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Involvement of the melanocortin MC_4 receptor in stress-related behavior in rodents

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Abstract

The melanocortin subtype 4 (MC_4) receptor has been postulated to be involved in stress and stress-related behavior. We made use of melanocortin MC_4 receptor agonists and antagonist to investigate the relationship between the melanocortin MC_4 receptor and stress related disorders. The nonspecific melanocortin receptor agonist α-melanocyte stimulating hormone (α-MSH) and the melanocortin MC_4 receptor agonist, Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH-(4-10)-NH2 (MT II) dose-dependently and significantly reduced the number of licking periods in the rat Vogel conflict test, suggesting that stimulation of the melanocortin MC_4 receptor causes anxiogenic-like activity in rats. We synthesized a peptidemimetic melanocortin MC_4 receptor selective antagonist, Ac-D-2Nal-Arg-2Nal-NH2 (MCL0020), which has high affinity for the melanocortin MC_4 receptor with IC_{50} values of 11.63 ± 1.48 nM, in contrast, the affinities for melanocortin MC_1 and MC_3 receptors were negligible. In addition, MCL0020 significantly attenuated the cAMP formation induced by α-MSH in COS-1 cells expressing the melanocortin MC_4 receptor without affecting basal cAMP contents. Thus, we considered MCL0020 to be a selective melanocrotin MC_4 receptor antagonist among melanocortin receptors. Restraint stress significantly reduced food intake in rats, and i.c.v. administration of MCL0020 dose-dependently and significantly attenuated restraint stress-induced anorexia without affecting food intake. Swim stress induced reduction in the time spent in the light area in the mouse light/dark exploration test, and MCL0020 significantly prevented it. Taken together our findings suggest that the melanocortin MC_4 receptor might be related to stress-induced changes in behavior, and blockade of the melanocortin MC_4 receptor may prevent stress-induced disorders such as anxiety.

Keywords: Melanocortin MC_4 receptor; Melanocortin; Anxiety; MCL0020; MT II (Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH-(4-10)-NH2)

1. Introduction

Brain melanocortins are derived from pro-opiomelanocortin (POMC) by enzymatic processing and are involved in a wide range of physiological events, including memory and/or learning (De Wied and Jolles, 1982), thermoregulation (Murphy et al., 1983), analgesia (Vrinten et al., 2000, 2001), stress responses (Dunn et al., 1979; De Barioglio et al., 1991; Von Frijtag et al., 1998; Adan et al., 1999) and feeding behavior (Poggioli et al., 1986).

To date, five types of receptor subtype for melanocortin (MC_1–MC_5) have been cloned (Chhajlani and Wikberg, 1992; Chhajlani et al., 1993; Mountjoy et al., 1992; Gantz et al., 1993a,b). Among them, melanocortin MC_3 mRNA has been found in distinct areas of the brain as well as placental and gut tissues, while melanocortin MC_4 receptor has been found only in the brain (Gantz et al., 1993a,b). The melanocortin MC_4 receptor is of interest in terms of the central regulation of feeding behavior and energy expenditure (Fan et al., 1997; Huszar et al., 1997; Jonsson et al., 2001).

In addition to feeding behavior, there are several lines of evidence that brain melanocortins are involved in stress responses and stress-related disorders such as anxiety and depression, and that melanocortin MC_4 receptor may

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mediate the physiological effects caused by stress. It has been reported that α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) induce excessive grooming behavior in rats (De Barioglio et al., 1991; Adan et al., 1999), and that antiserum to ACTH reduces novelty-induced grooming (Dunn et al., 1979). Injection of ACTH activates the hypothalamus–pituitary–adrenal axis (Von Frijtag et al., 1998). Moreover, α-MSH and ACTH have been shown to exert anxiogenic-like behavior (File and Clarke, 1980; Corda et al., 1990; Panksepp and Normansell, 1990; Gonzalez et al., 1996). In accord with findings hitherto, melanocortin MC_4 receptor agonists induces grooming behavior in rats, and an melanocortin MC_4 receptor antagonist attenuates melanocortin MC_4 receptor agonists-induced grooming as well as novelty-induced grooming (Adan et al., 1999). Moreover, the selective melanocortin MC_4 receptor antagonist, HS014, blocks immobilization stress-induced anorexia in rats (Vergoni et al., 1999). Therefore, it is postulated that the brain melanocortinergic system has an important role in stress-induced changes in neurochemical and behavioral responses, and stress-related disorders such as anxiety.

