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Inhibition of β-Catenin/Tcf Signaling by Flavonoids

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ABSTRACT

Functional activation of β-catenin/T-cell factor (Tcf) signaling has been implicated in human carcinogenesis. We identified the inhibitory effect of various polyphenolic flavonoid compounds against β-catenin/Tcf signaling in β-catenin-activated cells. Genistein, kaempferol, isorhamnentin, and baicalein inhibited the transcriptional activity of β-catenin/Tcf in HEK293 cells transiently transfected with a constitutively active mutant β-catenin gene. To investigate the inhibitory mechanism, electrophoresis mobility shift assay, immunoprecipitation, and Western blot experiments were performed. The shift assay showed that the binding of Tcf complexes with its specific DNA-binding sites was suppressed by four kinds of flavonoids. Immunoprecipitation analysis also showed that the binding of β-catenin to Tcf-4 was also disrupted by these flavonoids. Western blot analysis showed a decreased level of β-catenin in nucleus caused by genistein. Genistein also decreased phosphorylation of Akt and GSK3β. Taken together, these results suggest that the polyphenolic flavonoids genistein, kaempferol, isorhamnentin, and baicalein are negative regulators of β-catenin/Tcf signaling and their inhibitory mechanism is related to the decreased binding of β-catenin/Tcf complexes to consensus DNA. J. Cell. Biochem. 110: 1376–1385, 2010.

KEY WORDS: β-CATENIN/Tcf SIGNALING; INHIBITOR; GENISTEIN; KAEMPFEROL; ISORHAMNENTIN; BAICALEIN

β-Catenin contributes to cell–cell adhesion in cooperation with the cytoplasmic domain of E-cadherin [Mareel et al., 1997]. Also, β-catenin possesses transcriptional activity in cooperation with T-cell factor (Tcf)/lymphoid enhancer factor (Lef) transcription factor in the nucleus [Bullions and Levine, 1998]. Cytosolic β-catenin is phosphorylated by Axin-adenomatous polyposis coli (APC)–glycogen synthase kinase (GSK)-3β complex and recognized by beta-transducin repeat-containing protein (β-TrCP), an F-box component of the E3 ubiquitin ligase complex that promotes ubiquitination of β-catenin and increases its susceptibility to degradation by an ubiquitin–proteasome system [Rubinfeld et al., 1996]. In normal cells, β-catenin in the cytoplasm is rapidly degraded by the proteasome [Aberle et al., 1997]. However, mutational inactivation of the APC gene or β-catenin mutations at regulatory amino-terminal serine residues stabilizes cytosolic β-catenin protein by blocking degradation. Also, activated disheveled (Dsh) by Wnt stimulation blocks the ability of GSK-3β to phosphorylate β-catenin [Aberle et al., 1997]. When the failure of this degradation in cells occurs, β-catenin accumulates in the cytoplasm and enters the nucleus to transactivate the Tcf/Lef transcription factor leading to upregulation of many target genes responsible for cell proliferation [Rubinfeld et al., 1996]. Some of the genes activated by β-catenin/Tcf signaling include c-jun, c-myc, fibronectin, cyclin D1, and fra-1 [He et al., 1998; Mann et al., 1999; Tetsu and McCormick, 1999].

Activated β-catenin/Tcf signaling by the accumulation of β-catenin in the nucleus has been implicated in human carcinogenesis including colorectal cancer (CRC), melanoma, hepatocellular carcinoma, and gastric carcinoma [Morin et al., 1997; Fujie et al., 2001; Woo et al., 2001]. At least 60% of sporadic CRC contain one APC mutation and almost half show abnormalities in both APC alleles [Powell et al., 1992]. Studies have reported the detection of APC mutations in 12 of 46 gastric cancers, with β-catenin nuclear localization occurring in both diffuse- and intestinal-type gastric cancers at a higher rate [Nakatsuru et al., 1992; Clements et al., 2002]. This means that the dysregulation of β-catenin plays a crucial

Abbreviations used: APC, axin-adenomatous polyposis coli; BCA, bicinchoninic acid; CBP, CREB-binding protein; CRC, colorectal cancer; DMSO, dimethyl sulfoxide; Dsh, disheveled; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK, glycogen sythase kinase; IC_{50}, half maximal inhibitory concentration; Lef, lymphoid enhancer factor; NF-κB, nuclear factor-κB; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TBE, Tris base boric acid EDTA; TBS, Tris-buffered saline; TBST, Tris-buffered saline and Tween-20; Tcf, T-cell factor.

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role in some cancer cells. Therefore, we hypothesized that reduced β-catenin/Tcf transcriptional activity may lead to suppressed tumor growth in β-catenin activated type of cancer.

Genistein is one of several known isoflavones, which are found in a number of plants such as soybeans and soy products like tofu. Besides functioning as antioxidants, many isoflavones have been shown to interact with animal and human estrogen receptors, causing effects in the body similar to those caused by the hormone estrogen. The polyphenols, kaempferol, and isorhamnetin are presumably responsible for various health benefits of broccoli. Baicalein (5,6,7-trihydroxyflavone) is a flavonoid originally isolated from the roots of *Scutellaria baicalensis*. This flavonoid inhibits certain types of lipoxygenases [Deschamps et al., 2006] and acts as an anti-inflammatory agent [Hsieh et al., 2007]. Although flavonoid compounds distributed widely in plant-based foods exert diverse biological effects in cultured cells and in vivo, the molecular mechanisms underlying these effects are generally unknown. In this report, we investigated the inhibitory effect of flavonoids using SW480 and HEK293 cells transiently transfected with a constitutively active mutant β-catenin gene and reporter gene constructs containing either an optimized (TOPflash) or mutant (FOPflash) Tcf-binding element. We demonstrate here that the flavonoids, genistein, kaempferol, isorhamnentin, and baicalein act as inhibitors of β-catenin/Tcf signaling. The mechanism of reduced β-catenin/Tcf transcriptional activity for genistein, kaempferol, isorhamnentin, and baicalein is due to the decreased binding of β-catenin/Tcf complexes with consensus DNA. Moreover, decreased distribution of β-catenin in nucleus caused by decreased phosphorylation of Akt and GSK3β is partial mechanism of reduced β-catenin/Tcf transcriptional activity by genistein.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENTS

The HEK293 and SW480 cell lines were purchased from Korean Cell Line Bank (Seoul, Korea). Genistein, kaempferol, isorhamnentin, and baicalein were purchased from Sigma–Aldrich (St. Louis, MO) and dissolved in dimethylsulfoxide (DMSO) prior to use. TOPflash and FOPflash were provided by Hans Clevers (Hubrecht Institute, Netherlands) and β-catenin mutant S33Y gene was provided by Eric R. Fearon (University of Michigan Medical School, Ann Arbor).

