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The anorexic effect of alpha-melanocyte-stimulating hormone is mediated by corticotrophin-releasing factor in chicks

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Abstract

Alpha-melanocyte-stimulating hormone (alpha-MSH) is recognized as an anorexic peptide in the brain of vertebrates, but its mechanism of action has not been identified in birds. Therefore, we investigated whether the anorexic effect of alpha-MSH is mediated by corticotrophin-releasing factor (CRF) in the domestic chick. Firstly, we found that intracerebroventricular (ICV) injection of alpha-MSH dose dependently increased plasma corticosterone (CORT) concentration. This effect was partly attenuated by co-injection of astressin, a CRF receptor antagonist, demonstrating that alpha-MSH stimulated CORT secretion by activating CRF neurons. The alpha-MSH-elicited CORT release was not attenuated by the injection of agouti-related protein, an endogenous melanocortin-4 (MC4) receptor antagonist, suggesting that alpha-MSH stimulated CRF neurons through MC4 receptor-independent pathways. Finally, we found that the anorexic effect of alpha-MSH was partly attenuated by astressin. The present results suggest that the anorexic effect of alpha-MSH in the chick brain is mediated in part by activation of CRF neurons.

Keywords: Agouti-related protein; Alpha-melanocyte-stimulating hormone; Chick; Corticosterone; Corticotrophin-releasing factor; Feeding

1. Introduction

To date, many peptides that regulate feeding behavior have been found in the brain of mammals (Friedman and Halaas, 1998; Leibowitz and Wortley, 2004). One of them, alpha-melanocyte-stimulating hormone (alpha-MSH) is well known as an anorexic peptide because central administration of alpha-MSH decreases food intake (Poggioli et al., 1986). Alpha-MSH inhibits feeding behavior via the melanocortin-4 (MC4) receptor and its effect is opposed by agouti-related protein (AGRP) (Poggioli et al., 1986; Rossi et al., 1998), a naturally occurring antagonist of the MC4 receptor (Fong et al., 1997). Since central administration of AGRP itself stimulates feeding behavior (Rossi et al., 1998), endogenous alpha-MSH is proposed to play an important role in the inhibition of feeding in mammals as an endogenous agonist of the MC4 receptor. In support of this, targeted disruption of the MC4 receptor induces hyperphagia, hyperinsulinemia, hyperglycemia and obesity in mice (Huszar et al., 1997).

Alpha-MSH is also recognized as an inhibitory feeding peptide in birds (Kawakami et al., 2000; Tachibana et al., 2001; Strader et al., 2003). As shown in mammals (Rossi et al., 1998), antagonism of the MC4 receptor by central administration of AGRP stimulated feeding behavior in chicks (Tachibana et al., 2001) and ring doves (*Streptopelia risoria*) (Strader et al., 2003), suggesting that alpha-MSH is an important endogenous inhibitory feeding peptide in birds. However, the mechanism through which alpha-MSH exerts its anorexic actions has not been identified in avian species.

In mammals, the physiological actions of alpha-MSH involve interactions with corticotrophin-releasing factor (CRF), a hypothalamic signal of hypothalamus–pituitary–adrenal (HPA) axis, because alpha-MSH stimulates CRF release from hypothalamic explants (Dhillo et al., 2002). It has also been reported that MC4 receptor mRNA is co-expressed with

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CRF mRNA in neurons in the hypothalamic paraventricular nucleus (PVN) of rats (Lu et al., 2003). Since CRF is one of the inhibitory feeding factors in the brain (Morley and Levine, 1982), it has been regarded as a candidate for mediating the anorexic effect of alpha-MSH in mammals. In support of this, MC4 receptor agonist-induced anorexia is attenuated by blockade of the CRF receptor (Lu et al., 2003).

CRF is also recognized as an anorexic peptide in the chick because it inhibits feeding behavior after central administration (Furuse et al., 1997; Denbow et al., 1999). Similarly, anorexic effects of alpha-MSH and CRF have also been observed in fish (Volkoff et al., 2005), demonstrating that these functions have been well conserved during vertebrate evolution. These facts imply that the mechanisms underlying alpha-MSH induced anorexia are also similar between vertebrate species. Therefore, it is possible that the anorexic effect of alpha-MSH might be mediated by CRF neurons in chicks as is the case in mammals (Lu et al., 2003). However, there is currently little information available about the relationships between CRF and alpha-MSH in the chick brain.