We have synthesized Ac-D-2Nal-Arg-2Nal-NH2 (2-Nal = 3-(2-naphthyl)-L-alanine; D-2-Nal = 3-(2-naphthyl)-D-alanine), MCL0020), a peptidemimetic selective melanocortin MC_4 receptor antagonist. In the present study, we investigated involvement of the melanocortin MC_4 receptor in stress-induced behaviors such as anorexia and anxiety using melanocortin MC_4 receptor agonists and antagonist.

2. Materials and methods

2.1. Animals and surgery

Male ICR mice (20–30 g, Charles River, Yokohama, Japan) were housed 10/cage. Male Sprague–Dawley rats (220–240 g, Charles River, Yokohama, Japan) were housed 3/cage and used for Vogel conflict test in rats. For the stress-induced anorexia study, male Wistar rats (200–300 g, Charles River, Yokohama, Japan) were used. Animals were maintained under a 12 h light/dark cycle (light on 7:00 a.m.) in a temperature- and humidity-controlled holding room. Food and water were available ad libitum. In experiments for i.c.v. infusion, rats were surgically equipped with a single cannula placed above the lateral ventricle. Animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and placed in a stereotaxic apparatus (Narishige, Tokyo, Japan) where a 7-mm long, 23-gauge stainless steel guide cannula was placed to within 1 mm of the ventricle and anchored to the skull with screws and dental cement. The implantation coordinates were 1.0 mm posterior to the bregma, 1.2 mm lateral to the midline, and 4.5 mm ventral to the cortical surface according to the rat brain atlas of Paxinos and Watson. After a 7-day postsurgical recovery period, cannula patency was confirmed by gravity flow through an 8-mm, 30-gauge injector inserted through the guide to 1 mm beyond its tip. α-MSH, Ac-[Nle^4,Asp^5,D-Phe^7,Lys^{10}]α-MSH-(4-10)-NH2 (MT II) or MCL0020 was injected (10 μl/rat) through the cannula at 10 μl/min. All studies were reviewed by Taisho Pharmaceutical. Animal Care Committee and have met the Japanese Experimental Animal Research Association standards, as defined in the *Guidelines for Animal Experiments* (1987).

2.2. Materials

MCL0020 was synthesized in Taisho Medicinal Research Laboratories. Purity of compound was confirmed by Nuclear Magnetic Resonance and Thin Layer Chromotography, and molecular weight was checked by mass spectrometry. [^{125}I][Nle^4,D-Phe^7]α-Melanocyte stimulating hormone ([Nle^4,D-Phe^7]α-MSH) (specific radioactivity: 81.4 TBq/mmol) and a cAMP assay system were purchased from Amersham International (Buckinghamshire, England). COS-1 cells (CV-1 cell line transformed with an origin defective mutant of SV40 which codes for wild type T antigen) were purchased from American Type Culture Collection (Rocksville, MD, USA). α-MSH, [Nle^4,D-Phe^7]α-MSH and MT II were purchased from Peninsula Laboratories (Belmont, CA, USA). All other chemicals used in this study were obtained commercially, and of the highest purity available. For the in vitro study, MCL0020 was dissolved in 0.1% dimethylsulfoxide, and dimethylsulfoxide (0.1%) itself did not affect both the binding assay and cAMP levels. For behavioral studies, MT II and α-MSH were dissolved in buffered artificial cerebrospinal fluid (ACSF; 137.9 mM NaCl, 3.37 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1.45 mM NaH_2PO_4, 4.85 mM Na_2HPO_4, pH 7.4) containing 0.1% bovine serum albumin, and MCL0020 was dissolved in 10% dimethylsulfoxide in ACSF, and the vehicle did not affect behavior.

