Potential anti-obesity effects of a long-acting cocaine hydrolase

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Abstract

A long-acting cocaine hydrolase, known as CocH3-Fc(M3), engineered from human butyrylcholinesterase (BChE) was tested, in this study, for its potential anti-obesity effects. Mice on a high-fat diet gained significantly less body weight when treated weekly with 1 mg/kg CocH3-Fc(M3) compared to control mice, though their food intake was similar. There is no correlation between the average body weight and the average food intake, which is consistent with the previously reported observation in BChE knockout mice. In addition, molecular modeling was carried out to understand how ghrelin binds with CocH3, showing that ghrelin binds with CocH3 in a similar mode as ghrelin binding with wild-type human BChE. The similar binding structure mode explains why CocH3 has a similar catalytic activity against ghrelin.

Introduction

Obesity is related to the worldwide increasing rates of the disease and burden of the associated co-morbidities, such as type 2 diabetes, cardiovascular disease, and cancer [1]. Over one third of adult population in the world are in the risk of overweight-caused diseases, *e.g.* diabetes mellitus, hypertension, coronary heart disease, stroke, gall bladder disease, osteoarthritis, and dyslipidaemia [2-4]. More than 30 billion dollars have been spent on reduced-calorie and multitudinous diets in the United States per year [5]. Unfortunately, the diets used did not show significant efficacy in weight loss [6]. It is necessary to have an effective pharmacological treatment which can counteract the increased appetite during fasting. Unfortunately, so far, FDA-approved pharmaceutical agents for obesity treatment are accompanied by serious adverse/toxic effects. Due to the problems of serious adverse and toxic effects, a number of pharmaceutical agents, such as aminorex, dexfenfluramine, fenfluramine, and phenylpropanolamine, have been withdrawn from the market [7, 8]. Currently available FDA-approved anti-obesity drugs, including phentermine, lorcaserin, orlistat, and phentermine/topiramate ER, still have a variety of adverse side effects, such as insomnia, headache, diarrhea, dizziness, depression, and many others [9]. Hence, it is highly desired to develop a new, safe, and truly effective pharmacological agent for obesity treatment.

Ghrelin, a gastric peptide hormone known as the “hunger hormone” discovered in 1999,[10] is a 28-amino acid peptide (GSSFLSPEHQKAQQRKESKKPPAKLQPR) with Ser3 side chain acylated by a fatty acid (*n*-octanoic acid). So, ghrelin is an *n*-octanoylated peptide. The 28-amino

acid peptide without acylation (*n*-octanoylation) is known as desacyl-ghrelin [11]. This acylation reaction, catalyzed by an enzyme known as ghrelin O-acyltransferase (GOAT), is essential for the physiological activity of ghrelin with growth hormone secretagogue receptors (GHSRs) in the central nervous system that mediate hyperphagia and adiposity [12]. It is believed that ghrelin is produced in the stomach and released primarily from cells in the stomach and travels to the brain through blood circulation. In the brain, ghrelin interacts with both hypothalamus (physiological eating center) and pleasure centers of the brain to arouse hunger [13]. So far, ghrelin is the only known hormone stimulating hunger and food intake [1], telling you when to eat. In addition, ghrelin level naturally changes dramatically during the course of a day. Particularly, ghrelin level rises steeply with fasting or before a meal, and decreases after a meal [13]. Hence, ghrelin is emerging as a novel, potentially attractive anti-obesity drug target [7].

Drug discovery efforts centralized on ghrelin aim to reduce the appetite of overweight people by different approaches, including regulation of ghrelin release, ghrelin receptor antagonism, and reducing active ghrelin production by inhibition of GOAT [14-18]. However, these efforts still have not generated a clinically useful drug so far.

