p-Synephrine: A Novel Agonist for Neuromedin U2 Receptor

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In the brain, Neuromedin U2 receptor (NMU2R) is prominent in the hypothalamic regions and is known to be associated with regulation of several important physiological functions, including food intake, energy balance, stress response, and nociception. In this article, by random screening of compounds using the model of high-throughput screening for NMU2R stable expression, NMU2R negative and NMU2R short hairpin RNA (shRNA) knockdown HEK293 cell lines, for the first time, we discovered that *p*-synephrine, which is the primary protoalkaloid in *Citrus aurantium* (bitter orange) and is widely used in weight loss and weight management products, is a highly potent and selective NMU2R agonist. In NMU2R activating ability experiments, *p*-synephrine was found binding to NMU2R with high efficacy and potency; the efficacy, 50% of the maximum possible effect (EC₅₀) and potency values were determined to be 7.207, 6.604 and 0.227 μmol/L for the NMU2R, respectively. Our researches have important theoretical value for elucidating the mechanisms of *p*-synephrine in body weight and energy balance regulation. These data provide further evidence for widespread roles for *p*-synephrine and its receptors in the brain.

Key words *p*-synephrine; neuromedin U2 receptor; agonist

para-Synephrine (*p*-synephrine) was first isolated as a natural product from the leaves of various *Citrus* trees, and its presence noted in different *Citrus* juices, by STEWART in the early 1960s. In Traditional Chinese Medicine (TCM), the *Citrus aurantium* peel and whole, dried, immature fruit was used for a variety of clinical applications, including indigestion, diarrhea and dysentery, constipation, and as an expectorant.¹⁾ According to TCM, Zhi shi (immature bitter orange) was one of the best herbs to treat gastrointestinal disorders characterized by stagnation and accumulation.¹⁾ In addition, in South American folk medicine, bitter orange had also been used to treat insomnia, anxiety, and epilepsy.²⁾ In modern medicine, *Citrus aurantium* extract and its primary constituent *p*-synephrine were widely used in weight management products and thermogenic agents. For the mechanism above all, its purported effects were on metabolic processes, including an increase in basal metabolic rate and lipolysis as well as mild appetite suppression.^{2–4)}

Through the phytochemical method, *p*-synephrine was identified as the primary pharmacologically active protoalkaloid (biogenic amine) in most bitter orange peel and its extracts,^{5,6)} which was a phenylethanolamine derivative with the hydroxy group in the *para* position on the benzene ring of the molecule (Fig. 1A). *p*-Synephrine comprised greater than 85% of the total protoalkaloids in bitter orange extract. Other minor protoalkaloidal constituents included octopamine, hordenine, tyramine, and *N*-methyltyramine.⁶⁾

Based on previous receptor-binding researches, *p*-synephrine was believed to act primarily as an agonist binding to beta-3 adrenergic receptor, which resulted in increased thermogenesis and lipolysis.^{7,8)} And *p*-synephrine was likely also to be binding to α - as well as β -1 and β -2 adrenoreceptors resulted in cardiovascular effects including increased cardiovascular contractility, heart rate and bronchodilation.^{9,10)} However, Stohs *et al.* indicated that receptor binding studies

involving tissues from humans and animals indicate that *p*-synephrine exhibits poor binding affinities or non-specificities for α -adrenoreceptor subtypes as well as β -1 and β -2 adrenoreceptors.¹¹⁾

Neuromedin U2 receptor (NMU2R), one of the two neuromedin U (NMU) receptors, was G protein-coupled receptors (GPCRs) formerly known as “orphan” receptor FM3/GPR66 and FM4.^{12–14)} And NMU2R was predominantly expressed in central nervous system (CNS), with the highest level in the arcuate and paraventricular nuclei (PVN) of the hypothalamus, nuclei accumbens, hippocampus, thalamus, medulla oblongata, and spinal cord.¹⁵⁾ Over the past several years, significant progress had been made toward understanding the mecha-

Fig. 1. *p*-Synephrine (A) and *m*-Synephrine (B)

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nisms and physiological roles of NMU system in the brain.¹⁶ NMU2R played an essential role in the regulation of food intake and energy homeostasis,^{17–19} and had been proposed to be an important CNS target of metabolic diseases such as eating disorders, obesity, and type 2 diabetes.^{19,20} However, the existing NMU analogs, antibodies, and small interfering RNAs had limited clinical value due to their poor pharmacokinetic properties. Extensive efforts to generate a CNS-penetrable small-molecule agonist at NMU2R by peptidomimetic approaches had been unsuccessful thus far.

