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IGF-I Plus E2 Induces Proliferation via Activation of ROS-Dependent ERKs and JNKs in Human Breast Carcinoma Cells

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Induction of 17β-estradiol (E2) and insulin-like growth factor-I (IGF-I) has been detected in breast carcinoma, however the interaction between E2 and IGF-I in the proliferation of breast carcinoma cells is still unclear. In the present study, we found that IGF-I enhances the E2-induced proliferation in MCF-7 human breast carcinoma cells in accordance with stimulation of colony formation via a soft agar assay. Activation of insulin receptor substrate-1 (IRS-1) protein and extracellular signal-related kinases (ERKs) and c-Jun N-terminal kinases (JNKs), but not p38 mitogen-activated protein kinase (MAPK), via phosphorylation induction was detected in MCF-7 cells treated with IGF-I plus E2 (E2/IGF-I). E2/IGF-I-induced proliferation was blocked by chemical inhibitors of ERKs (PD98059) and JNKs (SP600125). An increase in the expression of c-Jun protein was detected in E2/IGF-I-treated MCF-7 cells, and this was inhibited by PD98059 and SP600125. Transfection of the dominant negative MEKK and JNK plasmids significantly reduced E2/IGF-I-induced proliferation with suppression of c-Jun protein expression. An increase in peroxide production was detected in E2/IGF-I-treated cells, and *N*-acetyl-L-cysteine (NAC) and Tiron (TIR) addition significantly inhibited E2/IGF-I-induced cell proliferation with blocking of the phosphorylation of ERKs and JNKs, and the expression of c-Jun protein. Additionally, 3-OH flavone, baicalein, and quercetin showed effective inhibitory activities against E2/IGF-I-induced proliferation through suppressing proliferative events such as phosphorylation of IRS-1, ERKs, and JNKs proteins, and induction of c-Jun protein and colony formation. These results indicate that IGF-I interacts with E2 to promote the proliferation of breast carcinoma cells via ROS-dependent MAPK activation and c-Jun protein expression. The structure-related inhibition of E2/IGF-I-induced proliferative events by flavonoids is elucidated.

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Both estrogen (E2) and insulin-like growth factor-I (IGF-I) are important mediators of various cellular responses and are associated with the progression of a number of human cancers, notably breast cancer. The mechanisms of E2 are believed to occur through activation of estrogen receptor (ER) transcriptional activity and potentially through non-genomic mechanisms by activation of intracellular signaling pathways (Hall et al., 2001; Keshamouni et al., 2002; Tang et al., 2004). In addition to E2, binding of IGF-I to the IGF-I receptor (IGF-R) leads to dimerization of the receptor, activation of its tyrosine kinase followed by phosphorylation of substrate proteins such as insulin receptor substrate proteins (IRS-1 through -4) and Src-homology collagen (SHC). Phosphorylated IRS-1 and SHC in turn recruit different SH-2-containing proteins to activate specific intracellular signaling pathways such as the MAPK and PI3K cascades, both of which are important for IGF-I-induced responses (Lassarre and Ricort, 2003; Kim et al., 2004). Utilizing ERKO mice demonstrated that ER is necessary for IGF-I induction of uterine proliferation (Klotz et al., 2002), while inhibition of IGF-IR with anti-IGF-IR antibodies or antisense RNA abrogated the effect of E2 on cell growth (Chen et al., 1996; Dupont et al., 2000). Although a possible linkage between E2 and IGF-I has been postulated, the molecular mechanism is still unclear.

Reactive oxygen species (ROS), including hydroxyl radicals (OH), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2), are considered to be important in the formation of cancer and various diseases. Accumulation of excess ROS production and oxidative stress resulting in cell death have been associated with several degenerative diseases such as atherosclerosis, Alzheimer's disease, and Parkinson's disease (Guo et al., 2005; Thiruchelvam et al., 2005; Houstis et al., 2006; Zhu et al., 2006). Recent evidence indicates that low levels of ROS generated by growth factors and cytokines can activate redox-sensitive kinases, leading to gene expressions for various cellular functions. Low exogenous concentrations of H_2O_2 stimulated cell proliferation through activation of PI3K and JNKs in human hepatoma cells (Liu et al., 2002). Basic fibroblast growth factor (bFGF) was found to induce c-fos expression via ROS generation in chondrocytes (Lo and Cruz, 1995).

Abbreviations: E2, 17β-estradiol; IGF-I, insulin-like growth factor-I; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated protein kinase; ER, estrogen receptor; IRS-1, insulin receptor substrate-1; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species.

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Platelet-derived growth factor (PDGF) also induced ROS production and MAPK activation, accompanied by promotion of cell proliferation in lens epithelial cells (Chen et al., 2004a). However, the role of ROS in E2- and IGF-I-induced cell proliferation is still undefined.

Flavonoids are benzo-γ-pyrone derivatives extensively found in plants, foods, and vegetables, and the average intake through the diet is about 1000 mg daily. Flavonoids consist of phenolic and pyrane rings and are divided into six subclasses: flavones, flavanones, flavonols, flavanonols, isoflavones, and anthocyanidins. Several beneficial health effects such as antioxidant, anti-inflammation, and antitumor activities of flavonoids have been identified (Lin et al., 2003; Chen et al., 2004b; Nguyen et al., 2004; Shen et al., 2004a), and are attributed to their capacity to eliminate free radicals (Lozano et al., 2005; Harris et al., 2006; Lu et al., 2006). Flavonoids such as genistein, daidzain, and quercetin are known to exhibit estrogenic and antiestrogenic activities which mimic the effect of estrogen in physiological actions such as antiosteoporosis, protection against cardiovascular and neural degenerative diseases (Hintz and Ren, 2004; Wattel et al., 2004; Mahn et al., 2005). In the present study, we investigated the mechanisms of E2 and IGF-I on the proliferation and transformation of MCF-7 breast carcinoma cells. Structure–activity relationships of flavonoids on E2 and IGF-I-induced proliferation and colony formation are elucidated.