In light of the aforementioned physiological and biological mechanisms of ghrelin, an ideal therapeutic approach would directly inactivate ghrelin itself and convert ghrelin into a peptide form (desacyl-ghrelin) which is devoid of appetite-inducing properties by using an efficient ghrelin-metabolizing enzyme. For example, inhibition of GOAT would decrease the production of (active) ghrelin, but cannot inactivate ghrelin that has already been produced in the body. A ghrelin-metabolizing enzyme would directly inactivate ghrelin by converting ghrelin to desacyl-ghrelin and, thus, more effectively decrease the appetite. Despite of extensive effort that aims to regulate the ghrelin level in the body, there is no report of a therapeutic candidate which can be used to directly inactivate ghrelin by converting ghrelin to desacyl-ghrelin.

Recent studies have revealed the possibility of ghrelin hydrolysis in human butyrylcholinesterase (BChE). It was first reported that ghrelin can be hydrolyzed to desacyl-ghrelin by BChE [19], but the kinetic parameters for BChE-catalyzed ghrelin hydrolysis were not determined. Lockridge *et al.* [20] demonstrated that the BChE knockout mice became overweight on a high-fat diet compared to the control mice, suggesting that BChE is essential for ghrelin inactivation. Most recently reported *in vitro* and *in vivo* studies [21, 22] provided more convincing evidence for the role of human BChE in hydrolyzing ghrelin to desacyl-ghrelin [21].

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4 It was evident that plasma ghrelin level significantly dropped in mice that received BChE (or
5 mutant) gene transfer [22]. The effective enzymes examined in the gene transfer study [22]
6 included our previously reported cocaine hydrolases (CocHs): human BChE mutants
7 A199S/F227A/S287G/A328W/Y332G (CocH3) and A199S/F227A/S287G/A328W/E441D
8 (CocH2) [23-25]. CocH2 and CocH3 *etc.* were designed in our lab to improve the catalytic
9 efficiency of human BChE against cocaine for treatment of cocaine addiction and overdose [23,
10 26-32], and were not expected to improve the catalytic efficiency against ghrelin. Hence, it is not
11 surprising to know that CocH3 has a moderate catalytic efficiency ($k_{cat}/K_m = 4.8 \times 10^5 \text{ min}^{-1} \text{ M}^{-1}$)
12 against ghrelin compared to that ($k_{cat}/K_m = 6.1 \times 10^5 \text{ min}^{-1} \text{ M}^{-1}$) of wild-type human BChE, and
13 that CocH2 has a slightly lower catalytic efficiency ($k_{cat}/K_m = 2.2 \times 10^5 \text{ min}^{-1} \text{ M}^{-1}$) against
14 ghrelin [33]. All of the data imply that these CocHs, particularly CocH3, might be valuable for
15 treatment of both cocaine abuse and obesity.
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18 It has also been demonstrated that the more promising CocH3 is capable of efficiently
19 degrading not only cocaine itself, but also its toxic metabolites (norcocaine and cocaethylene)
20 [34, 35]. Recently, we have designed, prepared, and tested a long-acting CocH3 form [36], a
21 fusion protein in which CocH3 is fused with the N-terminus of a triple mutant (*i.e.*
22 A1V/D142E/L144M) of the Fc region of human immunoglobulin G1 (IgG1). The obtained
23 fusion protein, denoted as CocH3-Fc(M3) and regarded as a catalytic antibody analog, is as
24 active as the unfused CocH3 in terms of the catalytic activity (because the Fc fusion is not
25 expected to change the catalytic activity of CocH3 against any substrate), but has a considerably
26 longer biological half-life (*e.g.* $t_{1/2} = \sim 107$ hours in rats) like an antibody [36]. In the animal
27 behaviour tests, a single dose of CocH3-Fc(M3) was able to accelerate cocaine metabolism in
28 rats even after 20 days and, thus, block cocaine-induced hyperactivity and toxicity for a long
29 period [36].
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32 Further, based on the promising preclinical data for cocaine abuse treatment [36] and the
33 newly reported catalytic activity of CocH3 against ghrelin [33], we would like to know whether
34 repeated dosing of CocH3-Fc(M3) could be effective for obesity treatment and why CocH3 is
35 active against ghrelin. For these purposes, in the present study, we examined the effects of
36 weekly dosing of 1 mg/kg CocH3-Fc(M3) on the body weights of C57BL/6 mice fed a high-fat
37 diet. In addition, we also performed molecular modeling of the CocH3-ghrelin binding in
38 comparison with the corresponding wild-type human BChE-ghrelin binding. The obtained data
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4 suggest that CocH3-Fc(M3) is indeed promising for obesity treatment, and that it is possible to
5 develop a therapeutic enzyme effective for treatment of both cocaine abuse and obesity.
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9 **Materials and Methods**

