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Apigenin induces apoptosis and impairs head and neck carcinomas EGFR/ErbB2 signaling

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1. ABSTRACT

The development of head and neck squamous cell carcinomas (HNSCCs) is a multistep process progressing from precancerous lesions to highly malignant tumors. A critical role in HNSCCs development and progression is played by EGFR family members including EGFR and ErbB2. The aim of this study was to investigate the effect of apigenin, a low molecular weight flavonoid contained in fruits and vegetables, on growth and survival and on EGFR/ErbB2 signaling in cell lines derived from HNSCCs of the tongue (CAL-27, SCC-15) or pharynx (FaDu). Using sulforhodamine B assay, FACS analysis and activated caspase-3 detection by immunofluorescence, we here demonstrate that apigenin dose-dependently inhibits survival and induces apoptosis of HNSCC cells. Further, by performing western blotting with antibodies specific for phosphorylated EGFR, ErbB2, Erk1/2 and Akt we demonstrate that apigenin reduces ligand-induced phosphorylation of EGFR and ErbB2 and impairs their downstream signaling. On the whole, our results suggest that apigenin properties might be exploited for chemoprevention and/or therapy of head and neck carcinomas.

2. INTRODUCTION

Apigenin (4',5,7,-trihydroxyflavone) is a low molecular weight flavonoid contained in fruits and vegetables such as orange, grapefruit, onion, parsley, basil, celery, tea leaf, licorice root, wheat sprouts (1, 2). Apigenin has been shown to possess anti-cancer properties due to its ability to inhibit the growth of a variety of human cancer cell lines *in vitro* including prostate, breast, lung, colon, ovarian, cervical, thyroid, liver, hematologic cancers and melanoma (1-22). Several reports have demonstrated that apigenin is able to modulate cell cycle progression in cancer cells (3, 5-8, 12, 16). Indeed, exposure of cancer cells to apigenin resulted in G0/G1 and G2/M arrest through suppression of cyclin B-associated cdc2 activity and phosphorylation of Rb, induction of the cdk inhibitors p21 and p27 and downregulation of cyclin D1, D3 and cdk4 (3, 5-8, 12, 16, 23). Moreover, inhibition of cell proliferation by apigenin was reported to involve inhibition of PI3K/Akt activity in prostate and breast cancer cells (4, 5, 8, 21), reduction of ELK-1 phosphorylation and c-FOS expression in prostate cancer cells (5), depletion of ErbB2/HER2/neu protein in breast cancer cells (8), impairment of mitogen activated protein (MAP) kinase

phosphorylation by Epidermal Growth Factor Receptor (EGFR) in thyroid cancer cells (15) and down-modulation of the constitutive expression of NF-kappaB/p65 in a prostate cancer cell line (16). In addition, apigenin was reported to trigger apoptosis of cancer cells through the activation of both the intrinsic and extrinsic apoptotic pathways (10-14). Accumulating evidence has also demonstrated that apigenin can inhibit motility and invasion of cancer cells by repressing FAK/Src signaling and tumor angiogenesis by repressing HIF-1 alpha and VEGF expression (17-20).

The incidence of head and neck squamous cell carcinomas (HNSCCs) is increasing worldwide and despite advances in treatment, the survival rate of patients with this type of cancer has not substantially changed over the last two decades (24). The development of HNSCCs is a multistep process progressing from precancerous lesions to highly malignant tumors (25, 26). A critical role in HNSCC development and progression is played by EGFR family members, including EGFR, ErbB2, ErbB3, and ErbB4 (27-29).

The aim of this study was to investigate the effect of apigenin on growth and survival and on EGFR/ ErbB2 signaling in tongue and pharynx cancer cells.