2.3. Melanocortin receptor expression constructs, cell cultures and transfection

Melanocortin MC_4, MC_1 and MC_3 receptor cDNAs were isolated by reverse transcription-polymerase chain reaction from the human hippocampus, WM-266-4 cells (human melanoma cell line) and rat hypothalamus, respectively, as reported previously (Chaki et al., 2003). Melanocortin MC_4 and MC_3 receptor cDNAs were cloned into expression vector pcDLΔPE and melanocortin MC_1 receptor cDNA into pTARGET. COS-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37 °C. The melanocortin receptor cDNAs inserted into expression vectors were separately transfected into COS-1 cells using lipofectin (GIBCO BRL) according to the

protocol provided by manufacturer (Felgner et al., 1987). At 72 h after transfection, COS-1 cells expressing melanocortin MC_1, MC_3 or MC_4 were used in pharmacological experiments.

2.4. [^{125}I][Nle^4,D-Phe^7]α-MSH binding

COS-1 cells expressing the melanocortin receptor were washed with phosphate buffered saline, scraped and pelleted by centrifugation. Cell pellets were homogenized with 50 mM Tris–HCl buffer (pH 7.4) containing 2 mM EDTA, 10 mM $CaCl_2$ and 100 μM phenylmethylsulfonylfluoride, and centrifuged at 48,000 × *g* for 20 min at 4 °C. The pellet was washed twice with the buffer, and the final pellet was suspended in the assay buffer (50 mM Tris–HCl buffer (pH 7.4) containing 2 mM EDTA, 10 mM $CaCl_2$, 100 μM phenylmethylsulfonylfluoride and 0.1% bovine serum albumin), and served as crude membrane preparation for binding studies. Protein concentration was determined according to the method reported by Bradford (1976). Binding assays of [^{125}I][Nle^4,D-Phe^7]α-MSH were done according to Schioth et al. (1998). Membranes were incubated with [^{125}I][Nle^4,D-Phe^7]α-MSH (0.2 nM) for 120 min at 25 °C, and the reaction was terminated by rapid filtration over a GF/C filter presoaked with 0.5% bovine serum albumin, after which the filters were washed three times with the buffer. The radioactivity was quantified in a γ-counter. Nonspecific binding was determined in the presence of 1 μM [Nle^4,D-Phe^7]α-MSH. Specific binding was determined by subtracting nonspecific from total binding. In the competition assay, concentration of the test compound that caused 50% inhibition of the specific binding (IC_{50} value) was determined from each concentration-response curve.

2.5. Determination of cAMP

COS-1 cells transiently expressing the melanocortin MC_4 receptor and grown in a six-well plate were used. The culture medium was removed, the cells were washed with phosphate buffered saline, and 1 ml of DMEM containing 1 mM isobutylmethylxanthine, a phosphodiesterase inhibitor, was added. The cells were incubated with α-MSH (10 nM) and/or various concentrations of MCL0020 for 15 min at 37 °C. The culture medium was then aspirated and the cells were washed with phosphate buffered saline. Two milliliter of ice-cold 65% ethanol was added, and the cells were scraped from the wells. The supernatant was collected by centrifugation at 15,000 rpm for 15 min at 4 °C. cAMP formed in the cells was determined using a commercially available cAMP EIA system.

2.6. Stress-induced anxiogenic-like behavior in mice

The swim stress consists of placing mice in a 20-cm tall, 13-cm wide cylindrial plastic container containing 10 cm of water maintained at 25 ± 1 °C. Duration of the swim stress was 10 min, and the light/dark exploration test was done 10 min after the swim stress. The apparatus consisted of two polyvinylchloride boxes (20 × 20 × 14 cm) covered with Plexiglas; one of these boxes was darkened with cardboard. The light compartment was illuminated using a desk lamp (400 lx) placed 17 cm above the box, and the dark compartment provided the only room illumination. An opaque plastic tunnel (5 × 7 × 10 cm) separated the dark and light compartments. During the observation, the experimenter unaware of drug treatment always sat in the same place, next to the apparatus. The mice were individually tested in 5-min sessions in the apparatus described above. Each mouse was placed in the center of the light area to start the test session. The amount of time spent in the light area was recorded for 5 min after the first entry into the dark area. A mouse whose four paws were in the next box was considered as having changed boxes. All mice were naïve to the apparatus. MCL0020 was administered i.c.v. 30 min prior to application of the swim stress. Each for three doses of compounds was used to generate dose–response reactions.