Materials and Methods

Chemicals

All structurally related compounds including flavone, 3-OH flavone, 3-OCH_3 flavone, 5-OH flavone, 5-OCH_3 flavone, 7-OH flavone, 7-OCH_3 flavone, baicalein, baicalin, quercetin, quercitrin, and rutin were obtained from Sigma Chemical (St. Louis, MO) and dissolved in DMSO. 17β-Estradiol, IGF-I, and tamoxifen were purchased from Sigma. ICI182780 was purchased from Tocris Cookson (Ellisville, MO). PD98059 and SP600125 were purchased from Calbiochem (La Jolla, CA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCHF-DA) was obtained from Molecular Probe (Eugene, OR). [Methyl-^{3}H]-thymidine (25 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). Antibodies against ERKs, JNKs, p38, c-Jun, ER and α-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific ERK, JNK, p38 and ERα antibodies were from Cell Signaling Technology (Beverly, MA). Anti-phosphotyrosine antibody (PY20) was obtained from Transduction Laboratories (Lexington, KY).

Cell culture

The MCF-7 human breast carcinoma cell line was obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in modified Eagle's medium (MEM) supplemented with 5% heat-inactivated fetal bovine serum and 100 U penicillin–streptomycin at 37°C in a humidified incubator containing 5% CO_2. All culture reagents were purchased from Life Technologies (Gaithersburg, MD).

Cell proliferation assay

MCF-7 cells were plated at a density of 1×10^4 cells per 24-well plates. Cells were grown arrested in MEM supplemented with 5% dextran-coated charcoal-treated FBS (DCC-FBS; Hyclone) for an additional 24 h and were then subjected to the indicated treatment for 3 days. Cells were trypsinized and then measured using a Z1 Coulter particle counter (Beckman Counter, Hialeah, FL).

[^{3}H]-thymidine incorporation assay

DNA synthesis was measured by [^{3}H]-thymidine incorporation. In brief, 1×10^4 cells/well were grown arrested in MEM containing 5% DCC-FBS for 24 h and then treated with different doses of E2 for a further 3 days. Cells were incubated and labeled with 1 μCi of [^{3}H]-thymidine per well for 4 h and then washed three times with PBS followed by treatment with cold 10% trichloroacetic acid and 1% SDS/0.015N NaOH for 30 min at 4°C to lyse the cells. Cell-bound radioactivity was quantified in a Beckman LS 7000 liquid scintillation counter.

Cellular fractionation

The nucleus and membrane fractions were prepared as described (Ko et al., 2002) with some modifications. Nuclear fraction was washed three times in the isotonic buffer (10 mM Tris–HCl pH 7.4, 0.5% Triton X-100, 0.25 M sucrose, 10 mM $MgCl_2$, 1 mM PMSF) to discard the cytoplasmic contamination, and the membrane and nucleus were lysed in RIPA buffer (150 mM NaCl, 1% TritonX-100, 1 mM sodium fluoride, 1 mM sodium orthovanadate 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The purity of nuclear fraction was assessed by detecting the expression of a nuclear protein PARP via Western blotting.

Western blotting

Cells lysates were prepared by suspending cells in lysis buffer (50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 0.025% sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride), and equal amounts of protein were prepared and separated on 8% SDS-polyacrylamide gels, and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membrane was blocked with 1% bovine serum albumin at room temperature for 1 h and then incubated with specific indicated antibodies for a further 3 h. Expression of protein was visualized by incubating with the colorimetric substrates, nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

Transient cell transfections

The dominant-negative expression vectors of MEKK and JNK, and a control vector (pcDNA3) were transfected into MCF-7 cells using Transfast™ transfection reagent (Promega, Madison, WI) for 6 h. After transfection, cells were serum-starved for a further 24 h and stimulated with E2/IGF-I, and then cells were lysed for analysis.

Measurement of ROS production

Intracellular ROS production was monitored by flow cytometry using an oxidant-sensitive DCHF-DA probe. This dye is a stable compound that rapidly diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped within cells. Hydrogen peroxide and low-molecular-weight peroxide produced by cells oxidize DCHF to the highly fluorescent compound, 2',7'-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by cells. In the present study, MCF-7 cells were incubated with DCHF-DA (100 μM) for 10 min in the dark followed by E2/IGF-I treatment in the presence or absence of NAC for 30 min. After incubation, cells were resuspended in ice-cold PBS and detected using a 525-nm (FL1-H) band-pass filter by FACScan flow cytometry (Becton Dickenson, San Jose, CA).

Soft agar assay

Cells were plated in 1 ml MEM containing 0.35% agarose and 5% DCC-FBS, and then overlaid with 1 ml of 0.7% agarose. Cultures were maintained for 3 weeks and refreshed with MEM supplemented with 5% DCC-FBS and subjected to the indicated treatment twice per week. Colonies were observed and photographed using a light microscope, and the number of colonies in each well was measured. Each value is derived from three independent experiments, and results are expressed as the mean ± SE.

Statistical analyses

Values are expressed as the mean ± SE. The significance of the difference from the respective controls for each experimental test condition was assayed using Student's *t*-test for each paired experiment. A P value of <0.01 or <0.05 was regarded as indicating a significant difference. The measurements are derived from three independent MCF-7 cultures, and there are three replicate wells in each time.

Results

IGF-I enhances E2-induced proliferation and colony formation in MCF-7 cells

Human MCF-7 breast carcinoma cells have been shown to express estrogen receptors (ERs). In the present study, the

addition of E2 significantly induced cell proliferation characterized by increasing cells number and [^{3}H]-thymidine intensity in DNA, and this indicates that E2 at a dose of 10 nM expresses the optimal proliferative effect for study (Fig. 1A and B). Inhibition of E2-induced proliferation by the ER antagonists, tamoxifen (Tam) and ICI182780 (ICI), was detected, and this suggests that E2-induced proliferation occurs through activation of ERs in MCF-7 cells. Interestingly, incubation of MCF-7 cells with IGF-I (10 ng/ml) enhanced the proliferative effect of E2 in MCF-7 cells (Fig. 1D). Data of the soft agar assay showed that the colony number was increased in E2- and IGF-I-treated cells, and IGF-I plus E2 (E2/IGF-I) significantly stimulated colony formation (Fig. 1E). The number of colonies under different treatments was quantitated from three independent experiments, and the number of colonies was significantly increased in E2/IGF-I -treated MCF-7 cells (Fig. 1E; lower part). We further analyze the expression of ERα protein in both membrane and nucleus in the presence or absence of E2/IGF-I treatment by Western blotting. As illustrated in Figure 1F, E2/IGF-I induces an increase in the phosphorylated ERα protein in both nuclear and membrane fractions. No change in the expression of total ERα protein was observed in both fractions with or without E2/IGF-I stimulation. The expression of PARP protein in the nuclear, but not membrane, fraction was detected to verify the purity of nuclear and membrane fractions. These data supported the notion that IGF-I and E2 work together to stimulate the proliferation of breast carcinoma cells, and activation of non-genomic pathway may be involved.