Based on the overlap in the pharmacology of NMU2R and *p*-synephrine, we supposed that *p*-synephrine regulated food intake and energy homeostasis by active NMU2R on the central nervous system. In other words, *p*-synephrine was likely to be an agonist for NMU2R. Hence, in this article, we used an effective and reliable microplate assay (NMU2R/pCDNA3.1(+)-3×CRE/3×MRE/SRE-LUC/pGL3) reported by us firstly to investigate effect of *p*-synephrine on the activation of NMU2R. The microplate assay used luciferase as the reporter that enables simple and rapid screening of natural product library to identify new NMU2R agonists, and we had found flavonoid glycoside (icariin, hesperidin and nobiletin) could activate the reporter gene response evidently.^{21–23} Further, the false-positive results were ruled out with negative cell line and RNA interfere method.

MATERIALS AND METHODS

Materials HEK293 cells stably expressing NMU2R (named as HEK293-NMU2R cells) were presented from the professor Hu Yinghe (East China Normal University). The HEK293 negative cell line was constructed by our laboratory. White 96-well assay plates were purchased from Corning Costar, Bright-Glo™ assay system reagent and pGL3 vector were purchased from Promega, Fugene HD transfection reagent were purchased from Roche, and Dulbecco's modified Eagle's medium (DMEM) cell culture medium and fetal bovine serum (FBS) were purchased from Hyclone. NMU and forskolin were obtained from Sigma. G418 was from Gibco and the Standard *p*-synephrine was purchased from ChromaDex Inc. Primary antibodies for NMU2R, β -actin and horseradish peroxidase (HRP) labeled second antibodies were purchased from Santa Cruz. All additional chemical reagents (AR) in this study were obtained from Chongqing Chem. Ltd.

Negative Cell Line Generation and Luciferase Assay HEK293 cells were transiently transfected with the pCDNA3.1(+) null vector and a plasmid containing MRE/CRE/SRE-LUC. Wild-type HEK293 cells were cultured at 37°C in DMEM with 10% fetal bovine serum to be grown to 70–90% confluence. Transfection was carried out in 6-well dishes with Fugene HD transfection reagent (Roche) using the protocol provided by the supplier. The ratio of null vector and MRE/CRE/SRE-LUC plasmid is 1:5. One day before the luciferase assay, $2\text{--}10 \times 10^4$ cells were placed into a 96-well plate with 100 μL regular medium. After the cells attached, 11 μL compounds at different concentrations or vehicle were added. With a continuous incubation of 6–8 h, Bright-Glo assay reagent (Promega) was added to the media at 110 μL /well, and luminescence was measured using the Reporter (Turner Design).

Plasmid Construction, RNA Interference and Trans-

fection Based on the rat *Nmu2r* (AF272363) gene, an oligonucleotide sequence for *Nmu2r* short hairpin RNA (shRNA) was selected to knock down *Nmu2r* expression in HEK293-NMU2R cells. The human *Nmu2r*-specific shRNA [5'-GCGCAACTACCCCTT CTTGTT-3'(1#), 5'-GCCCCATGTGG ATCTACAATTT-3'(2#)] plasmids were provided by GeneCopoeia Inc. Constructed plasmid were transfected into HEK293-NMU2R cells using FuGENE HD transfection reagent (Promega). Negative-control shRNA was used as control.

Immunoblot Analysis Cells were washed with cold phosphate buffered saline (PBS) and lysed using a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (v/v) Triton X-100, 0.1% sodium dodecyl sulfate, protease inhibitors, 1 mM Na_3VO_4 , and 2.5 mM $\text{Na}_4\text{P}_2\text{O}_7$. Lysates were sonicated, and aliquots were taken for the determination of protein concentration, and samples were stored at -80°C . Protein from total cell extracts (10–20 μg protein) were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes for Western blot. Blotted proteins were probed using antibodies for NMU2R and β -actin (Santa Cruz) according the supplier's protocols. Horseradish peroxidase-conjugated IgG was used as second antibody with ECL advance (Millipore, U.S.A.) for detection.