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11 **Materials.** Male C57BL/6 mice (26–30 g) were ordered from Harlan (Indianapolis, IN).
12 High-fat diet F3282 was ordered from Bio-Serv (Flemington, NJ). The purified CocH3-Fc(M3)
13 protein used in this study was prepared in our previous study [36]. Briefly, the CocH3-Fc(M3)
14 protein was expressed in stable CHO-S cells (developed in our lab using a lentivirus-based
15 method) that can stably produce the CocH3-Fc(M3) protein. The protein production was
16 performed in an agitated bioreactor BioFlo/CelliGen 115 (Eppendorf, Hauppauge, NY). CocH3-
17 Fc(M3) in the culture medium was purified by using the aforementioned protein A affinity
18 chromatography which was performed on ÄKTA Avant 150 system (GE Healthcare Life
19 Sciences, Pittsburgh, PA). The purified protein was dialyzed in a storage buffer and stored at
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30 **Animal tests.** Male C57BL/6 mice were given high-fat diet F3282 and water ad libitum, and
31 maintained on a 12 h light/12 h dark cycle, with the lights on at 8:00 a.m. at a room temperature
32 of 21–22°C. Experiments were performed in a same colony room in accordance with the Guide
33 for the Care and Use of Laboratory Animals as adopted and promulgated by the National
34 Institutes of Health. Mice were randomly divided into two groups. Mice in the treatment group
35 (n=7) were administered intravenously (IV) with a buffer solution (phosphate-buffered saline
36 buffer) containing 1 mg/kg CocH3-Fc(3M) once a week, *i.e.* IV administration of 1 mg/kg
37 CocH3-Fc(3M) on days 0, 7, 14, 21, 28, 35, and 42. Mice in the control group (n=5) were
38 administered IV with the same buffer solution without the enzyme once a week. So, the only
39 difference in the injected solution between the two groups is the existence of 1 mg/kg CocH3-
40 Fc(3M) for the treatment group. The mice in the two different groups were caged separately.
41 Body weights were recorded daily for 44 days, and food intake was measured by weighting the
42 food served and the food remaining in each cage.
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53 **Molecular modeling.** The initial structure of the human BChE binding with ghrelin was
54 based on the crystal structure (PDB code: 1P0I, 2.1 Å resolution) [37] of BChE complexed with
55 butanoic acid. In the initial BChE-ghrelin binding structure, the carbonyl group of on the *n*-
56 octanoylated Ser3 side chain of ghrelin was superimposed with that of butanoic acid in the active
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4 site. The initial structure of the Coch3-ghrelin complex was built from the modeled human
5 BChE-ghrelin structure. Hydrogen atoms of the enzyme (human BChE or Coch3) and ghrelin
6 were added by using the HBUILD module [38] implemented in the CHARMM program [39].
7 Protonation states of all acidic and basic amino acids were determined by surrounding
8 environment under physiological pH condition (pH 7.4). The enzyme-ghrelin complex was
9 solvated by a water droplet with 22 Å radius by the standard superimposing protocol at the center
10 of the oxygen atom (O γ) of Ser198 sidechain, and solvent water molecules within 2.8 Å of any
11 crystal atoms were removed. A modified TIP3P water model [40, 41] was used for the solvent.
12 The all-atom additive CHARMM potential function (CHARMM36) [42] was used for the
13 protein/peptide atoms, and the topology and parameters of *n*-octanoylated Ser3 of ghrelin was
14 evaluated using the CHARMM generalized force field (CGenFF) [43] *via* the Paramchem Web-
15 interface (<https://cgenff.paramchem.org/>). The used non-bonded interaction truncation cut-off
16 was 13 Å.
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18 Stochastic boundary molecular dynamics (MD) method [44] was used with the oxygen atom
19 (O γ) of Ser198 side chain as the reference centre. The reaction region was a sphere with a radius
20 (*r*) of 20 Å, and the buffer region extended over $20 \text{ Å} \leq r \leq 22 \text{ Å}$. All atoms located longer than
21 22 Å away from the hydroxyl oxygen on Ser198 side chain of the enzyme was fixed. All bonds
22 involving hydrogen atoms were constrained by using the SHAKE algorithm [45]. The initial
23 structure for the entire stochastic boundary system was optimized using the steepest descent (SD)
24 and adopted-basis Newton- Raphson (ABNR) methods. A 1-fs time step was used for integration
25 of the equation of motion. The solvated system of the enzyme-ghrelin complex was gradually
26 heated from 50.0 K to 298.15 K in MD simulation for 100 ps and then production MD until the
27 MD trajectory became stable (for at least 1 ns). The last snapshot of the stable MD trajectory was
28 energy-minimized by using the ABNR method to generate the final structure of the enzyme-
29 ghrelin binding complex for binding mode analysis.
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31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 **Results**

