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Roles of *Rheum palmatum* in the regulation of food intake and neuropeptides expression

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Abstract

Hypothalamus is the center of regulating appetite and energy homeostasis. Neuropeptide Y (NPY) and Agouti related peptide (AgRP) are orexigenic neuropeptides, which are responsible for appetite stimulation. Pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) are anorexigenic neuropeptides. These neuropeptides are involved in satiety. Events of uncontrolled appetite can lead to metabolic diseases, such as anorexia and obesity. Despite the issues related to these metabolic diseases, effective treatment for neither anorexia nor obesity has been developed. Currently available treatments for anorexia and obesity can cause severe side effects. For this reason, natural compounds that can potentially regulate appetite were screened in this study. Natural compounds have vast chemical diversities and are an excellent source for developing new pharmaceuticals. Thus, the objective of this study is to discover natural compounds that can either stimulate or suppress appetite. Twenty natural compounds were selected from New Natural Material Bank. Out of twenty natural compounds, *Rheum palmatum* standized sample 1 (RPSS1) induced neuropeptide Y (NPY) expression. RPSS1 increased NPY expression by four folds in mHypoA-NPY/GFP cell line. However, food intake and body weight of the mice was reduced when RPSS1 was intracerebroventrically (icv) injected into the 3rd ventricle. In addition, there was no change in any of the neuropeptides mRNA expression in the hypothalamus. The *in vivo* results suggest that RPSS1 controls food intake by not regulating neuropeptides but by other mechanism in hypothalamus.

Keywords: hypothalamus, food intake, neuropeptides, *Rheum palmatum*

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1. INTRODUCTION

Energy homeostasis and food consumption are controlled in the hypothalamus of the brain. The arcuate nucleus of the hypothalamus contains two types of neurons that regulate food intake. Orexigenic neurons express neuropeptides called neuropeptide Y (NPY) and agouti-related protein (AgRP), which stimulate appetite [1-4]. Anorexigenic neurons, which express pro-opimelanocortin (POMC) and cocaine-and-amphetamine-regulated transcript (CART). These anorexigenic neurons are known to suppress appetite. Abolishing NPY and AgRP neurons in the brain reduced both food intake and body weight [3]. Regulating appetite is extremely important since uncontrolled appetite can lead to metabolic diseases. For example, prolonged appetite suppression can lead to anorexia.

There are various types of anorexia, however, anorexia nervosa is the most common eating disorder. A severe case of anorexia can lead to osteoporosis, malnutrition, stunned growth and abnormal development [5]. The cause of anorexia nervosa remains unknown as it seems to be various. A common feature of anorexia nervosa is that patients conceive the idea that their body is abnormally large. For this reason, anorexia nervosa patients restrain from eating. Since anorexia nervosa patients have misperception of their body shape, it is considered to be a psychiatric disorder [6]. There are numerous treatments available for anorexia nervosa; from residential hospitalization programs to outpatient care. Study done by Baran *et al* indicates limitations of anorexia treatments. In this study, eating habit, body weight and depression were observed in 22 anorexia patients during hospitalization and up to 29 months after discharge [7]. The results suggested that many anorexia patients were discharged without full recovery and short hospitalization often leads to re-hospitalization. This indicates that a short-term treatment for anorexia nervosa is not effective. Suggesting that many anorexia nervosa patients may require constant and long-term monitoring. Apart from nutritional re-

habilitation, many antidepressants, such as selective serotonin reuptake inhibitors, tricyclic antidepressants, antipsychotics, and antiepileptic drugs, were used on anorexia nervosa patients [6]. However, none of the drugs seem to have any effect on their body weight and appetite. Therefore, it is critical to develop an efficacious cure or treatment for anorexia nervosa patients.

On the other hand, if appetite is consistently stimulated, obesity could be promoted. In the United States, the incidence of obesity has dramatically increased since 1980's [8-10]. Obesity is a worldwide issue that can lead to various chronic diseases, such as cardiovascular diseases, diabetic mellitus, osteoarthritis and even cancer. Ongoing research is extensively trying to discover treatment for obesity. For example, current studies indicate that intracerebroventricular (icv) injection of unsaturated fatty acids into diet-induced obese (DIO) mice model reduced inflammation of hypothalamus, insulin resistance and adiposity [11, 12]. However, human clinical trial is incomplete. Therefore, potential for unsaturated fatty acids as obesity treatment remain debatable. In addition, it is known that serotonin plays a role in appetite regulation [12-14]. Short-term clinical administration of serotonergic drug, lorcaserin, reduced body weight significantly. However, when lorcaserin was administered over a long period of time, the result was contradictory. Participants did lose body weight after 1 to 2 year treatment [14, 15]. However, many did not participate completely to the trial, and lots of participants did not lose weight and some even gained. As stated earlier, much research for developing a cure for obesity is in progress. Thus, it is vital to develop a cure for obesity.

Regulating appetite is the key to finding a cure for anorexia and obesity. Many candidates can be selected to develop treatment for these metabolic diseases. However, natural compounds were selected in this research. Natural compounds have been used from ancient China and it is still being used in modern medicine and even throughout European and Western society. Natural compounds have enormous chemical diversity, and this makes natural compounds a good source for developing

new pharmaceuticals. Approximately half of drugs approved by Food and Drug Administration (FDA) are synthesized from natural compounds or its derivatives. Another characteristic of natural compounds is that it can react with various types of bioactive molecules [16]. Natural compounds can act as selective ligands in disease state. These ligands can react with target molecules and convert disease state into healthy one. Natural compounds are not only used for drug development, but used to study the targets and pathways involved in disease. Furthermore, new target can be identified with use of natural compounds [17]. The precise components and doses present in natural compounds can differ from one extract to another. This means that effects of natural compounds may not be consistent. Therefore there is controversy in modern science regarding the effect of natural compounds. Despite the controversy, many researchers rely on natural compounds to develop cures or treatments for a variety of diseases. One of many reasons for this is that natural compounds have fewer side effects than chemically synthesized pharmaceuticals [18].

Pharmaceutical companies use high throughput screening (HTS) for drug development [17, 19]. Enzyme or receptor-based assays are used to screen thousands of compounds at a time to uncover possible candidates. However, this poses a problem for natural compounds because a crude extract of natural compounds contain hundreds of various compounds. For example, tannins cause a major problem. Tannins can bind non-specifically to proteins. This characteristic of tannins produces faulty positive results. For this reason, natural compounds cannot be used in HTS, unless it is detanninized [19]. Isolating bioactive compounds from raw source is another problem when dealing with natural compounds [17, 19]. Natural compounds are usually available in small quantities [17]. Several to hundreds of grams are often required for preclinical development. Furthermore, kilograms of raw material are most likely to needed for clinical use. The amount of raw material required for lead compound extraction would vary on potency of the compound and its target [19]. This characteristic of natural compounds would delay the development process of pharmaceuticals [17]. However, this

can be overcome with synthetic chemistry [19].