TRANSFECTION AND LUCIFERASE ASSAY

Transient transfection was performed using Lipofectamine Plus reagent (Invitrogen. Carlsbad, CA). Briefly, 1×10^4 HEK293 cells distributed in the medium were dispensed into wells of a 96-well plate. After 24 h, cells were transfected with 0.10 μg of the TOPflash or FOPflash luciferase reporter constructs, 0.04 μg of pcDNA β-catenin S33Y gene, and 0.08 μg of pGL4.74 (Renilla luciferase) gene for normalization. After 24 h of transfection, inhibitors were added to the medium. Cells were incubated for another 24 h, lysed, and collected for assays of luciferase activity using Dual-GIo™ Luciferase Assay System (Promega, Madison, WI) with a luminescence multi reader (Zenyth 1100; ANTHOS, Austria). TOPflash luciferase activity was normalized to Renilla luciferase activity and expressed as relative value compared with control.

IMMUNOPRECIPITATION

HEK293 cells transfected with β-catenin mutant gene were trypsinized and whole cell protein was obtained by lysing the cells on ice for 20 min in 700 μl of lysis buffer (0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 1% Noniodet P-40 (NP-40), 0.5 M phenylmethylsulphonyl fluoride (PMSF), 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). The lysates were then sonicated for 20 s and centrifuged at 15,000*g* for 10 min, and the supernatant was saved. Protein concentration was determined using a Bicinchonic acid (BCA) kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA). Cell lysates (150 μg) were incubated with 2 μl primary antibody (anti-Tcf-4 antibody; Abcam, Cambridge, MA) overnight at 4°C. Then, 15 μl Protein A/G Plus (GenDEPOT, Barker, TX) was added and the complex was incubated for 4 h at 4°C. The pellet was washed three times with lysis buffer. The immunoprecipitated complexes were released with 2× sample buffer for Western blot analysis.

ISOLATION OF NUCLEAR EXTRACTS

Nuclear extracts were prepared using a nuclear extraction kit (Panomics, Fremont, CA) according to the manufacturer's instructions. HEK293 cells transfected with β-catenin mutant gene were treated with lysis buffer (Panomics) for 10 min at room temperature with vigorous mixing and then scraping. After centrifugation, the pellet was resuspended in extraction buffer (Panomics) and incubated on ice for 1 h. Nuclear extracts were used as described in the following two sections.

ELECTROPHORESIS MOBILITY SHIFT ASSAY

EMSA was performed using a gel shift kit according to the manufacturer's protocol (Panomics). Nuclear extract (4 μg) was incubated for 5 min at room temperature with poly dI-dC (1 μg), 5× binding buffer, and, if needed, inhibitors. Then, biotin-labeled β-catenin/Tcf binding probe (5′-CTTTGATCTTACC-3′) was added and incubated for 30 min at 15°C in a thermal cycler. The samples were analyzed by non-denaturing 6% polyacrylamide gel electrophoresis (PAGE) in 0.5× TBE buffer at 120 V for 55 min. Separated proteins were transferred to Biodyne B nylon membrane (Pall Life Science, Port Washington, NY) for 1 at 300 mA. Target proteins were visualized by autoradiography using streptavidin-horse radish peroxidase and substrate solution.

TOTAL CELL LYSATE EXTRACTION AND WESTERN BLOT ANALYSES

Cells (1×10^6) were harvested and suspended with 1.0 ml of ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 ng/ml PMSF, 0.03% aprotinin, and 1 μM sodium orthovanadate) and incubated on ice for 30 min. The protein content of the final extracts was estimated using the BCA kit according to the manufacturer's protocol (Bio-Rad). Total cell lysates were used for measuring phosphorylation of Akt and GSK-3β. Anti-phospho-Akt (Ser 473) antibody (Cell Signaling Technology, Beverly, MA) and anti-phospho-GSK-3β (Ser 9) antibody (Cell Signaling Technology) were used. Nuclear extracts were used to measure β-catenin level. The extracts were subjected to 12% sodium dodecyl sulfate (SDS)–PAGE. The separated proteins were transferred to a

nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After transfer, the membrane was saturated by incubation, at 4°C for 1 h with 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS)–0.1% Tween-20 (TBST) and then incubated with antibody overnight at 4°C. After washing with TBST, the membrane was incubated with an anti-rabbit immunoglobulin coupled with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). After 60 min of incubation at room temperature, the membrane was washed three times with TBST and the blots were developed using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). Normalization was performed using tubulin antibody (Santa Cruz Biotechnology).

RNA ISOLATION AND POLYMERASE CHAIN REACTION

SW480 cells and HEK293 cells transfected with β-catenin mutant gene were treated with or without inhibitors for 24 h and total RNA was extracted by homogenization in Trizol reagent (Invitrogen) according to the manufacturer's instructions. Synthesis of cDNA was performed as previously described [Yang et al., 2003]. Briefly, 50 μg of total RNA was reverse-transcribed to double stranded cDNA using oligo(dT) primers (iNtRON, Gyeonggi-Do, Korea) and reverse transcriptase (iNtRON). For real-time Polymerase Chain Reaction (PCR), 40 ng of cDNA was mixed with 5 μM of each primer in IQ SYBR Green Super-Mix (Bio-Rad). Real-time PCR was performed using a CFX 96 Real-time system (Bio-Rad) with the following thermal profile: 95°C, 3 min; (95°C, 10 s; 56°C, 10 s; 72°C, 20 s) × 39 cycles; 95°C, 10 s; 65°C, 20 min; 95°C, 15 s. All amplifications were run in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the endogenous control. The conditions were optimized to show single peaks in the melting curve. ΔC_T was computed by subtracting the C_T (the number of cycles to reach the threshold) for GAPDH from the C_T value for each gene. The expression level for each gene expressed in units of GAPDH was then taken as $2^{-\Delta C_T}$. These units were normalized so that the GAPDH mRNA level was 10,000. Primer pairs were designed for c-myc, cyclin D1, axin 2, and GAPDH. The information for each probe was obtained from the Stanford Online Universal Resource for Clones and ESTs (SOURCE; http://www.source.stanford.edu), which compiles information from several publicly accessible databases including UniGene, dbEST, Swiss-Prot, GeneMap99, RHdb, GeneCards, and LocusLink. The sequences of primers (Bionics, Korea) used in the real-time PCR were as follows:

GAPDH: 5′-CGTGCCGCCTGGAGAAACC-3′ and 5′-TGGAAGAGTGGGAGTTGCTGTTG-3′; c-myc: 5′-CTTCTCTCCTTCCTCGGACTC-3′ and 5′-CCTCATCTTCTTGCTCTTCTTCAG-3′ cyclin D1: 5′-TCTTTCCAGAGTCATCAAGTGTG-3′ and 5′-TCCTCCTCAGTGGCCTTG-3′; axin 2: 5′-TCTGTGGAGAAGAAATTCCATACA-3′ and 5′-ATTCGTCACTCGCCTTCTTG-3′.

