The Inhibitory Effect of Baicalin on the Short-Term Food Intake in C57BL/6J Mice

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Abstract − Appetite is inhibited by the anorexigenic neuropeptides POMC (proopiomelanocortin) and CART (cocaine-amphetamine-regulated transcript) in the hypothalamus. The present study was performed to examine the inhibitory effects of baicalin against food intake and the upregulation of POMC/CART. Short-term food intake (48 h) was significantly inhibited by treatment with baicalin (10 mg/kg, $p < 0.05$) in C57BL/6 mice. Immunohistochemical analysis showed that baicalin upregulated POMC and CART levels in the arcuate nucleus of the hypothalamus. These effects were also examined using an *in vitro* system. pPOMC-Luc or pCART-Luc plasmids were transformed into mouse N29-2 neuronal and human SH-SY5Y cells, and the activities of baicalin were examined in these cells. Baicalin increased POMC and CART promoter-driven luciferase activity in a dose-dependent manner without cytotoxic effects. These results suggest that baicalin downregulates short-term food intake while upregulating POMC and CART expression.

Keywords: Baicalin, *Scutellaria baicalensis*, POMC, CART, Food intake

INTRODUCTION

Feeding is regulated strictly by appetite-related neuro-peptides, which are expressed in the feeding center in the hypothalamus (Schwartz *et al*., 2000; Morton *et al*., 2006; Murphy and Bloom, 2006). The main factors involved in control of feeding are the opposing controls between the orexigenic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons and the anorexigenic proopiomelano-cortin (POMC)/cocaine-amphetamine-regulated transcript (CART) neurons, which originate from the arcuate nucleus to the paraventricular nucleus (Kalra and Kalra, 2003; O'Rahilly and Yeo, 2004; Higuchi *et al*., 2006; Murphy and Bloom, 2006).

Among the anorexigenic neuropeptides, melanocortin is a series of peptides that are cleaved to POMC, the ligand of melanocortin receptors (Eberle, 2000). Melanocortin receptors (MCRs) belong to a superfamily of seven-trans-membrane G protein-coupled receptors, consisting of five subtypes, MC1R-MC5R (Gantz and Fong, 2003; Chai *et*

al., 2007). MC3 (MC3R) and MC4 (MC4R) expressed in the brain play important roles in maintaining body weight (Fan *et al*., 1997; Ollmann *et al*., 1997; Catania, 2008; Fu and van den Pol, 2008). MC4R is an established regulator of food intake and body weight, and blockade of this receptor causes obesity (Cone, 2000; O'Rahilly *et al*., 2004). These results indicate that MC4R signals play an important role in the regulation of food intake and obesity.

In contrast, excess ligand binding to these receptors can reduce obesity through reduced food consumption (Irani and Haskell-Luevano, 2005; Emmerson *et al*., 2007). Thus, modulators that promote POMC and CART expression are potential new lead compounds for controlling obesity (Vicentic and Jones, 2007; Coll and Loraine-Tung, 2009). Despite their potential, however, compounds that upregulate the expression of POMC and CART have not been explored in detail. To search for new lead compounds that up-regulate the expression of POMC and CART, more than 2,300 plant extracts were tested for their transcription promoting activity.

Baicalin is known to have multiple biological functions, including antibacterial (Huang *et al*., 2009), anti-inflammatory (Lin *et al*., 2008), antiviral (Li *et al*., 2000; Wang *et al*.,

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2004), and antitumor effects (Chan *et al*., 2000; Wang *et al*., 2008; Li-Weber, 2009). However, the relationships between food intake and the anorexigenic neuropeptides have not been reported.

In this study, we isolated baicalin from the root of *Scutellaria baicalensis* Georgi (Labiatae) using activity guided purification and examined its effects on anorexigenic neuropeptides POMC/CART and on feeding in C57BL/6 mice.

MATERIALS AND METHODS

Plant material

Roots of *Scutellaria baicalensis* Georgi were purchased from an herb market in Seoul. The plant was authenticated and deposited (#SJ-0143) at the Jung San Herbarium, Hwaeseong-City, Republic of Korea.

