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ORIGINAL ARTICLE

Kaempferol Downregulates Insulin-like Growth Factor-I Receptor and ErbB3 Signaling in HT-29 Human Colon Cancer Cells

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Background: Novel dietary agents for colon cancer prevention and therapy are desired. Kaempferol, a flavonol, has been reported to possess anticancer activity. However, little is known about the molecular mechanisms of the anticancer effects of kaempferol. The aim of this study was to determine the inhibitory effect of kaempferol on growth factor-induced proliferation and to elucidate its underlying mechanisms in the HT-29 human colon cancer cell line.
Methods: To assess the effects of kaempferol and/or growth factors [insulin-like growth factor (IGF)-I and heregulin (HRG)-β], cells were cultured with or without 60 μmol/L kaempferol and/or 10 nmol/L IGF-I or 20 μg/L HRG-β. Cell proliferation, DNA synthesis, and apoptosis were determined by a cell viability assay, a [^{3}H]thymidine incorporation assay, and Annexin-V staining, respectively. Western blotting, immunoprecipitation, and an in vitro kinase assay were conducted to evaluate expression and activation of various signaling molecules involved in the IGF-I receptor (IGF-IR) and ErbB3 signaling pathways.
Results: IGF-I and HRG-β stimulated HT-29 cell growth but did not abrogate kaempferol-induced growth inhibition and apoptosis. Kaempferol reduced IGF-II secretion, HRG expression and phosphorylation of Akt and extracellular signal-regulated kinase (ERK)-1/2. Kaempferol reduced IGF-I- and HRG-β-induced phosphorylation of the IGF-IR and ErbB3, their association with p85, and phosphatidylinositol 3-kinase (PI3K) activity. Additionally, kaempferol inhibited IGF-I- and HRG-β-induced phosphorylation of Akt and ERK-1/2.
Conclusions: The results demonstrate that kaempferol downregulates activation of PI3K/Akt and ERK-1/2 pathways by inhibiting IGF-IR and ErbB3 signaling in HT-29 cells. We suggest that kaempferol could be a useful chemopreventive agent against colon cancer.
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Key Words: Kaempferol, Insulin-like growth factor-I receptor, ErbB3, HT-29 human colon cancer

INTRODUCTION

Colon cancer is one of the leading causes of cancer-associated deaths worldwide.[1,2] The incidence of colon cancer is strongly related to lifestyle, particularly dietary habits.[3] Over the past several decades, there has been an increased interest in the utility of plant-derived polyphenols based upon their broad spectrum of biological properties, including antioxidative, anticarcinogenic, and cardioprotective activities.[4] The important advantage with phytochemicals is that they are generally non-toxic and have wide human acceptance.[3]

Flavonoids are polyphenolic compounds that are distributed widely in fruits and vegetables. The six major subgroups of flavonoids commonly found in the human diet are flavonols, flavones, anthocyanidins, catechins, flavonones and isoflavones.[5] Their wide-ranging biological activities include antioxidant, anti-inflammatory and anti-cancer effects.[6] Studies have shown that several flavonoids have potent anti-cancer activity against colon cancer cell lines.[7-11] Individual intake of flavonoids varies depending upon the type of diet consumed. In Western popul-

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ations, estimated daily intake of flavonoids is in the range of 20-50 mg per day.[12]

Kaempferol (3,4',5,7-tetrahydroxyflavone) is a flavonol (Fig. 1A) present in fruits and vegetables, including onions, kale, broccoli, apples, cherries, berries, tea and red wine.[13,14] Kaempferol has broad biological properties including anticancer effects. For example, kaempferol induces apoptosis and cell cycle arrest in various cancer cell lines, including colon cancer cells.[15-18] We have reported that kaempferol induces cell cycle arrest and apoptosis in HT-29 colon cancer cells.[9,19] The gastrointestinal tract is the first line of contact with food and these studies suggest that kaempferol may have preventive/therapeutic benefits in colon cancer. Nevertheless, the underlying mechanisms of kaempferol in human colon cancer cells are not fully understood. It has been reported that various human colon cancer cell lines, including HT-29 cells, produce polypeptide growth factors such as transforming growth factor-α/epidermal growth factor (EGF) receptor, transforming growth factor-β, and platelet-derived growth factor.[20] In addition, we have observed previously that HT-29 cells express other growth factors, including insulin-like growth factor (IGF)-II[21] and heregulin (HRG).[22] These observations suggest that colon cancer cells produce autocrine and/or paracrine growth factors that stimulate cell growth. Therefore, inhibiting the signaling growth factor may be a potential strategy for preventing colon cancer.

