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Orally administered H-Dmt-Tic-Lys-NH-CH_2-Ph (MZ-2), a potent μ-/δ-opioid receptor antagonist, regulates obese-related factors in mice ☆

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ABSTRACT

Orally active dual μ-/δ-opioid receptor antagonist, H-Dmt-Tic-Lys-NH-CH_2-Ph (MZ-2) was applied to study body weight gain, fat content, bone mineral density, serum insulin, cholesterol and glucose levels in female *ob*/*ob* (B6.V-Lep<ob>/J homozygous) and lean wild mice with or without voluntary exercise on wheels for three weeks, and during a two week post-treatment period under the same conditions. MZ-2 (10 mg/kg/day, p.o.) exhibited the following actions: (1) reduced body weight gain in sedentary obese mice that persisted beyond the treatment period without effect on lean mice; (2) stimulated voluntary running on exercise wheels of both groups of mice; (3) decreased fat content, enhanced bone mineral density (BMD), and decreased serum insulin and glucose levels in obese mice; and (4) MZ-2 (30 μM) increased BMD in human osteoblast cells (MG-63) comparable to naltrexone, while morphine inhibited mineral nodule formation. Thus, MZ-2 has potential application in the clinical management of obesity, insulin and glucose levels, and the amelioration of osteoporosis.

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1. Introduction

The opioid system is one of the key homeostatic mechanisms participating in the development of obesity by affecting the neural reward system through feeding behavior and body weight regulation (Atkinson, 1987; Berestov, 1983; Cota et al., 2006; Kas et al., 2004; Khawaja et al., 1989; Tabarin et al., 2005; Yeomans and Gray, 2002). While opioid agonists increase food intake and body weight gain (Fields, 2007; McCormack and Denbow, 1989), these endpoints are decreased by various opioid antagonists (Cole et al., 1997; Jarosz and Metzger, 2002; Levine et al., 1991; Recant et al., 1980; Shaw et al., 1991), indicating that opioid antagonists may have a potential role in the management of obesity.

Among the multiplicity of opioid receptors, the μ-opioid (MOP) receptor appears to be a key element in the neuronal reward pathway within the central nervous system responsible for craving and addiction to various drugs, such as morphine and its derivatives, as well as alcohol and excess food consumption (Avena et al., 2008; Wang et al., 2006). This observation was verified by MOP receptor knockout mice which had decreased food anticipation (Kas et al., 2004; Papaleo et al., 2007; Tabarin et al., 2005) and a reduced response to the anorectic effects of opioid antagonists (Zhang et al., 2006). These data suggest that MOP receptor is a responsible factor in the reward pathway for feeding behavior in mammals. Furthermore, MOP receptor knockout mice have higher body weight than control animals, which may be a result of the up-regulation of neuropeptide Y expression as a compensatory pathway (Han et al., 2006). Studies using positron emission tomography in obese individuals showed reductions in striatal dopamine D_2 receptors similar to that observed in drug addicted subjects, indicating that overeating in obese individuals shares similarities with the loss of control and compulsive drug taking behavior observed in drug-addicted subjects (Wang et al., 2004).

In our efforts in developing opioid antagonists that elicit a reduction in and impinge upon addiction, withdrawal and the development of tolerance, we described an effective dual functioning μ-/δ-opioid (MOP/DOP) receptor antagonist H-Dmt-Tic-Lys-NH-CH_2-Ph (MZ-2) (Balboni et al., 2006). MZ-2 inhibited analgesia and tolerance to morphine in mice after intracerebroventricular, subcutaneous and oral administration (Jinsmaa et al., 2008). In the present study we investigated whether a single oral dose of MZ-2 (10 mg/kg/day) can elicit an effect on obesity in

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ob/*ob* mice, a well established in vivo model system (Recant et al., 1980), in comparison to wild type lean mice. Female mice were used because obesity is more prevalent in female than in male population (Gellner and Domschke, 2008; Ogden et al., 2006). Using an exercise paradigm, the mice were divided into two groups: one with voluntary access to exercise wheels and another without the wheels as representative of physically active and sedentary life styles, respectively. Each group was administered MZ-2 or saline orally for three weeks and body weight gain, body fat content and bone mineral density were recorded, and then housed for additional two weeks without treatment but measuring the same endpoints.

2. Materials and methods

2.1. Chemicals

Minimal Essential Medium (MEM) with Earle's salt and non-essential amino acids was from Invitrogen Corp., (Carlsbad, CA, USA), penicillin–streptomycin solution, L-glutamine, sodium pyruvate, alizarin red, morphine sulphate pentahydrate, β-glycerophosphate, dexamethasone, and 2-phospho-L-ascorbic acid from Sigma (Louis, MO, USA), AlamarBlue solution from Biosource International Inc. (Camarillo, CA, USA), Insulin (Mouse) Ultrasensitive EIA Kit from Alpco Diagnostics (Salem, NH, USA), fetal bovine serum from HyClone (Logan, UT, USA), and a protease inhibitor cocktail from Roche Diagnostics (Indianapolis, IN, USA). Naltrexone hydrochloride was purchased from Tocris Bioscience (Ellsville, MO, USA). The reagents for determination of cholesterol, glucose and triglycerides in serum were from Olympus America Inc., (Melville, NY, USA), and the reagents for determination of HDL, LDL, and free fatty acids were from The Genzyme Corporation (Cambridge, MA, USA). MZ-2 (H-Dmt-Tic-Lys-NH-CH_2-Ph) was synthesized as described earlier (Balboni et al., 2006).