2.7. Stress-induced decrease in food intake in rats

Rats were fasted for 17 h, and then were exposed to restraint stress by placing them in a stress cage made of acryl glass then placing it in an upside-down position for 90 min. Food was provided right after cessation of the restraint stress and food intake was measured for the next 1 h. MCL0020 was administered i.c.v. 30 min prior to exposure to restraint stress.

2.8. Vogel test in rats

A modification of the method of Vogel et al. (1971) was used. Briefly, groups of 7–12 rats were deprived of drinking water but not of food for 48 h prior to the conflict session, then, they were placed in a plexiglas conflict test box (Neuroscience, Tokyo) with a stainless steel grid floor. Each box was placed in a sound-attenuated ventilated chamber. Water was provided through a stainless steel drinking tube extending 1 cm into the box, 3 cm above the floor. The drinking tube and the grid floor were connected to a constant-current shock generator and to a licking time-counter. Drinking attempts were punished with an electric shock (0.4 mA), and the impulses were released, every 2 s of cumulative licking time-counter output. Each shock lasted for 0.5 ms and if the rat was drinking when an impulse was released, it received a shock. Unpunished drinking was measured in as separate group of animals with the current intensity set to 0 mA. The number of shocks was recorded automatically during a 5-min period. MT II or α-MSH was administered i.c.v. 30 min prior to starting the test.

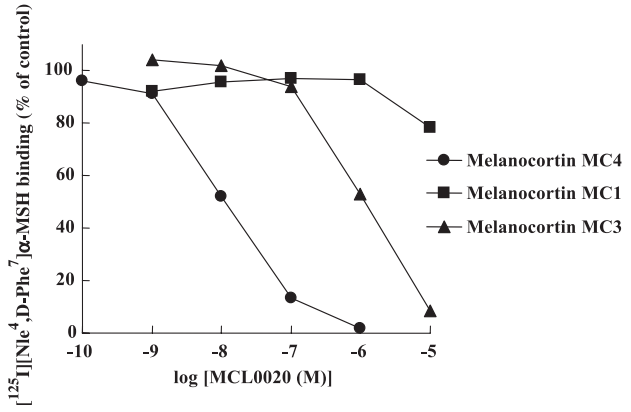

Fig. 1. Inhibition of [Nle^4,D-Phe^7]α-MSH binding to recombinant melanocortin MC_4 (●), MC_1 (■) and MC_3 (▲) by MCL0020. Receptor binding assay of melanocortin receptors was done as described in Materials and methods. Results are mean value of three separate experiments, each done in duplicate.

2.9. Statistical analysis

Data from in vivo experiment were analyzed by one-way analysis of variance (ANOVA) and significant differences between groups were determined, using Dunnett's test.

3. Results

3.1. Receptor binding and cAMP production

MCL0020 inhibited [^{125}I][Nle^4,D-Phe^7]α-MSH binding to membranes of COS-1 cells expressing human melanocortin MC_4 receptor with IC_{50} values of 11.63 ± 1.48 nM (Fig. 1). In contrast, MCL0020 did not show affinity for the melanocortin MC_1 receptor even at 10 μM, and showed moderate affinity for the melanocortin MC_3 receptor with an IC_{50} value of 1115 ± 25.6 nM (Fig. 1). With regard to selectivity over melanocortin MC_1 and MC_3 receptors, MCL0020 is one of the most selective compounds hitherto identified, more selective than HS014 and HS024, both of which was reported to be melanocortin MC_4 receptor selective antagonists (Kask et al., 1998), and equally selective to HS028 as reported by Skuladottir et al. (1999). Both α-MSH and MT II increased cAMP formation in the melanocortin MC_4 receptor expressed in COS-1 cells with potency consistent with the result previously reported (data not shown). MCL0020 concentration-dependently attenuated 10 nM of α-MSH-stimulated cAMP formation in melanocortin MC_4 receptor expressing COS-1 cells (Fig. 2A). On the other hand, MCL0020 per se did not affect basal cAMP formation in COS-1 cells expressing the melanocortin MC_4 receptor, hence, MCL0020 was devoid of agonist activity at the melanocortin MC_4 receptor (Fig. 2B).