QUANTITATIVE ANALYSIS

Blots were quantified using a Gel Doc 2000 densitometer (Bio-Rad). In all experiments, statistical significance was calculated for the data from three or four independent experiments using one-way ANOVA or Student's *t*-test (Sigma Plot, LaJolla, CA). The error bars represent the standard deviations of the mean.

RESULTS

GENISTEIN, KAEMPFEROL, ISORHAMNENTIN, AND BAICALEIN DOWNREGULATE β-CATENIN/Tcf SIGNALING

The structures of genistein, kaempferol, isorhamnentin, and baicalein are shown in Figure 1. These compounds possess a

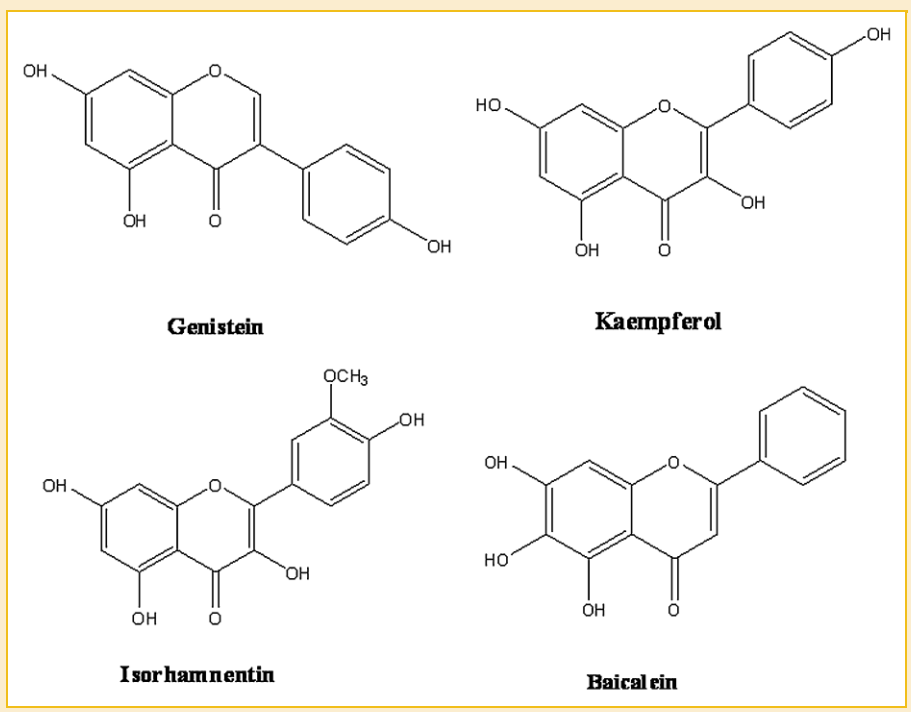

Fig. 1. Structures of genistein, kaempferol, isorhamnentin, and baicalein.

common phenylbenzopyrone structure (C6–C3–C6). But, they are categorized according to the substitution state and direction of the phenyl ring mainly into flavanols (kaempferol and isorhamnentin), flavone (baicalein), and isoflavone (genistein). To investigate whether genistein, kaempferol, isorhamnentin, and baicalein modulated β-catenin/Tcf signaling, we used HEK293 cells transiently transfected with constitutively activated mutant β-catenin gene. Cells were also transiently transfected with the reporter plasmids containing either an optimal Tcf binding site (TOPflash) or mutant binding site (FOPflash) and Renilla luciferase (pGL4.74) to normalize for transfection efficiency. HEK293 cells have low transcriptional activity of β-catenin/Tcf for the low endogenous amounts of β-catenin protein. However, when they were cotransfected with β-catenin mutant gene, the Tcf signaling luciferase activity was elevated about 10-fold. Inhibitors were treated with successively increasing doses for 24 h. Figure 2A shows that genistein, kaempferol, isorhamnentin, and baicalein suppressed β-catenin/Tcf transcriptional activity in these cells in a concentration-dependent manner. Baicalein, genistein, kaempferol, and isorhamnentin (each 0.5 μM) inhibited β-catenin/Tcf signaling to 48–59%, 45–53%, 45–55%, and 38–40%, respectively, compared with the control. To confirm the inhibitory effects of flavonoid compounds on β-catenin/Tcf transcriptional activity in cancer cells, we also performed luciferase assay with SW480 colon cancer cell line, which has inactivating mutation of the APC gene and shows a constitutively active transcriptional activity of β-catenin/Tcf. Genistein, kaempferol, isorhamnentin, and baicalein also suppressed β-catenin/Tcf transcriptional activity in SW480 cells in a concentration-dependent manner (Fig. 2B).