Screening strategy was used to identify natural compounds that induced expression of NPY, which could have potential to stimulate appetite. 20 natural compounds were selected from New Natural Material Bank. Initial screening process indicated that six natural compounds induced NPY expression. Two natural compounds were selected from the six, since it induced NPY expression significantly. One natural compound is extracted from *Rheum palmatum* and another is *Eucommia ulmoides* extract. However, effect of selected natural compounds as appetite stimulant is debatable. Therefore, further study is required in order to confirm that these natural compounds can stimulate appetite. Extract of *R. palmatum* has anti-oxidant, anti-inflammatory and anti-cancer activities [20-23]. *E. ulmoides* extract has anti-oxidant effect, inhibits lipid accumulation in liver and is used for treating osteoarthritis [24-26].

These natural compounds have diverse characteristics and properties. However, research in appetite regulation of these natural compounds has not been approached yet. Therefore the objective of this research is to discover natural compounds that can regulate appetite. As stated above, discovering natural compounds that can stimulate and suppress appetite could be developed into treatment for anorexia and obesity, respectively. Initial screening indicated that some natural compounds induced NPY expression, suggesting potential to stimulate appetite. Therefore, we hypothesize that *R. palmatum* extracts will stimulate appetite via increasing NPY expression in the hypothalamus. Further investigation is required to confirm that extracts of *R. palmatum* can stimulate appetite, and other natural compounds that can suppress appetite must be discovered as well. Consequently, these natural compounds can be developed into treatment for anorexia and obesity.

2. MATERIAL AND METHODS

2.1 Natural compounds

All natural compounds were provided by New Natural Material Bank (Wonkyang University, Rep. of Korea) free of charge. Standized sample are fractions of crude extract. Natural compounds used in this study are listed as followed; NNMBS003 (*Pueraria lobata* standized sample 2), NNMBS026 (*Rubus coreanus* standized sample 1), NNMBS027 (*Rubus coreanus* standized sample 2), NNMBS039 (*Morus alba* standized sample 2), NNMBS058 (*Curcuma aromatic* standized sample 2), NNMBS095 (*Scutellaria baicalensis* standized sample 1), NNMBS096 (*Scutellaria baicalensis* standized sample 2), NNMBS100 (*Rheum palmatum* extract), NNMBS101 (*Rheum palmatum* standized sample 1), NNMBS102 (*Rheum palmatum* standized sample 2), NNMBS136 (*Lycium chinense* standized sample 1), NNMBS137 (*Lycium chinense* standized sample 2), NNMBS156 (*Citrus unshiu* extract) , NNMBS157 (*Citrus unshhiu* standized sample 1), NNMBS158 (*Citrus unshiu* standized sample 2), NNMBS170 (*Cinnamomum cassia* standized sample 1), NNMBS173 (*Eucommia ulmoides* standized sample 1), NNMBS208 (*Morinda offincinalis* standized sample 2), NNMBS230 (*Alpinia katsumadai* standized sample 2), NNMBS251 (*Cudrania tricuspidata* standized sample 2). All natural compounds were dissolved in dimethyl sulfoxide (DMSO) at concentration of 50 μg/ml and stored at -20 ^{o}C.

2.2 Cell line culture

mHypoA-NPY/GFP, N41 and N43/5 mouse hypothalamic cell lines were purchased from Cellutions Biosystems, Inc. Cell lines were maintained at recommended conditions. Cell lines were maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Cell lines were incubated at 37 ^{o}C with 5% CO_2 and moderate

humidity.

2.3 Animals

Six week old C57BL/6J male mice were purchased from Koatech, Inc. Four mice were housed in individually ventilated cage (ivc) at first. Three to four days later, mice were housed individually. Mice were housed in temperature of 22~24 ^{o}C, 45% (±5) humidity and light-dark (12:12-h) cycle. All mice had *ad libitum* access to distilled water and standard laboratory rodent chow. All animal experiments were approved by the Animal Care and Use Committee of Daegu Gyeungbuk Institute of Science and Technology.

2.4 Flow cytometry

Cells were prepared as described below. Media was removed and cells were washed with 800 μl of Dulbecco's Phosphate Buffered Saline (DPBS). DPBS was removed, and 200 μl of trypsin/EDTA was added and incubated for 5 min. 800 μl of DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin was added to each well. Cell suspension was collected and transferred to a 1.5 ml eppendorf-tube. Cell suspension was centrifuged at 3,000 rpm for 2 min. Supernatant was removed and re-suspended in 300 μl of DPBS containing 2% FBS. Cell suspension was transferred to 5 ml round-bottom tube. GFP intensity was measured with Gallios Flow cytometry (Beckman Coulter). A dot plot and histogram were analyzed with Kaluza software.

2.5 MTS assay

Cells were seeded in 96 well plate, including blank (BD Science). An equal amount of media was placed around the cells to prevent cells on the edge from drying. Cells were treated with *R. palmatum* and *E. ulmoides* extracts (50 and 100 μg /ml) 24 hours after seeding. 3 hour before the time point, 10

μl of cell titer blue was inserted to each well. 96 well plate was covered with a tin foil and incubated for further 3 hours. Fluorescence was measured with Synergy HT microplate reader (Bio Teck) at excitation wavelength of 560 nm and emission wavelength of 590 nm.

2.6 Quantitative RT-PCR

Total cellular RNA was isolated with Trizol reagent, based on the guanidinium thiocyanate-phenol-chloroform extraction method, following the manufacturer's instructions (Life Technologies). First strand cDNA was synthesized from 3 μg (1.5 μg for tissue sample) of RNA in a total volume of 20 μl using GoScript™ reverse transcription system (Promega). Quantitative RT-PCR reactions were performed with SYBR® premix *Ex Taq* II (Takara). Primers sequences are as follows: NPY forward 5' CAG AAA ACG CCC CCA GAA 3' and reverse 5' AAA AGT CGG GAG AAC AAG TTT CAT T 3'; AgRP forward 5' CTG CAG ACC GAG CAG AAG A 3' and reverse 5' TGC GAC TAC AGA GGT TCG TG 3'; POMC forward 5' GAA CAG CCC CTG ACT GAA AA 3' and reverse 5' ACG TTG GGG TAC ACC TTC AC 3'; CART forward 5' CGA GAA GAA GTA CGG CCA AGT CC 3' and reverse 5' GGA ATA TGG GAA CCG AAG GTG G 3'; GAPDH forward 5'ATC ACT GCC ACC CAG AAG AC 3' and reverse 5'ACA CAT TGG GGG TAG GAA CA 3'.