The IGF system includes IGF-I, IGF-II, IGF-binding proteins and the IGF-I receptor (IGF-IR). This system plays an important role in the growth of various cancer cells, including colon cancer cells.[23,24] The IGF-IR and its ligands IGF-I and IGF-II play critical roles in the regulation of cellular proliferation, apoptosis and transformation.[25] Ligand binding to the IGF-IR triggers multiple signaling pathways, including the Ras/Raf/extracellular signal-regulated kinase (ERK) pathway implicated in receptor-mediated mitogenesis and transformation and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway implicated in the transmission of cell survival signals.[26,27]

The ErbB family of type I receptor tyrosine kinases has four members, including EGF receptor (EGFR; ErbB-1; HER1 in

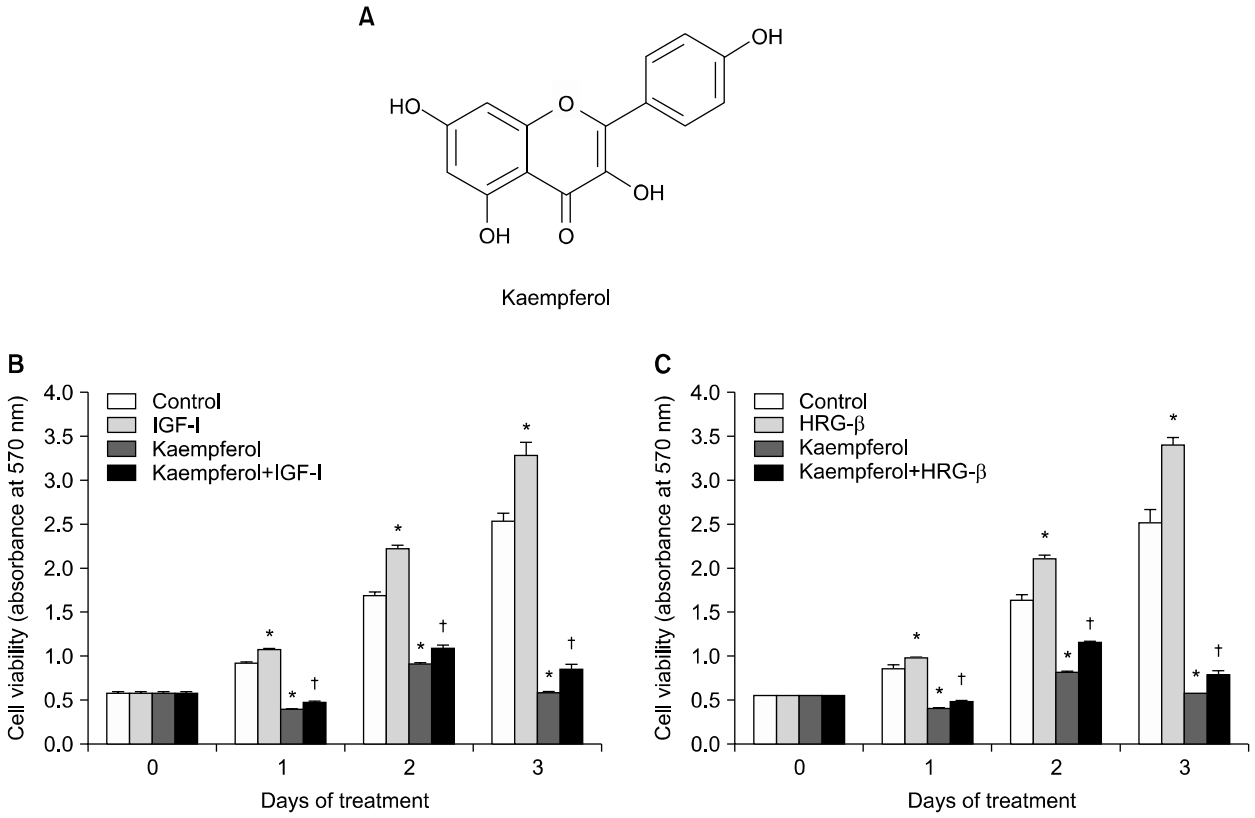

Figure 1. Effect of kaempferol on cell viability in growth factor-treated HT-29 cells. (A) Chemical structure of kaempferol. (B, C) HT-29 cells were plated and serum-starved. After serum starvation, the cells were treated with 0 or 60 μmol/L kaempferol with or without growth factors [B: 10 nmol/L insulin-like growth factor-I (IGF-I); C: 20 μg/L heregulin (HRG)-β]. Viable cell numbers were estimated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Each bar represents the mean±SEM (n=6). *$P < 0.05$ as compared with the untreated cells. †$P < 0.05$ as compared with the cells treated with kaempferol.

humans), ErbB2 (HER2; c-*neu* in rodents), ErbB3 (HER3) and ErbB4 (HER4).[28] Activation of these receptors regulates a number of processes including cell proliferation, differentiation and survival. Excess ErbB signaling is associated with the development of a wide variety of human cancers including colon cancer.[29-31] HRG is a potent mitogen of colon cancer cells and autocrine released HRG generates growth factor independence and prevents apoptosis.[32,33]

Our recent study indicated that inhibiting the IGF-IR signaling pathway may be one of the mechanisms by which luteolin, 3',4',5,7-tetrahydroxyflavone, inhibits cell cycle progression and induces apoptosis in HT-29 human colon cancer cells.[34,35] In the present study, we examined whether the growth inhibitory effects of kaempferol are related to changes in IGF-I-IGF-IR or HRG-ErbB3 signaling in the HT-29 human colon cancer cell line. We found that kaempferol markedly inhibited growth factor-induced cell proliferation. Kaempferol also inhibited activation of the PI3K/Akt and ERK-1/2 pathways.