Tcf COMPLEX BINDING TO DNA IS DECREASED BY FLAVONOIDS

The direct binding of transcription factor with DNA is an important step for factor activity. Therefore, we performed EMSA to investigate whether the binding of Tcf complexes to DNA is decreased by inhibitor treatment in cells. Nuclear extracts of mutant β-catenin gene-transfected cells were prepared and incubated with β-catenin/Tcf binding consensus DNA and inhibitors in vitro. Because nuclear extract was prepared without inhibitor treatment and the inhibitors were added during incubation in EMSA, the effect of inhibition was likely to be ascribed, not to an indirect effect to diminish β-catenin/Tcf–DNA complex formation, but to a direct blocking of complex formation. When excess unlabeled Tcf-binding oligonucleotide was competitive with labeled Tcf-binding oligonucleotide, the retarded bands disappeared (Fig. 3A). This result suggested that the shifted DNA protein complex was specific for Tcf complexes binding to the consensus DNA. As shown in Figure 3B, baicalein, genistein, kaempferol, and isorhamnentin showed a direct inhibitory effect against the β-catenin/Tcf–DNA complex formation in an in vitro assay with an IC_{50} value of 0.2, 10, 5, and 5, respectively. Baicalein (which has a flavone structure) exhibited an IC_{50} value of 0.2 μM in an in vitro assay, showing a more potent inhibitory effect on the formation of the β-catenin/Tcf–DNA complex than flavanols and isoflavone. The change of the phenyl ring into a benzene ring lacking a hydroxyl group caused a marked increase in the inhibitory activity. The IC_{50} value of 0.2 μM was identical to that obtained in the luciferase activity assay shown in Figure 2, which was determined in a cell-based system. This result was consistent with the notion that the inhibition of β-catenin/Tcf signaling in the cell-based luciferase activity assay was totally due to the direct interference of interaction between dimer and DNA in case of baicalein. Meanwhile, in case of genistein, kaempferol, and isorhamnentin (IC_{50} of 0.2, 0.5, and 0.2 μM, respectively, in the cell-based luciferase activity assay), inhibition of β-catenin/Tcf signaling was likely partially due to the interference of direct interaction between dimer and DNA. To demonstrate the specificity of inhibitors for the interaction of Tcf and its recognition site, AP-1 transcription factor and the corresponding DNA probe was used as control. Our inhibitors did not suppress Jun/Fos-DNA complex (Fig. 3C).

FLAVONOIDS DIMINISH THE NUCLEAR ASSOCIATION OF β-CATENIN WITH Tcf-4

To examine the possibility that the suppressed association of β-catenin with Tcf-4 leads to suppressed β-catenin/Tcf signaling, Tcf-4 and β-catenin were coimmunoprecipitated using an anti-Tcf-4 antibody and blotted with anti-β-catenin antibody. HEK293 cells transfected with β-catenin mutant genes were treated with inhibitors, and nuclei were isolated. Figure 4 shows that inhibitors suppressed the association of β-catenin with Tcf-4 in concentration-dependent manners.

GENISTEIN-MEDIATED INHIBITION OF β-CATENIN/Tcf SIGNALING IS RELATED TO β-CATENIN UPSTREAM COMPONENTS

Tcf signaling activation results from accumulation of nuclear β-catenin [Rubinfeld et al., 1996]. We examined the possibility that the decreased formation of the β-catenin/Tcf complex and the suppressed binding to DNA result from the decreased distribution of nuclear β-catenin products by inhibitors. Western blot analysis was performed to determine the change in the amount of β-catenin in nuclear fractions. Compared with control, genistein decreased β-catenin levels in nucleus (Fig. 5). Reductions into 26.3% and 16.6% in nuclear β-catenin levels were observed with 5 and 10 μM of genistein for 24 h, respectively. Baicalein, kaempferol, and isorhamnentin showed only marginal inhibition in nuclear β-catenin levels at 10 μM. However, the amount of β-catenin protein in the nucleus altered by 5 μM each of baicalein, kaempferol, and isorhamnentin showed no significant difference compared with control. To investigate whether genistein, kaempferol, isorhamnentin, and baicalein inhibited β-catenin/Tcf signaling via the upstream β-catenin degradation machinery in a cell, phosphorylation levels of Akt and GSK-3β were assessed using Western blot. Phosphorylated Akt (p-Akt) inhibits GSK-3β and dephosphorylation, and activation of GSK-3β enhances phosphorylation of β-catenin, which is known to facilitate ubiquitylation and proteasome-mediated degradation of β-catenin [Aberle et al., 1997]. Presently, phosphorylation of Akt and GSK-3β was inhibited only by genistein in a dose-dependent manner when compared with control (Figs. 6 and 7).

TARGET GENE OF β-CATENIN, c-Myc, AXIN 2, AND CYCLIN D1

c-Myc, axin 2, and cyclin D1 are β-catenin target genes [He et al., 1998; Tetsu and McCormick, 1999; Mann et al., 1999]. Therefore, we