2.7 Cannualtion surgery

Mice were habituated in a cage for a week. Mice were anaesthetized with mixture of tiletamine/zolezepam, xylazine and saline (100ml/Kg). Anaesthetized mice were positioned to stereotaxic instrument with incisor bar and skull position was leveled. A hole was drilled into the skull on the midline and stainless steel 26 gauge guide cannula was inserted into the brain according to following coordinates (M/L: 0.0 mm, A/P: 1.8 mm, D/V: 5.0 mm for 3rd ventricle; M/L: 0.2 mm, A/P: 1.5 mm, D/V: 5.8 mm for arcuate nucleus). The cannula was secured with 3 micro screws and dental

cement. After the surgery, the mice were exposed to heat lamp to reduce them hyperthermia from anesthesia.

2.8 Intracerebroventicular injection

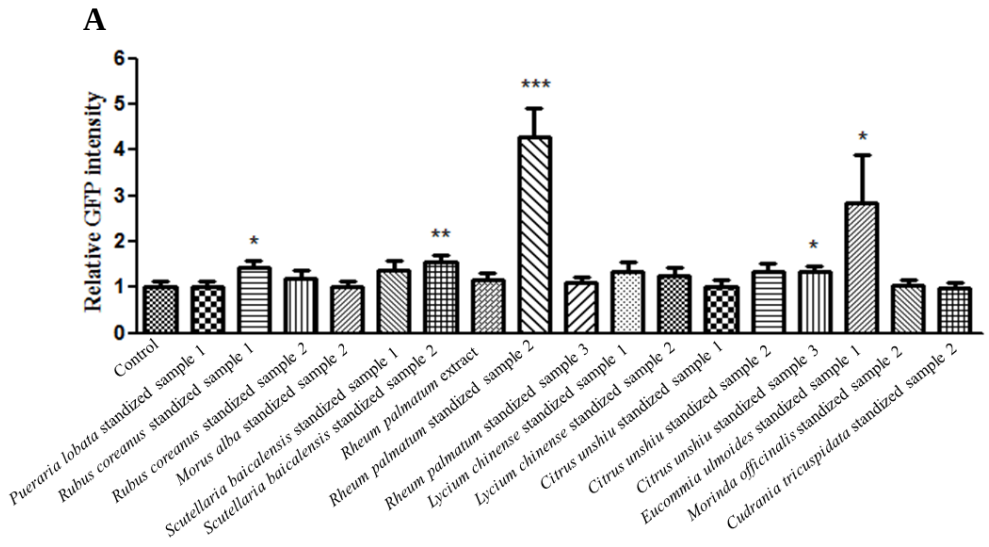
Cannula placement was confirmed with intracerebroventricular (icv) injection of 0.25 nmole NPY (Sigma). Food intake was measured for 1 hour after NPY injection. Icv injection was performed with microliter syringe (Hamilton) attached to PE-tubing and a stainless steel 33 gauge internal cannula. NPY injection was performed a week after cannulation surgery to allow mice to recover from the surgery. Mice that ate more than 0.4 g of chow were decided to have correct placement of cannula. Further icv injection was continued after confirming that food intake of mice has recovered. Icv injection of vehicle and *R. palmatum* extract was performed in following order. First, vehicle (mixture of DMSO and saline) was injected to all mice. Then food intake and body weight was measured for 3 days. *R. palmatum* extract was injected to all mice, and food intake and body weight was measured for 3 days. All icv injection was performed during the light cycle and ended just before the dark cycle.

2.9 Food intake and body weight measurement

Food intake and body weight of individual mice were measured at 6 pm of each day. For body weight measurement, a plastic bowl was placed on the scale and set to zero. Each mouse was placed in the plastic bowl and body weight was recorded. For measuring food intake, each mouse was provided with approximately 10 g of standard rodent chow. Weight of entire food rack and chow were measured on a scale and recorded. Any small chow pellets on the corn cob were collected and measured with rest of the chow. Small chow pellets were discarded after food intake measurement and replaced with new chow pellets.

3. RESULTS

Flow cytometry was performed to select natural compounds that could potentially stimulate appetite. For preliminary screening, mHypoA-NPY/GFP cell line was treated with 20 different natural compounds for 24 hours. Out of 20 natural compounds, only two induced NPY expression at a relatively high level (Fig. 1A). One of the natural compound is *R. palmatum* standized sample 1 (RPSS1) and the other is *E.ulmoides* standized sample 1 (EUSS1). RPSS1 induced NPY by four folds and EUSS1 induced NPY by three folds. Natural compounds that induced NPY expression most or significantly were selected from 20 natural compounds to execute secondary screening. Secondary screening further confirmed that RPSS1 and EUSS1 induced NPY expression (Fig. 1B). During preliminary screening, natural compounds were treated to NPY/GFP reporter cell line at dose of 100 μg/ml. Some natural compounds caused undesirable cytotoxicity, which was observed from naked eyes with light microscope (data not shown). Therefore 50 μg/ml was determined as maximum dose. Cytotoxicity of both RPSS1 and EUSS1 were performed prior to continuing further experiments. Extracts of RPSS1 and EUSS1 were treated to three different cell lines, mHypoA-NPY/GFP, N41 and N43/5 cell lines. Both RPSS1 and EUSS1 did not cause cytotoxicity in any of the cell lines (Fig. 2A, B and C).