Purification of baicalin from the roots of *S. baicalensis*

The dried roots of *S. baicalensis* (1.2 kg) was finely powdered with a blender, extracted with 80% aqueous methanol (6 L) three times at 80°C for 4 h, and filtered (Whatman No. 2, 30 cm in diameter). The combined filtrate was concentrated in vacuum at 45°C and gave a yield of about 65% (based on the weight of the dried material). The extract (780 g) was sequentially partitioned into *n*-hexane (8 L×3), ethyl acetate (8 L×3), and water-soluble (8 L) portions for subsequent bioassay. The organic solvent- soluble fractions were concentrated to dryness by rotary evaporation at 40°C, and the water-soluble fraction was concentrated at 50°C.

Due to its potent activity, the water-soluble extracts (500 g) were subjected to open-column chromatography (Diaion HP-20, column size: 11×120 cm, resin wet volume 2 kg; Mitsubishi Chemical Co. Ltd., Tokyo, Japan) and eluted successively with a stepwise gradient of distilled water (DW)-methanol with increasing proportions of methanol. The respective fractions were evaporated to dryness.

The water-soluble fraction gave five subfractions (W1-W5). Subfraction W4 (about 26 g) isolated by removal of the solvent was chromatographed on a Lobar A column chromatography (low-pressure liquid chromatography: LPLC) and successively eluted with a stepwise gradient of water-methanol with increasing proportions of methanol. W4 gave eight subfractions (W41-W48). Subfraction W43 (9.3 g) was recrystallized in DW. Finally, the potent active principle 1 (W431, about 3.2 g) was obtained on recrystallization using DW. W431 was obtained as light yellow needles and had the molecular formula $C_{21}H_{18}O_{11}$ (Fig. 1) as determined by LC-EI-MS (m/z) and ^{1}H-NMR. The proton
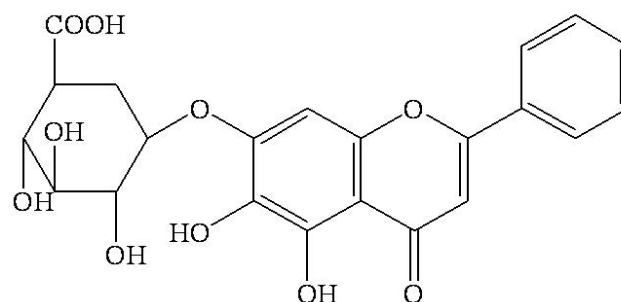
Baicalin

Fig. 1. Structure of baicalin.

signals and MS data of this compound were largely consistent with those reported by Sujuan *et al*. (2005).

Baicalin: Light yellow crystals, EI-MS *m/z*: 447 [M]$^+$, ^{1}H-NMR (DMSO-*d*6) δ: 12.60 (1H, s, −OH), 8.65 (1H, s, −OH), 8.07 (2H, m), 7.61 (3H, m), 7.04 (1H, s), 7.01 (1H, s).

Animal experimental procedure

Five-week-old male C57BL/6J mice (Daehan Biolink Co., Ltd., Seoul, Republic of Korea) were individually housed under a 12 h/12 h dark/light cycle (lights on from 06:00 to 18:00) at 22°C under specific pathogen-free conditions. Mice were fed a normal diet (Cargill Agri Purina Inc., Seoul, Republic of Korea) and were 6 weeks old at the time of the experiments. Mice were deprived of food for 24 h (from 15:00 to 15:00) and then injected (every 24 h) intraperitoneally with either baicalin (10 mg/kg body weight) or vehicle alone (olive oil). After intraperitoneal injection, mice were returned immediately to their home cages and presented a preweighed amount of food. Food intake was measured serially 0-3, 3-24, and 24-48 h after injection. All of the animal protocols used in this study were approved by the Committee for Animal Experiments of Jung San Bio Technology.