2.2. Animals

Five week old female *ob*/*ob* (B6.V-Lep<ob>/J homozygous) and age matched lean control mice (C57BL/6) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival to our facility mice were housed one per cage in a 12 h light/dark cycle and temperature-controlled room with free access to food and water. Animals were acclimatized for one week before starting the experiments. All animal procedures were carried out according to protocols approved and on file by the Animal Care and Use Committee (ACUC) at the National Institute of Environmental Health Sciences.

2.3. Monitoring of body weight, food intake and physical activity of animals

Animals were divided into two groups, one with sedentary life style and the other with voluntary access to exercise wheels. Animals were treated orally either with MZ-2 (10 mg/kg) dissolved in saline or with saline alone (vehicle group) once daily for three weeks one half hour before the dark cycle (19:00 h). Each treatment group consisted of 5 animals. Body weight and food consumption were recorded daily before treatment. Voluntary exercise on wheels was recorded automatically with the use of stainless steel rodent activity wheels (34.5 cm diameter) (Mini Mitter, Bend, OR, USA). The wheel formed a portion of the lid of the cage; the other portion contained a food hopper and water bottle holder. Mice were placed on wheels for one week before the experiment began in order to acclimate them to this novelty in their housing regime. Revolutions of wheels were counted by magnetic switches connected to the Vital View 3000 data acquisition system (Mini Mitter, Bend, OR, USA). Wheel revolution counts were collected every 5 min 24 h a day for the entire duration of the experiment (five weeks). Wheel activity data were exported into MS Excel spreadsheet program for data reduction and analysis.

2.4. Determination of body composition and bone mineral density

Body composition and bone mineral density were measured three times during the course of the study: before dosing, at the termination of drug administration (three weeks), and at the end of study (five weeks). Measurements were based on dual-energy X-ray absorptiometry (DEXA) with the use of PIXImus2 Mouse Densitometer (GE Medical Systems, Madison, WI). Prior to measurements, mice were anesthetized with isoflurane gas. Bone mineral density (g/cm^2) was measured from a region of interest (ROI) that encompassed the entire body without the head. At the end of the study mice were euthanized with CO_2, and blood was taken for analyses.

2.5. Analyses of serum

Serum insulin levels were determined with the use of Insulin (Mouse) Ultrasensitive EIA Kit (Alpco Diagnostics, Salem, NH, USA), following the manufacturers protocol. Assays of serum glucose, triglycerides, cholesterol, HDL, LDL, and free fatty acids were performed in duplicates on an AU400e Clinical Chemistry analyzer (Olympus America Inc. Irving, TX, USA).

2.6. Cell culture

The MG-63, human osteoblast-like cells, (CRL-1427) were obtained from the ATCC (Manassas, VA, USA). MG-63 cells were cultured as monolayers in MEM with Earle's salt, non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate, supplemented with10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The effect of 30 μM MZ-2, morphine or naltrexone on cell proliferation was determined with the use of AlamarBlue assay (Nakayama et al., 1997). Cells were seeded in 96 well plates (5×10^4 cells/well in 0.1 ml medium) and grown overnight as described above. The medium was changed with fresh medium containing test compounds and cells were incubated for 24 h. Then 10 μl of the AlamarBlue solution was added to each well and after 1 h incubation fluorescence (λ_{Ex} 530 nm, λ_{Em} 590 nm) was measured with the use of Microplate Fluorescence Reader FL600 (BioTek Instruments, Winooski, VT, USA). In order to measure the effect of the compounds on mineral nodule formation, cells were seeded in 24 well plates ($2.5–3\times10^5$ cells/well) and grown for one day. When cells reached ~70% confluency, they were treated with an osteogenic medium [β-glycerophosphate (2 mM), dexamethasone (10 nM), 2-phospho-L-ascorbic acid (50 μM)] with or without MZ-2, morphine or naltrexone (30 μM) for 30 days. Medium was changed every 2–3 days. Effect of compounds on bone nodule formation was determined by alizarin red staining, a common histochemical technique used to detect calcium deposits in mineralized tissues and cultures (Lievremont et al., 1982). Medium was aspirated, cells were washed with phosphate buffered saline, fixed with 70% ethanol and stained with 1% alizarin red for 30 min following washing with demineralized water, and viewed under a light microscope. Quantitative analysis of mineralization was performed according to (Gregory et al., 2004) with a minor modification. Briefly, cells stained with alizarin red were dispersed in 10% acetic acid and transferred into 2 ml tubes. After heating for 10 min at 85 °C, cell dispersion was cooled on ice and centrifuged at 15,000×*g* for 15 min. Aliquots of supernatants were transferred to a new tubes and pH was adjusted to 4.1–4.5 with 10% ammonium hydroxide. Optical density of solution was measured at 405 nm and amount of alizarin red bound was calculated from standard curve obtained with the use of standard alizarin red solutions.

2.7. Statistical analysis

Results were expressed as the means ± S.E.M. Statistical analysis of body weight gain was performed with repeated measures ANOVA.