3.MATERIALS AND METHODS

3.1. Reagents

DMSO, apigenin, sulforhodamine B (SRB) and recombinant EGF were purchased from Sigma Aldrich (Milano, ITA). Antibodies against ERK1/2 (C-14), phospho-ERK (E-4), Akt1 (B-1) and phospho-AKT (Ser 473) were obtained from Santa Cruz Biotechnology (CA, USA). Antibodies against EGFR and phosphorylated EGFR (pY992, pY1045, and pY1068), ErbB2 and phosphorylated ErbB2 (pY1222) and anti-activated caspase-3 were purchased from Cell Signaling Technology (MA, USA). The goat anti-rabbit IgG Alexa fluor-594-conjugated secondary antibody was from Invitrogen (Milan, Italy) and the goat anti-mouse or -rabbit IgG peroxidase-conjugated secondary antibodies were from Sigma Aldrich.

3.2. Cell lines and treatments

Cell lines derived from HNSCCs of the tongue (CAL-27, SCC-15) or pharynx (FaDu) were maintained in RPMI containing 10% fetal bovine serum, 100 u/ml penicillin, and 100 µg/ml streptomycin. Cells were grown at 37°C in a humidified incubator with an atmosphere of 5% CO_2. Apigenin was dissolved in DMSO. For treatments, cells were incubated for the indicated times in the presence of apigenin (dose range 6-100 µM) or vehicle control (DMSO ≤0.1%).

3.3. Sulforhodamine B (SRB) cell proliferation assay

Cells were seeded at 4 x 10^3/well in 96-well plates and incubated at 37°C to allow cell attachment. After 24 hours, the medium was changed and the cells were treated with apigenin or DMSO and incubated for 48 hours. Cells were then fixed with cold trichloroacetic acid (final concentration 10%) for 1 hour at 4°C. After 4 washes with distilled water, the plates were air-dried and stained for 30 min with 0.4% (wt/vol) SRB in 1% acetic acid. After 4 washes with 1% acetic acid to remove the unbound dye, the plates were air-dried and cell-bound SRB was dissolved with 200 µl/well of 10 mM unbuffered Tris base solution, pH 10. The optical density (O.D.) of the samples was determined at 540 nm using a spectrophotometric plate reader. The percentage survival of the cultures treated with apigenin or DMSO was calculated by normalization of their O.D. values to those of untreated control cultures (30). The experiments were performed in triplicate and repeated three times. The concentrations of apigenin required to reduce cell survival by 50% (IC_{50}) were calculated from dose-response data using GraphPad Prism software.

3.4. FACS analysis

Asynchronized log-phase growing cells (60% confluent, about 2.5 x 10^5/well in 6-well plates) were treated with apigenin or DMSO in complete culture medium. After 48 hours, adherent as well as suspended cells were harvested, centrifuged at 1500 rpm for 10 min and washed twice with cold phosphate buffered saline (PBS). Cell pellets were resuspended in 70% ethanol for 1 hour at −20°C. Cells were then washed twice with cold PBS, centrifuged at 1500 rpm for 10 min, incubated for 1 hour in the dark with propidium iodide (25 µg/ml final concentration in 0.1% citrate and 0.1% Triton) and analyzed by flow cytometry using a FACSCalibur cytometer running CellQuest software.

3.5. *In situ* detection of apoptosis

For *in situ* detection of programmed cell death, CAL-27 and FaDu cells were seeded at 5 × 10^3 cells/well in 8-well chamber-slides and, after 24 hours, treated with 50 µM of apigenin or vehicle control. After 48 hours, cells were fixed in 4% formaldehyde for 15 min, washed and incubated with an anti-activated caspase-3 polyclonal antibody for 1 hour. After additional washings the cells were labeled with a goat anti-rabbit IgG Alexa fluor-594-conjugated antibody for 30 min (31). After a third washing the cells were incubated with 0.1µg/ml Hoechst 33342 and mounted under a coverslip with glycerol. Cells treated for 24 hours with staurosporine 1 µM were used as positive control. The percentage of apoptotic cells was calculated by determining the ratio between the cells positive for activated caspase-3 and the total number of cells present in five randomly chosen microscopic fields. Cell counts were done in a blinded fashion.