53 **Effects of Coch3-Fc(M3) on the body weight and food intake.** As shown in Figure 1, the
54 average body weight of high-fat diet-fed mice in the treatment group (n=7) was not significantly
55 different from that in the control group (n=5) within the first 10 days. After 11 days, the average
56 body weights became different between the two groups, and the difference became larger and
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larger. The data suggest that the weekly dosing of 1 mg/kg CochH3-Fc(M3) significantly affected the body weights of high-fat diet-fed mice. Notably, the enzyme did not significantly influence the average body weight of high-fat diet-fed mice within the first 10 days, but the effect of the enzyme on the average body weight became significant after 11 days.

Figure 1. Body weights of high-fat diet-fed mice. Mice in the treatment group (n=7) were administered (IV) with a buffer solution containing 1 mg/kg CochH3-Fc(3M) once a week. Mice in the control group (n=5) were administered IV with the same buffer solution without the enzyme.

We also tried to identify the possible correlation between the average body weight and the average daily food intake of the high-fat diet-fed mice. Surprisingly, there was no significant difference in the average food intake between the two groups, as shown in Figure 2. The average daily food intake within the observation period (44 days) was always ~3.0 g per mouse for both groups. So, CochH3-Fc(M3) did not significantly influence the total food intake per day. According to these results, the total food intake per day is not the only factor affecting the body weight, and there is no correlation between the average body weight and the average food intake per day. These data are consistent with the previous observation reported by Lockridge *et al.* [20]. They observed that the BChE knockout mice were obese on a high-fat diet, and that the BChE knockout mice did not consume more food compared to the wild-type mice [20]. Based on

the observation, Lockridge *et al.* [20] concluded that the weight gain of BChE knockout mice was the result of reduced fat utilization, rather than the result of increased food intake. Our data with the enzyme administration in this study further support their conclusion.

Figure 2. Average food intake (g per mouse) of high-fat diet-fed mice. Mice in the treatment group (n=7) were administered (IV) with a buffer solution containing 1 mg/kg CochH3-Fc(3M) once a week. Mice in the control group (n=5) were administered IV with the same buffer solution without the enzyme.

