

**Mu-opioid receptor agonist diminishes POMC gene expression and anorexia by  
central insulin in neonatal chicks**

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## Abstract

Pro-opiomelanocortin (POMC) neurons in the hypothalamus are direct targets of peripheral satiety signals, such as leptin and insulin in mammals. The stimulation of these signals activates hypothalamic POMC neurons and elevates POMC-derived melanocortin peptides that inhibit food intake in mammals. On the other hand, it has been recognized that  $\beta$ -endorphin, a post-translational processing of POMC, acts in an autoreceptor manner to the  $\mu$ -opioid receptor (MOR) on POMC neurons, diminishing POMC neuronal activity in mammals. Recently, we found that central insulin functions as an anorexic peptide in chicks. Thus, the present study was done to elucidate whether  $\beta$ -endorphin affects the activation of POMC neurons by insulin in neonatal chicks. Consequently, quantitative real-time PCR analysis shows that intracerebroventricular (ICV) injection of insulin with  $\beta$ -endorphin significantly decrease brain POMC mRNA expression when compared with insulin alone. In addition, co-injection of MOR agonist ( $\beta$ -endorphin or DAMGO) significantly attenuates insulin-induced hypophagia in chicks. These data suggest that  $\beta$ -endorphin regulates the activity of the central melanocortin system, and its activation may provide an inhibitory feedback mechanism in the brain of neonatal chicks.

Key words: Food intake;  $\beta$ -Endorphin; Insulin; Central nervous system; Neonatal chick; Melanocortin system

Identifying peripheral signals that form in the central nervous system (CNS) is just one of several important steps in understanding how energy balance is regulated in most vertebrates. Also, it is critical to understand the neural circuitry upon which these peripheral signals act. This has been made more complicated by the large number of neurotransmitters that has been linked to the control of food intake and energy expenditure. These functional circuits focus upon the systems that receive the most direct input from peripheral satiety signals. In mammals, one of the systems contained in the arcuate nucleus (ARC) in the hypothalamus is a logical starting point to begin unraveling the complicated neural underpinnings of the regulation of energy balance.

Although the ARC contains a number of neuropeptide systems that can influence energy balance, it is the primary site in which a variety of ligands for the receptors such as the melanocortin system are produced [18]. The central melanocortin system is the downstream mediator of insulin and leptin that act in the brain to reduce food intake and body weight [2, 22, 24]. Recently, we found that insulin functions as an appetite-suppressive peptide in the CNS of chicks, and that this anorexic effect of insulin was due to  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH), a post-translational processing of pro-opiomelanocortin (POMC) via melanocortin 4 receptor [19]. POMC is a precursor that gives rise to several biologically active neuropeptides, including  $\alpha$ -MSH and  $\beta$ -endorphin [10, 12, 20]. It has been recognized that  $\beta$ -endorphin acts in an autoreceptor manner to the  $\mu$ -opioid receptor (MOR) on POMC neurons, diminishing POMC neuronal activity in response to elevated POMC-derived melanocortin peptides that inhibit food intake in mammals [6, 21].

Our hypothesis was that if there is a similar inhibitory feedback mechanism for

the autoregulation of POMC in chicks, co-injection of MOR ligand should attenuate the brain POMC gene expression and the insulin-induced anorexia. To investigate this hypothesis, the effects of MOR agonist with insulin on the expression of POMC mRNA and food intake were examined in neonatal chicks.

5 Day-old male Single Comb White Leghorn chicks (Akita Co. Ltd., Hiroshima, Japan) were maintained in a room with 24-h lighting and at a temperature of 30°C. They were given free access to a commercial starter diet (Nichiwa Sangyo Co. Ltd., Kobe, Japan) and water during the pre-experimental period. They were distributed into experimental groups based on their body weight so that the average body weight  
10 (53.5±1.2 g) was as uniform as possible for each treatment. The birds were reared individually in experimental cages and had ad libitum access to food up to the time of experiments. The handling of birds was performed in accordance with the regulations of the Animal Experiment Committee of Hiroshima University.