MATERIALS AND METHODS

1. Materials

Kaempferol, essentially fatty acid-free bovine serum albumin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 7-amino-actinomycin D (7-AAD), phosphatidylinositol, transferrin and anti-β-actin antibody were obtained from Sigma (St. Louis, MO, USA); selenium and DMEM/Ham's F-12 nutrient mixture (DMEM/F-12 were purchased from Gibco BRL (Gaithersburg, MD, USA)); [methyl-^{3}H]thymidine, protein A-Sepharose and horse-radish peroxidase-conjugated anti-rabbit and anti-mouse IgG were from Amersham (Arlington Heights, IL, USA); antibodies against HRG, IGF-IRβ, p85 and ErbB3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against Akt, phospho (P)-Akt, ERK-1/2 and P-ERK-1/2 were from Cell Signaling Technology (Beverly, MA, USA); anti-phosphotyrosine-RC20 (PY20) antibody linked to horse-radish peroxidase was purchased from BD Transduction Laboratories (Palo Alto, CA, USA); anti-IGF-II antibody was from Amano International Enzyme (Troy, VA, USA); recombinant human HRG-β and recombinant human IGF-II were purchased from R&D Systems, (Minneapolis, MN, USA); phycoerythrin-conjugated Annexin V was from BD Pharmingen, (Franklin Lake, NJ, USA); and [γ-^{32}P]ATP was obtained from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA, USA).

2. Cell culture

HT-29 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM/F12 supplemented with 100 mL/L fetal bovine serum, 100,000 U/L penicillin and 100 mg/L streptomycin. Cell monolayers were serum-starved in DMEM/F12 supplemented with 5 mg/L transferrin, 5 μg/L selenium and 0.1 g/L bovine serum albumin (serum-free medium) for 24 hours at 24 hour after plating. After serum starvation, the cells were incubated in serum-free medium containing various concentrations of kaempferol with or without growth factors (10 nmol/L IGF-I or 20 μg/L HRG-β) for the indicated time periods. Viable cell numbers were estimated by the MTT assay. Conditioned medium (24 hours) was collected and subjected to Western blot analysis to estimate IGF-II production.

3. [^{3}H]Thymidine incorporation assay

To determine DNA synthesis, HT-29 cells were plated in 96-well plates, serum-starved and treated with kaempferol and/or growth factors in the presence of [^{3}H]thymidine. After 3 hours, the cells were harvested onto glass-fiber filters and the incorporated [^{3}H]thymidine was quantified using a scintillation counter (Beckman Coulter Inc, Fullerton, CA, USA).

4. Fluorescence-activated cell sorting analysis

HT-29 cells were plated in 24-well plates, serum-starved and treated with kaempferol and/or growth factors as described above. After 24 hours, the cells were trypsinized and loaded with phycoerythrin-conjugated Annexin V and 7-AAD. Annexin V-positive and 7-AAD-negative cells (early apoptotic cells) were counted by flow cytometry (Becton Dickinson, Franklin Lake, NJ, USA) and expressed as a percentage of the total cell number.

5. Western blot analysis and immunoprecipitation

Total cell lysates were prepared, and immunoprecipitation studies were conducted as described previously.[22] Briefly, total cell lysates were incubated with anti-IGF-IRβ antibody or anti-ErbB3 antibody overnight. The immune complexes were collected by adding protein A-Sepharose followed by centrifugation. Total cell lysates, 24-hour conditioned media, and the immunoprecipitated proteins were subjected to Western blot analysis with their relevant antibodies.

6. In vitro kinase assay

ERK-1/2 activity was measured using a p44/p42 MAP Kinase Assay Kit (nonradioactive) in accordance with the manufacturer's instruction (Cell Signaling Technology, Beverly, MA, USA). Briefly, total cell lysates were immunoprecipitated with immobilized phospho-p44/p42 mitogen activated protein kinases (MAPK)

antibody (bead slurry). The immunoprecipitated proteins were incubated with ATP and Elk-1 fusion protein (an ERK-1/2 substrate). The resulting P-Elk-1 was analyzed by Western blotting with anti-P-Elk-1 antibody. The immunoprecipitated proteins (mentioned above) were incubated with phosphatidylinositol and [γ-^{32}P]ATP as described previously to detect PI3K activity.[22] The resulting ^{32}P-labelled phosphatidylinositol 3-phosphate was separated by thin-layer chromatography and visualized by autoradiography.

7. Statistical analysis

Results are expressed as means±SEM. Differences between groups were assessed via the Student's *t* test, utilizing SAS statistical software ver. 9.2 (SAS Institute, Cary, NC, USA).

RESULTS

1. Kaempferol abrogates the growth stimulatory effects of exogenous IGF-I and HRG-β in HT-29 cells

We reported previously that kaempferol induces cell cycle arrest and apoptosis in HT-29 colon cancer cells.[9,19] In the present study, we first assessed whether exogenous growth factors inhibit the growth inhibitory effect of kaempferol. We cultured HT-29 cells in the absence or presence of exogenous growth factors, including 10 nmol/L IGF-I or 20 μg/L HRG-β, with or without 60 μmol/L kaempferol. Consistent with our previous results, kaempferol significantly reduced the viable cell number. IGF-I and HRG-β increased viability of HT-29 cells throughout the entire 3-day incubation period. IGF-I and HRG-β slightly increased the viable cell number of kaempferol-treated cells (Fig. 1B and 1C).