3.6. Preparation of cell lysates and Western Blotting

About 1 x 10^6 FaDu cells were seeded in 100 mm tissue culture dishes 24 hours prior to the addition of 50 µM of apigenin or vehicle control. After 32 hours of treatment, the cells were starved for 16 hours in serum-free medium containing 0.2% bovine serum albumin in the presence of 50 µM of apigenin or DMSO and then either left unstimulated or stimulated for 2.5 or 5 minutes with 100 ng/ml of EGF. At the endpoint of the experiment, the cells were harvested, washed twice with cold PBS and lysed in RIPA lysis buffer (Triton X-100 1%, SDS 0.1%, NaCl 200 mM, Tris HCl 50 mM pH 7.5, PMSF 1 mM,

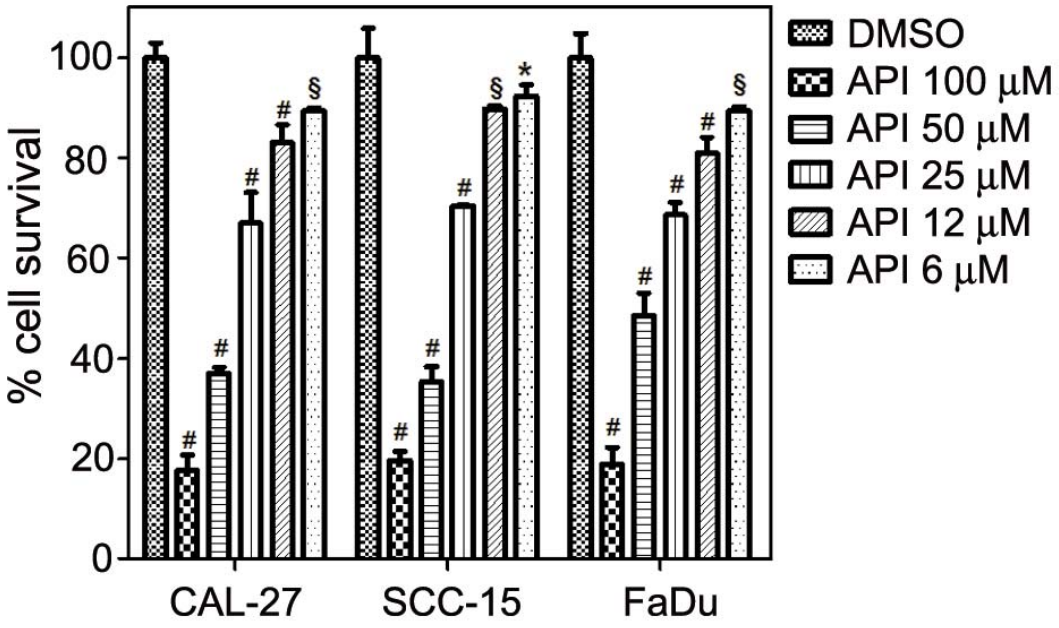

Figure 1. Effect of apigenin on HNSCC cell survival. Effects of apigenin (API) on survival of tongue (CAL-27 and SCC-15) and pharynx (FaDu) cancer cells as assessed by SRB assay. The percentage survival of the cultures treated with apigenin or DMSO as vehicle control was calculated by normalization of their O.D. values to those of untreated control cultures. Results reported are mean ± SD values from three experiments performed in triplicates. **#:** $p \leq 0.0001$; **§:** $p \leq 0.001$; *: $p \leq 0.05$.

NaOV 1mM). After 30 minutes at 4°C, the mixtures were centrifuged at 12,000 g for 15 minutes and the supernatants were analyzed by western blotting (32, 33).