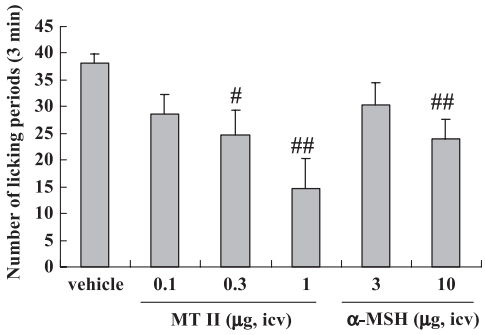

Fig. 3. Effect of α-MSH and MT II on number of licks in Vogel test in rats. Data represent mean ± S.E. ($n = 7–12$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus vehicle group (Dunnett's test).

3.2. Effect of melanocortin MC_4 receptor agonists in Vogel proconflict test in rats

Intracerebroventricular administration of α-MSH (10 μg) induced a decrease in the number of punished licking

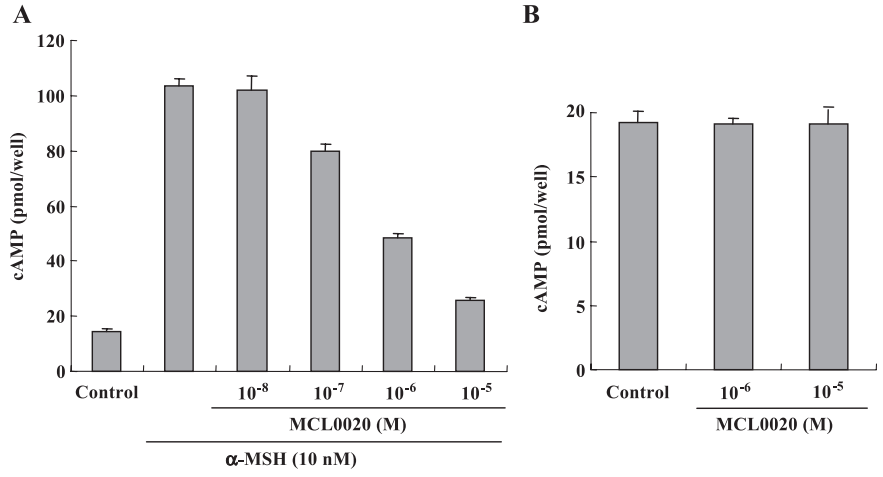

Fig. 2. Effect of MCL0020 on basal and α-MSH-induced increase in cAMP accumulation in COS-1 cells expressing melanocortin MC_4 receptor. MCL0020 was incubated with or without 10 nM of α-MSH for 15 min, and then cAMP formed in the cells was measured, as described in Materials and methods. Results are mean ± S.E. obtained from three experiments.

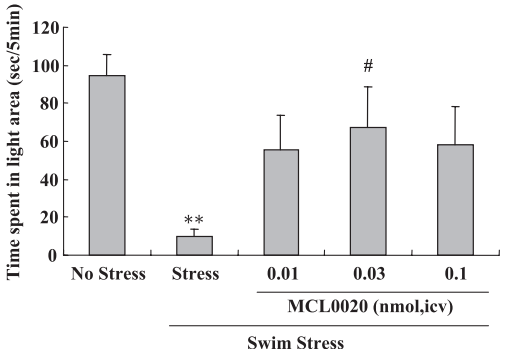

Fig. 4. Effect of MCL0020 on stress-induced reduction of time spent in light area in light/dark exploration test in mice. MCL0020 was administered intracerebroventricular. Data represent mean ± S.E. ($n = 10$). $^{**}P < 0.01$ versus nonstress group (Dunnett's test). $^{\#}P < 0.05$ versus swim stress group (Dunnett's test).

periods in a dose-dependent and significant manner (Fig. 3), which was in good agreement with the reported data (Corda et al., 1990). A similar and rather potent proconflict effect was seen in case of the i.c.v. administration of the melanocortin MC_4 receptor agonist, MT II (Fig. 3). The suppression of punished licking produced by α-MSH occurred at doses that failed to change unpunished behavior, while MT II also slightly reduced unpunished licking period at higher doses (data not shown).