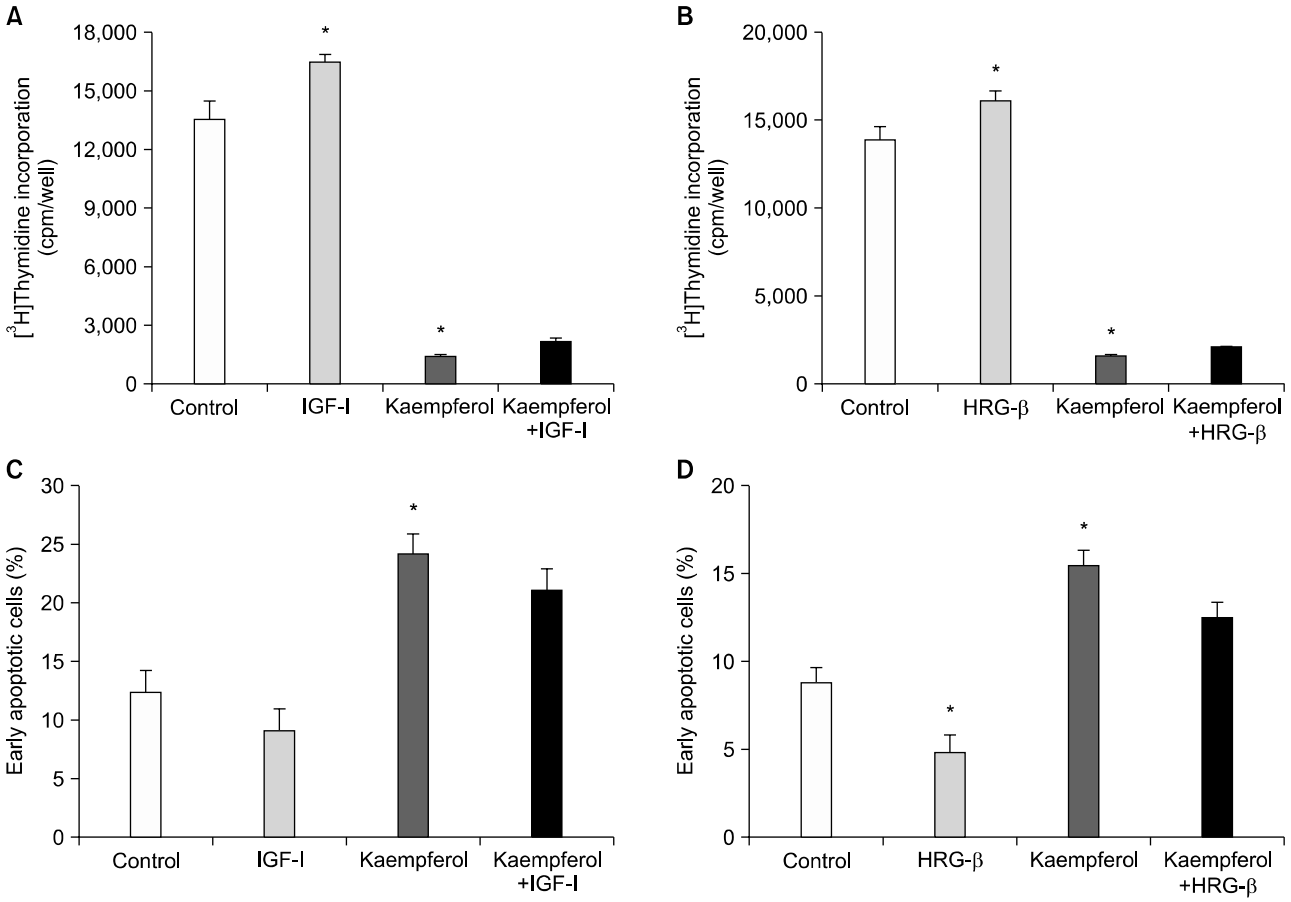

Figure 2. Effect of kaempferol and/or growth factors on DNA synthesis and apoptosis in HT-29 cells. (A, B) HT-29 cells were plated in 96-well plates, serum-starved and treated with 0 or 60 μmol/L kaempferol and/or growth factors [A: 10 nmol/L insulin-like growth factor-I (IGF-I); B: 20 μg/L heregulin (HRG)-β)] in the presence of [^{3}H]thymidine. The cells were incubated for 3 hours and then harvested, and DNA synthesis was measured by incorporation of [^{3}H]thymidine into DNA. (C, D) HT-29 cells were plated in 24-well plates, serum-starved, and treated with 0 or 60 μmol/L kaempferol and/or growth factors (C: 10 nmol/L IGF-I; D: 20 μg/L HRG-β). At 24 hours after treatment, the cells were trypsinized and loaded with Annexin V and 7-amino-actinomycin D (7-AAD). The percentages of early apoptotic cells (Annexin V$^+$/7-AAD$^-$) were quantified by flow cytometry. Each bar represents the mean±SEM (n=6). *P < 0.05 as compared with the untreated cells.