The purpose of the present study was to investigate whether CRF is involved in the anorexic effect of alpha-MSH in the chick. To determine the interaction between alpha-MSH and CRF neurons, we firstly examined the effect of intracerebroventricular (ICV) injection of alpha-MSH on corticosterone (CORT) secretion. Secondly, we examined whether the anorexic effect of alpha-MSH and its effects on CORT secretion are attenuated by astressin, a CRF antagonist, and/or AGRP.

2. Materials and methods

2.1. Animals

Day-old layer-type male chicks (*Gallus gallus*, Julia strain) were purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan). Birds were maintained in a room kept at 30 °C with continuous lighting. They were given free access to water and a commercial diet (Toyohashi Feed and Mills Co., Aichi, Japan) throughout the experiment unless indicated otherwise. Experimental procedures followed the guidance for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

2.2. ICV injection

Alpha-MSH, human AGRP (86–132) (both purchased from Peptide Institute, Osaka, Japan), and astressin (Sigma Chemical CO., USA) were dissolved in a 0.1% Evans Blue solution, which was prepared in a saline solution. The control group was injected with the same volume of this Evans Blue solution. The injected volume was 10 μl in all experiments. Human AGRP and astressin are reported to be useful as antagonists for MC4 (Tachibana et al., 2001) and CRF receptor (Saito et al., 2005) in chicks.

ICV injection was performed according to the method previously reported (Davis et al., 1979). Briefly, the head of the chick was inserted in an acrylic device which positioned a hole in a plate overlying the skull immediately over the left lateral ventricle. A microsyringe was then inserted into the left lateral ventricle through the hole and the drug was injected. This method did not appear stressful for the chicks since the ICV injection of 0.85% saline, which was used for control injections in the present study, did not affect feeding behavior (Furuse et al., 1999) and CORT release (Saito et al., 2005) when compared with non-injected chicks. Therefore, we did not anesthetize chicks for the injection and the birds could move and eat immediately afterwards.

At the end of each experiment, chicks were sacrificed with an intraperitoneal overdose of sodium pentobarbital and then their brains were removed. Confirmation of drug injection was made by observation of the presence of Evans Blue dye in the lateral ventricle. The results obtained from chicks, which did not have Evans Blue dye in the lateral ventricle, were not used.

2.3. Experiment 1: Effect of alpha-MSH on CORT secretion

Plasma CORT concentration was measured at 10 and 30 min after the ICV injection of alpha-MSH in different experiments. Chicks (6 days old) were subdivided into 4 groups. Three of these were injected with either 0 (control), 10 or 100 pmol alpha-MSH under an ad libitum feeding condition. Food and water were not available to the chicks after the injection. At 10 min after the injection, a blood sample was obtained from the jugular vein. The blood collection was finished within 15 s. Chicks in the fourth group did not receive injections but underwent blood sampling in order to assess the pre-injection level of CORT. The blood was centrifuged at 4 °C, 9000×*g* for 4 min, to obtain the plasma. Plasma CORT concentration was measured using a commercial kit (Corticosterone Correlate-EIA Kit, Assay Designs, Inc., USA). The number of chicks in the 10-min study was as follows: intact, 6; 0 pmol, 6; 10 pmol, 6; 100 pmol, 7. In a second trial, 4-day-old chicks were used. The procedures followed were identical to those of the first trial except that blood sampling was done at 30 min following the injection. Five chicks were used in each group.

2.4. Experiment 2: Effect of CRF receptor antagonist on alpha-MSH-elicited CORT release

The CRF antagonist astressin was used to investigate whether the CORT-releasing effect of alpha-MSH is mediated by CRF. Each chick (6 days old) was injected with saline (control), 100 pmol alpha-MSH or 100 pmol alpha-MSH plus 6 nmol astressin under an ad libitum feeding condition. The same dose of astressin administered alone did not affect plasma CORT concentration under these same conditions in a previous study (Saito et al., 2005). Food and water were then removed and blood samples were collected at 30 min after the injection. Plasma CORT was measured as described for Experiment 1. The number of chicks was as follows: saline, 6; alpha-MSH alone, 6; alpha-MSH plus astressin, 5.

