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Functional Evaluation of THIQ, a Melanocortin 4 Receptor Agonist, in Models of Food Intake and Inflammation

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Abstract: The central melanocortinergic system plays an important role in regulating different aspects of energy homeostasis and the immunomodulatory response. In the present study, we evaluated the *in vivo* activities of food intake suppression and anti-inflammatory activity of THIQ, which has been proposed to possess high and selective melanocortin-4 receptor agonistic activity *in vitro*. The results showed that THIQ (0.1, 0.3 and 1 nmol/rat, intracerebroventricularly) is less effective in reducing food intake and body weights of rats than the non-selective melanocortin receptor agonist melanotan II. Electron paramagnetic resonance measurements in mice brain tissue showed that THIQ at doses of 0.001 and 0.01 nmol/mouse (intracisternally) increased the concentration of nitric oxide, which is not typical for melanocortin receptor agonists. In an experimental brain inflammation model, THIQ only weakly antagonized lipopolysaccharide-induced nitric oxide overproduction in brain tissue at a dose of 0.01 nmol/mouse. Our findings provide new insight into the *in vivo* pharmacological profile of the *in vitro* selective melanocortin-4 receptor agonist THIQ and give grounds for caution when interpreting and predicting melanocortin receptor selective agonist activity *in vivo*.

Melanocortin-4 (MC4) receptor belongs to a family of G-protein-coupled receptors that bind melanocyte-stimulating hormones (MSH) and regulate physiologically important responses of the immune system and energy homeostasis [1,2]. MC4 receptor is known to play a central role in appetite regulation and energy expenditure [3,4]. In addition, the involvement of the MC4 receptor in regulation of sexual activity and inflammatory responses has also been suggested [5–7]. Over the past several years, selective agonists and antagonists for the MC4 receptor have been synthesized with the goal to treat health disorders such as obesity and sexual dysfunction [1–3,5,8].

Among others, researchers at Merck reported the discovery of an orally active non-peptide MC4 receptor mimetic, called THIQ (fig. 1) [9]. MC receptor-binding data in Chinese hamster ovary cells expressing the relevant receptor showed high selectivity of THIQ for the MC4 receptor. THIQ bound to MC4 receptors with more than 1300-fold selectivity over MC1, more than 1100-fold over MC3 and more than 350-fold over MC5 receptors [9]. Moreover, THIQ stimulated 3',5'-cyclic monophosphate (cAMP) generation, giving maximally 95–97% of the stimulation of cAMP induced by α-MSH in a human MC1 receptor assay, as well as THIQ was similarly active as α-MSH also in human and rat MC4 receptor assays. However, for the MC3 receptor and MC5 receptors THIQ only reached 32% and 61%, respectively, of the maximal α-MSH stimulation on cAMP [9]. Thus, THIQ could be considered as a full agonist at MC1 and MC4 receptors *in vitro*.

The pharmacological activity of THIQ has also earlier been tested in experimental models for male sexual activity in rats [10] and mice [11]. It was shown that central administration of THIQ induced MC4 receptor-mediated erectogenic effects in both species [10]. Moreover, the cardiovascular responses of the compound were also evaluated in rats. It was found that THIQ at a dose of 10 mg/kg after intraperitoneal administration increased both heart rate and mean arterial pressure [12]. In the same study, THIQ decreased food intake in rats over a 24-hr period only at the highest dose tested (10 mg/kg, intraperitoneally) [12]. The effect of THIQ on food intake has been studied in mice after intracerebroventricular administration at doses of 3, 10 and 32 nmol/mouse. As expected for a highly selective MC4 receptor agonist, THIQ dose-dependently inhibited food intake in wild-type mice and had no effect in MC4 receptor knock-out mice [13].