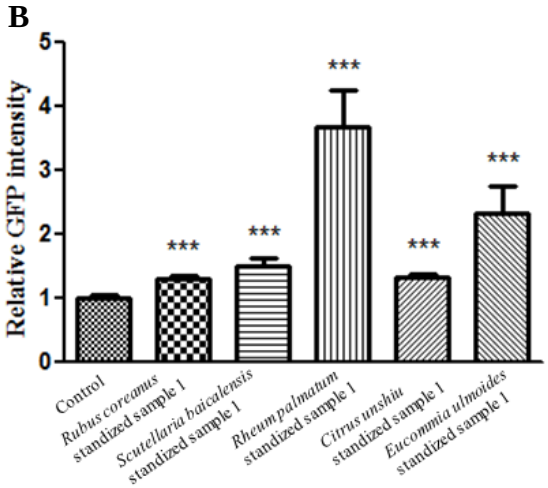
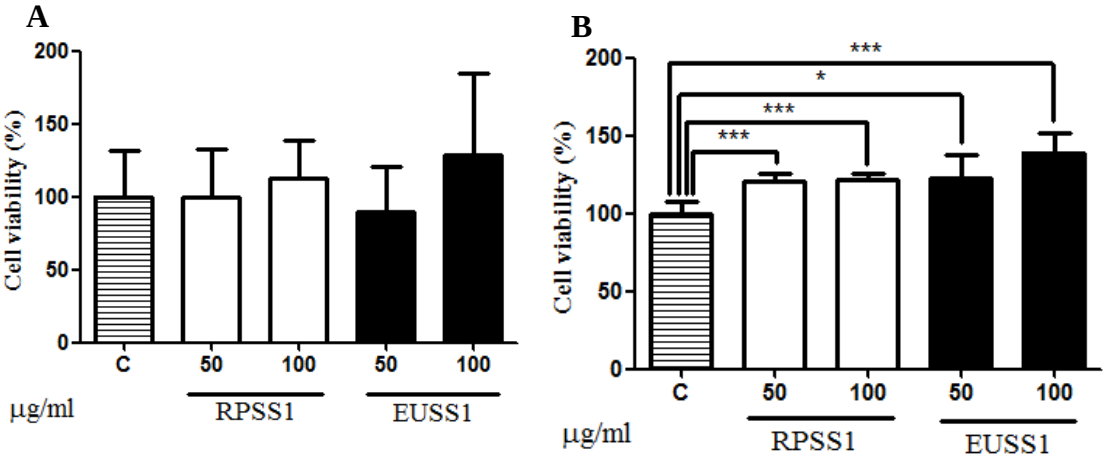


Fig. 1 Screening for natural compounds that induce NPY expression.
(A) mHypoA-NPY/GFP reporter cell line was treated with 50 μg/ml of 20 natural compounds for 24 hours (*n*=3). (B) mHypoA-NPY/GFP reporter cell line was treated with *Rubus coreanus* standized sample 1, *Scutellaria baicalensis* standized sample 1, *Rheum palmatum* standized sample 1, *Citrus unshiu* standized sample 1, *Eucommia ulmoides* standized sample 1 (50 μg/ml). GFP intensity was measured with flow cytometry (*n*=4). Results are means ± S.E.M., **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs control.



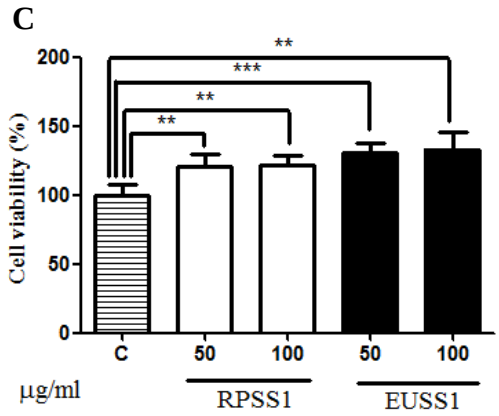


Fig. 2 Cytotoxicity of *Rheum palmatum* standized sample 1 (RPSS1) and *Eucommia ulmoides* standized sample 1(EUSS1).
Extracts of *Rheum palmatum* standized sample 1 (RPSS1) and *Eucommia ulmoides* standized sample 1 (EUSS1) were treated to (A) mHypoA-NPY/GFP, (B) N41 and (C) N43/5 cell lines for 24 hours (*n*=5). Results are means ± S.E.M., **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs control.

Consecutively, dose-dependent experiments were performed to discover optimum dose of RPSS1 and EUSS1. Dose-dependent experiments indicated that RPSS1 and EUSS1 induced NPY expression

highest at dose of 50 μg/ml (Fig. 3A). Then time-dependent experiments were performed to find optimum time of NPY induction. Result of time-dependent experiment showed that NPY expression was induced as early as 3 hours (Fig. 3B) and highest at 24 hours (Fig. 1). To confirm that RPSS1 and EUSS1 extracts can induce NPY expression in NPY producing neurons, these natural compounds were treated to N41 hypothalamic cell lines. Results of qRT-PCR suggest that RPSS1 induced NPY expression by nearly three folds and EUSS1 induced NPY expression by one and an half folds in hypothalamic cell lines (Fig. 4A). In addition, correlation of neuropeptides expression was one of the many interests. Therefore extracts of RPSS1 and EUSS1 were treated to POMC producing N43/5 hypothalamic cell lines. POMC mRNA expression did not change significantly after RPSS1 and EUSS1 treatment (Fig. 4B). Thus, extract of RPSS1 and EUSS1 induce orexigenic neuropeptide NPY and do not effect expression of anorexigenic neuropeptide POMC.

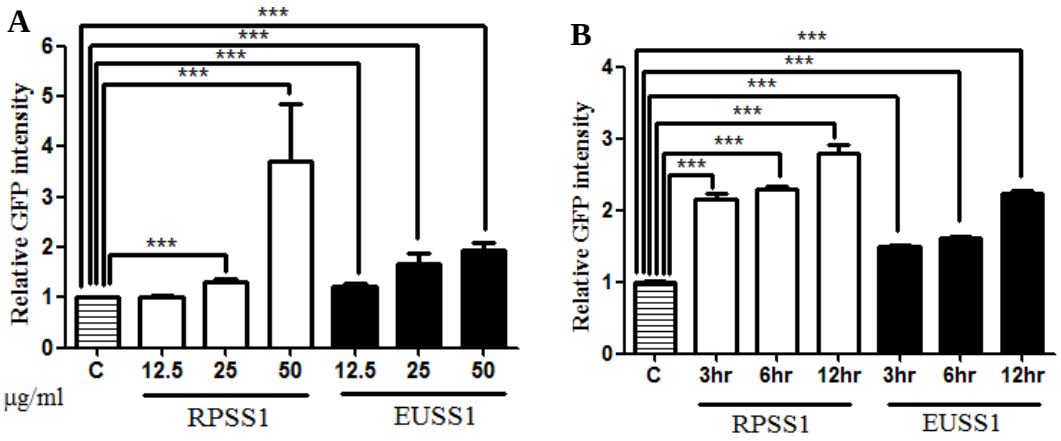


Fig. 3 Dose- and time-dependent effect of *Rheum palmatum* standized sample 1 (RPSS1) and *Eucommia ulmoides* standized sample 1 (EUSS1) on NPY expression.
(A) mHypoA-NPY/GFP cell line was treated with DMSO or 12.5, 25 and 50 μg/ml of *Rheum palmatum* standized sample 1 (RPSS1) and *Eucommia ulmoides* standized sample 1 (EUSS1) for 24 hours (*n*=4~5). (B) mHypoA-NPY/GFP cell line was treated with DMSO or *Rheum palmatum* standized sample 1 (RPSS1) and *Eucommia ulmoides* standized sample 1 (EUSS1, 50 μg/ml) for 3, 6 and 12 hours (*n*=4).