Activation of ERKs and JNKs in E2/IGF-I-treated MCF-7 cells

We first examined if activation of MAPKs is involved in E2/IGF-I-induced proliferation of MCF-7 cells. Data of Figure 2A show that activation of IRS-1, ERKs, and JNKs was detected in E2/IGF-I-treated MCF-7 cells via the induction of protein phosphorylation. No change in the expression of phosphorylated p38 protein was detected. As the same part of the experiment, E2 alone slightly induces the expression of phosphorylated ERKs, but not IRS-1 and JNKs, protein, and IGF-I addition significantly stimulates the level of phosphorylated ERKs, IRS-1, and JNKs- protein in the presence of E2 (Fig. 2B). Incubation of MCF-7 cells with the ERK inhibitor, PD98059, or the JNK inhibitor, SP600125, significantly reduced the proliferation induced by E2/IGF-I (Fig. 2C). Furthermore, PD98059 and SP600125 addition significantly reduced the expression of phosphorylated ERKs and JNKs protein, respectively (Fig. 2D and E). These data indicate that activation of ERKs and JNKs via stimulation of IRS-1 protein

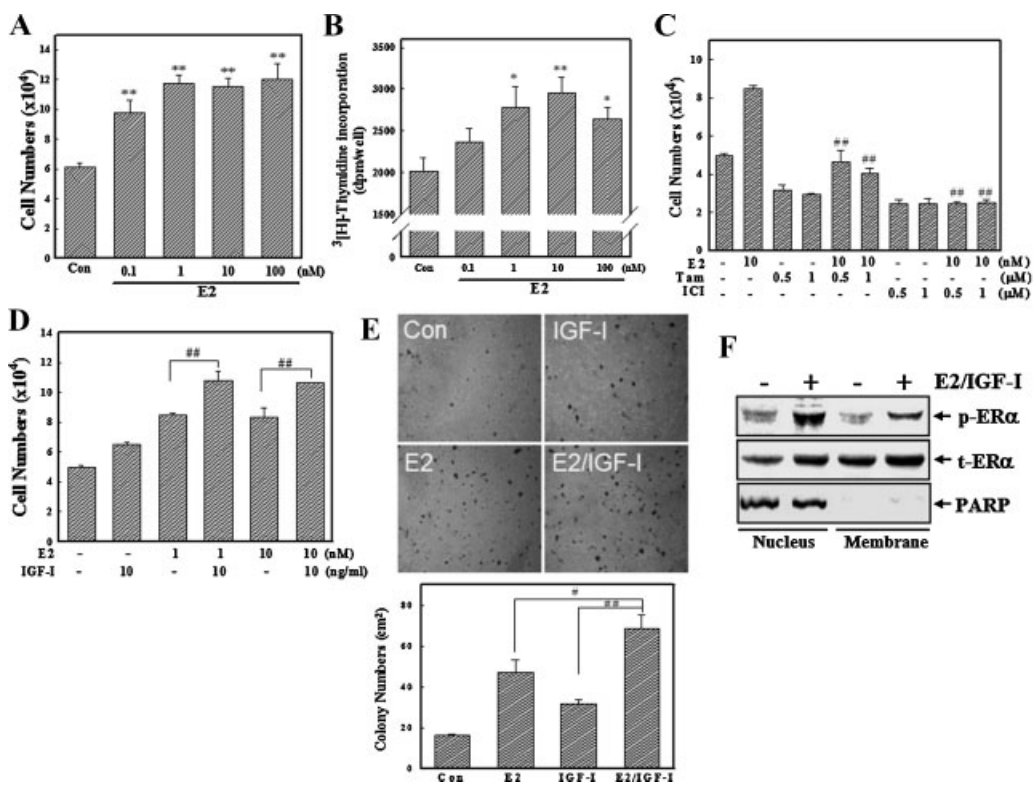

Fig. 1. IGF-I enhancement of E2-induced cell proliferation and colony formation. MCF-7 cells at 1×10^4 cells/well were treated with different concentrations of E2 (0.1, 1, 10, and 100 nM) for 3 days, and the proliferation of cells were determined by a Coulter particle counter (A) and [^{3}H]-thymidine incorporation assay (B) as described in "Materials and Methods". C: Cells were pretreated with the ER antagonist, tamoxifen (Tam; 0.5, 1 μM) or ICI182780 (ICI; 0.5, 1 μM), for 30 min followed by the addition of E2 (10 nM) for a further 3 days, and cell numbers were measured by a Coulter counter analysis. D: Cells were treated with E2 (1 and 10 nM), IGF-I (10 ng/ml), or their combination for 3 days, and cell numbers were measured by a Coulter counter. E: MCF-7 cells were under different treatments in 0.35% agarose containing 5% DCC-FBS overlaid with 0.7% agarose. Cell colonies were observed using a light microscope after 21 days of incubation (left part). The number of colonies was measured under microscopic observation (right part). Values derived from three independent experiments were analyzed, and expressed as the mean ± SE. $^*P < 0.05$, $^{**}P < 0.01$ significantly different from the control group, $^{\#\#}P < 0.01$ significantly different from the E2-treated or indicated groups, as analyzed by Student's t-test. F: MCF-7 cells were treated with E2/IGF-I for 15 min, and the expression of total and phosphorylated ERα and PARP protein in both nuclear and membrane fractions were detected by Western blotting. Data of Western blotting is a representative of three-independent experiments.