In our previous studies, we aimed to characterize the MC receptor responses that regulate inflammatory processes in the brain. In these studies, we used low-affinity MC4 receptor peptide ligands and the results indicated leading MC3 receptor role for the anti-inflammatory effects of peptides [14]. THIQ is a small non-peptide molecule with high affinity and selectivity for the MC4 receptor and its anti-inflammatory activity has not been evaluated thus far. Therefore, to get additional information about MC receptor role in regulation of brain inflammation in the present study we tested the *in vivo* nitric oxide inhibiting potency of THIQ in a lipopolysaccharide (LPS)-induced brain inflammation model in mice.

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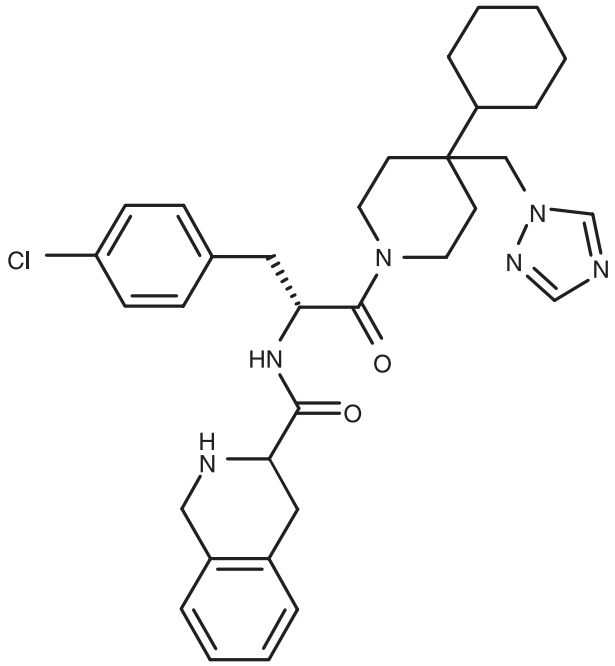

Fig. 1. Structural formula of THIQ.

Moreover, we also studied the effects of THIQ in food intake experiments in rats. In feeding experiments, THIQ induced a less pronounced suppression of food consumption than melanotan II (MTII), a non-selective MC receptor agonist. Interestingly, even though the MC3 receptor is thought to be the main inflammation-regulating MC receptor in the brain [14], THIQ, within a narrow dosage window, was found to have some activity in inhibiting LPS-induced nitric oxide overproduction.

Materials and Methods

Materials. Melanotan II, LPS (*Escherichia coli* 055:B5), sodium diethyldithiocarbamate, $FeSO_4$, sodium citrate, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). THIQ was synthesized at the Department of Pharmaceutical Biosciences of Uppsala University, Sweden, by Dr.chem. F. Mutulis [15].

Animals. Male ICR mice, weighing 23–25 g, were housed under standard conditions (21–23°C, a 12-hr light:dark cycle) with unlimited access to food and water. In feeding experiments, adult male Wistar rats, weighing 290–320 g, were housed individually in hanging plastic cages (55 × 33 × 19 cm) with free access to food pellets (diet R3 for mice and R70 for rats, Lactamin, Sweden) and water *ad libitum* at controlled temperature (20 ± 1°C) and light (a 12-hr light:dark cycle). All experimental procedures were carried out in accordance with guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved Ethics Council of Animal Protection at the Veterinary and Food Service, Riga, Latvia.

Intracerebroventricular administration. Rats were anaesthetized with ketamine 100 mg/kg, xylazine 10 mg/kg, intraperitoneally, and an 8-mm long 24-gauge stainless – steel cannula was implanted into the left lateral ventricle 1.3 mm caudal to the bregma, 1.7 mm lateral from midline and 3.2 mm below the skull at the point of entry [16]. Cannulas were anchored to the skull with a screw and kept in place with dental cement (Fulldent, Switzerland). After surgery the cannula was closed with a stylet. Rats were handled and weighed during the recovery period (at least 7–10 days) to minimize non-specific stress. The substances were dissolved in saline and administered by a 31-gauge stainless-steel injector, projecting 1.0 mm below the tip of the guide cannula. The injector was connected to a gastight 10 µl Hamilton syringe (Reno, NV, USA) by polyethylene tubing (0.58 mm i.d.; 1.27 mm o.d.). Drugs were infused by infusion pump (World Precision Instruments, Sarasota, FL, USA) at a speed of 1 µl/min. (together 5 µl). The movement of an air bubble inside the PE20 polyethylene tubing confirmed the solution flow. The needle was left in place for 15 sec., then the cannula was closed with a stylet and rats were returned to their home cage.