For immunoblot analysis, 50 μg of cell lysates were resolved in 10% SDS-PAGE and then transferred to nitrocellulose membranes. After blocking, the membranes were incubated with specific primary antibodies at the concentration of 1-2 μg/ml overnight at 4°C. After washing, the filters were incubated with goat anti-mouse or -rabbit IgG peroxidase-conjugated antibodies and developed by chemiluminescence as previously described (32, 33). Densitometric analysis of autoradiographic bands was performed using the Image J software (National Institutes of Health, USA) after blot scanning.

3.7. Statistical analysis

Data distribution of cell survival and FACS analyses were preliminarily verified by the Kolmogorov-Smirnov test, and data sets were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test. Differences were regarded as significant when p value was less than 0.05. Differences in number of apoptotic cells were evaluated by a two-tailed t-test.

4. RESULTS

4.1. Apigenin inhibits survival of HNSCC cells

Survival of tongue (CAL-27 and SCC-15) and pharynx (FaDu) cancer cells was quantified after exposure to increasing doses of apigenin (6 to 100 μM) or vehicle control for 48 hours. Apigenin decreased cell survival to similar extents in the three cell lines, while vehicle control treatments had no effect (Figure 1). The effect of apigenin was dose-dependent and reached statistical significance at all tested doses. In particular, at 100 μM apigenin, survival of CAL-27, SCC-15 and FaDu cells decreased up to 90%, 93% and 90% respectively. The concentrations of apigenin required to reduce cell survival by 50% (IC_{50}) were 36.4 ± 1.0 μM, 38.3 ± 1.0 μM and 41.6 ± 1.1 μM for CAL-27, SCC-15 and FaDu.

4.2. Apigenin induces apoptosis of HNSCC cells

The inhibition of survival observed in apigenin-treated HNSCC cells could be due to both reduced proliferation and increased cell death. Therefore, in order to evaluate the effect of apigenin on apoptosis and cell cycle distribution of HNSCC cells, we performed FACS analysis of DNA content. Figure 2 shows a representative experiment in which the effects of increasing doses of apigenin are compared to those obtained with DMSO vehicle only. The mean results of three independent experiments are reported in Table 1. On the whole, our results demonstrate that in all three cell lines apigenin induced a dose-dependent increase in the percentage of cells in sub G0, meaning cells with a hypodiploid DNA content typical of apoptosis. Such effect was associated with a decrease of the percentage of cells in G0/G1 and G2/M.

To further corroborate the effect of apigenin on apoptosis, CAL-27 and FaDu cells were labeled with an anti-activated caspase-3 polyclonal antibody after treatment with apigenin (50 μM) or DMSO for 48 hours or, as

Table 1. Effects of apigenin on the cell cycle progression in HNSCC

		mean[1]	***p***	**mean**	***p***	**mean**	***p***	**mean**	***p***
CAL-27	DMSO	0,685		58,530		28,720		11,790	
	API 100[2]	53,115	*3*	24,045	*3*	23,085		0,540	*3*
	API 50	23,740	*4*	49,470	*4*	26,560		2,700	*3*
	API 25	3,610		49,080	*4*	37,240		9,815	
	API 12	1,245		55,595		28,985		13,280	
	API 6	0,913		51,440		35,465		10,085	
SCC-15	DMSO	1,175		70,625		19,375		11,495	
	API 100	58,360	*3*	24,765	*3*	15,355		1,555	*3*
	API 50	33,880	*3*	46,460	*5*	13,420		6,550	*5*
	API 25	3,750		71,365		16,825		8,070	
	API 12	1,745		61,080		31,380	*5*	6,470	
	API 6	2,210		56,430		31,125	*5*	8,710	
FaDu	DMSO	3,070		62,360		24,925		10,175	
	API 100	57,860	*3*	27,375	*3*	13,055		1,650	*3*
	API 50	20,680	*3*	47,420	*4*	24,985		6,815	
	API 25	4,105	*5*	48,610	*4*	30,044		18,060	
	API 12	2,585		54,540		31,015		11,500	
	API 6	1,365		53,675		34,065		10,285	

[1] Percentage of cells in sub G0, G0/G_1, S and G2/M phase were calculated using CellQuest software. The data are representative of three experiments. [2]API= Apigenin, doses of apigenin are in µM. *3*: p≤0.0001; *4*: p≤0.001; *5*: p≤0.05.