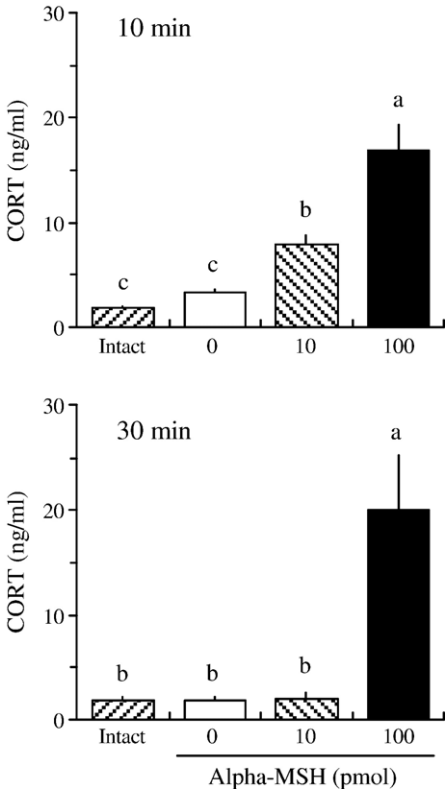

Fig. 1. Plasma CORT concentration at 10 min (upper panel) and 30 min (lower panel) after ICV injection of alpha-MSH. The number of chicks at 10 min was as follows: intact, 6; 0 pmol, 6; 10 pmol, 6; 100 pmol, 7, and at 30 min, the number of chicks in each group was 5. Values are expressed as means±S.E.M. Groups with different letters are significantly different ($P<0.05$).

2.5. Experiment 3: Effect of AGRP on alpha-MSH-elicited CORT release

The involvement of MC4-R on the CORT-releasing effect of alpha-MSH was assessed using AGRP. Each chick (6 days old) was injected with saline (control), 100 pmol alpha-MSH alone or 100 pmol alpha-MSH plus 480 pmol AGRP under an ad libitum feeding condition. This dose of AGRP was decided according to the previous study (Tachibana et al., 2001) that 480 pmol AGRP was sufficient to block the anorexic effect of alpha-MSH. In addition, this dose of AGRP did not affect plasma CORT release in chicks (data not shown). Food and water were then removed and blood samples were collected at 30 min after the injection. The plasma CORT concentration was measured as noted in Experiment 1. The number of chicks was as follows: saline, 6; alpha-MSH alone, 6; alpha-MSH plus AGRP, 5.

2.6. Experiment 4: Effect of CRF receptor antagonist on alpha-MSH-induced suppression of food intake

Astressin was used to determine whether the anorexic effect of alpha-MSH is mediated by CRF. After 3-h food deprivation, each chick (4 days old) was injected with saline (control), 10 pmol alpha-MSH or 10 pmol alpha-MSH plus 6 nmol astressin. This dose of astressin did not affect food intake of chicks in a previous study (Tachibana et al., 2001). Food was then returned and intake was measured at 30 and 60 min after the injection. The number of chicks was as follows: saline, 11; alpha-MSH alone, 9; alpha-MSH plus astressin, 9.

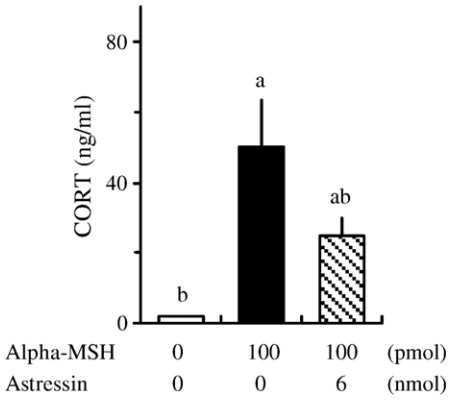

Fig. 2. Effect of blockade of the CRF receptor on alpha-MSH-elicited CORT release. The doses of alpha-MSH and astressin were 100 pmol and 6 nmol, respectively. The number of chicks was as follows: saline, 6; alpha-MSH alone, 6; alpha-MSH plus astressin, 5. Values are expressed as means±S.E.M. Groups with different letters are significantly different ($P<0.05$).

2.7. Data analysis

Data from Experiments 1–3 were statistically analyzed with one-way analysis of variance (ANOVA) and Fisher's PLSD test as a post hoc test. Data from Experiment 4 were analyzed with two-way repeated ANOVA and Fisher's PLSD test. Statistical significance was set at $P<0.05$. The results are shown as the means±S.E.M.

3. Results

3.1. Experiment 1: Effect of alpha-MSH on CORT secretion

Plasma CORT concentration after ICV injection of alpha-MSH is shown in Fig. 1. Alpha-MSH significantly increased plasma

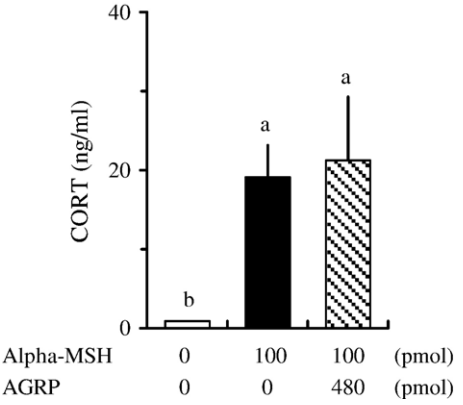

Fig. 3. Effect of AGRP on alpha-MSH-elicited CORT release. The doses of alpha-MSH and AGRP were 100 and 480 pmol, respectively. The number of chicks was as follows: saline, 6; alpha-MSH alone, 6; alpha-MSH plus AGRP, 5. Values are expressed as means±S.E.M. Groups with different letters are significantly different ($P<0.05$).