Feeding experiments. The effects of THIQ and MTII on food intake and rat body weight were tested after an overnight fast. At the start of the experiment (5:00–5:30 p.m.), the animals were placed in clean cages with free access to water, but without food pellets. After 24 hr, the rats were weighed and tests compounds MTII or THIQ at doses 0.1, 0.3 and 1 nmol/rat were injected intracerebroventricularly a volume of 5 µl of saline. The control group animals received an identical injection volume. After drug administration, rats had free access to food pellets and water during the entire observation period. Food intake was measured at 3 (dark phase), 15 (overnight) and 24 hr after intracerebroventricular administration of the compounds. The change in body weight was measured at the start and the end of the experiment.

Determination of nitric oxide production. The determination of nitric oxide content in mice brain tissue was carried out by the electron paramagnetic resonance (EPR) method as described by Vanin et al. [17]. THIQ was administered intracisternally with or without LPS in a total injection volume of 10 µl. The concentration of LPS and THIQ ranged from 0.0001 to 1 nmol per animal. Control mice received an intracisternal injection of 10 µl saline. Spin trap reagents were administered 0.5 hr before animal decapitation. Thus, all animals received intraperitoneal injections of diethyldithiocarbamate (400 mg/kg) followed by subcutaneous injections of ferrous citrate, prepared directly in the syringe just before use (40 mg/kg ferrous sulfate + 200 mg/kg sodium citrate) 5.5 hr after drug administration. Decapitation was carried out 6 hr after LPS administration according to a previously evaluated brain inflammation model [18]. The brains were dissected and immediately frozen in liquid nitrogen.

The frozen brain tissues were slightly thawed to allow sample preparation by compacting the tissue into plastic tubes to form rods, 20 mm long and 4 mm in diameter, which were then immediately frozen in liquid nitrogen. EPR spectra of the samples were recorded at liquid nitrogen temperature using an EPR spectrometer Radiopan SE/X2544 (Radiopan, Poznan, Poland) as described previously [19]. Measurement parameters were as follows: X-band operation, 25 mW microwave power, 9.24 GHz microwave frequency, 100 kHz modulation frequency, 5 G modulation amplitude, 0.5 × 104 receiver gain, time constant 1 sec. The content of nitric oxide was computed from the third component (Ix) at g = 2031. The nitric oxide concentration (ng/g of tissue) was calculated according to Vanin et al. [17].

Statistical analysis. All results are expressed as mean ± S.E.M. The statistical significance was calculated by the Student's t-test. The cumulative food intake data were analysed by analysis of variance (ANOVA). P-values of < 0.05 were considered significant.

Results

Comparative effects of THIQ and MTII on food intake.

After the overnight fast, the food intake in control rats 3, 15 and 24 hr after the start of the experiment was 3.3 ± 0.3, 15.7 ± 1.1 and 17.4 ± 1.0 g, respectively (fig. 2). As seen in