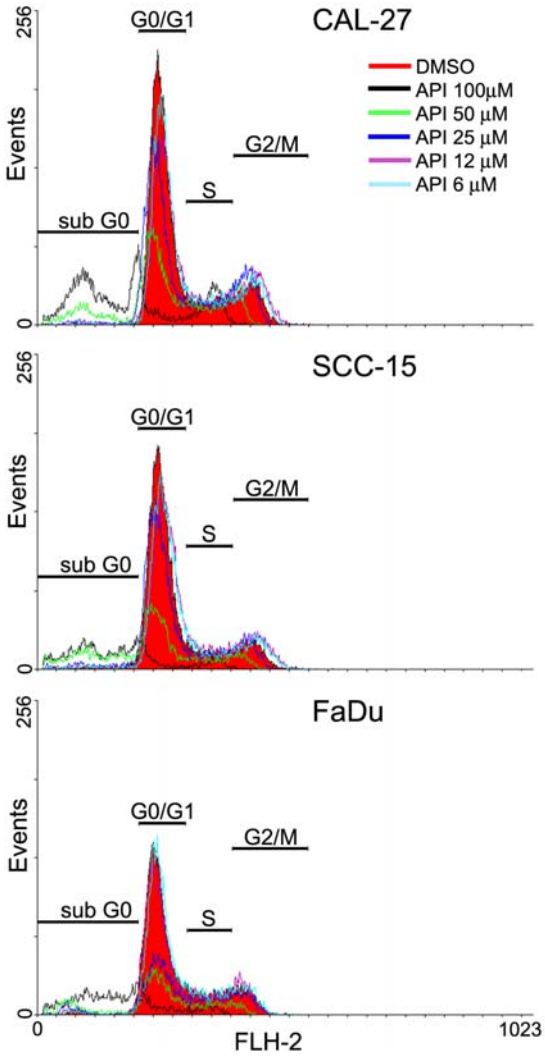

Figure 2. Effects of apigenin on apoptosis and cell cycle distribution of HNSCC cells. Apigenin (API) induced the increase of the sub G0 peak and the decrease of G0/G1 and G2/M peaks in a dose-dependent manner in asynchronized log-phase growing HNSCC.