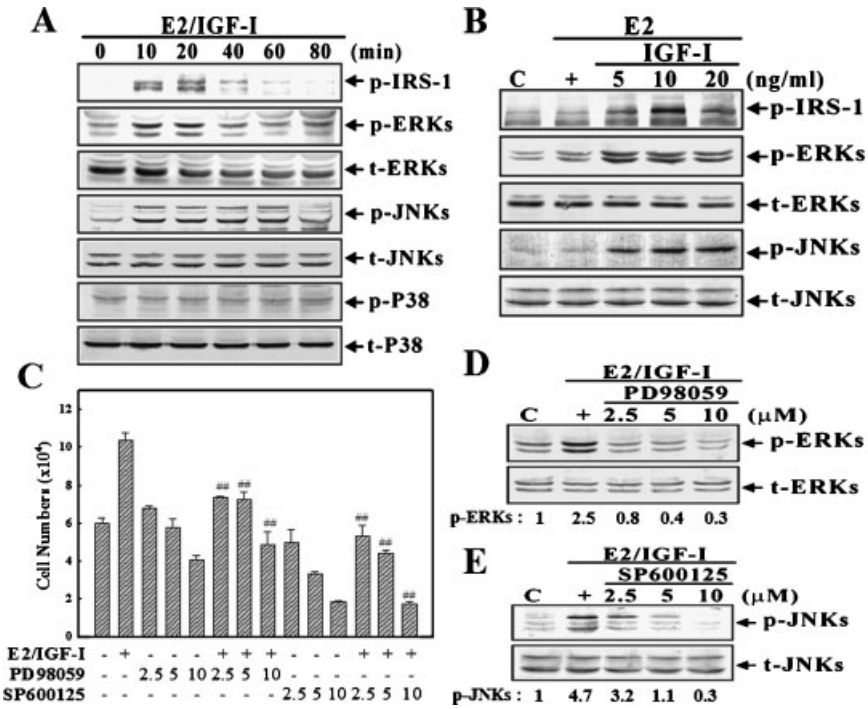

Fig. 2. IGF-I enhancement of E2-induced proliferation via upregulation of IRS-1 and JNK protein phosphorylation. A: MCF-7 cells were serum-starved for 18 h and treated with IGF-I (10 ng/ml) plus E2 (10 nM) (E2/IGF-I) for different time periods, and cell lysates were determined by Western blotting using specific antibodies. B: Cells were treated with E2 (10 nM) alone or combined with IGF-I (5, 10, and 20 ng/ml) for 20 min, and the expression of the indicated protein was detected by Western blotting. C: Cells were treated with E2/IGF-I in the presence or absence of PD (2.5, 5, and 10 μM) or SP (2.5, 5, and 10 μM) for 3 days, and cell numbers were measured by Coulter counter analysis. Values derived from three independent experiments were analyzed, and expressed as the mean ± SE. $^{\#\#}P < 0.01$ significantly different from E2/IGF-I-treated groups, as analyzed by Student's *t*-test. D and E: PD98059 and SP600125 inhibited E2/IGF-I-induced phosphorylation of ERKs, JNKs and cell proliferation. Cells were pretreated with either PD98059 (2.5, 5, and 10 μM) or SP600125 (2.5, 5, and 10 μM) for 30 min followed by the addition of E2/IGF-I for 30 min; cell lysates were determined for phosphorylated and total ERKs (D) and JNKs (E) by Western blotting, respectively. The intensity of each protein was quantitated by densitometric analysis and expressed as folds of control.

phosphorylation participates in E2/IGF-I-induced proliferation in MCF-7 cells.

Induction of c-Jun protein expression is involved in E2/IGF-I-induced proliferation in MCF-7 cells

Both c-Jun and c-Fos genes have been shown to be active in the proliferation of cells, therefore we investigated if induction of c-Jun and c-Fos gene expressions is involved in E2/IGF-I-induced MCF-7 proliferation. A time-dependent increase in c-Jun protein expression was detected in E2/IGF-I-treated MCF-7 cells by Western blotting; however, no change in c-fos protein was found (Fig. 3A). Additionally, E2 alone slightly induced c-Jun protein expression which was enhanced by IGF-I addition in a dose-dependent manner (Fig. 3B). Interestingly, incubation of cells with the respective ERK and JNK inhibitors, PD98059 and SP600125, significantly blocked E2/IGF-I-induced c-Jun protein expression (Fig. 3C). A specific blockage of intracellular ERKs and JNKs via the respective transfection of the dominant negative MEKK (DN-MEKK) or JNK (DN-JNK) plasmid inhibited E2/IGF-I-induced proliferation in accordance with reducing c-Jun protein expression in MCF-7 cells (Fig. 3D). Curcumin has been shown to be an inhibitor of c-Jun gene expression (Han et al., 2002; Park et al., 2005). The addition of curcumin significantly inhibited E2/IGF-I-induced proliferation with a concomitant reduction in c-Jun protein expression (Fig. 3E). These data indicate that induction of c-Jun protein expression, which is located downstream of ERK and JNK activation, is involved in E2/IGF-I-induced proliferation of MCF-7 cells.

E2/IGF-I induced proliferative events through stimulation of ROS production

It is important to elucidate if ROS production is involved in E2/IGF-I-induced proliferation of MCF-7 cells. Data on DCHF-DA via a flow cytometric analysis showed that an increase in intracellular peroxide levels was detected in E2/IGF-I-treated cells, and that this was blocked by the addition of the antioxidant, *N*-acetyl-L-cysteine (NAC) (Fig. 4A). In the same part of the experiment, DCF fluorescent intensity was induced in E2/IGF-I-treated cells, which was attenuated by NAC (Fig. 4B). Reduction of intracellular peroxide level elicited by E2/IGF-I has also been observed in the presence of another chemical antioxidant Tiron (TIR) treatment (data not shown). Induction of peroxide production and DCF fluorescent intensity by H_2O_2 was described as a positive control. Additionally, pre-incubation of MCF-7 cells with the antioxidants, NAC and Tiron (TIR) inhibited E2/IGF-I-induced IRS-1, ERK, and JNK protein phosphorylation and c-Jun protein expression (Fig. 4C). Accordingly, suppression of E2/IGF-I-induced proliferation by NAC and TIR was identified in MCF-7 cells (Fig. 4D) accompanied by the inhibition of cell proliferation (Fig. 4C and D). These data support the notion that stimulation of ROS production may act as an initiator of E2/IGF-I-induced proliferation.