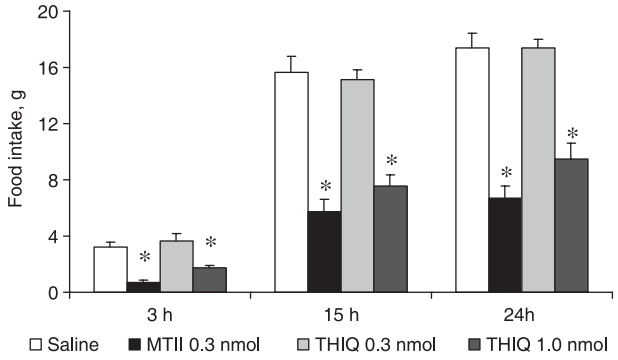

Fig. 2. Influence of THIQ and melanotan II (MTII) on food intake. THIQ at doses of 0.3 and 1 nmol/rat and MTII at a dose of 0.3 nmol/rat were administered intracerebroventricularly. Food intake was detected 3, 15 and 24 hr after injection. Food consumption was significantly suppressed 3 hr after injection of MTII at a dose of 0.3 nmol/rat. THIQ significantly inhibited food intake at a dose of 1 nmol/rat. Both compounds at the active doses suppressed food consumption in rats over the observation period. *$P < 0.05$ compared to control.

fig. 2, 3 hr after injection of MTII at a dose of 0.3 nmol/rat, food consumption was significantly suppressed and reached only about 20% of the control level. We found that at all the test time-points 0.3 nmol/rat of THIQ did not induce any statistically significant changes in food intake. However, at a dose of 1 nmol/rat THIQ significantly inhibited food intake to about half of the control level (fig. 2). Both compounds at the active doses suppressed food consumption in rats over the observation period, including at night when rodents usually consume the greatest amount of food pellets.

The rat body weight was established before intracerebroventricularly injection of the drug and at the end of the experiment. Body weight (290–320 g) before the experiment was taken as zero control value. As seen in fig. 3, the body

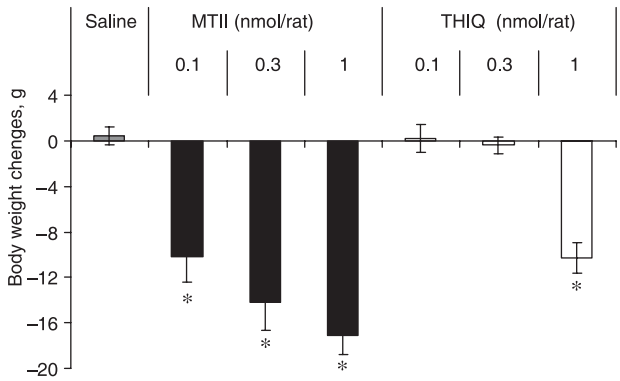

Fig. 3. Influence of THIQ and melanotan II (MTII) on body weight. THIQ and MTII at doses of 0.1, 0.3 and 1 nmol/rat were administered intracerebroventricularly and body weight was measured 24 hr after administration. MTII statistically significantly reduced body weight in all tested doses. THIQ caused a statistically significant reduction in body weight at a dose of 1 nmol/rat. This reduction was similar to the MTII effect at a dose of 0.1 nmol/rat. *$P < 0.05$ compared to control.

weights increased slightly over the 24 hr evaluation period for the saline-treated control rats, while for the MTII treated ones the body weights were dose-dependently reduced at all MTII doses tested. The most pronounced decrease was observed after administration at dose of 1 nmol/rat, which resulted in weights being 15–20 g lower than that of control rats (fig. 3). THIQ administration did not influence rat body weights at doses of 0.1 and 0.3 nmol/rat, while at 1 nmol/rat it caused a statistically significant reduction of the animal body weight, amounting to 10.3 ± 1.3 g. This reduction was similar to the decrease in rat body weight induced by MTII at a 10-time lower dose (0.1 nmol/rat).

Effects of THIQ on nitric oxide production.

The effect of THIQ was first evaluated on basal nitric oxide production in mice brain tissue after intracisternal injection at doses of 0.001, 0.01 and 1 nmol/mouse. Six hours after intracisternal vehicle administration, the normal control level of nitric oxide ranged from 50–70 ng/g (fig. 4). Administration of THIQ at doses of 0.001 and 0.01 nmol/mouse caused a statistically significant increase in nitric oxide levels of 46% and 34%, respectively. In contrast, a higher dose of 0.1 nmol/mouse THIQ did not show any significant influence on the basal nitric oxide level.