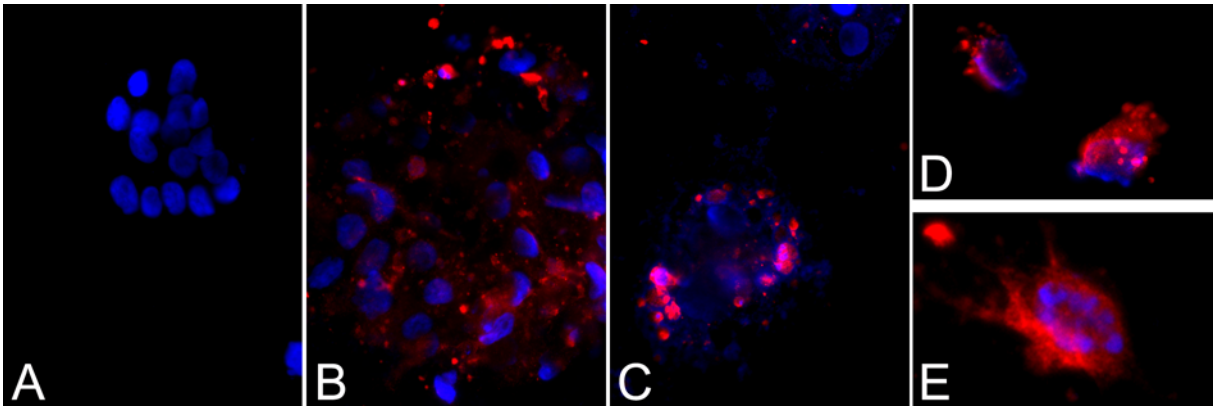

Figure 3. *In situ* detection of apoptosis. Induction of apoptosis by apigenin in FaDu cells as assessed by immunolabeling with an anti-activated-caspase 3 polyclonal antibody: A) vehicle control, B) staurosporine (1 μM, 24 h), C), D) and E) apigenin (50 μM, 48 h). A, B and C, original magnification x200. D and E, original magnification x500. Nuclei were counterstained with Hoechst.

positive control, with staurosporine (1 μM) for 24 hours. Figure 3 shows representative pictures of immunolabeled FaDu cells. According to activated caspase 3 positivity, the treatment with DMSO had no relevant effect on the induction of apoptosis in both CAL-27 (2.0%) and FaDu (2.6%) cells. In comparison, the percentage of apoptotic cells was 31% (p=0.0002) and 34% (p=0.0025) for apigenin-treated CAL-27 and FaDu, respectively, while treatment with staurosporine resulted in about 90% of apoptosis in either cell line.

4.3. Apigenin reduces ligand-induced phosphorylation of EGFR and ErbB2 and impairs their signaling

EGFR and ErbB2 play a critical role in HNSCCs development (27-29). ErbB receptors dimerization triggers their kinase activity leading to the phosphorylation of specific residues of tyrosine located in the receptors' C-terminal domain, ultimately resulting in cell proliferation, inhibition of apoptosis, enhanced migration, invasion and angiogenesis (34, 35). Therefore, we investigated whether apigenin could modulate the phosphorylation status of EGFR and ErbB2 in unstimulated or EGF-stimulated HNSCC cells. To this end, FaDu cells were serum starved and treated with either DMSO vehicle or apigenin 50 μM for 48 hours prior to stimulation with 100 ng/ml of EGF. FaDu cell lysates were then analyzed by western blotting using antibodies specific for the phosphorylated Y992, Y1045 and Y1068 of EGFR and Y1222 of ErbB2. Phosphorylation of Y992 and Y1045 of EGFR mediates recruitment of PLCγ and Cbl, respectively, while phosphorylation of Y1068 of EGFR and Y1222 of ErbB2 is essential for activation of Ras/Raf/Erk1/2 through the recruitment of Shc and Grb2 (27, 34, 35). Following serum starvation, FaDu cells grown in the presence of either DMSO or apigenin did not exhibit detectable levels of phosphorylation of EGFR and ErbB2 at the tyrosine residues recognized by our antibodies (Figure 4A). EGF stimulation of FaDu cells grown in DMSO induced after 2.5-5 minutes a strong phosphorylation at Y1045 and Y1068 of EGFR and at Y1222 of ErbB2, while phosphorylation at Y992 of EGFR was detected, albeit very weakly, only after 5 minutes (Figure 4A). By comparison, FaDu cells grown in apigenin and stimulated with EGF showed consistently lower levels of EGFR and ErbB2 phosphorylation at all tyrosine residues analyzed. Of note, apigenin treatment induced a reduction of both amplitude and duration of ligand-induced EGFR/ErbB2 phosphorylation (Figure 4A).

Next, we investigated the effect of apigenin on the activation status of signal transducers downstream of EGFR and ErbB2, namely Erk1/2 and AKT. Our results indicate that treatment of FaDu cells with apigenin, but not with the vehicle DMSO, caused a consistent reduction of AKT phosphorylation and a sustained reduction of Erk1/2 phosphorylation upon EGF stimulation (Figure 4B).