Immunocytochemistry

Briefly, at 48 h after baicalin administration, the mice were perfused transcardially under deep CO_2 anesthesia with physiological saline followed by formalin. After perfusion, their brains were removed, postfixed overnight with formalin at 4°C, and submerged in 30% sucrose at 4°C overnight; then, sections were cut at a thickness of 20 μm on a Leica Cryostat (Leica, Wetzlar, Germany). Sections were mounted on slide glasses and blocked with 5% fetal
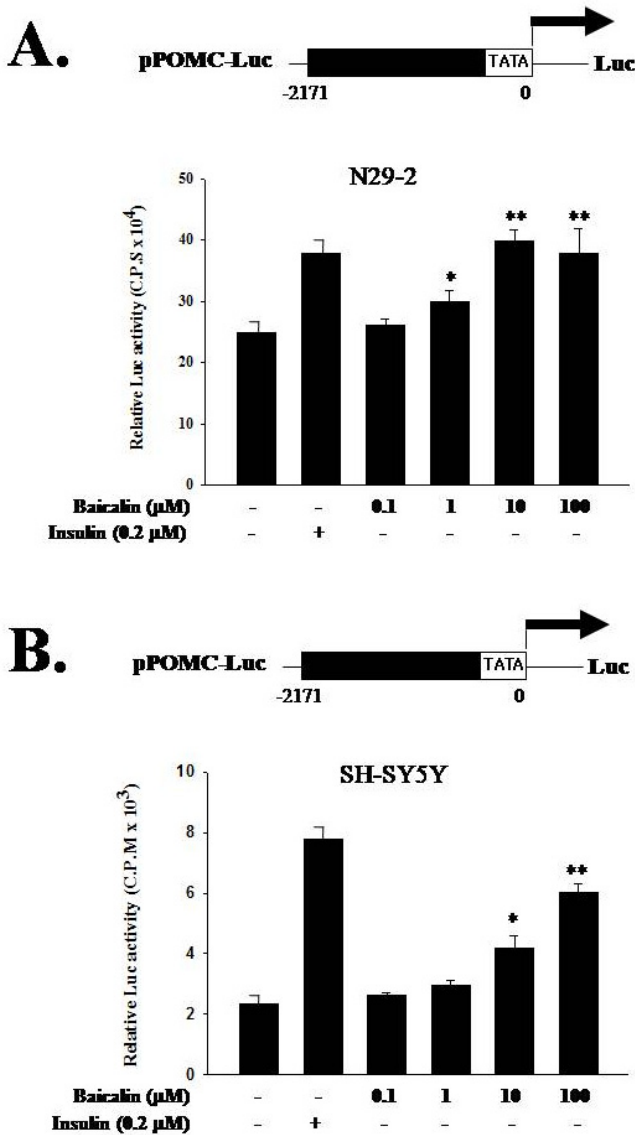
Fig. 2. Baicalin upregulated human POMC promoter [5'-flanking region of the human POMC (−2,171 k/b)] -driven luciferase activity in a dose-dependent manner. Luciferase activity was upregulated by treatment with baicalin in N29-2 cells (A) and SH-SY5Y cells (B). *$p < 0.05$, **$p < 0.01$.

Fig. 3. Baicalin was also involved in the expression of CART in N29-2 cells (A) and SH-SY5Y cells (B). pCART-Luc vector containing the 5'-flanking region of the human CART promoter (−2.0 k/b) region. In N29-2 (A) and SH-SY5Y (B) cells, level of CART-promoter induced luciferase expression by baicalin treatment. *$p < 0.05$, **$p < 0.01$.