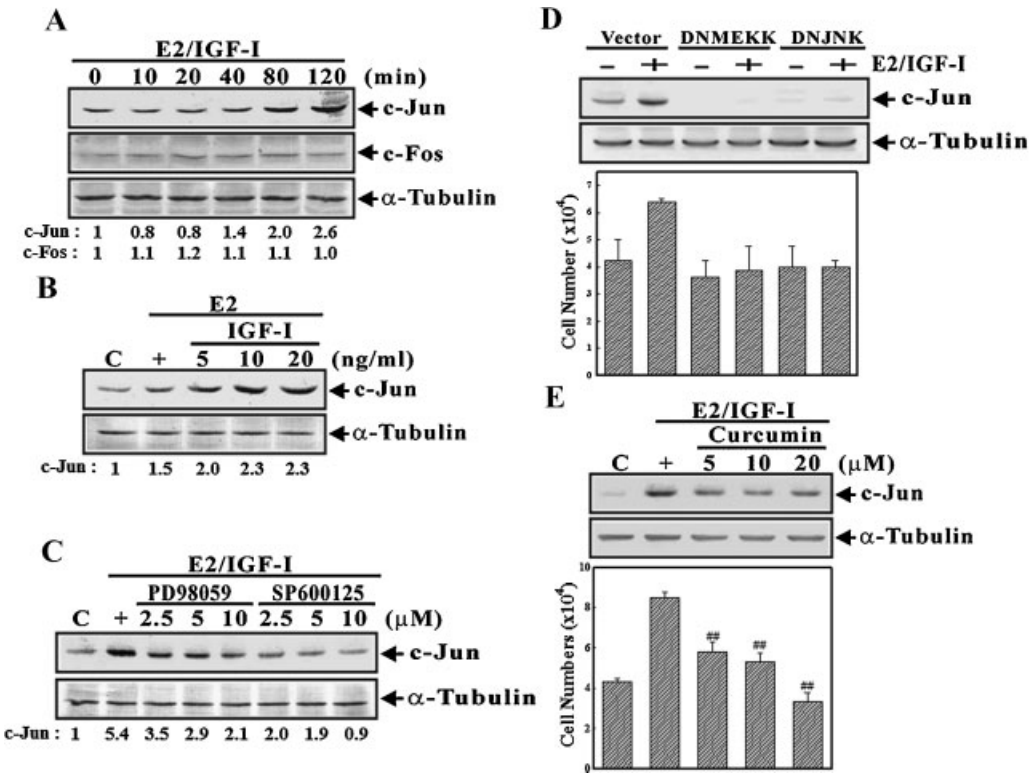

Fig. 3. IGF-I enhancement of E2-induced c-Jun protein expression and cell proliferation is located downstream of the ERKs and JNKs pathway. A: MCF-7 cells were treated with E2/IGF-I for different time intervals, and expression of the indicated proteins was detected by Western blotting. B: Cells were treated with E2 (10 nM) alone or combined with IGF-I (5, 10, and 20 ng/ml) for 120 min, and the expression of the indicated protein was detected by Western blotting. C: Cells were treated with E2/IGF-I in the presence of PD98059 (2.5, 5, and 10 μM) and SP600125 (2.5, 5, and 10 μM) for 120 min, and c-Jun protein in cell lysates was detected by Western blotting. D: Cells were transfected with a dominant negative MEKK (DNMEKK) or JNK (DNJNK) for 16 h as described in "Materials and Methods", respectively, followed by E2/IGF-I treatment for 120 min. The expression of the indicated protein was analyzed by Western blotting (upper part), and cell numbers were detected by Coulter counter analysis (lower part). E: Cells were treated with curcumin (5, 10, and 20 μM) for 30 min followed by the addition of E2/IGF-I for a further 120 min, and the expression of c-Jun protein was detected by Western blotting (upper part), and cell numbers were detected by Coulter counter analysis (lower part). Quantification of the indicated protein under different treatments by densitometric analysis was performed, and expressed as folds of control. Data of Western blotting were carried out at least three times, the results are representative of all of the data.

3-OH flavone is an effective inhibitor of E2/IGF-I-induced proliferation

Our previous studies showed that flavonoids exhibit several important biological properties including apoptosis induction, anti-inflammation, and antioxidant activities. We further examined the effects of flavones on E2/IGF-I-induced proliferation of MCF-7 cells. As illustrated in Fig. 5A, the basic structure of flavone contains two benzene rings (A and C) linked by a heterocyclic pyran ring in the middle. First, native flavone and seven mono-substituted flavones including 2′-OCH_3, 3-OH, 3-OCH_3, 5-OH, 5-OCH_3, 7-OH, and 7-OCH_3 flavones were used in the present study. Among the eight tested compounds, 3-OH flavone expressed the most potent inhibitory effect on E2/IGF-I-induced proliferation, and others also expressed slight but significant inhibitory effects on E2/IGF-I-induced proliferation. The inhibitory percentage of 3-OH flavone on E2/IGF-I-induced proliferation was around 75%. When replacing the 3-OH group with 3-OCH_3 (3-OCH_3 flavone), the inhibitory percentage dropped from 75 to 30% (Fig. 5B). Data of the colony formation assay showed that treatment of MCF-7 cells with 3-OH flavone significantly reduced the number of colonies grown in soft agar stimulated by E2/IGF-I (Fig. 5C). We further analyze the effect of 3-OH flavone on the expression of c-Jun, and phosphorylated IRS-1 and ERKs protein by Western blotting. As illustrated in Fig. 5D, 3-OH, but not 3-OCH_3, flavone slightly reduces the expression of phosphorylated ERKs protein in the condition without E2/IGF-I stimulation (Fig. 5D; left part). In the presence of E2/IGF-I treatment, 3-OH, but not 3-OCH_3, flavone addition significantly inhibits E2/IGF-I-induced the phosphorylation of IRS-1 and ERKs protein in according with attenuating the expression of c-Jun protein (Fig. 5D; right part). Reduction of intracellular peroxide levels produced by E2/IGF-I was identified in 3-OH, but not 3-OCH_3, flavone-treated MCF-7 cells under fluorescent microscopic observation ($n = 3$; Fig. 5E).

