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Involvement of K^+-Cl^--Cotransport in the Apigenin-Induced Generation of Reactive Oxygen Species in IMR-32 Human Neuroblastoma Cells

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Abstract – Apigenin, a natural flavonoid found in a variety of vegetables and fruits, has been shown to possess many biological functions. In this study we investigated the role of apigenin in the production of reactive oxygen species (ROS) through the modulation of activity of K^+-Cl^--cotransport (KCC) in IMR-32 human neuroblastoma cells. Apigenin induced Cl^--dependent K^+ efflux, a hallmark of KCC activity, which was markedly prevented by different kinds of KCC inhibitors (calyculin-A, genistein and $BaCl_2$). These results indicate that KCC is functionally present, and activated by apigenin in the IMR-32 cells. Treatment with apigenin also induced a sustained increase in the level of intracellular ROS. The KCC inhibitors also significantly inhibited the apigenin-induced ROS generation. Taken together, these results suggest that apigenin can modulate ROS generation through the activation of a membrane ion transporter, KCC. These results further suggest that the alteration of KCC activity may play a role in the mechanism of degenerative diseases and/or carcinogenesis in neuronal tissues through the regulation of ROS production.

Key words □ K^+-Cl^--cotransport, Reactive oxygen species, Apigenin, IMR-32 cells

INTRODUCTION

Apigenin is a natural flavonoid found in high amounts in parsley, peppermint, lemon, perilla, berries, and fruits (Peterson and Dwyer, 1998). It has been shown to possess anti-inflammatory and anti-carcinogenic effects for skin, and free radical scavenging properties in many *in vitro* systems (Kim *et al.*, 1988). Studies have shown that apigenin possesses growth inhibitory properties against many human cancer cell lines, for examples, breast, colon (Wang *et al.*, 2000), skin (Caltagirone *et al.*, 2000), thyroid, leukemia cells, and solid malignant tumor cells (Fotsis *et al.*, 1998).

Flavonoids including apigenin, can protect against oxidative stress by scavenging reactive oxygen species (ROS) (Hanasaki *et al.*, 1994; Hu *et al.*, 1995; Jovanovic *et al.*, 2000). Since oxidative damage to biomolecules, such as DNA, proteins and polyunsaturated fatty acids, is thought to cause cancer and aging, considerable attention has been focused on the development of antioxidants to treat diseases associated with oxidative stress. However, there is also evidence that flavonoids are mutagenic and carcinogenic in both bacterial and mammalian experimental systems (Ochiai *et al.*, 1984; Pamukcu *et al.*, 1980; Rueff *et al.*, 1986).

Although K^+-Cl^--cotransport (KCC) has been first described in red blood cells as a swelling-activated K^+ efflux mechanism (Cossins and Gibson, 1997; Lauf *et al.*, 1992), functional and physiological evidence has also shown for the existence of KCC in epithelia (Amlal *et al.*, 1994; Greger and Schlatter, 1983), endothelium (Perry and O'Neill, 1993), vascular smooth muscle (Adragna *et al.*, 2000), heart (Yan *et al.*, 1996), skeletal muscle (Weil-Maslansky *et al.*, 1994), and neurons (Rivera *et al.*, 1999). Many studies have shown that KCC has been implicated in regulatory volume decrease (Lauf *et al.*, 1992), transepithelial salt absorption (Amlal *et al.*, 1994), myocardial K^+ loss during ischemia (Yan *et al.*, 1996), regulation of neuronal Cl^- concentration (Rivera *et al.*, 1999), and renal K^+ secretion (Ellison *et al.*, 1985). In addition, our recent reports have suggested that KCC is an essential mediator for the induction of apoptotic cell death in human hepatic cancer cells (Kim *et al.*, 2001). Human cervical carcinogenesis appears to be accompanied by up-regulation of KCC transcripts (Shen *et al.*, 2000). Interestingly, the activity of KCC has been associated with the

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generation of ROS in human cancer cells (Kim and Lee, 2001).