Statistical Analysis Data are shown as mean $\pm$ S.D. Statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* method using the software of Origin version 8.0. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Identification of *p*-Synephrine as a NMU2R Agonist In

Fig. 2. Response of HEK293-NMU2R Cells to *p*-Synephrine and NMU (Neuromedin)

After the cells were treated with different doses *p*-synephrine and 5 $\mu\text{mol/L}$ forskolin or 5 $\mu\text{mol/L}$ NMU and 5 $\mu\text{mol/L}$ forskolin, luciferase activity was determined using the Reporter (Turner Design) with Bright-Glo agents (Promega), treatment with equal volume vehicle (DMSO) taken as control. The results represent at least three independent experiments and are expressed as means $\pm$ S.D. ($n=5$, * $p < 0.05$, ** $p < 0.01$ vs. control).

Fig. 3. Effect of *p*-Synephrine and NMU (Neuromedin) on the 3×MRE/3×CRE/3×SRE-LUC Plasmid Transfected Cells, a Negative Cell Line

After the cells were treated with different doses *p*-synephrine and 5 μmol/L forskolin or 5 μmol/L NMU and 5 μmol/L forskolin, luciferase activity was determined, as described in Materials and Methods. The results represent at least three independent experiments and are expressed as means ± S.D. ($n=5$, * $p<0.05$, ** $p<0.01$ vs. control).

previous studies, we developed a stable cell line that could respond to both Gs and Gq-coupled NMU2R.^{21,22} Through combining with forskolin, the activating response would be increased. As shown in Fig. 2, the activation level of the combination of NMU with forskolin, an activator of CRE, was up to 4-fold level of forskolin alone, but the activating level of NMU alone was only up to 2-fold level of control. Likewise, Fig. 2 shows that *p*-synephrine is an active component, which could activate the reporter gene through NMU2R, and the activating levels of *p*-synephrine (1, 10, 100 μmol/L) were up to 3-, 4- and 6-fold level of forskolin alone, respectively. These results suggested that *p*-synephrine is an agonist for NMU2R.

Luciferase Assay of the Negative Cell Line To demonstrate ligands specificity, the negative cell line has been established. The negative cell line is transiently transfected with pcDNA3.1 null vector and 3×CRE/3×MRE/SRE-LUC/pGL3 plasmid. The experimental results have shown that, although forskolin could activate the negative cell line, 10, 100 μmol/L *p*-synephrine and 5 μmol/L NMU alone could not activate them (Fig. 3). Compared with forskolin, combination of *p*-synephrine or NMU with forskolin would not increase the reporter gene response.

NMU2R Activating Ability of *p*-Synephrine The assessment of functional properties is a crucial step in the screening of potential new drug candidates. To affirm the NMU2R activating ability of *p*-synephrine, HEK293-NMU2R cells were treated with various concentrations of *p*-synephrine (0.001, 0.01, 0.1, 1, 10, 100, 1000 μmol/L). Dose-dependent activity response curves for the samples are shown in Fig. 4A. The results showed that, analogous to NMU (Fig. 4B), *p*-synephrine is a strong agonist of NMU2R, which could activate this reporter system in a dose dependent manner. By the logarithmic curve fitting method, the formulas of *p*-synephrine and NMU doses and multiple of forskolin response had been got Eqs. 1 and 2. In the formula, y was multiple of forskolin response and x was *p*-synephrine doses. By Eq. 1, the *p*-synephrine effica-

A

B

Fig. 4. Dose Response Curves of *p*-synephrine and NMU (Neuromedin) in the HEK293-NMU2R Cells

After the HEK293-NMU2R cells were treated with different doses *p*-synephrine and 5 μmol/L forskolin (A) or different doses NMU and 5 μmol/L forskolin (B), luciferase activity was determined, as described in Materials and Methods. The results represent at least three independent experiments and are expressed as means ± S.D. ($n=5$)

cy, 50% of the maximum possible effect (EC_{50}), and potency values, which was the luciferase activity of NMU2R cells up to 2-fold of forskolin alone, were 7.207, 6.604, 0.227 μmol/L, respectively. The effective concentrations of *p*-synephrine were from 0.227 to 1000 μmol/L. And in this analysis systems, NMU efficacy, EC_{50} , and potency values were 8.45, 7.49, 0.6 μmol/L, respectively. The effective concentrations of NMU were from 0.6 to 1000 μmol/L.

$$y = 7.21 - \frac{5.94}{1 + \exp\left(\frac{x + 5.18}{0.75}\right)} \quad (\text{Eq. 1})$$

$$y = 8.45 - \frac{7.63}{1 + \exp\left(\frac{x + 5.13}{0.65}\right)} \quad (\text{Eq. 2})$$

Fig. 5. HEK293-NMU2R Cells Were Transfected with *nmu2r* shRNA (1# and 2#) Using Lipofectamine Reagent (Named as shNMU2R Cells)

After transfected 24h, the cells were collected. Western blotting was used to assay the change of NMU2R protein in HEK293 cells. β -Actin was taken as internal references, respectively. Experiments were repeated three times, band intensities were quantified, and S.D. values were calculated. * $p < 0.05$, ** $p < 0.01$ vs. control.