Differential inhibitory effects of flavonoids and the flavonoid glycoside on E2/IGF-I-induced proliferation

Our previous studies also suggested that glycoside addition might affect the biological activities of flavonoids; however the role of glycoside on flavonoid inhibition of E2/IGF-I-induced proliferation is still unclear. Two groups of flavones including baicalein (BE) and its glycoside, baicalin (BI; baicalein-7-*O*-glucuronide), and quercetin (QE) and its glycosides, quercitrin (QI; quercetin-3-*O*-rhamnoside) and rutin (RT; quercetin-3-*O*-rutinoside), were used in the present study. Results of Fig. 6A show that incubation of MCF-7 cells with BE and QE, but not their respective glycosides, BI, QI, or RT, significantly inhibited E2/IGF-I-induced proliferation. Data of the colony formation assay also supported BE and QE possessing the ability

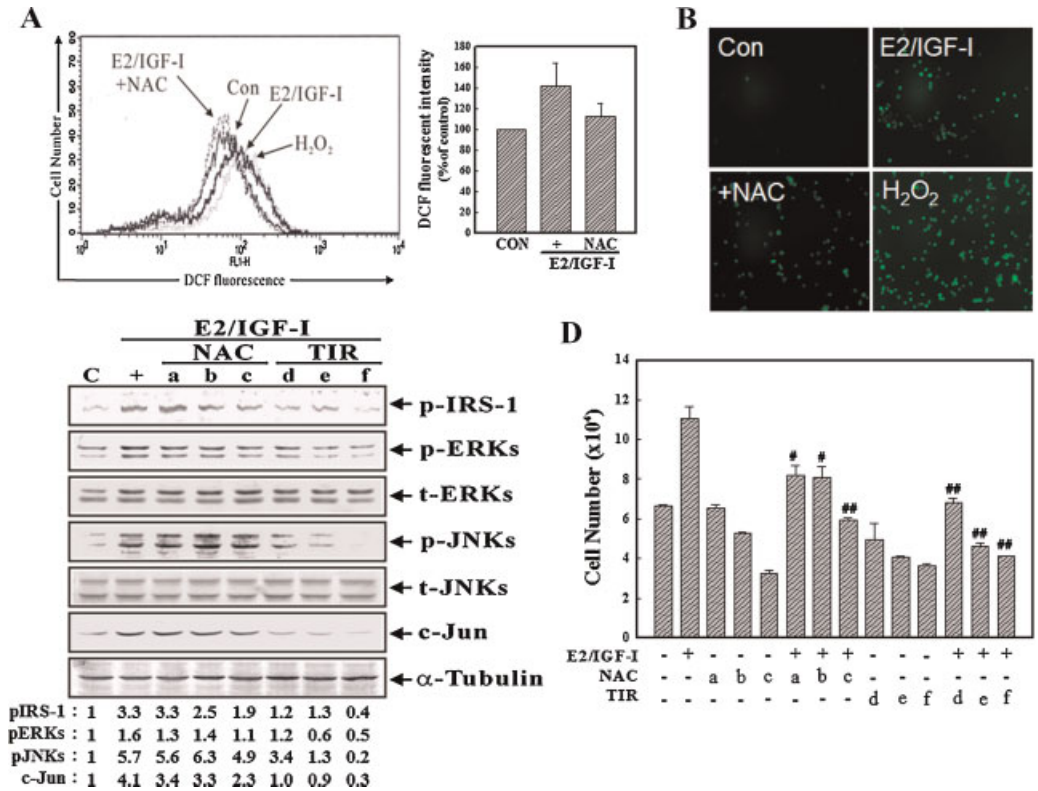

Fig. 4. Antioxidant NAC and TIR inhibition of E2/IGF-I-induced MAPK activation and cell proliferation. A: (Left part) MCF-7 cells were treated with E2/IGF-I in the presence or absence of *N*-acetyl-L-cysteine (NAC; 2 mM) for 30 min. Intracellular ROS production was detected using an oxidant-sensitive DCHF-DA probe and measured by flow cytometric analysis as described in "Materials and Methods". (Right part) The fluorescent intensity from three independent experiments was quantitated, and data were expressed as the mean ± SE. B: As described in (A), the DCF fluorescence in MCF-7 cells under different treatments was detected under fluorescent microscopic observation, C: Cells were pretreated with NAC (a: 0.5 mM; b: 1 mM; c: 2 mM) or TIR (d: 25 μM; e: 50 μM; f: 100 μM) for 30 min followed by E2/IGF-I treatment for either 20 (IRS-I, ERK, and JNK detection) or 120 min (c-Jun detection), and the expression of the indicated proteins was detected by Western blotting. The intensity of indicated protein was quantitated by densitometric analysis and expressed as folds of control. D: Cells were treated with E2/IGF-I in the presence or absence of NAC or TIR for 3 days, and cell numbers were measured by a Coulter counter. Values derived from three independent experiments were analyzed, and expressed as the mean ± SE. $^{\#\#}P < 0.01$ significantly different from E2/IGF-I-treated groups, as analyzed by Student's *t*-test. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to inhibit E2/IGF-I-induced colony formation (Fig. 6B). Reduction of E2/IGF-I-induced c-Jun protein expression was detected in MCF-7 cells treated with BE and QE but not their respective glycosides, BI, QI, or RT (Fig. 6C). Inhibition of peroxide production induced by E2/IGF-I was detected in MCF-7 cells treated with BE and QE, but not BI, QI, or RT, using fluorescent microscopic observations.

Discussion

Evidence underlying the cross-talk of E2 and IGF-I in the proliferation of breast carcinoma is herein provided. Treatment of MCF-7 cells with IGF-I enhanced E2-induced proliferation through phosphorylation of IRS-1, ERKs, and JNKs via a ROS-dependent pathway. 3-OH flavone, BE, and QE exhibited inhibitory effects against E2/IGF-I-induced proliferation among 16 tested compounds, and a tentative structure–activity relationship of flavonoids is illustrated.

E2 has been shown to play an important role in the formation of breast cancer through promoting cell cycle progression and increasing telomerase activity (Kimura et al., 2004; Han et al., 2005). The cellular actions of E2 can be mediated by both ligand-dependent transcriptional regulation (genomic effect) and/or through GPCR-associated membrane ER (non-genomic effect), which triggers different signaling cascades (Zivadinovic and Watson, 2005). Thomas et al. (2006) indicated E2 rapidly induced cPLA2 activation and increased calcium response was mediated by membrane ER. Lim et al. (2006) reported that E2 couples to G-proteins to mediate EGFR and AT-1 activation can act similar effects in ER-negative cells than in ER-positive cells. An increase in the expression of IRS-1 protein through formation of the ER-IRS-1 complex was observed in E2-induced proliferation (Morelli et al., 2004). Induction of IGF-I and IGF-IR proteins was detected in E2-treated cells (Lee et al., 1999), and direct binding and activation of IGF-IR by ligand-binding ER were reported (Kahlert et al., 2000). Zhang et al. (2005) indicated IGF-I induction of proliferation in MCF-7 cells was dependent on ER expression. Recent evidence reported that E2 and IGF-I synergized the proliferative actions on breast tumor cells (Hamelers et al., 2002), and cotreatment of E2 and IGF-I might induce ERKs activation and cyclin D1 expression leading to the progression of cells from the G1 to the S phase (Lai et al., 2001; Mawson et al., 2005). In the present study, we found that E2/IGF-I stimulated the expression of phosphorylated ERα protein in the membrane fraction of MCF-7 cells, and IGF-I addition enhanced the phosphorylated level of ERKs, IRS-1, and JNKs protein with stimulating MCF-7 cell proliferation in the presence of E2. It suggests that activation of the intracellular kinases cascade through stimulating non-genomic pathway may participate in E2/IGF-I-induced proliferation.