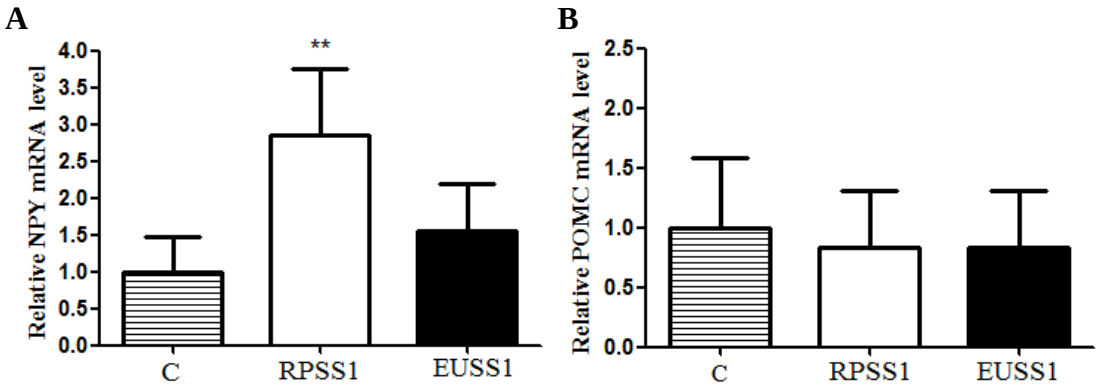


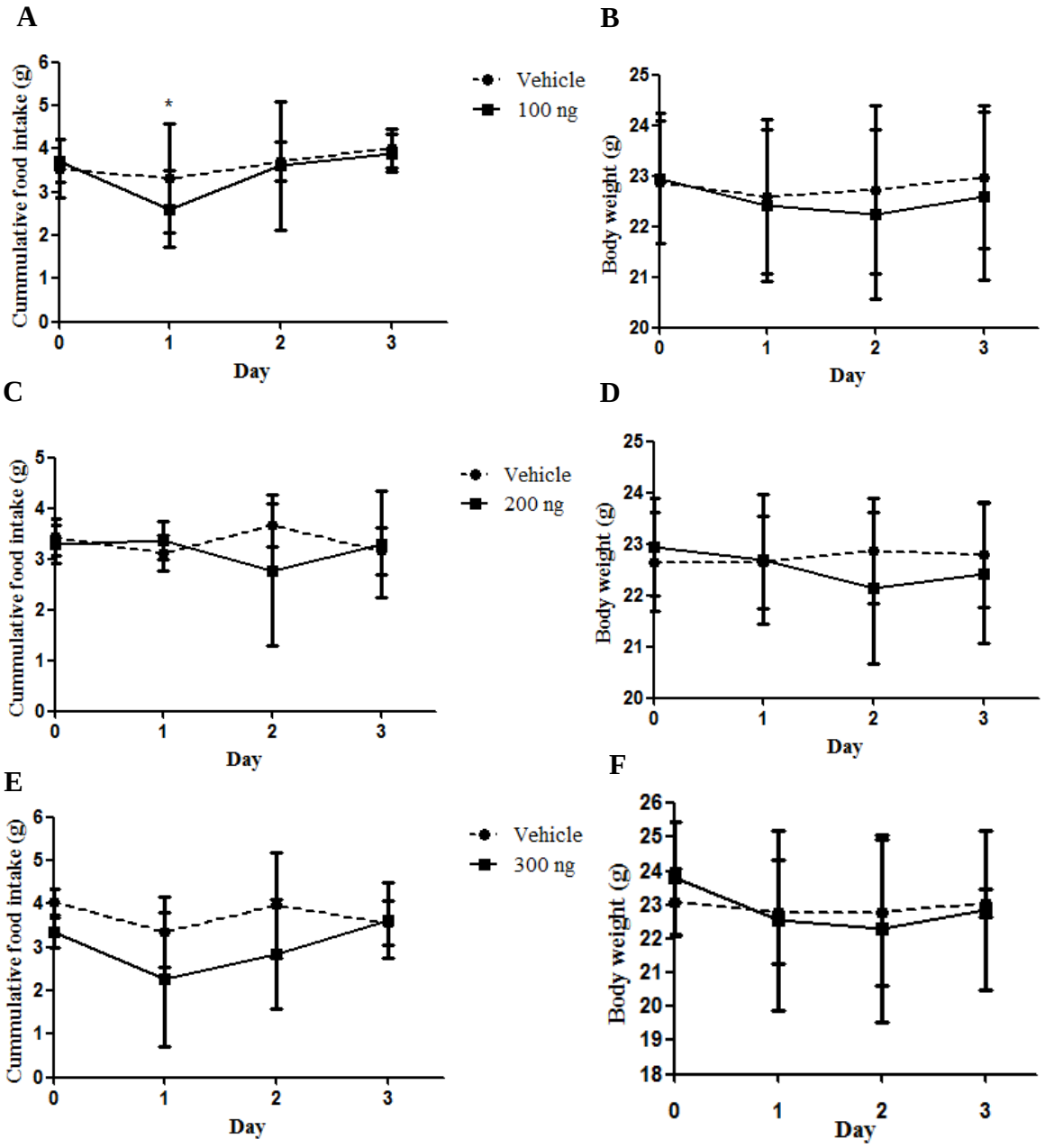
Fig. 4 *In vitro* effects of *Rheum palmatum* standized sample 1 (RPSS1) on neuropeptides expression. (A) N41 and (B) N43/5 cell lines were treated with *Rheum palmatum* standized sample 1(RPSS1, 50 μg/ml) for 24 hours (n=6~9). Results are means ± S.E.M., *P < 0.05, **P < 0.01, ***P < 0.001 vs control.