To test the effect of THIQ on brain inflammation processes, we used a mouse experimental model for acute brain inflammation in which the production of nitric oxide is stimulated

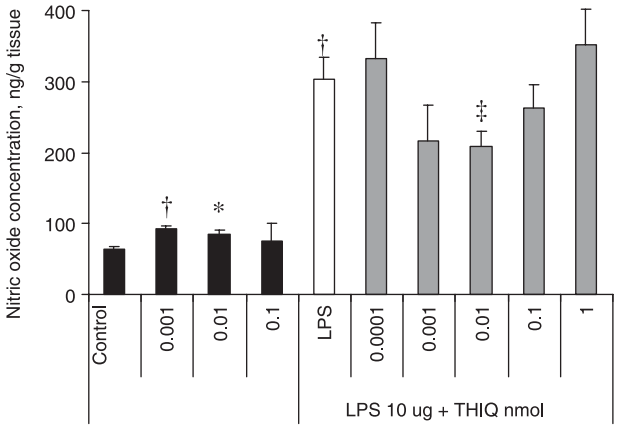

Fig. 4. Effects of THIQ on the levels of nitric oxide in the brains of lipopolysaccharide (LPS)-treated mice. THIQ was administered intracisternally with or without LPS (10 µg/mouse) in a total injection volume of 10 µl. The concentration of THIQ ranged from 0.0001 to 1 nmol per animal. Control mice received an intracisternal injection of 10 µl saline. THIQ increased basal nitric oxide production in mice brain tissue at doses of 0.001 and 0.01 nmol/mouse. Administration of LPS-induced increase in nitric oxide production in brain tissue. THIQ did not prevent the LPS-induced increase of nitric oxide at the lowest and highest doses. Statistically significant nitric oxide production inhibitory effect was observed at a dose of 0.01 nmol/mouse. *$P < 0.05$ compared to control nitric oxide level in mice brain. †$P < 0.001$ compared to control nitric oxide level in mice brain. ‡$P < 0.05$ compared to LPS-induced increase of the nitric oxide level.

by intracisternal injection of LPS. Administration of LPS after 6 hr induced approximately a 5-fold increase in nitric oxide production in the brain tissue, reaching a concentration of 300 ng/g tissue wet weight (fig. 4). THIQ administration to LPS-treated animals gave a U-type response curve over a broad dose range (0.0001–1.0 nmol/mouse), where the lowest and highest doses of THIQ did not prevent the LPS-induced increase of nitric oxide, while a statistically significant inhibitory effect on the nitric oxide production, amounting 30%, was observed at a THIQ dose of 0.01 nmol/mouse (fig. 4). Moreover, even at the most active dose of THIQ, nitric oxide levels still remained approximately three times higher than those of the control.

Discussion

In the present study, we found that a single injection of THIQ into rats induced a less pronounced suppression of food consumption than did MTII, a non-selective MC receptor agonist (figs 2 and 3). MTII has been know for almost two decades and has been studied in various pharmacological assays. Consistent with previous findings in mice [13] and rats [20], central administration of MTII was found to attenuate food intake. MTII at a dose of 0.3 nmol caused a reduction of short-term (3 hr) food intake and long-term (15 and 24 hr) feeding (fig. 2). Parallel examination of the two potent MC receptor ligands, MTII and THIQ, under the same experimental conditions revealed an unexpected effectiveness of the non-selective agonist MTII over the MC4-selective agonist THIQ after their intracerebroventricular administration in rats. In contrast to MTII, a statistically significant effect of THIQ on food intake was observed only at a higher dose – 1 nmol/rat (figs 2 and 3). Similar to previous reports in mice [13], the THIQ effect on food intake at a dose of 32 nmol/mouse was comparable with that of MTII at a dose of 1 nmol/mouse. In rats, MTII intracerebroventricularly inhibited food intake for up to 48 hr at a dose of 1 nmol/rat [9,20]. There are reported unpublished data about the effect of THIQ on food intake in rats [9]; however, the authors did not describe the administration route and dosage values. Our study is the first that compares the central effects of THIQ and MTII on food intake in rats. In parallel to its influence on food consumption, THIQ caused body weight reduction at a dose of 1 nmol/rat, and this effect was comparable with the effect of MTII at a 10-time lower dose (0.1 nmol/rat).