Fig. 6. Response of shNMU2R Cells to *p*-Synephrine

After the cells were treated with 10 μ mol/L *p*-synephrine and 5 μ mol/L forskolin or 5 μ mol/L NMU (neuromedin) and 5 μ mol/L forskolin for 6–8 h, the luciferase activity was determined as described in Materials and Methods. The data are expressed as means $\pm$ S.D. of three independent experiments. ($n=5$, ** $p < 0.01$ vs. HEK293-NMU2R cells treated with 10 μ mol/L *p*-synephrine and 5 μ mol/L forskolin, ## $p < 0.01$ vs. HEK293-NMU2R cells treated with 5 μ mol/L NMU and 5 μ mol/L forskolin.)

Knockdown of *Nmu2r* with shRNA in NMU2R Cells

To probe the direct action of *p*-synephrine on the NMU2R, we constructed the shRNA plasmids for NMU2R to silence the expression of NMU2R in HEK293-NMU2R cells. The level of NMU2R expression was analyzed with Western blot, and the interfered efficiency was shown as Fig. 5. The results from Western blotting showed that the two shRNA sequences both had a noticeable effect on the expression of *Nmu2r* in HEK293-NMU2R cells, but plasmid 1# had better knockdown efficiency than 2#. Therefore, to obtain the better cell clones, we used the plasmid 1#-transfected HEK293-NMU2R cells in further experiments and named as shNMU2R cells.

Effect of *p*-Synephrine on the shNMU2R Cells We also

checked the effect of *p*-synephrine on the shNMU2R cells. The results showed that, compared with NMU2R cells, in shNMU2R cells in which NMU2R had been knocked down, the activating level of both *p*-synephrine and NMU were significantly decreased (Fig. 6), suggesting that *p*-synephrine could selectively activate the *nmu2r* gene.

DISCUSSION

High-throughput screening was an important tool for discovering new drugs that target various biomolecular sites.²⁴⁾ A highly sensitive and simple ligand binding assay for membrane receptors, especially GPCRs, had always been challenging. Here, this assay constructed by us was based on HEK293 cells that were co-transfected with the human NMU2R gene and an integrated copy of a luciferase reporter gene driven by a SV40 promoter under the control of a 3 $\times$ MRE, 3 $\times$ CRE, and 3 $\times$ SRE enhancer elements (NMU2R/pcDNA3.1(+)-3 $\times$ CRE/3 $\times$ MRE/SRE-LUC/pGL3). The assay measured the stimulation of GPCRs by detecting changes in the luciferase levels.²⁵⁾ We had demonstrated the feasibility of using this assay to perform high-throughput screening of a small molecule library.^{21,22)} In this paper, we successfully used this screening system to first discover *p*-synephrine (Fig. 1A), which could strongly activate NMU2R in a dose dependent manner (Fig. 4). More than that, we also confirmed the specificity of *p*-synephrine on the negative cell line transfected with pcDNA3.1 null vector/ luciferase reporter gene plasmid or RNA interference *Nmu2r* expression cells (Figs. 3, 6). All of these results suggested that *p*-synephrine could selectively activate the *nmu2r* gene.

Once a “new” drug candidate had been discovered, one had to verify that it possesses the potential functional activity and therapeutic efficacy.²⁶⁾ Efficacy is a measure of the maximum biological effect that a drug can produce as a result of receptor binding. Potency of a drug refers to the amount of drug required to achieve a defined biological effect—the smaller the dose required, the more potent the drug, which is the same with EC_{50} .^{27,28)} The *p*-synephrine targeting NMU2R efficacy, EC_{50} , and potency values were 7.207, 6.604, 0.227 μ mol/L, respectively, which manifested the high potency and high efficacy. And the potency and efficacy of *p*-synephrine are essentially the same with that of NMU by this screening system.