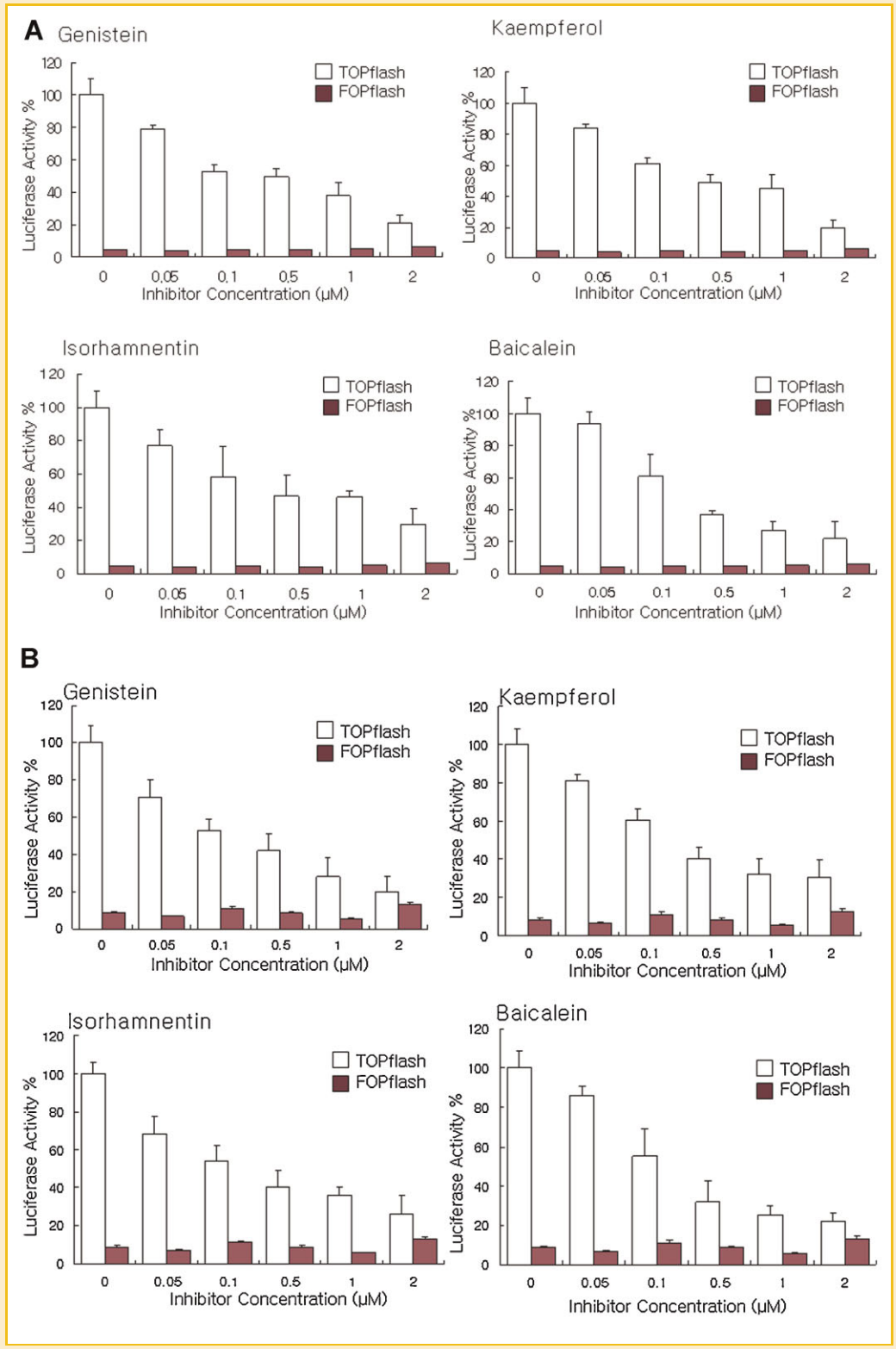

Fig. 2. Inhibition of β-catenin/Tcf signaling by genistein, kaempferol, isorhamnentin, and baicalein in HEK293 cells transfected with constitutively active mutant β-catenin gene (A) and in SW480 cells (B). HEK293 cells were cotransfected with β-catenin mutant gene, Renilla gene and reporter genes harboring Tcf-4 binding sites (TOPflash) or a mutant Tcf-binding site (FOPflash), respectively. Twenty-four hours after transfection, increasing amounts of flavonoids as indicated were added to the cells. Luciferase activity was determined after 24 h of treatment and normalized against values for the corresponding renilla activity. An equivalent volume of DMSO substituted for flavonoid was used as a vehicle control. Values represent means ± SD of four independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

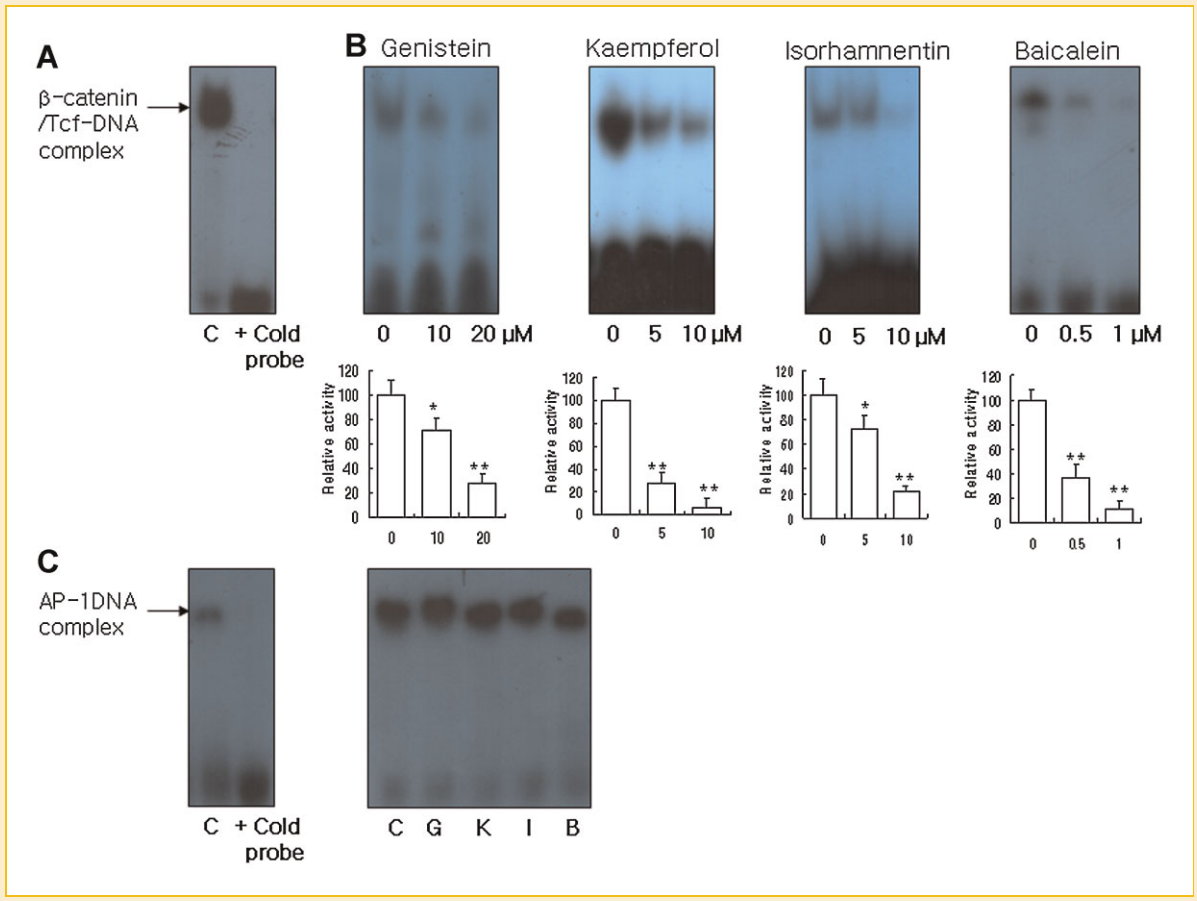

Fig. 3. Effect of genistein, kaempferol, isorhamnentin, and baicalein on the binding of Tcf complexes with DNA. HEK293 cells were transfected with constitutively active mutant β-catenin and nuclear extracts were isolated. EMSA was performed with 4 μg nuclear extracts with or without inhibitors. A: Lane 2 shows that 10-fold excess of unlabeled Tcf-binding oligonucleotide used as a competitor inhibits the binding of the bcatenin/Tcf to oligonucleotide containing Tcf-binding region. B: The lanes are the results of binding to a labeled Tcf-binding region. C: The lanes are the results of binding to a labeled AP-1 region (C: control, G: genistein, K: kaempferol, I: isorhamnentin, and B: baicalein). Quantitative analysis was performed using densitometry and results are expressed as relative activity to untreated control. Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t*-test as compared to the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