Thus, the main purposes of this study were to investigate (i) whether apigenin regulates the activity of KCC in a human neuroblastoma cell line and (ii) whether ROS production can be influenced by the apigenin-induced changes in KCC activity.

MATERIALS AND METHODS

Materials

The IMR-32 human neuroblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). The powders for Eagle's minimum essential medium (EMEM), trypsin solution, sodium pyruvate, calyculin-A, genistein, apigenin and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). Potassium-binding benzofuran isophthalate aceoxylmethyl ester (PBFI/AM), *N*-(6-methoxyquinolyl) acetoethyl ester (MQAE) and 2',7'-dichlorofluorescin diacetate (DCFH-DA) were from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). The stock solutions of drugs were sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

IMR-32 cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in an EMEM supplemented with 10% FBS, 200 IU/ml penicillin, 200 μg/ml of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

Measurement of intracellular K^+ concentrations

Intracellular K^+ levels were monitored with the K^+-sensitive fluorescent dye, PBFI/AM (Minta and Tsien, 1989). Cells were washed, and resuspended at a density of 4×10^5 cells/ml in Krebs-Ringer buffer. The cells were loaded with 5 μM PBFI/AM in Krebs-Ringer buffer containing 0.02% pluronic F-127, a nonionic surfactant, for 2 hr at 37°C. Unloaded dye was removed by centrifugation at 150×*g* for 3 min. The dual-wavelength excitation method for measurement of PBFI fluorescence was used. Fluorescence was monitored at 500 nm with excitation wavelengths of 340 and 380 nm in a stirred quartz cuvette. In the results relative changes in intracellular K^+ concentration were reported as the 340:380 fluorescence ratios.

Measurement of intracellular Cl^- concentrations

Relative changes in intracellular Cl^- concentration in the IMR-32 cells were monitored using the Cl^--sensitive indicator MQAE, developed by Verkman and colleagues (Verkman *et al.*, 1989). Experiments were performed, as described by West and Molloy (1996). Briefly, cells were washed twice and resuspended at a concentration of 4×10^5 cells/ml in Hank's solution. For loading MQAE into the cells, cells were incubated with the dye overnight at a final concentration of 5 mM at room temperature. Fluorescence (excitation wavelength set at 365 nm and the emission wavelength at 450 nm) was monitored in a well-stirred cuvette. Experiments were performed at room temperature to minimize fluorescent dye loss. Data are presented as relative fluorescence F_0/F, where F_0 is the fluorescence without Cl^- ions and F is the fluorescence as a function of time. The F_0/F values are directly proportional to intracellular Cl^- concentration (Shumaker *et al.*, 1999). All fluorescence values were corrected for background fluorescence which was separately determined using a HEPES-buffered KSCN solution containing 5 μM valinomycin to maximally quench the MQAE ion-selective signal (Shumaker *et al.*, 1999). In separate experiments the F_0 value was determined by bathing the cells with Cl^--free KNO_3 solution containing 10 μM tributyltin and 10 μM nigericin (Shumaker *et al.*, 1999).

Intracellular ROS measurement

Relative changes in intracellular ROS in the IMR-32 cells were monitored using a fluorescent probe, DCFH-DA (LaBel *et al.*, 1992). DCFH-DA diffuses through the cell membrane readily and is hydrolyzed by intracellular esterases to nonfluorescent 2',7'-dichlorofluorescin (DCFH), which is then rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly (Shen *et al.*, 1996). Cells were washed twice and resuspended at a concentration of 4×10^5 cells/ml in Hank's solution. For loading DCFH-DA into the cells, cells were incubated with the dye for 2 hr at a final concentration of 5 μM at 37°C. Fluorescence (excitation wavelength set at 485 nm and the emission wavelength at 530 nm) was monitored in a well-stirred cuvette.

Data analysis

All experiments were performed four times. Data were expressed as mean±standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and

Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered statistically significant.