CORT concentration in a dose-dependent manner [$F(3,21)=23.1$, $P<0.01$] at 10 min but after 30 min the only effective dose was 100 pmol [$F(3,16)=11.7$, $P<0.01$].

3.2. Experiment 2: Effect of CRF receptor antagonist on alpha-MSH-elicited CORT release

The effects of CRF receptor blockade on alpha-MSH-induced CORT secretion are shown in Fig. 2. Alpha-MSH and astressin treatments significantly altered the plasma CORT concentration [$F(2,14)=8.6$, $P<0.01$]. Alpha-MSH alone significantly raised the plasma CORT concentration as it did in Experiment 1. There was a tendency for this effect of alpha-MSH to be attenuated by co-administration of astressin. Thus, the difference in plasma CORT concentration in the alpha-MSH plus astressin group from that observed after injection of alpha-MSH alone was close to statistical significance ($P=0.058$). There was no significant difference in plasma CORT concentration between the saline and alpha-MSH plus astressin groups.

3.3. Experiment 3: Effect of AGRP on alpha-MSH-elicited CORT release

Fig. 3 represents the effect of AGRP on alpha-MSH-elicited CORT release. Alpha-MSH and AGRP treatments significantly influenced the plasma CORT concentration [$F(2,14)=8.6$, $P<0.01$]. Alpha-MSH alone significantly increased the plasma CORT concentration as noted in Experiments 1 and 2, and the value was almost identical to the Alpha-MSH plus AGRP treatment group. Thus, there was no significant effect of co-administration of AGRP on alpha-MSH-elicited CORT release.

3.4. Experiment 4: Effect of CRF receptor antagonist on alpha-MSH-induced suppression of food intake

Fig. 4 shows the effect of co-injection of astressin on the anorexic effect of alpha-MSH in chicks. Food intake was significantly influenced by alpha-MSH and astressin treatments [$F(2,26)=18.9$, $P<0.01$]. Alpha-MSH alone significantly reduced intake throughout the experimental period. The effect was attenuated by astressin treatment because the food intake of the alpha-MSH plus astressin group was significantly higher than that in the alpha-MSH alone group at all times determined, although the food intake was significantly lower than the control group.

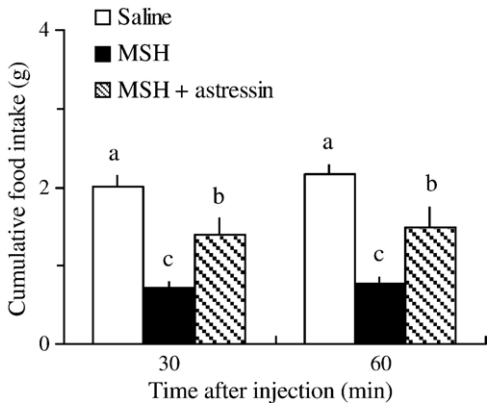

Fig. 4. Effect of astressin on alpha-MSH-induced anorexia. The doses of alpha-MSH and astressin were 10 pmol and 6 nmol, respectively. The number of chicks was as follows: saline, 11; alpha-MSH alone, 9; alpha-MSH plus astressin, 9. Values are expressed as means ± S.E.M. Groups with different letters are significantly different at each time point ($P<0.05$).