A [^{3}H]thymidine incorporation assay was conducted to explore the effect of kaempferol on growth factor- induced DNA synthesis in HT-29 cells. Kaempferol markedly inhibited [^{3}H]thymidine incorporation. IGF-I and HRG-β increased [^{3}H]thymidine incorporation but did not abrogate the inhibitory effect of kaempferol (Fig. 2A and 2B). To examine whether these growth factors inhibit kaempferol-induced apoptosis, we estimated the fractions of HT-29 cells undergoing apoptosis after treatment with kaempferol and/or growth factors using Annexin-V staining followed by flow cytometry. We observed an increase in the apoptotic cell number after treatment with 60 μmol/L kaempferol. IGF-I and HRG-β did not significantly suppress kaempferol-induced apoptosis (Fig. 2C and 2D).

2. Kaempferol reduces IGF-II secretion and HRG expression in HT-29 cells

As shown in Figure 1B, HT-29 cells can grow continuously without fetal bovine serum supplementation. Additionally, we have observed previously that HT-29 cells express IGF-II[21] and HRG.[22] These observations indicate that the autocrine and/or paracrine mechanisms for these growth factors exist in HT-29 cells. Therefore, we examined whether growth inhibitory effects

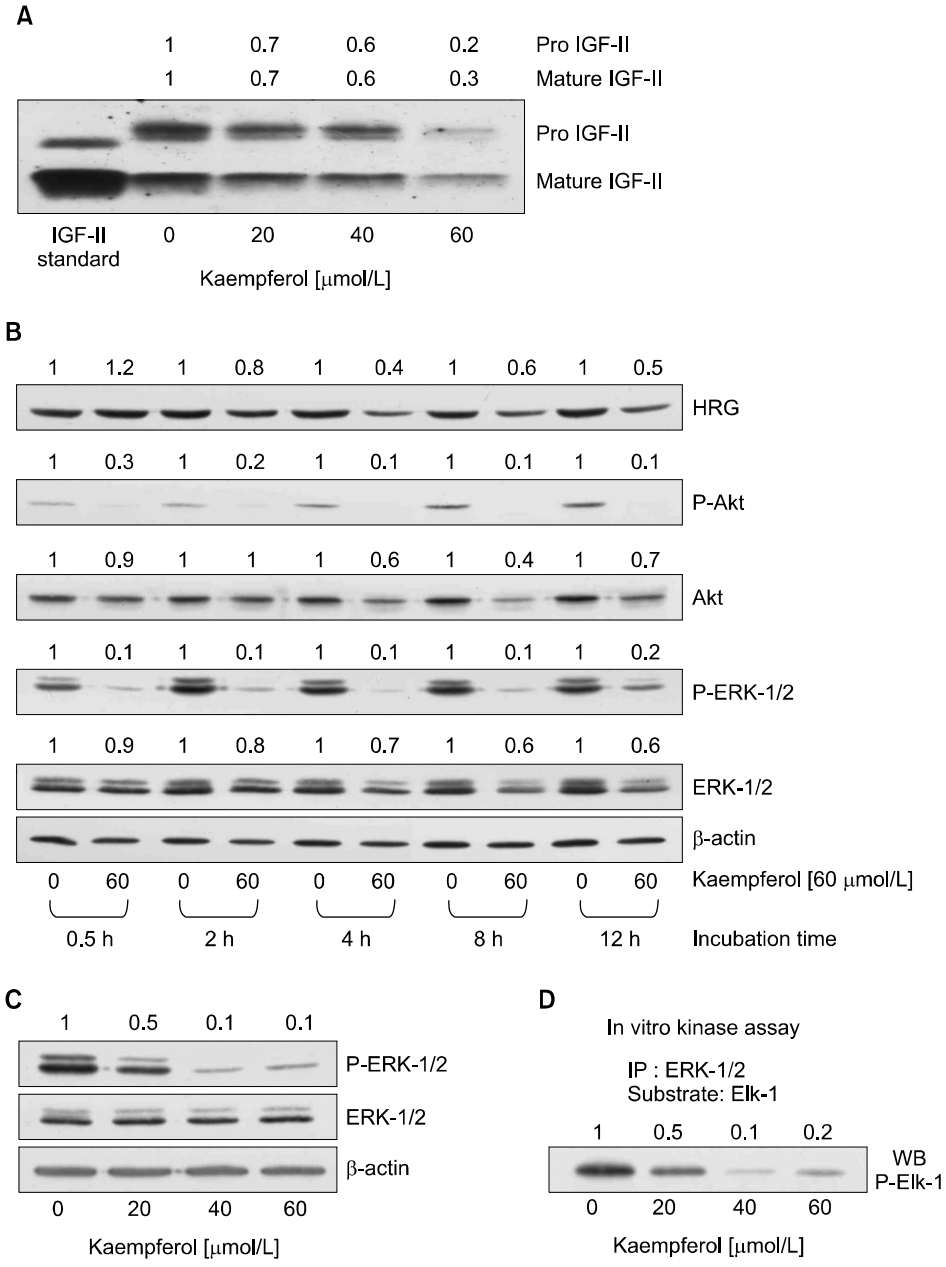

Figure 3. Effect of kaempferol on the expression of insulin-like growth factor (IGF)-II and heregulin (HRG) and the phosphorylation of Akt and extracellular regulated kinase (ERK)-1/2. HT-29 cells were serum-starved and treated with various concentrations (0, 20, 40, or 60 μmol/L) of kaempferol. (A) At 24 hours after treatment, 24 hour-conditioned media were collected and subjected to Western blot analysis with anti-IGF-II antibody. Media loaded onto the gel was adjusted for equivalent cell numbers. (B) At the indicated time point after kaempferol treatment, total cell lysates were prepared and subjected to Western blot analysis with their relevant antibodies. (C, D) At 2 hours after kaempferol treatment, total cell lysates were prepared. (C) Total cell lysates were subjected to Western blot analysis with the relevant antibodies. (D) Total cell lysates were incubated with immobilized P-ERK-1/2 antibody overnight (4°C). After centrifugation, the immunoprecipitated proteins were incubated with Elk-1 (an ERK-1/2 substrate) and ATP for 30 minutes (30°C). The resulting P-Elk-1 was analyzed by Western blot analysis with an anti-P-Elk antibody. IP, immunoprecipitation; WB, western blot analysis.

of kaempferol are associated with the production of these growth factors. HT-29 cells were treated with various concentrations (0-60 μmol/L) of kaempferol for 24 hours to assess its effect on IGF-II secretion in the cells. The conditioned media was subjected to Western blot analysis with an anti-IGF-II antibody. Kaempferol treatment reduced secretion of pro and mature IGF-II in a dose-dependent manner (Fig. 3A). The cells were treated with 0 or 60 μmol/L kaempferol and then harvested at different times for Western blot analysis to detect HRG expression. As shown in Fig. 3B, kaempferol treatment decreased HRG expression within 2 hours, and the effect was persistent during the 12-hour incubation period.

3. Kaempferol decreases Akt and ERK-1/2 phosphorylation

