyn would be necessary for binding with chlorogenic acid on the allosteric site. This docking result is consistent with the experimental result showing that chlorogenic acid only weakly bound with Fyn. Future investigations using X-ray crystallography to determine the structure of the inhibitor complex would elucidate the exact binding modes of these compounds with Fyn.

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