Enzyme-ghrelin binding. To understand why CochH3 is similar to wild-type human BChE in the catalytic efficiency against ghrelin, molecular modelling was performed on ghrelin binding with both CochH3 and wild-type human BChE. The obtained enzyme-ghrelin binding structures are depicted in Figure 3. As depicted in Figure 3(A), ghrelin binds with BChE in a mode suitable for hydroxyl oxygen (O^{γ}) on Ser198 side chain of BChE to initiate nucleophilic attack at the carbonyl carbon (C) on the *n*-octanoylated Ser3 side chain of ghrelin, with the C- O^{γ} distance being ~ 3.04 Å. Meanwhile, the carbonyl oxygen on the *n*-octanoylated Ser3 side chain of ghrelin is in the oxyanion hole (consisting of the backbone NH groups of Gly116, Gly117, and Ala199 of BChE), with a stable hydrogen bond with Gly117 backbone NH group of BChE (with an N $\cdots$ H distance of 2.02 Å). As depicted in Figure 3(B), CochH3-ghrelin binding mode is very similar to the BChE-ghrelin binding, with the C- O^{γ} distance being ~ 3.26 Å (about 0.22 Å longer). The carbonyl oxygen on the *n*-octanoylated Ser3 side chain of ghrelin is also in the

oxyanion hole of CocH3, with a slightly weaker hydrogen bond with Gly117 backbone NH group of CocH3 (with an N $\cdots$ H distance of 2.20 Å) compared to the wild-type human BChE. The similar enzyme-ghrelin bonding mode helps us to understand why CocH3 has a similar catalytic activity against ghrelin.

Figure 3. Enzyme-ghrelin binding structures obtained from molecular dynamics simulations and energy minimizations. (A) Wild-type human BChE binding with ghrelin. (B) CocH3 binding with ghrelin. Depicted in the figure are only residues of the enzyme and ghrelin close to the active site in which the big yellow mass represents the van der Waals surface of ghrelin.

Discussion

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4 It has been known that CocH3-Fc(M3), a long-acting form of CocH (human BChE mutant
5 with a considerably improved catalytic efficiency against cocaine), is highly effective for
6 treatment of cocaine abuse. The present study has further demonstrated the potential anti-obesity
7 effect of CocH3-Fc(M3) for the first time. Therefore, CocH3-Fc(M3) might be valuable for
8 treatment of not only cocaine abuse, but also obesity, which is a good news for the CocH (BChE
9 mutant)-based enzyme therapy development. On the other hand, the potential anti-obesity effect
10 of CocH3-Fc(M3) might be moderate, due to its relatively low catalytic efficiency against
11 ghrelin compared to that against cocaine. Further CocH-based enzyme therapy development
12 should be focused on rational design of a CocH3-Fc(M3) variant with a significantly improved
13 catalytic efficiency against ghrelin without decreasing the catalytic efficiency against cocaine.
14 Such a CocH3-Fc(M3) variant may be efficient in hydrolyzing both cocaine and ghrelin, and
15 therefore may be highly effective for treatment of both cocaine abuse and obesity. Further, the
16 CocH3-ghrelin binding structure obtained may be used as a starting point for future rational
17 design of the desired CocH3-Fc(M3) variant.
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32 **Conclusions**

33 It has been demonstrated that CocH3-Fc(M3) can significantly affect the body weights of
34 the high-fat diet-fed mice, but not the total food intake per day. There is no correlation between
35 the average body weight and the average food intake, which is consistent with the previously
36 reported observation in BChE knockout mice. In addition, in light of molecular modeling,
37 ghrelin binds with CocH3 in a similar mode as ghrelin binding with wild-type human BChE,
38 which explains why CocH3 has a similar catalytic activity against ghrelin compared to wild-type
39 human BChE. The obtained enzyme-ghrelin binding mode may be used as a starting point for
40 future rational design and discovery of a CocH3-Fc(M3) variant with a significantly improved
41 catalytic efficiency against ghrelin without decreasing the catalytic efficiency against cocaine.
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Additional Information

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