RESULTS

Activation of KCC by apigenin in IMR-32 cells

To identify whether apigenin activates KCC in IMR-32 cells, we examined the effect of apigenin on Cl^--dependent K^+ efflux which is a hallmark of the existence of KCC (Cossins and Gibson, 1997; Lauf *et al.*, 1992). Apigenin rapidly decreased intracellular Cl^- concentration in a dose-dependent manner, as shown in Fig. 1. This apigenin-induced Cl^- efflux was significantly prevented by treatment with KCC inhibitors, such as 25 nM calyculin-A (Kaji and Tsukitani, 1991), 200 μM genistein (Weaver and Cossins, 1996), and 10 mM $BaCl_2$ (Mercado *et al.*, 2000), as depicted in Fig. 2. Additionally, apigenin induced a slow and sustained decrease in intracellular K^+ concentration which was significantly prevented by treatment with these KCC inhibitors, as shown in Fig. 3. This apigenin-induced K^+ efflux was tightly associated with extracellular Cl^- concentration, since the condition using Cl^--free buffer almost completely inhibited the apigenin-induced K^+ efflux (Fig. 3). These results strongly indicate that apigenin positively regulates the activity of KCC in the IMR-32 cells.

KCC mediates the apigenin-induced ROS generation in IMR-32 cells

To determine that activation of KCC by apigenin may be associated with its ability to generate ROS in IMR-32 cells, we investigated (i) whether apigenin generates ROS measured by

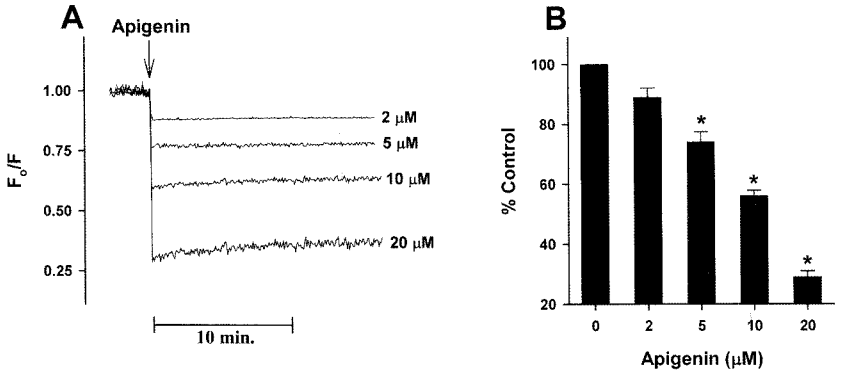

Fig. 1. Apigenin induces Cl^- efflux in a dose-dependent manner in IMR-32 human neuroblastoma cells. The data (A) show changes in intracellular Cl^- concentration as a function of time, measured by using the Cl^--sensitive indicator MQAE. In the data, F_o/F values are directly proportional to intracellular Cl^- concentration. In figure (A) the arrow shows the time point for addition of apigenin at a designated concentration. Quantitative changes (B) were expressed as percent changes of F_o/F value induced by apigenin compared to control condition in which the cells were treated with a drug-free vehicle. Each column represents the mean value of four replications with bars indicating SEM. *p<0.05 compared to control.

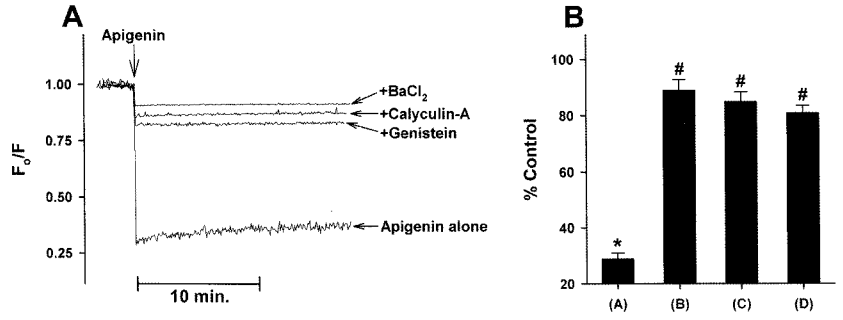

Fig. 2. The apigenin-induced Cl^- efflux is due to activation of KCC in IMR-32 human neuroblastoma cells. Data presentation is the same as Figure 1. In figure A, the arrow shows the time points for addition of apigenin (20 μM). In the figure B, (A), (B), (C) and (D) represent apigenin alone, 10 mM $BaCl_2$, 25 nM calyculin-A and 200 μM genistein, respectively. These KCC inhibitors were added 10 min before apigenin treatment. In the quantitative data each column represents the mean value of four replications with bars indicating SEM. *p<0.05 compared to control. #p<0.05 compared to apigenin alone.