bovine serum. Sections were then incubated with rabbit polyclonal anti-human POMC antibody (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and goat polyclonal anti-human CART antibody (1:200; Santa Cruz Biotechnology, Inc.) overnight at room temperature. Sections were washed with PBS and incubated for 1 h with biotin-conjugated mouse anti-rabbit (1:1,000; Santa Cruz Biotechnology, Inc.) and anti-goat secondary antibodies (1:1,000; Santa Cruz Biotechnology Inc.), and then treated with the avidin-biotin-peroxidase complex (ABC; Vector
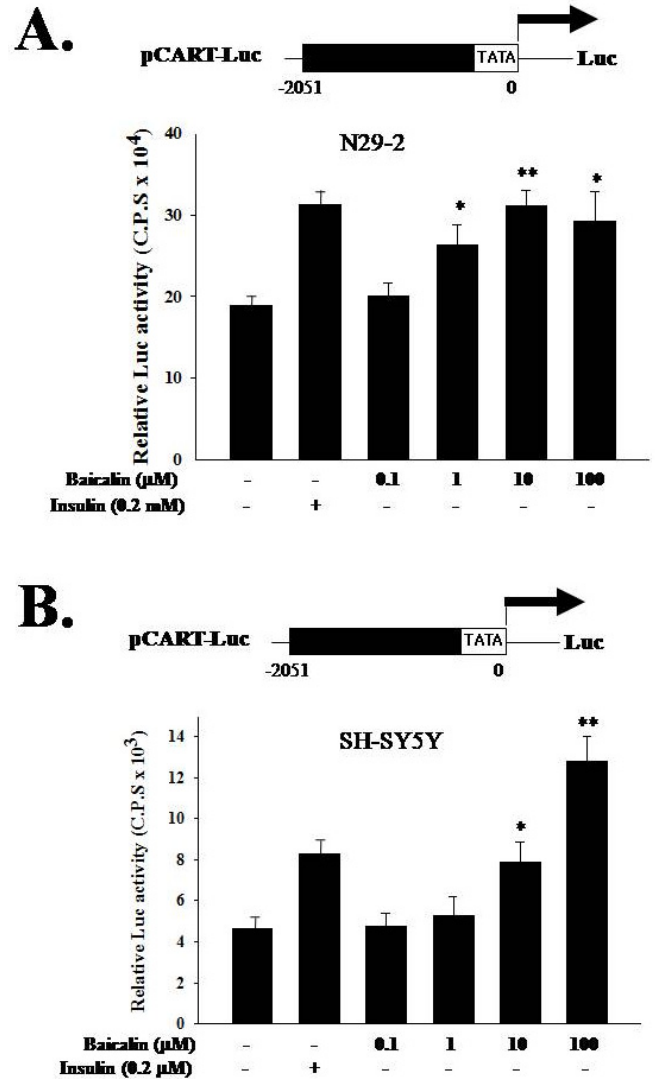
Laboratories, Burlingame, CA, USA). The slides were then detected with diaminobenzidine and hydrogen peroxide to visualize the peroxidase reaction. After stopping the reaction, the sections were mounted in mounting medium for further analysis.

Cell culture

Mouse N29-2 hypothalamic cells were purchased from Cellutions Biosystems (Toronto, ON, Canada). Human hepatoblastoma HepG2 cells and human neuroblastoma SH-SY5Y cells were obtained from the American Type
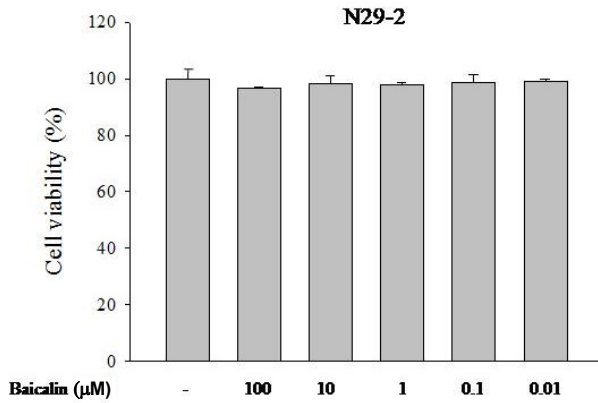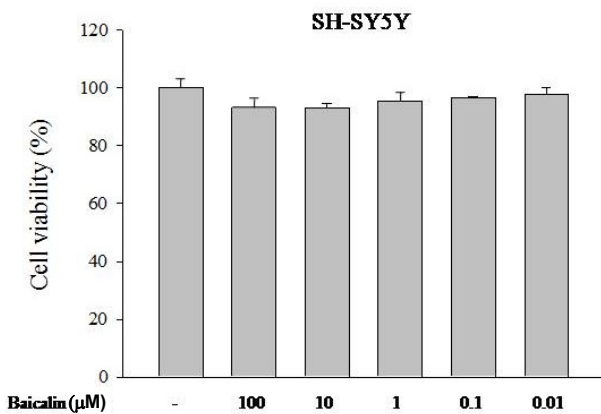
A.