Further confirmation was required in order to state that RPSS1 and EUSS1 have potential to stimulate appetite. Results of FACS and qRT-PCR indicate that RPSS1 induced NPY expression at higher level than EUSS1. For this reason, RPSS1 was selected for *in vivo* experiment. Thus, RPSS1 was icv injected into 3rd ventricle. However, results of *in vivo* experiments were opposite to *in vitro*. Prior to RPSS1 injection NPY test was performed. Most of the mice responded to icv injection of NPY (Table 1). Amount of RPSS1 that is to be icv injected was decided based on *in vitro* experiments. Throughout this study, 50 μg/ml of RPSS1 was used for *in vitro* experiments. Total of 2 ul is used for icv injection. Therefore equivalent amount of RPSS1 to 50 μg/ml is 100 ng. For this reason, 100 ng of RPSS1 was used for first injection. Food intake was reduced by 29.2 % (in day 1) when 100 ng of RPSS1 was injected to mice (Fig. 5A and G). This was repeated with dose of 200 ng and 300 ng. Similar to 100 ng, food intake was reduced by 12.6 % (in day 2) when 200 ng of RPSS1 was injected (Fig. 5C and G) and by 28.6% (in day 1) when 300 ng of RPSS1 was injected (Fig. 5E and G). However, reduction in food intake did not show dose-dependency. Body weight of mice was reduced by 0.54 g (in day 1) and by 0.72 g (in day 2) when 100 ng of RPSS1 was injected. (Fig. 5B and H). For 200 ng of RPSS1 injection, body weight was reduced by 0.18 g (in day 1) and by 0.73 g (in day 2) (Fig. 5D and H). Body weight was reduced by 1.24 g (in day 1) and by 1.49 g (in day 2) when 300 ng of RPSS1 was injected (Fig. 5F and H). Both food intake and body weight were recovered from icv

injection of RPSS1.

1h food Intake (g)	1	2	3	4	5	6	7	8	9	10	11	12
	0.98	0.66	0.84	1.35	1.06	0.86	0.47	0.12	0.75	1.07	1.28	1.5

Table 1 NPY test for the 3rd ventricle cannulation.
2 µl of NPY (0.25 nmole) icv injected into the 3rd ventricle. Food intake was measured for 1 hour. Numbers on the top row represents each individual mouse and number on the bottom row represent amount of chow eaten by each mouse for an hour after icv injection of NPY.



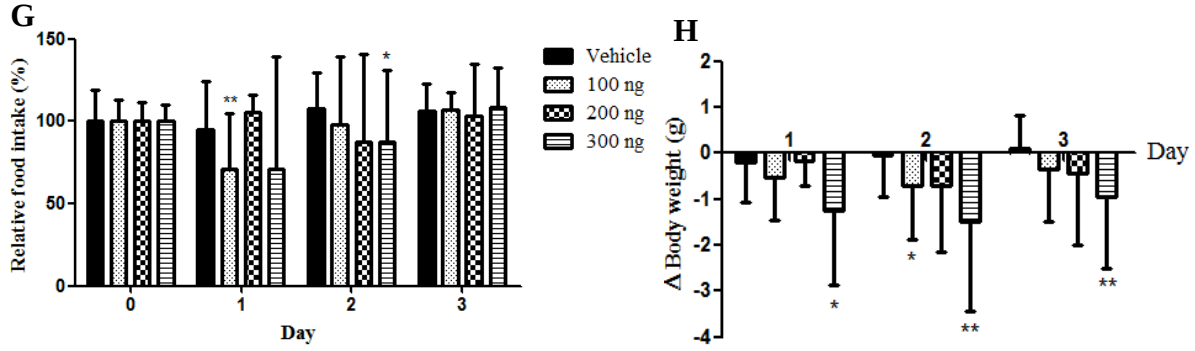


Fig. 5 Effect of *Rheum palmatum* standized sample 1 (RPSS1) on food intake and body weight by icv injection in the 3rd ventricle.

Rheum palmatum standized sample 1 (RPSS1, 100 ng) or vehicle was icv injected to the 3rd ventricle. (A) Food intake and (B) body weight was measured for every 24 hours (n=11). *Rheum palmatum* standized sample 1 (RPSS1, 200 ng) or vehicle was icv injected to the 3rd ventricle. (C) Food intake and (D) body weight was measured for every 24 hours (n=8~10). *Rheum palmatum* standized sample 1 (RPSS1, 300 ng) or vehicle was icv injected to the 3rd ventricle. (E) Food intake and (F) body weight was measured for every 24 hours (n=7~8). (G) Representation of food intake in (A), (C) and (E) as relative food intake. (H) Representation of body weight in (B), (D) and (F) as body weight difference. Results are means ± S.E.M., *P < 0.05, **P < 0.01, ***P < 0.001 vehicle vs 100 ng, 200 ng or 300 ng

RPSS1 was icv injected into 3rd ventricle. Injecting RPSS1 into 3rd ventricle is indirect method of injecting RPSS1 into the hypothalamus. Therefore RPSS1 maybe activated numerous types of neurons in the hypothalamus. Arcuate nucleus of the hypothalamus contains neurons expressing anorexigenic and orexigenic neuropeptides. Injecting RPSS1 directly into the arcuate nucleus of the hypothalamus might induce NPY and stimulate appetite. Coordinate of cannula was changed so that RPSS1 can be directly injected into the arcuate nucleus of the hypothalamus. NPY test was performed after allowing the mice to recover from surgery for a week. Most of the mice did respond when NPY was icv injected. However, response was not as dramatic as when NPY injected into 3rd ventricle. Mice ate average of 0.42 g of chow for one hour (Table 2). Amount of RPSS1 that is to be injected into the arcuate nucleus was decided depending on previous icv injection. 200 ng was decided to be injected into the arcuate nucleus as 100 ng may not produce significant effect. Food intake was reduced (28.1% in day 1) when 200 ng of RPSS1 icv injected into the arcuate nucleus. In addition, there was no significant change in body weight. Similar to icv injection into 3rd ventricle, food intake and body weight were recovered after from RPSS1 icv injection.

1h food	1	2	3	4	5	6
Intake (g)	0.19	0.37	0.99	0.34	0.41	0.23

Table 2 NPY test for the arcuate nucleus cannulation.
2 μl of NPY (0.25 nmole) icv injected into the arcuate nucleus. Food intake was measured for 1 hour. Numbers on the top row represents each individual mouse and number on the bottom row represent amount of chow eaten by each mouse for an hour after icv injection of NPY.