Several growth factors such as vascular endothelial growth factor (VEGF), nerve growth factor (NGF), transforming

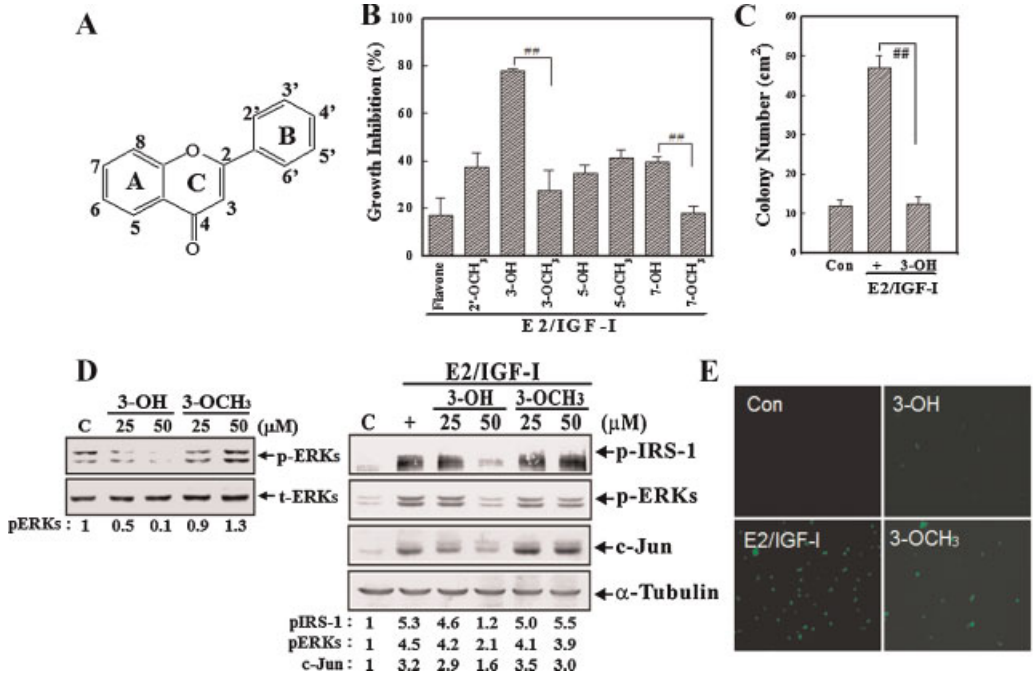

Fig. 5. Effect of 3-OH flavone and 3-OCH_3 flavone on E2/IGF-I-induced events in MCF-7 cells. A: The chemical structure of flavones used in the study. B: MCF-7 cells were treated with the indicated flavones (25 μM) for 30 min followed by incubation with E2/IGF-I for an additional 3 days, and cell numbers were measured by a Coulter counter. C: Cells were treated with E2/IGF-I in the presence or absence of 3-OH flavone (25 μM) for a 21-day incubation, and colony formation was detected by a soft agar assay as described in "Materials and Methods"; the number of colonies under different treatments was counted under microscopic observation. Values derived from three independent experiments were analyzed, and expressed as the mean±SE. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ significantly different from indicated groups, as analyzed by Student's *t*-test. D: (Left part) Cells were alone treated with 3-OH flavone or 3-OCH_3 flavone (25 and 50 μM) for 30 min, and the expression of phosphorylated and total ERKs protein was examined by Western blotting. (Right part) MCF-7 cells were pretreated with 30 min followed by E2/IGF-I treatment, and the expression of indicated proteins was analyzed by Western blotting using specific antibodies. The intensity of each protein was quantitated by densitometric analysis and expressed as folds of control. E: Cells were treated with 3-OH flavone or 3-OCH_3 flavone (25 μM) followed by the addition of E2/IGF-I for 30 min, and the fluorescence in cells was detected by fluorescent microscopic observation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

growth factor-β1 (TGF-β1) and interleukin-1β (IL-1β) were demonstrated to induce proliferation of cells via ROS production (Suzukawa et al., 2000; Colavitti et al., 2002; Hwang et al., 2004; Sturrock et al., 2006). Felty et al. (2005) indicated E2-induced mitochondria ROS-mediated stimulation of redox-sensitive kinase via intergrin pathway, but not ER, and generation of ROS by E2 leading to activate oxidant-sensitive transcription factors such as CRE, NRF-1 and TRE. In the present study, the intracellular peroxide level was slightly increased in MCF-7 cells in the presence of E2 or IGF-I treatment, and that was significantly elevated in E2/IGF-I treated cells (data not shown). Incubation of cells with the chemical free radical scavengers, NAC and TIR, significantly attenuated E2/IGF-I-induced proliferation and reduced the expression of phosphorylated IRS-1, ERK, JNK, and c-Jun proteins. This suggests that ROS induction leading to activation of the intracellular kinase cascade is an early event in E2/IGF-I-induced proliferation. A ROS-dependent pathway is thus identified in the E2/IGF-I-induced proliferation of breast carcinoma cells.