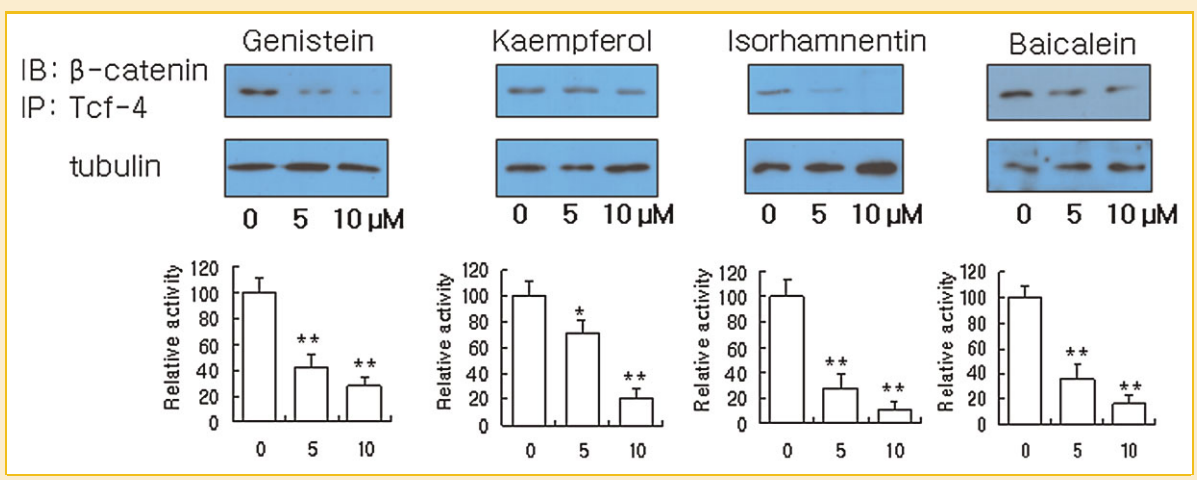

Fig. 4. The association of β-catenin with Tcf-4 is decreased by inhibitors. Genistein, kaempferol, isorhamnentin, and baicalein (5 and 10 μM) was treated in HEK293 cells for 24 h. Then, nuclear extracts were prepared and immunoprecipitation was performed with Tcf-4 antibody followed by immunoblotting with β-catenin monoclonal antibody. Tubulin was used as a control. Quantitative analysis was performed using densitometry and results were expressed as relative activity to untreated control. Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t*-test as compared to the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

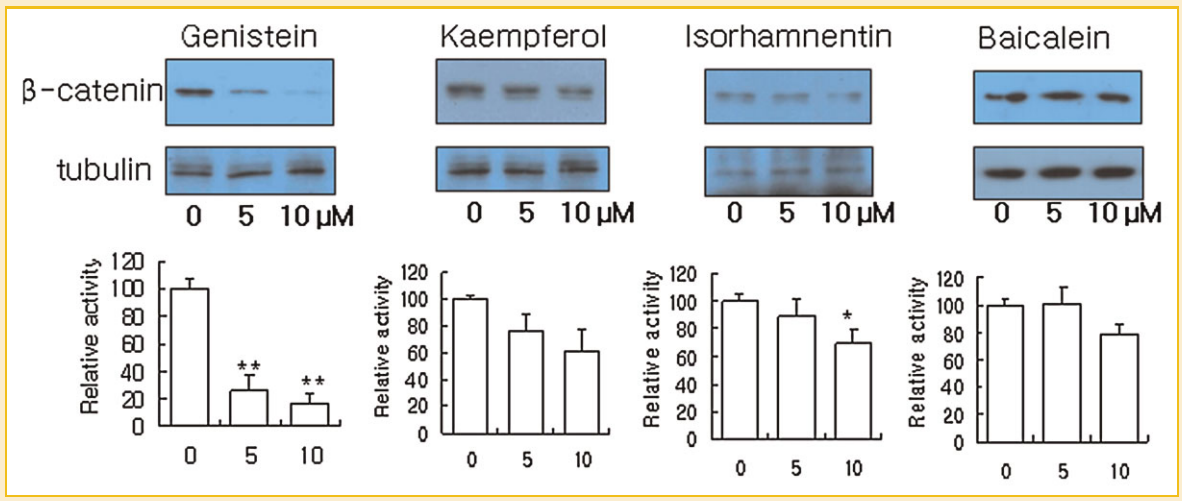

Fig. 5. β-catenin distribution in nucleus is decreased by genistein. Cells were treated with Genistein, kaempferol, isorhamnentin, and baicalein (5 and 10 μM) for 24 h and were trypsinized for preparing nucleus extracts. To demonstrate equivalent loading of the lines, anti-tubulin was used. Each cell fraction was used for Western blot with anti β-catenin antibody. All pictures are representative of three independent experiments. Quantitative analysis was performed using densitometry and results are expressed as relative activity to untreated control. Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t*-test as compared to the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

investigated whether genistein, kaempferol, isorhamnentin, and baicalein would downregulate the c-myc, axin 2, and cyclin D1 gene transcription by inhibiting β-catenin/Tcf signaling. In both SW480 cells and HEK293 cells transfected with β-catenin, c-myc, axin 2, and cyclin D1 were downregulated by 5 μM of genistein, kaempferol, isorhamnentin, and baicalein (Fig. 8). These results indicate that the functioning of β-catenin as a transcription activator is rendered inoperative by inhibitors.

DISCUSSION

Elevated β-catenin/Tcf signaling is intimately related to a number of malignancies, such as colon cancer. Mutations in the regulatory region of β-catenin or loss of APC function have been identified in human colon cancers [Morin et al., 1997; Korinek et al., 1997]. Activation of an abnormal APC/β-catenin/Tcf signaling pathway and alterations in cellular adhesion mediated through changes in