Accumulating evidence indicates that flavonols modify the activities of several intracellular signal transduction enzymes, including Akt and mitogen activated protein kinases (MAPKs).[36,37] Because Akt and ERK-1/2 play important roles in cell survival and are activated by IGF-I and HRG,[21,22,38,39] we next investigated whether the growth inhibitory effects of kaempferol were related to a decrease in activation of Akt and ERK-1/2. Activation of Akt and ERK-1/2 was determined by examining the degree of phosphorylation by Western blot analysis with anti-P-Akt and anti-P-ERK-1/2 antibodies. Akt and ERK-1/2 phosphorylation was remarkably reduced within 30 minutes in cells treated with 60 μmol/L kaempferol. The total levels of Akt and ERK-1/2 were also reduced in cells treated with kaempferol but the decreases were much smaller than those with phosphorylation of Akt and ERK-1/2 (Fig. 3B). To determine whether the decrease in ERK-1/2 phosphorylation was proportional to the kaempferol concentration, HT-29 cells were treated with various concentrations (0-60 μmol/L) of kaempferol. As shown in Figure 3C, P-ERK-1/2 levels decreased dose-dependently by kaempferol treatment. The in vitro kinase assay revealed that ERK-1/2 activity also decreased following kaempferol treatment (Fig. 3D).

4. Kaempferol inhibits IGF-I- and HRG-β-induced activation of the IGF-IR and ErbB3 signaling pathways

Because exogenous IGF-I and HRG-β failed to abrogate the growth inhibitory effects of kaempferol (Fig. 1 and 2), we next investigated whether kaempferol inhibits growth factor receptor signaling. The receptor tyrosine kinase subunits become tyrosine phosphorylated after activation by the ligands. To determine whether kaempferol downregulates growth factor-induced phosphorylation of tyrosine residues, cells were treated with 0 or 60 μmol/L of kaempferol for 2 hours, and total cell lysates were prepared after 0, 1, or 5 minutes of stimulation with IGF-I (Fig. 4) or HRG-β (Fig. 5). Total cell lysates were immunoprecipitated using anti-IGF-IRβ antibody, and the immunoprecipitated proteins were subjected to Western blot analysis with anti-PY20. Adding IGF-I drastically increased IGF-IRβ phosphorylation within 1 minute, indicating that the receptor was activated by IGF-I. Levels of the IGF-IR protein remained unchanged during 5 minutes of IGF-I stimulation. IGF-I similarly induced IGF-IR activation in kaempferol-treated cells, but to a lesser degree, indicating that kaempferol inhibited IGF-IR activation (Fig. 4A). As shown in Fig. 5A, following the addition of HRG-β alone, ErbB3 levels remained same and phosphorylation of ErbB3 was detected at 1 minute and 5 minutes, indicating that ErbB3 was stimulated by HRG-β. ErbB3 phosphorylation decreased slightly in cells treated with 60 μmol/L kaempferol (Fig. 5A).

Because activation of IGF-IR or ErbB3 signaling leads to activation of PI3K, we next investigated whether kaempferol influences the IGF-I- or HRG-β-induced association of the p85 regulatory subunit of PI3K with IGF-IR or ErbB3 in HT-29 cells. Immunoprecipitation analysis results revealed that kaempferol treatment reduced the levels of IGF-IR- and ErbB3-associated p85 in serum-starved HT-29 cells (Fig. 4A and 5A). IGF-I (Fig. 4A) and HRG-β (Fig. 5A) stimulated the association of p85 with IGF-IR or ErbB3 within 1 minute, which was inhibited by kaempferol treatment. However, the decreased association of p85 with these receptors by kaempferol was small as time passed, indicating that kaempferol delayed activation of IGF-IR and ErbB3 signaling. Furthermore, kaempferol decreased both basal and IGF-I- and HRG-β-induced PI3K activity in HT-29 cells (Fig. 4A and 5A).