B.

Fig. 4. MTT assay of baicalin. Baicalin does not have a cytotoxic effect in N29-2 or SH-SY5Y cells at concentrations of 0.01, 0.1, 1, 10, and 100 μM.

Culture Collection (ATCC No. CRL-2266). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (Lonza, Bucheon-Si, Gyeonggi-Do Republic of Korea) in the presence of antibiotics at 37°C with 5% CO_2 in air.

Construction of pPOMC-Luc and pCART-Luc vectors

For cloning of the pGL4.15-basic luciferase reporter vector, five proximal regions of the POMC and CART genes were generated by PCR using Taq DNA polymerase from HepG2 genomic DNA. The following primers were used for pPOMC-Luc: [Forward: 5'-CGG GGT ACC CGC CTA CCC AGT GAT TC-3', Reverse: 5'-ACTG AAG CTT CTG TCT CGG GAG GCT GCT C-3'] and for pCART-Luc [Forward: 5'-CGG GGT ACC GCT TAG AAT AGT TTA AAG ATC ACT GG-3', Reverse: 5'-ACT GGA GCT CGT TCT GAA ACT CGT CGT TG-3']. The amplified POMC DNA fragments were cloned into the *Kpn*I/*Hind*III sites of the pGL4.15-basic plasmid. The CART DNA fragment was cloned at the *Kpn*I/*Sac*I sites of the pGL4.15 vector.

Cell culture and luciferase assay

N-29-2 and SH-SY5Y cells were transfected using the FuGene6 Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Each 35-mm cell culture dish received equimolar amounts (2 μg) of reporter plasmids. After transfection for 8 h, the medium was replaced with fresh medium for 12 h. Cells were then selected by 2 μg/ml (final concentration) puromycin for 7 days, and 1×10^5 cells/well were seeded into 24-well plates for 12 h and then changed to serum-free media for 24 h. Baicalin (final concentration, 4.0-0.16 μM) was added. Six h after treatment with baicalin, cells were washed twice with phosphate buffered saline (PBS), followed by addition of 250 μl of lysis buffer (125 mM Tris, pH 7.8, 2 mM DTT, 1 mM EDTA, 10% glycerol, 1% Triton X-100) and incubated at room temperature for 10 min. The supernatant was collected after centrifugation of the crude lysate at 11,000$\times$ g for 2 min. Lysate (50 μl) was used for the luciferase assay using a VICTOR^{TM} light (PerkinElmer, Shelton, CT) according to the manufacturer's instructions. The amount of protein was calculated by Bradford assay, and insulin was included as a positive control. All experiments were repeated at least three times.

MTT assay for evaluating cell viability

MTT assay was performed 24 h after baicalin treatment. MTT solution (5 mg/ml) was prepared in PBS and filtered through a 0.2-μm filter. Then, the medium of each well was replaced with 50 μl of MTT plus 500 μl of DMEM without phenol red. Cells were incubated for an additional 4 h at 37°C with 5% CO_2, 95% air, 100% humidity. After 4 h, the MTT solution was removed and replaced with 250 μl of DMSO. The plates were further incubated for 5 min at room temperature, and the optical density (OD) of the wells was determined at a wavelength of 595 nm.