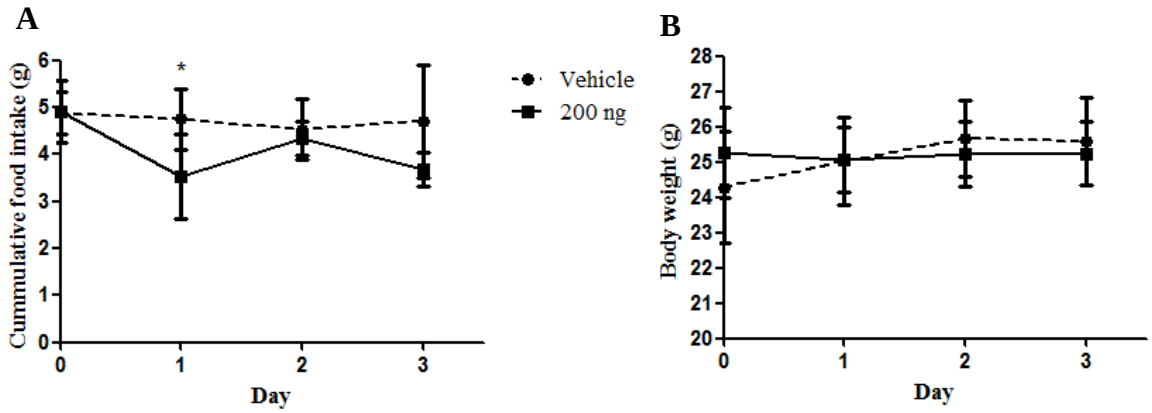


Fig. 6 Effect of *Rheum palmatum* standized sample 1 (RPSS1) on food intake and body weight by icv injection in the arcuate nucleus.
Rheum palmatum standized sample 1 (RPSS1, 200 ng) or vehicle was icv injected to 3rd ventricle. (A) Food intake and (B) body weight was measured for every 24 hours (n=6). Results are means ± S.E.M., *P < 0.05, **P < 0.01, ***P < 0.001 vehicle vs 200 ng

So far only natural compound that can potentially stimulate appetite have been investigated. As stated in the introduction, this natural compound can be developed and used as treatment for anorexia. Besides this, discovering and developing treatment for obesity would be much more powerful than anorexia. Obesity has become a worldwide issue since approximately 30% of the population is obese in United State alone [27]. Obesity can lead to hypertension, cardiovascular diseases, and sometimes even cancer. Therefore it is critical to find cure or treatment for obesity. However, same strategy used for discovering RPSS1 cannot be since POMC reporter cell line is not commercially available. Non-scientific literature was searched and six natural compounds that can potentially suppress appetite were selected. These natural compounds were treated to POMC producing hypothalamic cell line. Result of qRT-PCR indicate *Pueraria lobata* standized sample 2 (PLSS2), *Scutellaria baicalensis* standized sample 2 (SBSS2) and *Citrus unshiu* standized sample 1 (CUSS1) induced POMC mRNA

expression (Fig. 7A). Further analysis of POMC mRNA expression of these three natural compounds showed that PLSS2 induced POMC mRNA expression level by 1.3 folds, SBSS2 by 1.7 folds and CUSS1 by 1.4 folds (Fig 7B). These three natural compounds (PLSS2, SBSS2 and CUSS1) were treated to NPY producing hypothalamic cell lines to investigate whether these natural compounds induce NPY expression. Result of qRT-PCR show that PLSS2 and SBSS2 did not induce NPY mRNA expression. However, CUSS1 induced NPY mRNA expression by 8.6 folds (Fig. 7C). Results of qRT-PCR suggest that CUSS1 may stimulate and suppress appetite. CUSS1 might stimulate appetite as it induced NPY mRNA level greater than POMC mRNA expression. Therefore SBSS2 was selected for further *in vivo* experiments. *In vivo* experiment must be performed in order to confirm that CUSS1 can suppress appetite.

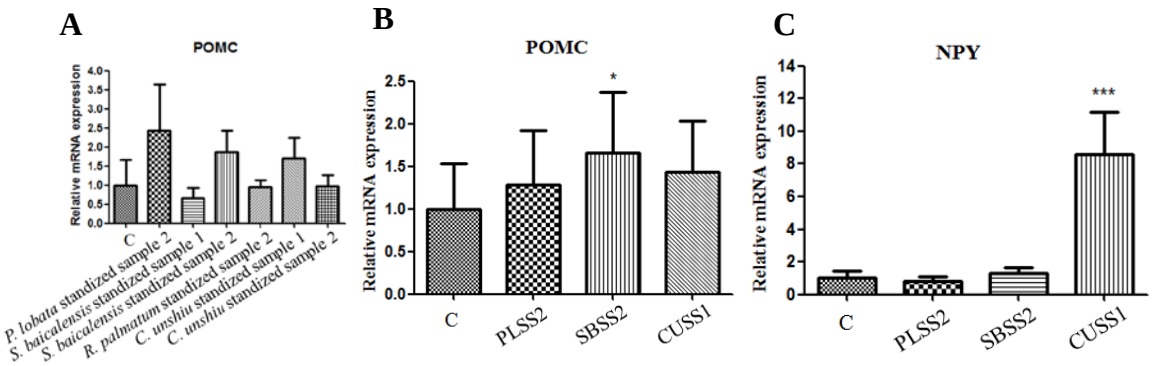


Fig. 7 Screening for natural compounds that induce POMC.
(A) Six natural compounds (50 μg/ml) were treated to N43/5 for 24 hours (*n*=3). (B) *Pueraria lobata* standized sample 2, *Scutellaria baicalensis* standized sample 2 and *Citrus unshiu* standized sample 2 (50 μg/ml) were treated to N43/5 for 24 hours (*n*=3~9). (C) *Pueraria lobata* standized sample 2, *Scutellaria baicalensis* standized sample 2 and *Citrus unshiu* standized sample 2 (50 μg/ml) were treated to N41 for 24 hours (*n*=6). Results are means ± S.E.M., **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs control

4. DISCUSSION

The hypothalamus is the center for regulating appetite and energy homeostasis [1-4]. The arcuate nucleus of the hypothalamus consists of two major anorexigenic and orexigenic neuropeptides that regulates feeding and satiety. However, appetite is a complex behavior controlled by various factors. Therefore mechanism of food intake is still not fully understood. Regulation of appetite is critical as malfunction in appetite control could cause metabolic diseases. For instance, anorexia can be induced when appetite is suppressed severely. On the other hand, constant stimulation of appetite can lead to obesity. Both appetite related metabolic disorders are major issues in current society. Therefore, it is vital to develop a treatment for these metabolic diseases.