The biological activities of flavonoids have extensively been investigated, however their structure–activity relationships are still unclear. Hydroxylation occurs in the metabolism of flavonoids, and previous studies suggested that the greater the number of hydroxyl groups, the more-potent antioxidant and anti-tumor activities flavonoids have (Cao et al., 1997; Babu et al., 2003). Our previous study found that hydroxylation at C4′ or C6 is important for the apoptosis-inducing activity of flavonoids (Ko et al., 2004). In addition, 3-OH flavone showed an effective antiproliferative effect against epidermal growth factor (EGF)-induced proliferation in A431 cells (Shen et al., 2004b). In the present study, the addition of 3-OH flavone effectively attenuated E2/IGF-I-induced proliferation. Reduction of intracellular proliferative events such as IRS-1 and ERK protein phosphorylation was detected in 3-OH flavone-treated cells in the presence of E2/IGF-I treatment. This suggests that hydroxylation at C3 may be essential for the antiproliferative effects of flavonoids. In addition to hydroxylation, glycosylated flavonoids occur extensively in plants, herbs, and fruits, and the addition of a glycoside has been shown to increase the hydrophilic property of flavonoids, and prevent flavonoids from enzymatic oxidation in plants (Regev-Shoshani et al., 2003). Plumb et al. (1999) reported that adding glycoside moieties to flavonol decreased its antioxidant properties. Our previous study demonstrated that aglycon flavonoids such as quercetin have more anti-inflammatory and cytoprotective activities than the respective glycosides, quercitrin and rutin, in several different cell types (Shen et al., 2003; Chow et al., 2005; Chen et al., 2006). Similarly, myricetin, but not its glycoside, myricitrin, possesses apoptosis-inducing activity in HL-60 cells (Ko et al., 2005a), and inhibits tumor invasion and migration in human colorectal carcinoma cells (Ko et al., 2005b). Results of the present study show that BE and QE, but not their respective glycosides, BI, QI, or RT, inhibit E2/IGF-I induced proliferation and colony formation with concomitant blocking of c-Jun protein expression. These data

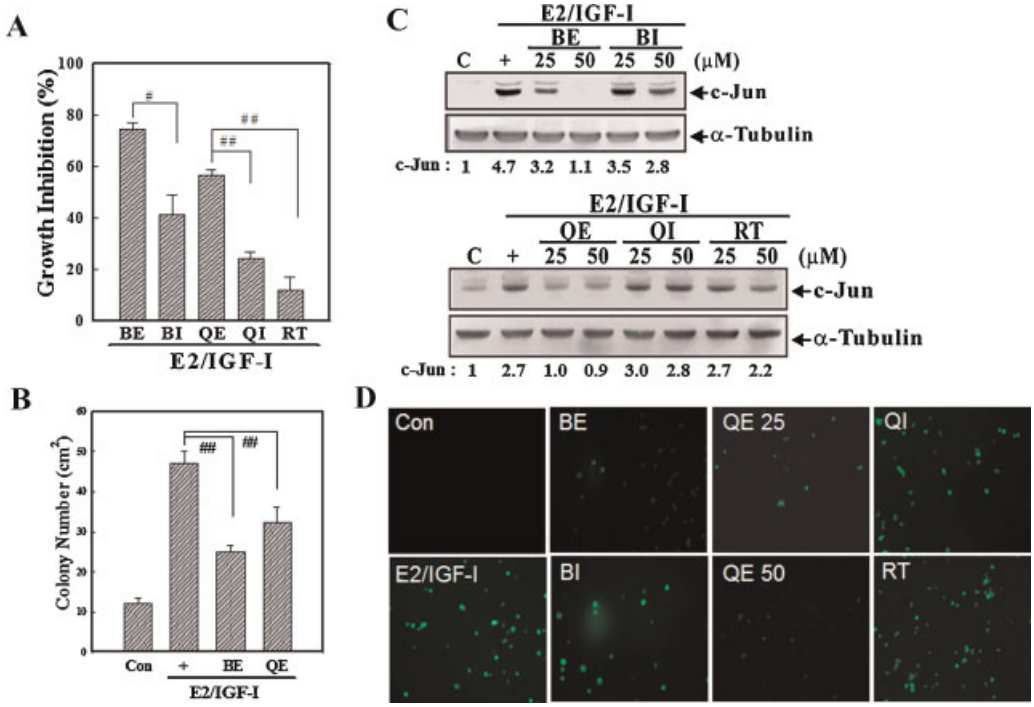

Fig. 6. Effects of BE, BI, QE, QI, and RT on E2/IGF-I-induced events in MCF-7 cells. A: MCF-7 cell were treated with the indicated flavones (25 μM) for 30 min followed by incubation with E2/IGF-I for a further 3 days, and the cell numbers were measured by a Coulter counter. B: Cells were treated with E2/IGF-I in the presence or absence of BE and QE (25 μM) for a 21-day incubation, and colony formation was detected by a soft agar assay as described in "Materials and Methods"; the number of colonies under different treatments was counted under microscopic observation. Data are presented as the mean ± SE of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ significantly different from the indicated group. C: Cells were treated with BE or BI (25 or 50 μM) for 30 min followed by E2/IGF-I treatment, and the expression of c-Jun proteins was analyzed by Western blotting (upper part). (Lower part): As described above, cells were treated with QE, QI, or RT (25 and 50 μM) for 30 min followed by E2/IGF-I treatment, and the expression of c-Jun proteins was analyzed by Western blotting using specific antibodies. Quantification of the c-Jun protein under different treatments was done by densitometric analysis and expressed as folds of control. Western blotting were done at least three times, the results are representative of all of the data. D: Cells were treated with BE, BI, QE, QI, or RT followed by the addition of E2/IGF-I for 30 min, and the fluorescence in cells was detected by fluorescent microscopic observation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

support the notion that glycosides act as a negative moiety in the biological actions of flavonoids. In addition, flavonoids such as genistein, apigenin and quercetin have been reported are potent inhibitors of tyrosine and serine/theronine kinases due to their antioxidant capacity (Agullo et al., 1997; Wijetunge et al., 2000). In the present study, 3-OH flavone, BE, and QE addition suppresses E2/IGF-1-induced proliferative events, and reduction of E2/IGF-1-induced intracellular peroxide was observed in 3-OH flavone, BE, and QE-treated MCF-7 cells by DCHF-DA staining analysis. These data indicate ROS-scavenging activity of 3-OH flavone, BE, and QE may contribute to their inhibitory effect against E2/IGF-1-induced proliferation of MCF-7 cells.

In conclusion, this study provides the first evidence to indicate that E2 and IGF-I participate in the proliferation and transformation of breast cancer via ROS-dependent activation of intracellular kinase cascades. Flavonoids such as 3-OH flavone, BE, and QE exhibit effective inhibitory activities against E2/IGF-I-induced proliferative events, and are worthy of further investigation.

Acknowledgments

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