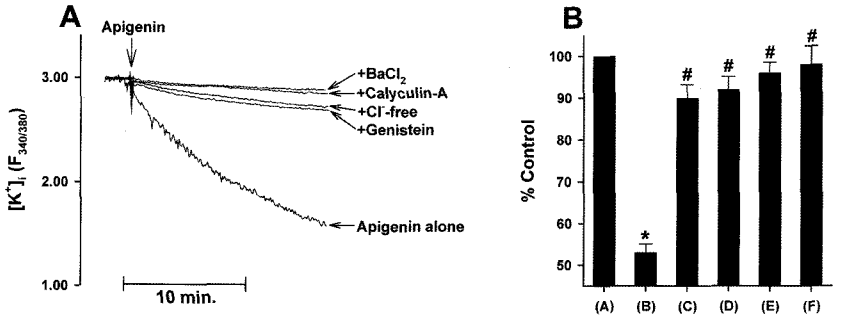

Fig. 3. Apigenin induces K^+ efflux through the activation of KCC in IMR-32 human neuroblastoma cells. The data show changes in intracellular K^+ concentration as a function of time, measured by using the K^+-sensitive fluorescent dye PBFI/AM. The arrow shows the time point for addition of apigenin (20 μM). KCC inhibitors (200 μM genistein, 25 nM calyculin-A and 10 mM $BaCl_2$) were added 10 min before apigenin treatment. For extracellular Cl^--free buffer solution, external Cl^- was replaced with gluconate. In the figure B, (A), (B), (C), (D), (E) and (F) represent control, apigenin alone, genistein, Cl^--free, calyculin-A and $BaCl_2$, respectively. In the quantitative data each column represents the mean value of four replications with bars indicating SEM. *$p<0.05$ compared to control. #$p<0.05$ compared to apigenin alone.

DCF fluorescence, and (ii) whether the KCC inhibitors can modulate the apigenin-induced ROS production. Apigenin (20 μM) induced a slow and sustained increase in intracellular ROS levels in the IMR-32 cells (Fig. 4). The KCC inhibitors, 25 nM calyculin-A, 200 μM genistein, and 10 mM $BaCl_2$ significantly inhibited the apigenin-induced ROS generation (Fig. 4). These results implicate that KCC may mediates the apigenin-induced ROS generation in IMR-32 cells.

DISCUSSION

KCC appears to respond to a variety of physiological stimuli in erythrocytes, including cell swelling, H^+ and urea (Hoffmann and Dunham, 1995; Lauf *et al.*, 1992). In normal high K^+-containing erythrocytes, KCC activation will result in net KCl efflux. KCC may contribute to cell shrinkage following swelling, and has therefore been implicated in regulatory volume decrease (Lee *et al.*, 2000). In addition to these physiological roles, inappropriate activation of KCC in red blood cells leads to excessive KCl loss, cell shrinkage and elevation of hemoglobin concentration, leading to deleterious rheological effects, including increased vascular resistance (Stuart and Ellory, 1988). KCC activity has, indeed, been shown to be inappropriately elevated in certain hemoglobinopathies (Joiner, 1993; Olivieri *et al.*, 1992). Therefore, KCC is important both physiologically and pathophysiologically, but regulatory mechanism of KCC is not much understood.