Statistic analysis

Data are expressed as means ± SE or SD. Differences between the values were considered significant at $p <$ 0.05. Body weight changes were analyzed by Student's *t*-test.
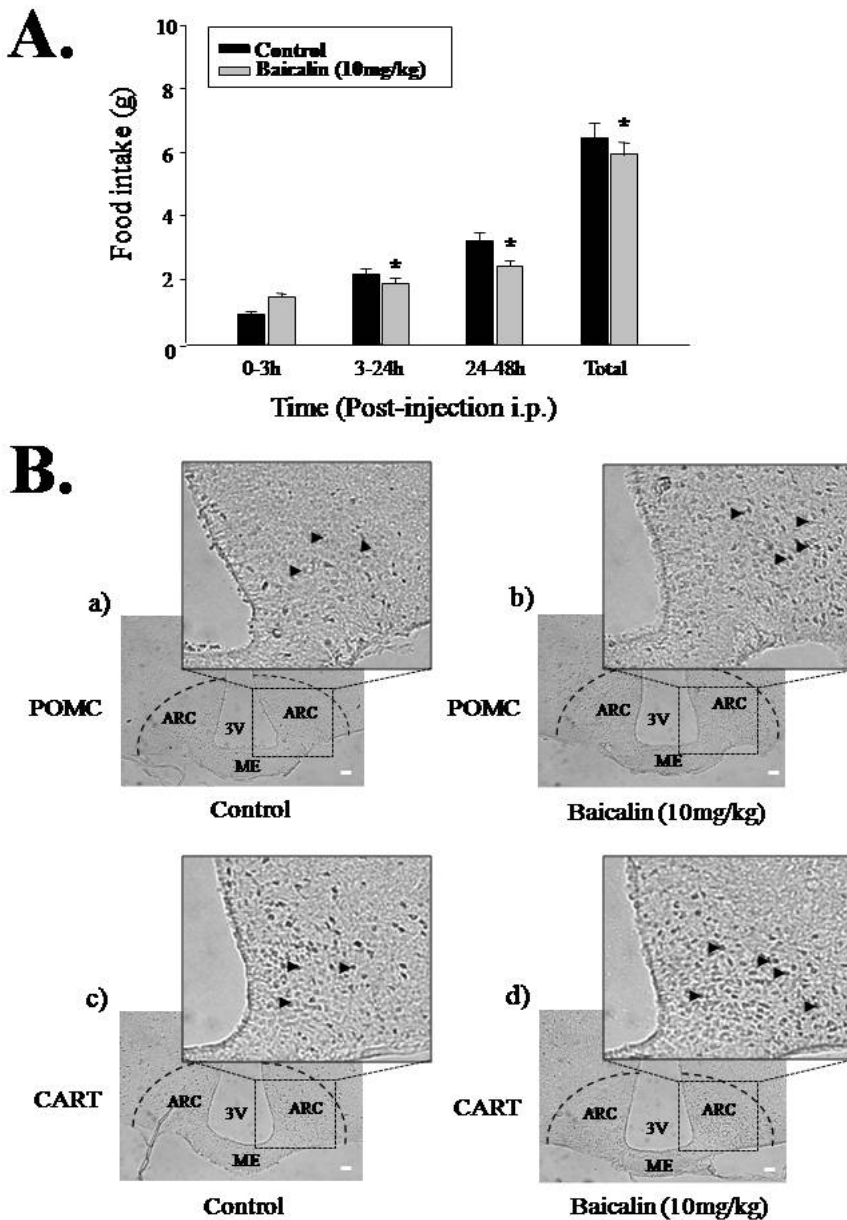
Fig. 5. Food intake of mice 3, 24, and 48 h after intraperitoneal (i.p.) injection of baicalin (10 mg/kg bw) and the expression of POMC and CART in the arcuate nucleus of the hypothalamus. (A) Note that the scale for the 0- to 3-h food intake values is expanded relative to the scale for the 3- to 24-h and 24- to 48-h values. Mice were fasted for 24 h before i.p. injection of baicalin (10 mg/kg). (B) Immunohistochemical analysis of POMC and CART in the arcuate nucleus of controls (a, c) and baicalin-treated (b, d) mice. Baicalin (10 mg/kg) considerably increased POMC and CART expression in the arcuate nucleus. All data are means ± SEM of 6 mice per group. ARC: arcuate nucleus, 3V: third ventricle, ME: median eminence, Scale bar: 100 μm, Arrows mean the POMC or CART positive cells. *p < 0.05 vs. CON group.