5. DISCUSSION

In this study we provide evidence that apigenin reduces cell survival and induces apoptosis of tongue and pharynx cancer cells. In addition we demonstrate that apigenin downregulates the phosphorylation of EGFR and ErbB2 at specific residues involved in activation of Ras/Raf/Erk1/2 and Akt (34-36) and, accordingly, reduces activation of Akt and Erk1/2 after EGF stimulation of HNSCC cells.

Different studies have shown a tight association between EGFR and ErbB2 overexpression and HNSCCs genesis and progression (27-29). In addition, clinical evidence indicates that the expression levels of EGFR and ErbB2 may be linked to chemotherapy and radiotherapy resistance, the major pathways involved in such resistance including Ras and/or Raf and Akt (27). Akt promotes cell survival and resistance to apoptosis by sequestering different protein targets, including the FOXO family of forkhead transcription factor and the pro-apoptotic protein Bad, as well as by activating the pro-survival transcription factor NF-κB (27). Persistent activation of Akt was also found to be frequent in human HNSCCs (37). Further, it was demonstrated that activated ERK1/2 are significantly

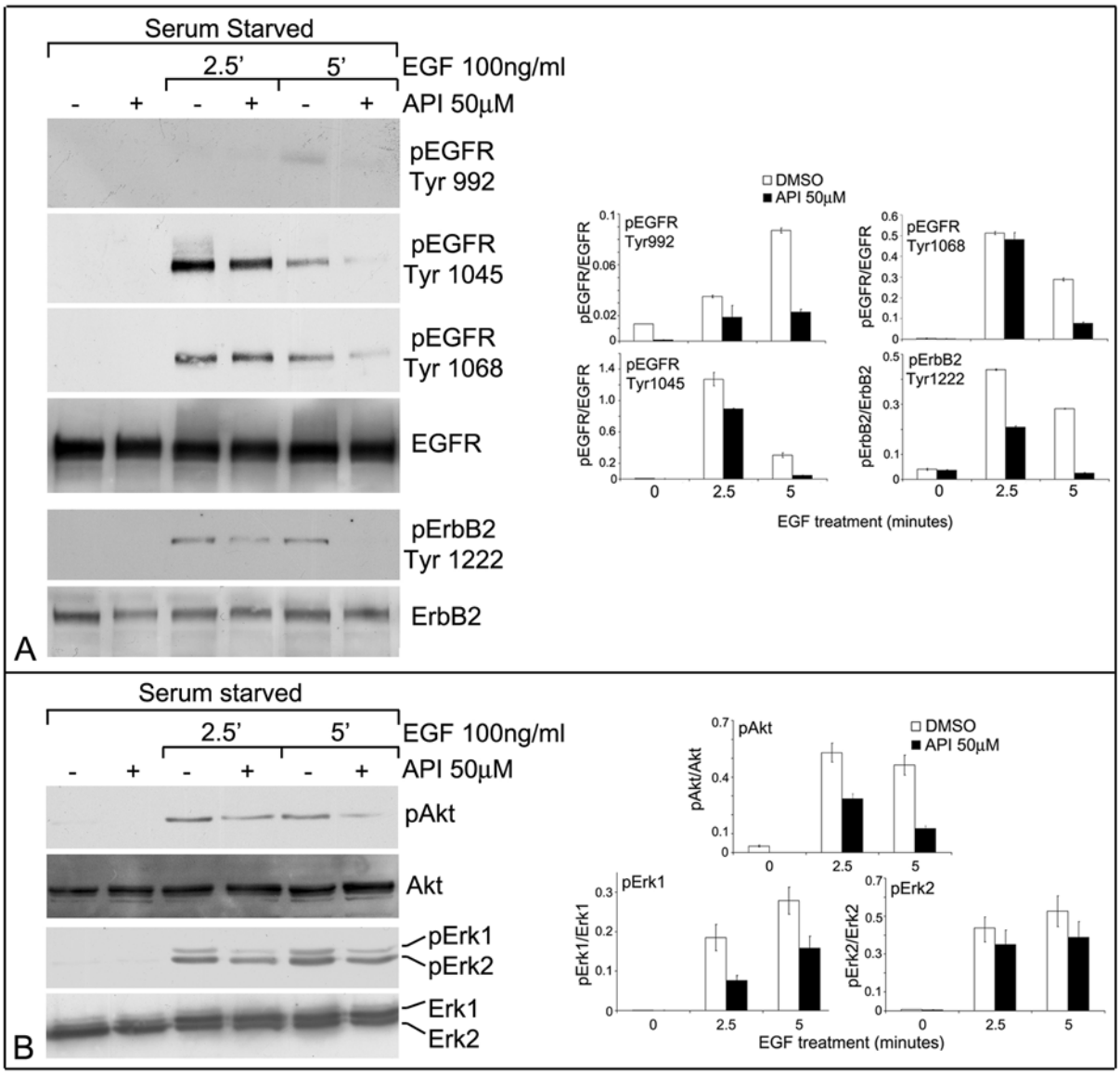

Figure 4. Downregulation of EGFR and ErbB2 phosphorylation and impairment of their downstream signaling. FaDu cells were treated for 32 hours with apigenin (50 μM) or DMSO, serum-starved for 16 hours and then stimulated for 2.5-5 minutes with 100 ng/ml of EGF in the continuous presence of apigenin or DMSO. Western blotting was then performed using: (A, left panel) antibodies specific for phosphorylated Y992, Y1045 and Y1068 of EGFR and Y1222 of ErbB2; anti-EGFR and anti-ErbB2 were used to determine total expression levels of the receptors; (B, left panel) antibodies specific for phospho-Akt and phospho-Erk1/2; anti Akt and anti-Erk1/2 were used to determine the total expression levels of each protein. The intensity of the bands obtained in two independent experiments was quantified using the ImageJ software after blot scanning, and densitometric ratios between the phosphorylated and total levels of each protein were reported in histograms (A and B, right panels).