We next investigated whether kaempferol decreases IGF-I- or HRG-β-induced phosphorylation of Akt, a downstream target of PI3K. Western blot analysis revealed that P-Akt levels increased time-dependently following IGF-I or HRG-β treatment without changes in total Akt levels, which were reduced by kaempferol treatment (Fig. 4B and 5B). In addition to Akt, activation of ERK-1/2 has also been implicated in mitogenic signaling by IGF-I- and HRG-induced activation of IGF-IR and ErbB3, respectively.[38,39] Kaempferol decreased both basal and IGF-I- and HRG-β-induced phosphorylation of ERK-1/2 in HT-29 cells (Fig. 4B and 5B).

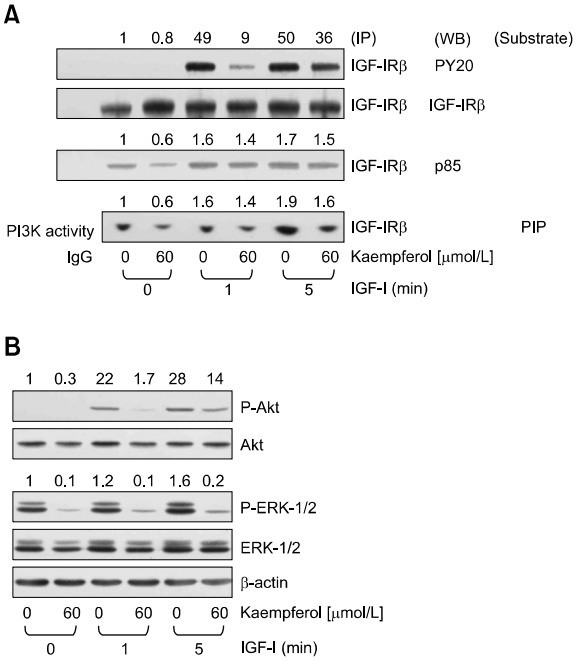

Figure 4. Effect of kaempferol on the insulin-like growth factor-I receptor (IGF-IR) signaling pathway. HT-29 cells were treated with 0 or 60 μmol/L kaempferol for 2 hours and lysed after 0, 1, or 5 minutes of stimulation with 10 nmol/L insulin-like growth factor-I (IGF-I). (A) Total cell lysates were incubated with anti-IGF-IRβ antibody and the immune complexes were precipitated with protein A-Sepharose. The immunoprecipitated proteins were subjected to Western blot analysis with the relevant antibodies. The immunoprecipitated proteins were incubated with phosphatidylinositol and [γ-^{32}P]ATP to detect phosphatidylinositol 3-kinase (PI3K) activity. The resulting ^{32}P-labelled phosphatidylinositol 3-phosphate (PIP) was separated by thin-layer chromatography and visualized by autoradiography. (B) Total cell lysates were subjected to Western blot analysis with their relevant antibodies. ERK, extracellular regulated kinase; IP, immunoprecipitation; WB, western blot analysis.

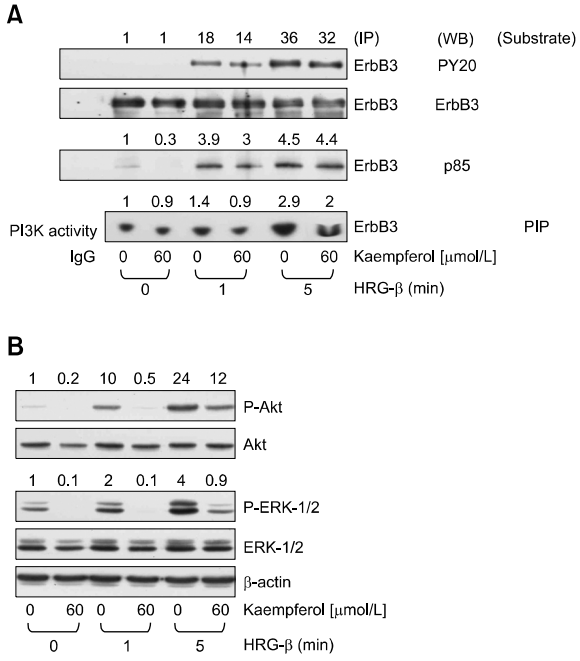

Figure 5. Effect of kaempferol on the ErbB3 signaling pathway. HT-29 cells were treated with 0 or 60 μmol/L kaempferol for 2 hours and lysed after 0, 1, or 5 minutes of stimulation with 20 μg/L heregulin (HRG)-β. (A) Total cell lysates were incubated with anti-ErbB3 antibody and the immune complexes were precipitated with protein A-Sepharose. The immunoprecipitated proteins were subjected to Western blot analysis and in vitro phosphatidylinositol 3-kinase (PI3K) assay as described in Figure 4. (B) Total cell lysates were subjected to Western blot analysis with their relevant antibodies. ERK, extracellular regulated kinase; IP, immunoprecipitation; PIP, phosphatidylinositol 3-phosphate; WB, western blot analysis.