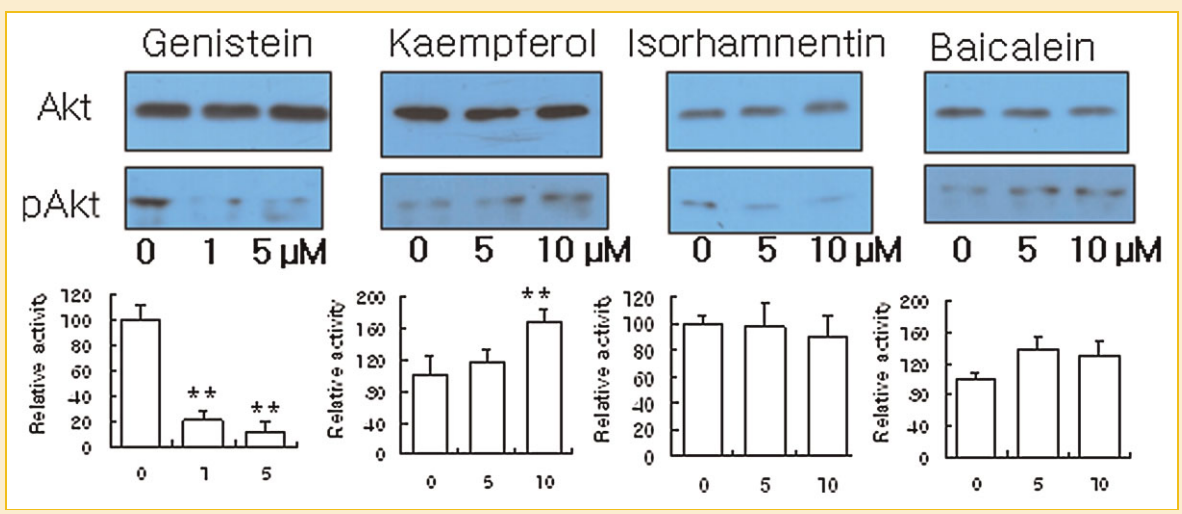

Fig. 6. Effect of genistein, kaempferol, isorhamnentin, and baicalein on phosphorylation of Akt in HEK293 cells transfected with constitutively active mutant β-catenin. HEK293 cells were transfected with β-catenin mutant gene for 24 h and then treated with genistein, kaempferol, isorhamnentin, and baicalein at indicated concentrations for 1 h. Total and phosphorylated levels of Akt were measured by Western blot analysis. Quantitative analysis was performed using densitometry and results were expressed as relative activity to untreated control. Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t*-test as compared to the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

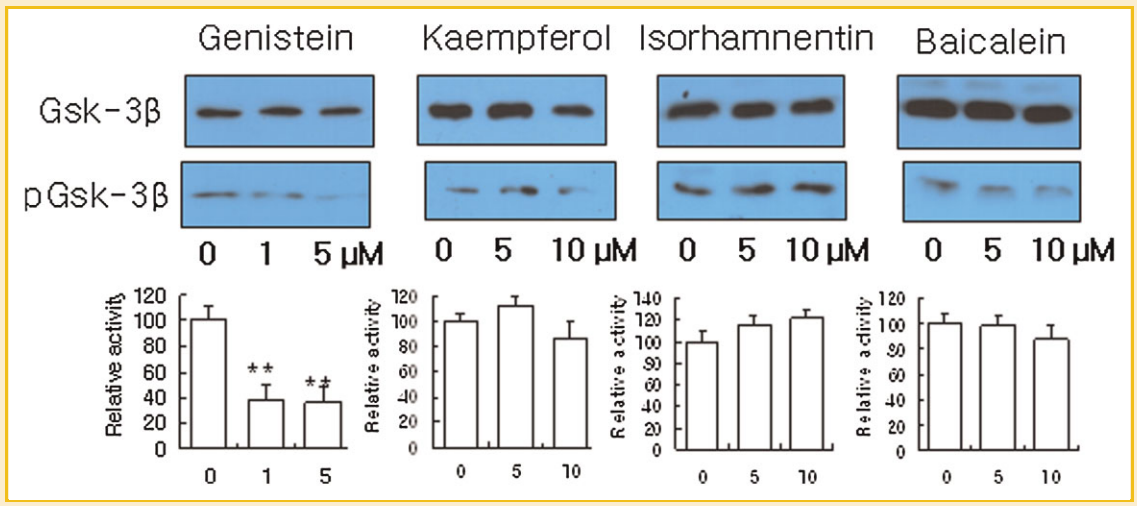

Fig. 7. Effect of genistein, kaempferol, isorhamnentin, and baicalein on phosphorylation of GSK-3β in HEK293 cells transfected with constitutively active mutant β-catenin. HEK293 cells were transfected with β-catenin mutant gene for 24 h and then treated with genistein, kaempferol, isorhamnentin, and baicalein at indicated concentrations for 1 h. Total and phosphorylated levels of GSK-3β were measured by Western blot analysis. Quantitative analysis was performed using densitometry and results are expressed as relative activity to untreated control. Results are shown as mean ± SD of three independent experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t*-test as compared to the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

β-catenin homeostasis within the colonic epithelium are initiating factors in the development of the majority of CRC [Morin et al., 1997].

Various studies on the inhibitory agent against β-catenin/Tcf signaling in cancer cell lines have been performed. Non-steroidal anti-inflammatory drugs [Dihlmann et al., 2001], nitric oxide-generating aspirin [Nath et al., 2003], curcumin [Jaiswal et al., 2002], Gleevec [Zhou et al., 2003], and epigallocatechin-3-gallate [Dashwood et al., 2002] were reported to inhibit β-catenin/Tcf signaling. Recently, it was suggested that a combination of tea plus sulindac shows effective inhibitory effect on intestinal neoplasia in mice via direct or indirect effects on the β-catenin/APC pathway [Orner et al., 2003]. We have previously reported the inhibitory effects of curcumin, curcumin analogs, quercetin, and naringenin against β-catenin/Tcf signaling [Lee et al., 2005; Park et al., 2005a,b]. As the importance of β-catenin as a cause of tumorigenesis increases, many more studies on β-catenin inhibitors and their inhibitory mechanisms are being conducted [Clapper et al., 2004]. In our previous reports, two kinds of flavonoids such as qercetin and naringenin inhibited β-catenin/Tcf transcription activity [Park et al., 2005a; Lee et al., 2005]. Therefore, we presently investigated the effect of other kinds of flavonoids on β-catenin/Tcf signaling. The results presented here demonstrate that four kinds of flavonoids including genistein, kaempferol, isorhamnentin, and baicalein inhibit β-catenin/Tcf transcription activity.

Flavonoids possess a variety of biological activities at non-toxic concentrations in organisms. The role of dietary flavonoids in cancer prevention has been widely discussed [Kuo, 1997; Gerritsen, 1998; Plaumann et al., 1996], as have the various mechanisms by which the flavonoids may affect tumorigenesis [Kuntz et al., 1999], including anti-oxidant activities [Bohm et al., 1998], scavenging effect on activated mutagens and carcinogens [Williamson et al., 1998]. However, the concrete and inclusive molecular mechanisms are not completely suggested. Genistein was known to inhibit Wnt-1-induced mammary epithelial proliferation and attenuate β-catenin signaling through promoting formation of the E-cadherin–β-catenin cell adhesion complex [Su and Simmen, 2009].