Porcine insulin was purchased from MP Biomedicals Inc. (Auroa, OH, USA).  
15  $\beta$ -Endorphin (human) and [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly<sup>5</sup>-ol]-enkephalin (DAMGO; a selective MOR agonist) was obtained from Sigma (St. Louis, MO, USA). The drugs were dissolved in a 0.1% Evans Blue solution, which was prepared in 0.85% saline. Saline containing Evans Blue was used as a control. After being deprived of food for 3 h, the birds (4-day-old) were ICV injected with solutions (10  $\mu$ l) using a microsyringe  
20 according to the methods used by Davis et al. [7]. Each chick was injected once only with either saline or drug(s). At the end of the experiments, birds were sacrificed by decapitation, followed by brain sectioning to identify the location of the drug injection. Data were deleted for individuals in which the presence of Evans Blue dye in the lateral ventricle was not verified. The number of birds used for data analysis is shown in each  
25 figure.

Birds were injected by ICV route with saline, insulin (100 ng) or insulin co-injected with  $\beta$ -endorphin (50 pmol). At 15, 30 and 60 min after the injection, the chicks were killed and the diencephalon collected. Total RNA was isolated from the dissected tissue using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. To rule out the possibility that PCR products would result from the amplification of genomic DNA containing the RNA sample, RNA samples were treated with DNase I using the DNA-free kit (Ambion, Austin, USA). Total RNA (300ng) was reverse transcribed at 42°C for 15min in 10  $\mu$ l of 1  $\times$  Prime Script buffer containing 50  $\mu$ M random hexamers and Prime Script RT Enzyme Mix I (Takara, Tokyo, Japan). The reaction product was subjected to quantitative RT-PCR performed according to the user instructions for the Light Cycler system (Roche Applied Science, IN, USA). Following a denaturation step at 95°C for 10 s, PCR was carried out with a thermal protocol consisting of 95°C for 5 s and 60°C for 20 s in a 20  $\mu$ l buffer containing 1 $\times$ SYBR Premix EX Taq (Takara, Tokyo, Japan) and 0.2  $\mu$ M of each primer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The primers used were as follows: POMC, 5'-aacagcaagtgccaggacc-3' (sense) and 5'-atcacgtacttgccgatgct-3' (antisense). GAPDH, 5'-gccgtcctctctggcaaa-3' (sense) and 5'-tgtaaaccatgtagttcagatcgatga-3' (antisense). They gave a single PCR product as verified by melting curve analysis, agarose gel electrophoresis. The critical cycle of each sigmoid PCR curve was calculated by the Light Cycler Software 3.5® (Roche Applied Science) as the PCR cycle corresponding to the maximum of the second derivative. For the data analysis, the average threshold cycle ( $C_T$ ) of each set of duplicates was calculated. To normalize the data, the  $\Delta C_T$  was calculated for each sample by subtracting the average  $C_T$  of GAPDH from the average  $C_T$  of POMC. For relative quantitation, the  $\Delta C_T$  was averaged for the defined control group and was then

subtracted from the  $\Delta C_T$  of each experimental sample to generate the  $\Delta\Delta C_T$ . The  $\Delta\Delta C_T$  was then used to calculate the approximate fold difference,  $2^{-\Delta\Delta C_T}$ .

Birds were injected with saline, insulin (100 ng), insulin co-injected with  $\beta$ -endorphin (25 or 50 pmol) or DAMGO (10 or 50 pmol). The doses of agonist  
5 applied here were decided according to the preliminary trials, in which they did not affect feeding behavior in fasted chicks. Food intake was measured at 30, 60 and 120 min after injection, and determined by measuring the reduction of diet from a pre-weighed feeder. The weight of feeders was measured using an electric digital balance of precision  $\pm 1$  mg.

10 The data were analyzed using the commercially available package, StatView (Version 5, SAS Institute, Cary, USA, 1998). The Tukey-Kramer test was used to determine overall statistical significance due to treatment. Differences were considered to be significant when P was less than 0.05. Results are presented as means  $\pm$  S.E.M.

15 As depicted in Fig. 1, POMC mRNA expression in insulin-treated chicks was significantly higher than that of the level in the saline chicks at 15 min post-injection ( $P < 0.05$ ), but not significantly different at 30 and 60 min post-injection ( $P > 0.1$ ). This is in good agreement with the previous studies that central injection of insulin augmented hypothalamic POMC gene expression in chicks [19]. On the other hand,  
20 co-injection with  $\beta$ -endorphin did not result in significant differences of POMC mRNA expression compared with saline treated chicks during the experimental period ( $P > 0.1$ ). The current findings support the hypothesis that there may be an inhibitory feedback mechanism for the autoregulation of POMC-producing neuron through MOR in chicks. Several reports implicated the existence of the feedback mechanism on the  
25 hypothalamic POMC system in mammals [5, 9, 11, 13, 15, 21], and activation of the

autoreceptor may provide ultrashort feedback loops that modulate the effects of adiposity signals on POMC neurons [6]. Anyway, the results of current studies affirmed that, similar to mammals [2], the reduced appetite by insulin was attributable to activation of the central melanocortin system in neonatal chicks.