DISCUSSION

The increasing acceptability of phytochemicals, particularly those from dietary sources, as cancer chemopreventive agents in recent years may be attributed to their high potency, low toxicity and a relatively safe profile compared to synthetic anticancer agents.[40] The anticarcinogenic effect of flavonoids has been the focus of many investigations. The flavonol kaempferol is abundant in onions, kale, broccoli, apples, cherries, berries, tea and red wine.[13,14] We demonstrated previously that kaempferol induces cell cycle arrest and apoptosis in HT-29 colon cancer cells.[9,19] Because human colon cancer cells produce autocrine and/or paracrine growth factors that stimulate cell growth,[20] the present study was conducted to examine whether kaempferol could counteract the stimulated cell growth induced by growth factors and to identify the mechanisms. Our results indicate that kaempferol (1) abrogates the growth stimulatory effects of IGF-I and HRG-β; (2) reduces secretion of IGF-II and expression of HRG; (3) decreases phosphorylation of Akt and ERK-1/2 and the activity of ERK-1/2; (4) inhibits IGF-I- and HRG-β-induced tyrosine phosphorylation of IGF-IR or ErbB3, and the association of p85 with IGF-IR or ErbB3, and PI3K activity in HT-29 cells. These results indicate that inhibiting IGF-II and HRG secretion as well as downregulating IGF-IR and ErbB3 signaling may be important factors underlying the inhibitory effects of kaempferol on growth of HT-29 cells.

PI3K is central to the coordinated control of multiple cell-signaling pathways leading to tumor development, including cell proliferation and apoptosis.[41] We found that kaempferol decreased IGF-IR or ErbB3-associated PI3K activity (Fig. 4 and 5). Lee et al. reported that kaempferol binds to PI3K directly and inhibits PI3K activation in JB6 P+ mouse epidermal cells.[42] Furthermore

kaempferol decreased the IGF-I- and HRG-β-induced association of p85 with IGF-IR or ErbB3 (Fig. 4A and 5A). Taken together, these results suggest that kaempferol inhibits PI3K activity directly by binding to PI3K and indirectly by decreasing the association between p85 and growth factor receptors.

Akt, a downstream target of PI3K, regulates a variety of cellular process (including cell-cycle regulation and apoptosis) by phosphorylating multiple substrates.[43] Activating of ErbB3 and IGF-IR signaling leads to activation of Akt.[21,22,38] We have reported previously that kaempferol reduces phosphorylation and activity of Akt in HT-29 cells.[19] Consistent with our results, several studies have indicated that kaempferol inhibits phosphorylation of Akt in human leukemia cells[44] and A549 lung cancer cells.[15] Deregulation of the PI3K/Akt signaling pathway can lead to an altered aspects of cell physiology that comprise the hallmarks of cancer.[44] Therefore inhibiting PI3K and subsequently inactivating Akt may contribute to the anti-cancer effect of kaempferol.

The MAPK family, which includes ERK-1/2, c-Jun N-terminal kinase, p38 and ERK5 subgroups, regulates proliferation and apoptosis.[45] Each MAPK is activated through a specific phosphorylation cascade. Activation of ERK-1/2 has been implicated in mitogenic signaling by IGF-I- and HRG-induced activation of IGF-IR and ErbB3, respectively.[38,39] The ERK-1/2 cascade is associated with cell differentiation, proliferation and survival. In the present study, we observed that kaempferol reduced phosphorylation and activity of ERK-1/2 (Fig. 3). It has also been reported that kaempferol inhibits phosphorylation of ERK-1/2 in SCC4 oral cancer cells.[46] In contrast, Nguyen et al. reported that activating ERK-1/2 is a requirement for kaempferol-induced apoptosis in A549 lung cancer cells.[15] Because it is possible to induce apoptosis through the activation of ERK-1/2,[47] future studies are needed to explore the detailed mechanisms between kaempferol-induced apoptosis and ERK-1/2 activation in various cell lines. In the present study, kaempferol decreased both basal and IGF-I- and HRG-β-induced phosphorylation of ERK-1/2 in HT-29 cells (Fig. 4B and 5B), indicating that inhibiting HT-29 cell growth by kaempferol may be mediated, at least in part, by the decrease in ERK-1/2 activation.

In summary, we demonstrated that IGF-I and HRG-β stimulated DNA synthesis and inhibited apoptosis but did not abrogate the growth inhibitory effect of kaempferol. Kaempferol inhibited IGF-II secretion and HRG protein expression as well as growth factor-stimulated phosphorylation of Akt and ERK-1/2. These results suggest that the growth inhibitory effect of kaempferol may be mediated by decreasing IGF-I/IGF-IR and HRG-β/ErbB3, PI3K/Akt pathway and ERK-1/2 signaling. However, other pathways may also play roles that require an in-depth investigation. Based on these observations, future in vivo studies using animal tumor models are needed to examine whether kaempferol could be a useful chemopreventive agent against colon cancer.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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