3.3. Effect of MCL0020 on swim stress-induced anxiogenic-like behavior in light/dark exploration tests in mice

Ten minutes of swim stress markedly reduced time spent in light area in the light/dark exploration test in mice (Fig. 4), suggesting that this test elicits anxiogenic-like effects in mice. MCL0020 dose-dependently and significantly attenuated swim stress-induced reduction of time spent in the light area (Fig. 4). In a preliminary study, MCL0020 did not affect locomotor activity at pharmacologically effective doses in mice, when assessed by the method previously reported (Okuyama et al., 1999) using a SCANET apparatus (Neuroscience, Japan) placed in a sound-proof box (data not shown).

3.4. Effect of MCL0020 on restraint stress-induced suppression of food intake in rats

Ninety minutes restraint stress led to a significant decrease in food intake during 1 h in the 17 h fasted rats (Fig. 5A). MCL0020 significantly reversed stress-induced anorexia (Fig. 5A). In contrast, MCL0020 itself did not affect food intake in fasted rats at the dose that reversed decrease in food intake induced by stress (Fig. 5B). Moreover, MCL0020 did not have a significant effect on food intake in free-feeding rats at 0.3 and 1.0 nmol i.c.v. (data not shown).

4. Discussion

In the present study, we found that melanocortin MC_4 receptor agonists induced anxiety-like behavior, and melanocortin MC_4 receptor antagonist attenuated stress-induced behavioral abnormalities such as anxiety and anorexia.

First, we tested affinity and agonistic/antagonistic activity of MCL0020, a newly synthesized peptidemimetic compound, at melanocortin receptor subtypes. MCL0020 potently inhibited [^{125}I][Nle^4,D-Phe^7]α-MSH binding to the melanocortin MC_4 receptor with an IC_{50} value of 11.63 nM, while it had more than 860- and 96-fold weaker affinities for melanocortin MC_1 and MC_3 receptors, respectively. Therefore, MCL0020 is specific for the melanocortin MC_4 receptor among melanocortin receptors, although interactions with melanocortin MC_2 and MC_5 receptors need to be ruled out. It has been reported that melanocortin MC_3 and MC_4 are abundantly expressed, and the melanocortin MC_5 receptor is scarcely expressed in the brain, while melanocortin MC_1 and MC_2 receptors are not expressed in the brain (Gantz et al., 1994). Therefore, in the brain, MCL0020 may exert its

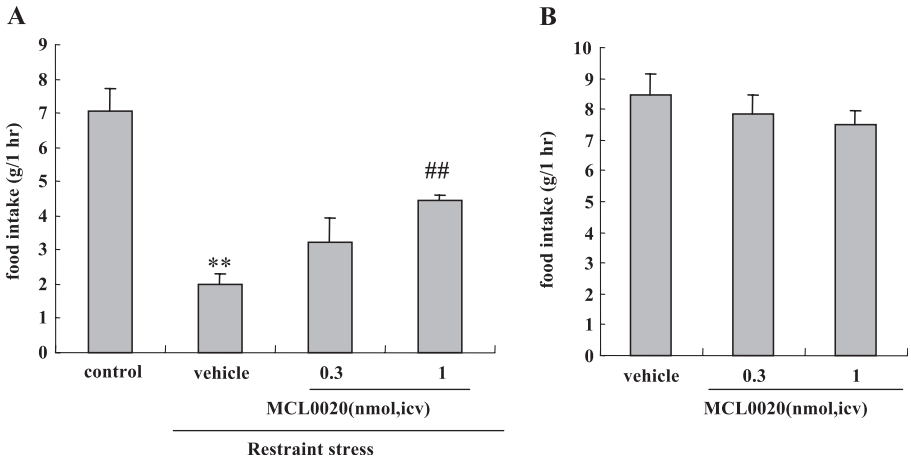

Fig. 5. Effect of MCL0020 on stress-induced reduction in food intake in fasted rats. Data represent mean ± S.E. ($n = 5–6$). $^{**}P < 0.01$ versus nonstress group (Dunnett's test). $^{\#\#}P < 0.01$ versus restraint stress group (Dunnett's test).

pharmacological effects by binding to the melanocortin MC_4 receptor. MCL0020 attenuated α-MSH-increased cAMP accumulation in melanocortin MC_4 receptor-expressing COS-1 cells, while MCL0020 itself did not increase basal cAMP levels. This finding clearly indicates that MCL0020 acts as an antagonist at the melanocortin MC_4 receptor. To date, several peptide antagonists for the melanocortin MC_4 receptor have been reported, including HS014, HS024 and HS028 (Kask et al., 1998; Skuladottir et al., 1999). Selectivity of these compounds for the melanocortin MC_4 receptor over the melanocortin MC_3 receptor is 17, 19 and 78, respectively, and HS028 as well showed affinity for the melanocortin MC_1 receptor. Therefore, MCL0020 is one of the most selective antagonists at the melanocortin MC_4 receptor over melanocortin MC_1 and MC_3 receptors hitherto identified. Therefore, it may prove beneficial to use MCL0020 in studies on melanocortin MC_4 receptor antagonists.