RESULTS

To search for active compounds that regulate the anorexigenic neuropeptides POMC and CART, we designed permanent cell assay systems using human POMC or CART promoter-driven luciferase (pPOMC-Luc and pCART-Luc, respectively) permanently transformed into N29-2 and SH-SY5Y cells (Fig. 2 and 3). Using these cell lines, about 2300 plant extracts were screened for their expression-promoting activity (data not shown), and the roots of *Scutellaria baicalensis* was selected for this study. Baicalin is a flavonoid isolated from the roots of *Scutellaria baicalensis* Georgi. To investigate whether baicalin leads

to stimulation of POMC and CART promoter activity, we tested human POMC (pPOMC-Luc) or CART (pCART-Luc) promoter-driven luciferase activity in both N29-2 and SH-SY5Y cells (Fig. 2 and 3). The 50% effective concentration (EC_{50}) of baicalin was 8.0 μM for POMC and 1.8 μM for CART in the N29-2 cells. Baicalin at 10 μM increased POMC promoter-driven luciferase activity by 164%, whereas it upregulated CART promoter-dependent luciferase activity by 168.4%. These luciferase activities were confirmed in SH-SY5Y cells to exclude the possibility that the promoter is nonfunctional in human cells. Treatment with baicalin (100 μM) induced significant increases in POMC and CART promoter-driven luciferase activities in

SH-SY5Y cells compared with controls. Baicalin did not have significant cell cytotoxicity in either cell line (Fig. 4). To confirm the effectiveness of baicalin against food intake, mice were deprived of food for 24 h before injection of baicalin (10 mg/kg) (Fig. 5A). No effects on food intake were observed at 3 h after intraperitoneal (i.p.) injection of either baicalin or vehicle control. Baicalin-treated mice showed significant reductions in food intake at 24 h (13%, $p < 0.05$) and 48 h (24%, $p < 0.05$) after i.p. injection compared with vehicle-treated controls.

To detect the upregulation of POMC and CART in the arcuate nucleus of the hypothalamus in the baicalin-administered mice, brain immunohistochemistry was examined to determine POMC and CART expression on brain sections (Fig. 5B). POMC and CART were detected in the arcuate nucleus of the hypothalamus in control mice. However, these molecules were considerably upregulated in the baicalin (10 mg/kg)-treated mice.

DISCUSSION

Baicalin is known to have many biological functions, including antimicrobial activities (Gao et al., 1999), antitumor effects (Chan et al., 2000; Wang et al., 2008; Li-Weber, 2009), anti-inflammatory (Lin et al., 2008), and antiviral activities (Li et al., 2000; Wang et al., 2004). However, the functional correlation between appetite inhibition and baicalin is currently unknown. pPOMC-Luc and pCART-Luc were expressed at considerable levels in both mouse N29-2 and human SH-SY5Y cells, but the baicalin treatment stimulated their expression levels significantly more than control levels in a dose-dependent manner (Fig. 2 and 3). Considering these effects of baicalin on anorexigenic neuropeptides, it was postulated that baicalin should affect the POMC/CART levels in the arcuate nucleus of the hypothalamus. In the CNS, POMC and CART are colocalized in the arcuate nucleus of the hypothalamus (Broberger, 1998; Elias, 1998; Koylu et al., 1998). The results of the present study indicated that neuronal cells treated with baicalin (10 mg/kg) showed elevated expression of POMC and CART in the arcuate nucleus of the hypothalamus (Fig. 5B). These results should be correlated with the short-term (48 h) reduced food intake in C57BL/6 mice, and they suggest that baicalin plays an important role in the upregulation of POMC and CART.

In summary, we demonstrated that baicalin decreased short-term food intake with the upregulation of POMC and CART in the arcuate nucleus of the hypothalamus. The patterns of anorexigenic neuropeptide gene expression were essentially similar in POMC and CART promoter-driven mouse and human neuronal cells. These observations suggested that the upregulation of POMC/CART in neuronal cells indicates a positive correlation with increased anorexigenic neuropeptide levels in the arcuate nucleus of the hypothalamus by baicalin. Although our results indicated that the effects of baicalin may not be particularly strong, it is considered a good supplement to reduce short-term food intake without cytotoxic effects. Further studies using models of prolonged food intake in a specific area of the hypothalamus may help us to understand the long-term effects of baicalin.

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