5           The effect of ICV injection of  $\beta$ -endorphin (25 or 50 pmol) on insulin-induced hypophagia for 120 min is shown in Fig.2. Central administration of insulin (100 ng) significantly decreased food intake of fasted chicks when compared with control during the experimental period ( $P < 0.01$ ). Although the differences were not significant when compared with the use of insulin alone, co-injection of  $\beta$ -endorphin showed a tendency  
10 to recover food intake from insulin-induced hypophagia in chicks. It is reasonable to suppose that  $\beta$ -endorphin diminished insulin-induced POMC mRNA expression and inhibited the release and/or synthesis of anorexic peptides, such as  $\alpha$ -MSH, derived from POMC-polypeptide precursor. However, it is known that  $\beta$ -endorphin is an endogenous ligand for both MOR and  $\delta$ -opioid receptor (DOR) [1, 16], and the  
15 stimulation for each receptor has an important role in feeding behavior in the CNS of chicks [3, 4]. Hence, we demonstrated whether co-injection of DAMGO (selective MOR agonist), as well as  $\beta$ -endorphin, attenuates the insulin-induced anorexia in chicks.

          The effect of co-injection of DAMGO, a selective MOR agonist (10 or 50  
20 pmol) on food intake in chicks is shown in Fig. 3. Similar to  $\beta$ -endorphin, co-injection of DAMGO showed a tendency to attenuate the insulin-induced suppression of eating response in a dose-dependent manner at 60 and 120 min, but significant differences were not detected at 30 min post-injection. It is affirmed that inhibitory regulation of POMC neuronal system in the CNS is mediated via MOR in chicks, similar to  
25 mammals.

Several reports indicate that  $\beta$ -endorphin facilitates feeding behavior in birds [8, 14, 17], and that the stimulation for either MOR or DOR in the CNS accelerates ingestive behavior in chicks [3, 4]. However, the stimulation for MOR, but not DOR, is important in the  $\beta$ -endorphin-induced hyperphagia in ad libitum chicks [23]. These facts imply that the orexigenic effect of  $\beta$ -endorphin in sated chicks has some relation to diminished activity of POMC neurons in the CNS.

Both MOR agonists attenuate insulin-induced anorexia, but do not completely abolish the effect of insulin (Figs. 2 and 3). It is reasonable to assume that central insulin could directly stimulate not only POMC, but also other anorexic peptide neurons, such as corticotropin-releasing hormone in the paraventricular nucleus. In fact, ICV injection of insulin (20 ng) significantly increased plasma corticosterone concentration at 60 min post-injection (unpublished data).

In conclusion,  $\beta$ -endorphin diminishes hypothalamic POMC gene expression and insulin-induced anorexigenic effects, especially the recovery effect on insulin-induced feeding behavior, is mediated via MOR. These data suggest that  $\beta$ -endorphin regulates the activity of the central melanocortin system, and its activation may provide ultrashort feedback loops in chicks. To further investigate that interaction signaling cascades in MOR and insulin signaling which plays a key role in central insulin action in energy homeostasis would clarify the mechanism of the inhibitory feedback in the brain of chicks. However, the results described here is the first report about the inhibitory feedback mechanism on the melanocortin system in chicks.