Intracerebroventricular administration of α-MSH and MT II reduced the number of licking periods in Vogel test on rats, hence stimulation of the melanocortin MC_4 receptor causes anxiogenic-like behavior. This result is in accord with a previous report that α-MSH and ACTH significantly produced decrease in punished licking periods in the conflict test (Corda et al., 1990). It was reported that the melanocortin MC_4 receptor is involved in pain, and melanocortin MC_4 receptor agonists increased the sensitivity to mechanical and cold stimulation, while melanocortin MC_4 receptor antagonists alleviated cold and mechanical allodynia in a neuropathic pain model of rats (Vrinten et al., 2000, 2001). Therefore, involvement of decrease in the pain threshold in this test using melanocortin MC_4 receptor agonists needs to be ruled out.

In a previous study, we reported that swim stress markedly reduced the time spent in light area in the light/dark exploration test in mice and in open arms in the elevated plus-maze task in rats, both of which were ameliorated by the administration of diazepam as well as CRF_1 receptor antagonists (Okuyama et al., 1999). These stress-induced anxiogenic-like behavior has been used an animal models of anxiety. In the present study, MCL0020 dose-dependently and significantly attenuated swim stress-induced anxiogenic-like effects in the light/dark exploration test in mice. This result suggests that melanocortin MC_4 receptor antagonists have anxiolytic-like activity in a rodent anxiety model. α-MSH was reported to reduce fever (Murphy et al., 1983), and effect on thermoregulation might affect the outcome of swim stress-induced anxiety behavior. Although effect of MCL0020 on thermoregulation has not been investigated, the involvement of this effect should be considered.

Stress leads to reduction in food intake in rodents. In the present study, 90 min restraint stress markedly reduced food intake in rats, and MCL0020 significantly attenuated restraint stress-induced anorexia. This result is consistent with the report that another melanocortin MC_4 receptor selective antagonist, HS014, blocked immobility stress-induced anorexia (Vergoni et al., 1999), albeit the experimental condition was different. In the present study, MCL0020 attenuated stress-induced anorexia without affecting food intake in nonstressed rats. In the current condition that rats were deprived of food for 17 h, hence MCL0020 may have attenuated anorexia by preventing stress, not by increasing basal food intake itself. In this respect, the melanocortin MC_4 receptor antagonist may have anti-stress activity.

There are several lines of evidence suggesting a relationship between stress-related behavior and the brain melanocortinergic system (Dunn et al., 1979; De Barioglio et al., 1991; Von Frijtag et al., 1998; Adan et al., 1999). Moreover, melanocortins have been shown to exert anxiogenic-like behavior (File and Clarke, 1980; Corda et al., 1990; Panksepp and Normansell, 1990; Gonzalez et al., 1996). By contrast, there are few reports showing that melanocortin MC_4 receptor antagonists elicit anti-stress activities and anxiolytic-like activities in experimental animal models (Adan et al., 1999; Vergoni et al., 1999). Moreover, recently, we found that a potent nonpeptide melanocortin MC_4 receptor antagonist showed anxiolytic-like and antidepressant-like activities in various animal models of anxiety and depression (Chaki et al., 2003).

In the present study, we took advantage of the newly synthesized melanocortin MC_4 receptor selective antagonist, MCL0020, and found that the melanocortin MC_4 receptor antagonist showed anti-stress and anxiolytic-like activity in rodent models. Taken together, the brain melanocortinergic system may be involved in stress-related behavior and disorders, and the melanocortin MC_4 receptor may mediate these events. Moreover, melanocortin MC_4 receptor antagonists may prove effective for treatment of subjects with anxiety and anorectic disorders.

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