The different activities of MTII and THIQ could be explained by the fact that MTII is an agonist that acts non-specifically on several MC receptors. In addition, non-peptide agonist THIQ and peptide agonist MTII might have different effects on MC4 receptor. Indeed, the studies on peptide and non-peptide agonist interactions with MC receptor showed that THIQ did not cause MC4 receptor internalization (the process that is linked to the receptor down-regulation) to the same degree as peptide agonists [21]. The differences between *in vitro* activity and *in vivo* potency have been described also in case of MC receptor antagonists. Thus, both HS024 and HS014, which bind to MC4 receptor with 10-fold different affinity, *in vivo* afforded similar food intake increasing activity [22].

The data obtained in brain inflammation experiments give evidence that THIQ administration influences nitric oxide concentration in mice brain (fig. 4). THIQ, administered together with LPS, possesses some anti-inflammatory activity and to some extent inhibits the LPS-induced increase in nitric oxide levels in a mouse model for brain inflammation (fig. 4). We [14,18] and others [23,24] have shown the significance of MC3 receptor signalling in the anti-inflammatory action of MC receptor agonists. In previous studies, we used peptides with a low affinity to MC4 receptor [14] and found good correlation between MC3 receptor-binding affinity and LPS-stimulated nitric oxide inhibition. Here, we tested THIQ in the same acute inflammation model *in vivo* that we used earlier to evaluate the effects of natural and synthetic MCs peptides. Our present study demonstrates only a weak inhibitory effect of THIQ (30%) on LPS-induced nitric oxide production in brain tissue. Moreover, the obtained dose-effect curve was U-shaped, reaching maximal nitric oxide-inhibition at a dose of 0.01 nmol/rat (fig. 4). At the highest-tested dose of 1 nmole/rat, THIQ showed a tendency to increase nitric oxide production. In contrast, in our previous experiments for MC receptor peptide agonists a dose-dependent effect was observed [14,18].

In our previous studies, none of the tested peptides influenced nitric oxide levels in the brains of mice [14,18]. In contrast, THIQ itself caused an increase in the nitric oxide level in control rats (fig. 4). This effect occurred at low THIQ doses and was statistically significant up to the highest dose of 1 nmol/rat. It is too early to speculate on the mechanism involved in THIQ-induced increase of nitric oxide production in control mice. In brain tissue, nitric oxide production is supposed to occur under many physiological conditions, and among others the activation of the neuronal isoform of nitric oxide synthase could be involved. One could hypothesize that the increase of nitric oxide levels, in respective tissues, after THIQ administration might explain the erectogenic effect of THIQ observed in male mice and rats [10,11]. However, although the erectogenic effect of THIQ has been demonstrated, the potency of MTII in sexual activity tests is more pronounced. Indeed, intracerebroventricular administration of MTII at doses of 0.3 and 1 μg increased the number of erections in rats [25], whereas THIQ at a dose of 20 μg intracerebroventricularly [10] did not afford such effect.

In summary, our data show that the MC4 receptor-selective compound THIQ only weakly antagonises LPS-induced nitric oxide overproduction in the brains of mice after intracisternal administration. In addition, THIQ is less effective than non-selective MC receptor agonist MTII in inhibiting food consumption in rats after intracerebroventricular administration. Our findings provide new insight into the *in vivo* pharmacological profile of the *in vitro* selective MC4 receptor agonist THIQ and prompt for caution when interpreting and predicting MC receptor-selective agonist activity *in vivo*.

Acknowledgements

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