p-Synephrine, the primary protoalkaloidal constituent of *Citrus aurantium* (bitter orange) and other *Citrus* species including seville oranges, mandarin oranges, clementines, Marrs sweet oranges, nova tangerines, grapefruits, etc., was widely used in weight loss and weight management products in combination with caffeine, polyphenolics, and other constituent.^{29–31)} These products were designed to promote thermogenesis and increase metabolism, suppress appetite, and/or slow the absorption of fats and carbohydrates.^{2–4)} However, due to lack of directly attributable adverse effects to *p*-synephrine and bitter orange extract, the safety of *p*-synephrine was frequently questioned, such as the potential cardiovascular hazards (strokes and cardiac arrhythmias).^{32–35)} In recent one or two years, Stohs *et al.* specially did some studies on the risk of *p*-synephrine.^{11,36–39)} They critiqued and reviewed over 20 studies involving a total of approximately 360 subjects that consumed *p*-synephrine alone or in combination with other ingredients, and drew a conclusion that bitter orange extract alone (*p*-synephrine) or in combination with other herbal in-

redients did not produce significant adverse events as an increase in heart rate or blood pressure, or alter electro-cardiographic data, serum chemistry, blood cell counts or urinalysis. On the other hand, Stohs believed that various researchers erroneously and inappropriately referenced studies involving *m*-synephrine (Fig. 1B), which was known as a synthetic phenylephrine with a hydroxyl group in the *meta*-position on the benzene ring,⁴⁰⁾ and was not present in standardised bitter orange reference materials from the National Institute of Standards and Technology.⁴¹⁾ The cardiovascular effects of *m*-synephrine and *p*-synephrine were clearly distinct as a result of their markedly different adrenergic receptor binding activities, with *m*-synephrine exerting markedly greater binding to various α -receptor subtypes as well as β -1 and β -2 receptors.^{9,42–44)} The well documented cardiovascular effects of *m*-synephrine cannot therefore be extrapolated to *p*-synephrine and bitter orange extract. So Stohs *et al.* indicated that receptor binding studies involving tissues indicate that exhibits poor binding affinities or non-specificities for α -adrenoreceptor subtypes as well as β -1 and β -2 adrenoreceptors.¹¹⁾

Considerable data supported a role for NMU and NMU2R in the regulation of the energy balance. Acute central NMU injection suppressed food intake,^{13,20,45)} increased energy expenditure and core body temperature, and stimulated locomotor activity.⁴⁶⁾ Intracerebroventricular injection of NMU immunoglobulin G (IgG) was shown to block the effects of centrally administered NMU on food intake in fasted rats and, more importantly, increases food intake in satiated rats.⁴⁷⁾ In addition, in long-term central NMU treatment, NMU2R-null mice provided evidence for an antiobesity and anorexigenic roles. Female NMU2R-null mice fed a high-fat diet were protected from central NMU-induced body weight loss compared with littermate wild-type mice.¹⁷⁾

Using rat fat cells and the β -antagonist, previous researches showed that *p*-synephrine was believed to act as an agonist binding to β -3 adrenergic receptor.^{7,48)} In humans, β -3 adrenergic receptor mRNA is expressed in adipose tissues, in gallbladder, and to a much lower extent in colon.⁴⁹⁾ However, NMU2R mRNA expression was found at the highest levels in the CNS regions of both rat and human tissues.¹⁵⁾ High expressions were observed in the ependymal cell layer along the wall of the third ventricle in the hypothalamus, CA1 region of the hippocampus, indusium griseum and septohippocampal nucleus.⁵⁰⁾

Based on the overlap in the pharmacology of NMU2R and *p*-synephrine and the localization of β -3 adrenergic receptor and NMU2R in human, we supposed that *p*-synephrine regulated food intake and energy homeostasis by active NMU2R on the central nervous system. In other words, *p*-synephrine was likely to an agonist for NMU2R. This article confirmed *p*-synephrine could strongly activate NMU2R in a dose-dependent manner using an effective and reliable microplate assay, and this result will enrich the *p*-synephrine pharmacology in the brain.

However, additional *p*-synephrine NMU2R receptor binding further studies are required. Furthermore, the relationship between the energy homeostasis effects of *p*-synephrine and NMU2R activation needed to make clear.

CONCLUSION

In conclusion, from the existing literature, it is known that *p*-synephrine had high lipolytic activity, and the activation of β -3 adrenergic receptor or NMU2R is known to contribute to the lipolytic activity. Different from the existing literature using fat cells, we use the model of high-throughput screening for NMU2R stable expression to random screen compounds, and try to find novel agonists for neuromedin U2 receptor. These data provide further evidence for widespread roles for *p*-synephrine and its receptors in the brain.

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