4. Discussion

We report here the observation that ICV injection of alpha-MSH stimulated CORT release in chicks dose dependently at 10 min (Fig. 1). The effect persisted at 30 min after the ICV injection at the highest (100 pmol) dose of alpha-MSH used. These findings support the idea of an interaction between alpha-MSH and the HPA axis in the brain of chicks. The CORT-releasing effect of centrally administered alpha-MSH observed in the present study resembles the findings of studies in the rat, where it appears to be mediated by activation of CRF neurons in the PVN (Dhillo et al., 2002; Lu et al., 2003). Based on these mammalian studies, we hypothesized that alpha-MSH stimulates CORT release via activation of CRF neurons in chicks. This hypothesis was partly supported by the present study because the CORT-releasing effect tended to be attenuated by astressin (Fig. 2). However, alpha-MSH-elicited CORT release could not be explained by CRF acting alone because astressin did not completely abolish the CORT-releasing effect (Fig. 2). Although the receptor-binding assay of astressin on chicken CRF receptor has not been investigated, ICV co-injection of astressin (700 pmol) completely abolished 21 pmol CRF-induced CORT release in chicks (data of our preliminary experiment, unpublished). This indicates that astressin might be useful for the antagonist of chicken CRF receptor. It is therefore possible that centrally administered alpha-MSH was either acting directly on the pituitary, or was interacting with the other adrenocorticotrophic hormone (ACTH) secretagogues. Candidates for the alternative ACTH secretagogues are arginine-vasotocin (AVT) and mesotocin (MT), the avian homologues of arginine vasopressin (AVP) and oxytocin, respectively, both of which have been reported to stimulate ACTH release in birds (Castro et al., 1986; Romero and Wingfield, 2001; Tachibana et al., 2004a). Since alpha-MSH stimulates AVP release from hypothalamic explants of rats (Dhillo et al., 2002), it is possible that alpha-MSH-elicited CORT release is induced by not only CRF but also AVT and MT neurons in chicks.

In addition to the CORT-releasing effect, the anorexic effect of alpha-MSH was also attenuated by co-injection of astressin (Fig. 4). This result demonstrated that alpha-MSH inhibited food intake via activation of CRF neurons in chicks as has been shown in mammals (Lu et al., 2003). We have reported previously that pituitary adenylyl cyclase-activating polypeptide, vasoactive intestinal peptide and ghrelin all inhibit food intake in chicks through activation of CRF neurons (Tachibana et al., 2004b; Saito et al., 2005). These facts suggest that CRF might play a mediator of the inhibition of food intake in the chick. On the other hand, astressin did not completely abolish the alpha-MSH-induced anorexia, suggesting that CRF-independent pathways are also associated with signaling the effect

of alpha-MSH. One of these may be the AVT system because central administration of AVT inhibits feeding behavior in chicks (Tachibana et al., 2004a) and there is the possibility that alpha-MSH stimulates AVT release, as mentioned above. Alternatively, the partial inhibitory effect of astressin on CORT-releasing and anorexic effects of alpha-MSH might be explained by the dose used in the present study. We used only one dose of astressin (6 nmol), and this dose may not have been optimal for complete inhibition. Further study will be needed to assess the involvement of CRF on the actions of alpha-MSH in the brain of chicks.

A mammalian study revealed that antagonism of the MC4 receptor attenuates stress-induced anorexia (Vergoni et al., 1999; Chaki et al., 2003), indicating that central alpha-MSH is involved in the stress response in mammals. In the present study, since alpha-MSH stimulated CORT release and anorexia via at least a partial activation of CRF neurons in chicks it is likely that central alpha-MSH might be involved in the response to stress in birds.

The alpha-MSH induced CORT release observed in the present study was not affected by co-administration of AGRP, a MC4 receptor antagonist (Fig. 3). The result demonstrates that alpha-MSH does not stimulate CORT release through the MC4 receptor, although it should be noted that we used only one dose of AGRP. This is the case for previous studies in rats. For instance, Dhillo et al. (2002) reported that intra-hypothalamic injection of AGRP increased plasma ACTH concentration and that AGRP did not attenuate alpha-MSH analogue-induced CORT release. The dose of AGRP used in the present study attenuated the anorexic effect of 120 pmol of alpha-MSH in chicks (Tachibana et al., 2001), suggesting that it was capable of antagonizing the 100 pmol dose used in the present study. However, the time courses of the effects of alpha-MSH on food intake and CORT release were different. For example, 10 pmol alpha-MSH inhibited feeding behavior 30 and 60 min after the injection (Fig. 4) while the CORT-releasing effect had disappeared at 30 min post-injection (Fig. 1). It is therefore possible that different melanocortin receptors are involved in mediating the anorexic and CORT-releasing effects of alpha-MSH. In mammals, melanocortin-3 (MC3), MC4 and melanocortin-5 (MC5) receptors are found in the brain (Adan and Gispen, 1997). Expressions of the MC4 and MC5 receptors are found in the chicken brain, but not that of the MC3 receptor (Takeuchi and Takahashi, 1998, 1999). It is therefore possible that another receptor but not MC3 receptor might mediate the alpha-MSH induced CORT release and anorexia in chicks.

In conclusion, the present results suggest that the anorexic effect of alpha-MSH is partially mediated by CRF neurons in the chick.

Acknowledgements

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