The development of treatment for anorexia is crucial since it has become another major metabolic disorder. Many teenagers and adolescents are refusing to eat due to insecurity of gaining weight. However, the development of treatment for obesity would be more significant than anorexia. Obesity is a worldwide issue that can lead to various chronic diseases, like cardiovascular diseases, cancer, osteoporosis and diabetes mellitus. Therefore, discovering natural compounds that can potentially suppress appetite would be much more valuable than those that can stimulate appetite. Screening process for natural compounds that can induce NPY was relatively straightforward since NPY/GFP reporter cell line is commercially available. However, hypothalamic reporter cell line producing POMC is not currently available. Thus, screening process for natural compounds that can potentially increase POMC expression will be more challenging than those that increased NPY. Once natural compounds that increased POMC mRNA expression has been selected. *In vitro* and *in vivo* experiments similar to this study can be performed.

The most intriguing finding of this study was that results of the *in vitro* and *in vivo* experiments being evidently opposite. Results of the *in vitro* study suggested that *R. palmatum* have potential to stimulate appetite. This hypothesis was confirmed with NPY mRNA expression in N41 cell lines, which is NPY producing hypothalamic cell line. Correlation of neuropeptides expression was further confirmed that *R. palmatum* is highly likely to stimulate appetite when POMC mRNA expression level was observed in N43/5 cell line. However, appetite was not stimulated when *R. plamatum* was icv injected into the 3rd ventricle. This method is an indirect injection to the hypothalamus. Injecting *R. palmatum* into the 3rd ventricle may have activated other neurons in hypothalamus, instead of NPY positive neurons in the arcuate nucleus. Therefore coordinate of cannula was changed and *R. palmatum* was directly injected into the arcuate nucleus. Coordinate for the 3rd ventricle is 1.8 mm posterior to the bregma, 0.0 mm from the midline and 5.0 mm ventral. However, coordinate for the arcuate nucleus was changed to 1.5 mm posterior to the bregma, 0.2 mm from the midline and 5.8 mm ventral [28]. Expectation of injecting *R. palmatum* into the arcuate nucleus was stimulation of appetite as it induced NPY mRNA expression in hypothalamic cell lines. However, injecting *R. palmatum* into the arcuate nucleus reduced food intake. It is uncertain whether *R. palmatum* regulate neuropeptides in the hypothalamus. Therefore neuropeptides mRNA expression in the hypothalamus needs to be analyzed.

An assumption can be made at this point on opposite results of *in vitro* and *in vivo* experiments. *In vivo* experiments are much more complex than in *vitro*. *In vitro* experiments only involve a single homogeneous cell line and its response to certain chemical or stimulation. *In vivo* experiments involve an entire organism, which is combination and organization of various heterogeneous cells. Therefore, there are more variations *in vivo* experiments than *in vitro*. In addition, *R. palmatum* that was used in this study is a mixture of various substances. Some substances in this sample have been identified and some not. Thus it is possible that one substance in *R. palmatum* standized sample can induce NPY and

another can act as an antagonist for Y1 receptor, which is receptor for NPY. To confirm this hypothesis, neuropeptides mRNA expression must be analyzed after injecting *R. palmatum* into either 3rd ventricle or arcuate nucleus. Following that, cAMP activity in the hypothalamus can be assessed. Y1 receptor is a G protein-coupled receptor and its activation can be analyzed with cAMP activity [29, 30]. Various g protein-coupled receptors can exist in the hypothalamus. Therefore assessing cAMP activity of Y1 receptor can pose a problem. However, this can be overcome with use of Y1 specific antagonist.

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요약문

식욕과 neuropeptides 발현 조절에 대한 대황 추출물의 역할

뇌의 시상하부는 식욕과 에너지 균형을 조절하는 중요 부분이다. 시상하부에서는 식욕에 관여하는 orexigenic 과 anorexigenic neuropeptide 들이 존재한다. Neuropeptide Y(NPY)와 Agouti related peptide (AgRP)는 orexigenic neuropeptide 라고 불리며 식욕 촉진을 담당하고 있다. Pro-opiomelanocortin (POMC)와 cocaine and amphetamine regulated transcript(CART)는 anorexigenic neuropeptide 이며 식욕 억제에 관여한다. 식욕이 제대로 조절 되지 않으면 여러 대사 질환을 유발 하기도 한다. 예를 들어 식욕이 과도하게 억제되면 거식증이 유발 될 수도 있고 반대로 식욕이 지속적으로 촉진되면 비만으로 이어질 가능성이 높다. 이러한 거식증 또는 비만에 대한 효과적인 치료제가 아직 개발되어 있지 않고 현재 개발 되어있는 신약들은 심각한 부작용을 유발 할 수 있다. 반면 천연물은 화학적인 다양성을 가지고 있어서 신약개발에 많은 기여를 한다. 하지만 천연물의 식욕 조절에 대한 연구가 많이 되어 있지 않기 때문에 본 연구에서는 식욕 조절에 관여하는 천연물을 찾고자 천연물신소재은행에서 20 가지의 천연물을 선택 하였다. 그 중 대황 추출물이 NPY/GFP reporter cell line 과 NPY 를 생성하는 다른 신경세포 주에서도 NPY 발현을 특이적으로 증가 시킴을 확인하였다. 하지만 대황 추출물을 3^{rd} ventricle 에 주입 했을 시 쥐의 먹이 섭취와 체중이 감소 하였다. 대황 추출물을 주입한 후 쥐의 시상하부에서 NPT, AgRP, POMC 그리고 CART 의 mRNA 발현을 관찰 했을 시 변화가 없었다. 이는 대황 추출물의 식욕 감소 효과가 neuropeptide 조절이 아닌 다른 기전으로 나타날 수 있음을 시사하며, 3^{rd} ventricle 로의 주입이 아닌 NPY 발현 신경세포가 밀집된 arcuate nucleus 로 주입하는 방법에 대한 추가 실험이 요구된다. 대황 추출물을 3^{rd} ventricle 대신 arcuate nucleus 에 직접 주입하여 식욕 촉진 여부가 확인 된다면 대황 추출물을 이용한 거식증 치료제 개발에 도움이 될 것으로 기대된다.

핵심어: 시상하부, 음식 섭취, neuropeptide, 대황