higher in tongue squamous cell carcinomas than in normal tongue tissues and are significantly associated with moderately or poorly differentiated grade carcinomas (38).

According to epidemiological and case-control studies, the consumption of plant flavonoids is inversely correlated with the development of cancer (39-43). Our results demonstrate that apigenin has promising chemopreventive and potentially therapeutic properties for head and neck carcinomas. For instance, similar to anti-EGFR/ErbB2 antibodies and small kinase inhibitors which have been shown to increase susceptibility of cancer cells to radio- or chemo-therapy, apigenin may act as a radiosensitizing or chemosensitizing agent by impairing EGFR/ErbB2 signaling in HNSCCs.

A further support to the potential of apigenin as an anti-cancer agent is provided by the evidence that apigenin treatment resulted in a significant decrease in cell viability of human prostate adenocarcinoma cells as

compared to normal or virally transformed human prostate epithelial cells (44). Still, *in vivo* preclinical studies employing animal models are needed to further corroborate these *in vitro* findings and to solve problems related to apigenin solubility and bioavailability. Indeed, the use of apigenin in its pure form is prevented by its instability and limited solubility in water or organic solvents (2). On the other hand, apigenin exhibits a higher elimination half-time in blood compared to other flavonoids and the slow pharmacokinetics might favor its accumulation in target tissues (2). Remarkably, 5-Fluorouracil (5-FU) buccal tablets have demonstrated their usefulness for locoregional chemotherapy of oral squamous cell carcinoma on reconstituted human oral epithelium and porcine buccal mucosa (45). Similarly, local delivery of apigenin might be envisaged for the treatment of tumors occurring in accessible sites such as the oral cavity. In this respect, our results and the recent finding that the combination with apigenin enhances the anticancer activity of 5-FU on breast cancer cells (46) provide the basis for evaluating the therapeutic potential of apigenin-5-FU-based combination regimens for locoregional treatments of oral carcinomas.

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6. ACKNOWLEDGEMENTS

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