Our experiment using luciferase reporter gene constructs containing either an optimized (TOPflash) or mutant (FOPflash) Tcf-binding element showed that β-catenin/Tcf-driven transcription was suppressed strongly by genistein, kaempferol, isorhamnentin, and baicalein in both SW480 cancer cells and HEK293 cells transiently transfected with constitutively active mutant β-catenin gene.

Next, we focused on its inhibitory mechanism. We conclude that the inhibitory mechanism of kaempferol, isorhamnentin, and baicalein is related to the upstream regulators of the β-catenin/Tcf pathway other than GSK-3b and Akt. Meanwhile, genistein affected the upstream components of the β-catenin/Tcf pathway by suppression of GSK-3β and Akt phosphorylation.

Therefore, the changes in the subcellular localization of β-catenin might be responsible for the downregulation of β-catenin/Tcf signaling by genistein, kaempferol, isorhamnentin, and baicalein. The amount of nuclear β-catenin proteins was changed by genistein, kaempferol, isorhamnentin, and baicalein in HEK293 cells transiently transfected with constitutively active mutant β-catenin gene (Fig. 4). To transcribe target genes, transcription factors, including Tcf complexes, must bind to consensus DNA. Thus, we investigated whether inhibitors affect the DNA-binding properties of the Tcf-4 complexes. Figure 3 shows that the binding of Tcf complexes with consensus DNA was blocked by genistein, kaempferol, isorhamnentin, and baicalein. Genistein showed the least potent blocking activity in EMSA while baicalein showed the most potent blocking activity. We suggest two possible inhibitory mechanisms by flavonoids. First, the nuclear localization of β-catenin may be decreased due to the suppression of GSK-3β and upstream Akt or

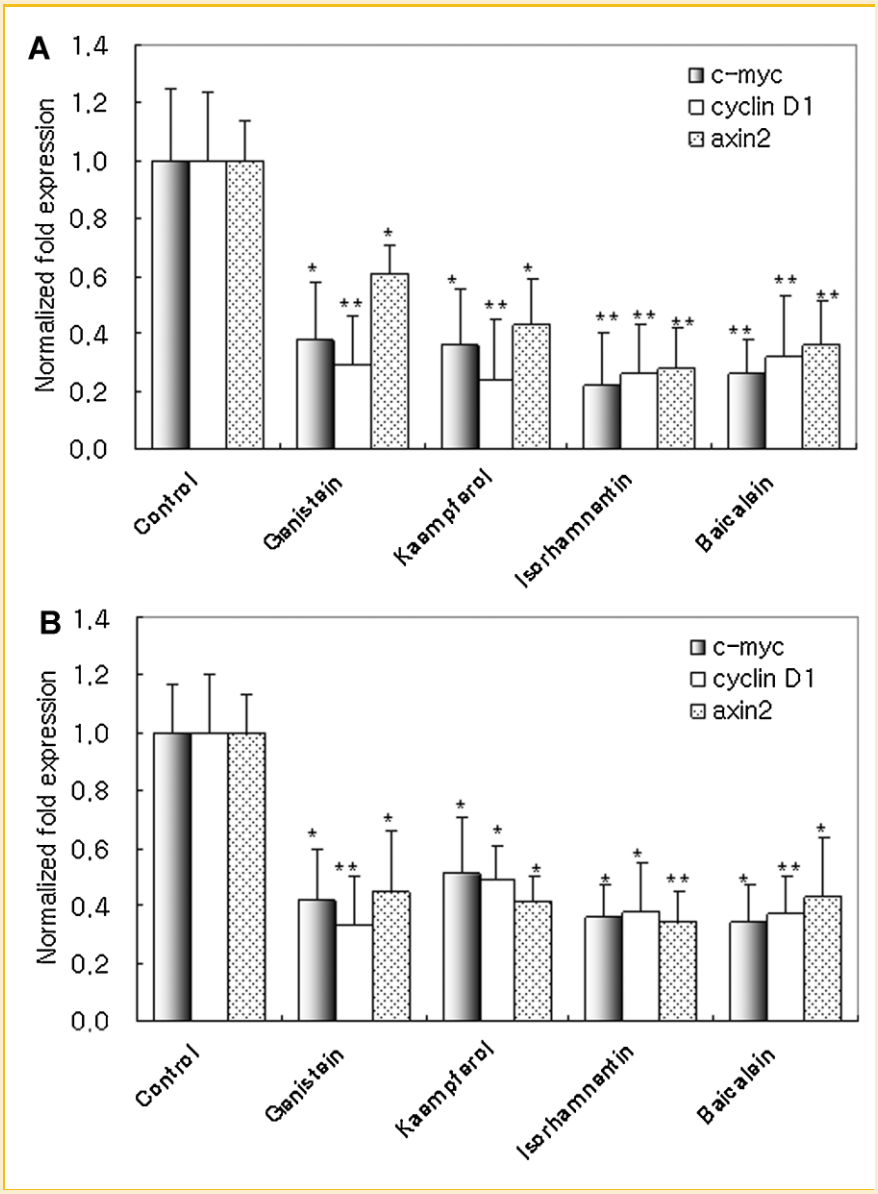

Fig. 8. Genistein, kaempferol, isorhamnentin, and baicalein downregulate the c-myc and cyclin D1 mRNA in HEK293 cells transiently transfected with constitutively active mutant β-catenin gene (A) and SW480 cancer cells (B). Cells were treated with genistein, kaempferol, isorhamnentin, and baicalein (10 μM) for 24 h and were trypsinized to isolate RNA and real time PCR was performed to study the change of amount of mRNA by inhibitors. Results were obtained using triplicate experiments. Asterisks indicate statistically significant difference between treatment and untreated control condition. $^{*}P < 0.01$ by Student's *t*-test as compared to the control.

other upstream regulations by inhibitors. Second, inhibitor directly blocks the complex formation of β-catenin/Tcf with DNA. Genistein, kaempferol, isorhamnentin, and baicalein behavior was consistent with the second mechanism, although with differing potency. The first mechanism might be partially responsible for the inhibitory mechanism of β-catenin/Tcf signaling by genistein, kaempferol, and isorhamnentin.

In conclusion, we have identified β-catenin/Tcf signaling as a target of flavonoid action, albeit the mechanism by which flavonoid inhibits Tcf activity is two-way. Given their function in inhibiting β-catenin/Tcf signaling, flavonoids may be interesting as a chemotherapeutic agent against tumorigenesis.

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