Excessively produced ROS may result in cellular damage

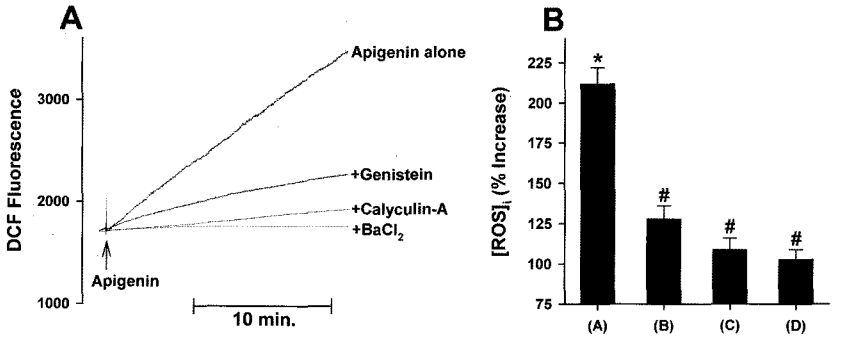

Fig. 4. Effects of KCC inhibitors on the ROS generation induced by apigenin in IMR-32 human neuroblastoma cells. The data (A) show changes in ROS levels as a function of time, which was measured by DCF fluorescence method. The arrow shows the time point for addition of apigenin (20 μM). KCC inhibitors (200 μM genistein, 25 nM calyculin-A and 10 mM $BaCl_2$) were added 10 min before apigenin treatment. In the figure B, (A), (B), (C) and (D) represent apigenin alone, genistein, calyculin-A and $BaCl_2$, respectively. Each column represents the mean value of four replications with bars indicating SEM. *$p<0.05$ compared to control. #$p<0.05$ compared to apigenin alone.

through their interaction with cellular macromolecules and structures (Yu, 1994). ROS have been shown to act as an important signaling molecule in the processes of apoptotic cell death (Bohler *et al.*, 2000), and cell differentiation (Suzukawa *et al.*, 2000). The mechanisms of these actions of ROS include activation of proteases and nucleases (Yu, 1994), altered gene expression (Schiaffonati and Tiberio, 1997), and changes in membrane permeability (Yu, 1994). Particularly, recent studies have implicated that ROS generation may be associated with KCC activity in red blood cells (Muzyamba *et al.*, 2000).

Although flavonoid compounds including apigenin, exhibit anti-inflammatory and antimutagenic properties (Choi *et al.*, 1994; Miyazawa and Hisama, 2003), they also induce genetic damage in a variety of prokaryotic and eukaryotic systems (Boos and Stopper, 2000; Silva *et al.*, 2000; Stopper *et al.*, 2005; Yamashita and Kawanishi, 2000). This inconsistency regarding the biological activity of flavonoids, especially as pro-oxidants resulting in DNA damage, has led many investigators to ascertain the exact nature of flavonoids (Snyder and Gillies, 2002). A number of studies provide evidence on dual biological activities of flavonoids in mutagenesis and carcinogenesis. The anti-mutagenic/pro-mutagenic and anti-oxidant/pro-oxidant activity largely depends upon the levels consumed as well as the physiological conditions of the body (Christine and Smith, 2000). Unfortunately, the potential toxic effects of excessive flavonoid intake are largely ignored.

Thus, the main aim of the present study was to investigate whether ROS production induced by apigenin is modulated by change in KCC activity in a human neuroblastoma cells. The results of the present study strongly suggest that KCC may be functionally present and activated by apigenin in IMR-32 cells, since apigenin induced Cl^--dependent K^+ efflux, a functional hallmark of KCC presence (Cossins and Gibson, 1997; Lauf *et al.*, 1992), which was significantly prevented by KCC inhibitors, calyculin-A, genistein and $BaCl_2$ (Figs. 1, 2 and 3). In addition, ROS production appear to require the apigenin-induced KCC activation, since apigenin markedly increased the level of ROS, and the KCC inhibitors completely antagonized the ROS production induced by apigenin (Fig. 4).

In conclusion, KCC is functionally present in a human neuroblastoma cell line, and ROS generation may be associated with the apigenin-induced KCC activation. These results further suggest that KCC may play a key role in the regulation of ROS production in neuronal tissues in which ROS are implicated in the mechanism of degenerative diseases (Floyd, 1999) and carcinogenesis (Storz, 2005).

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