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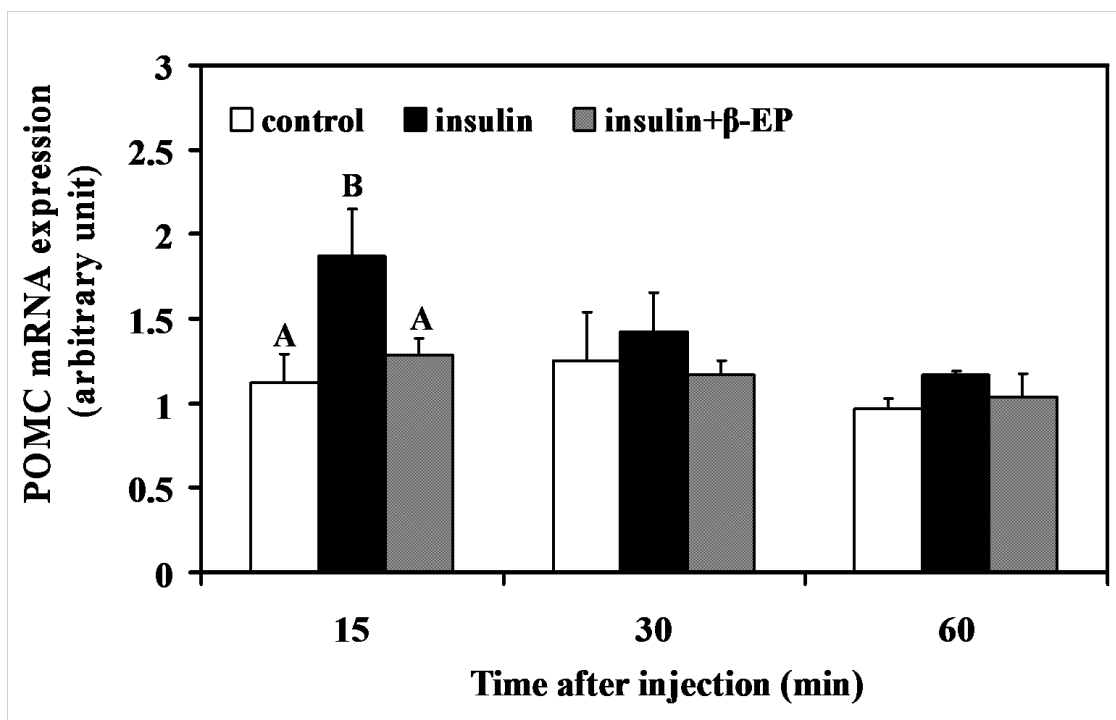
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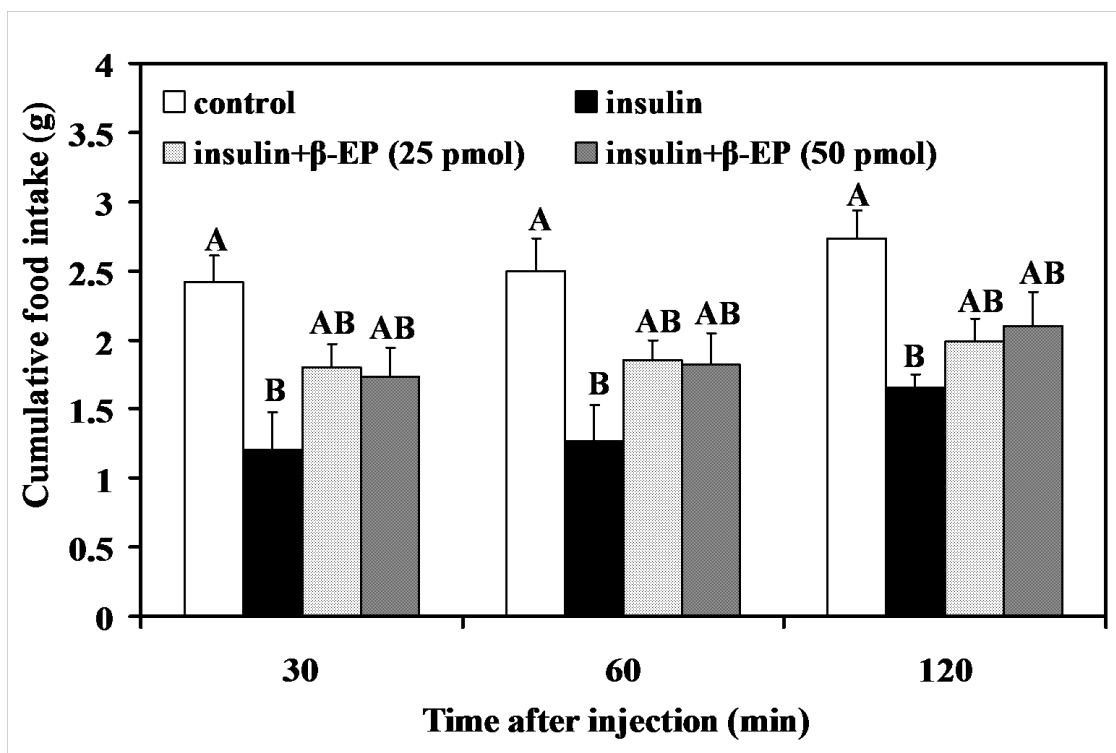
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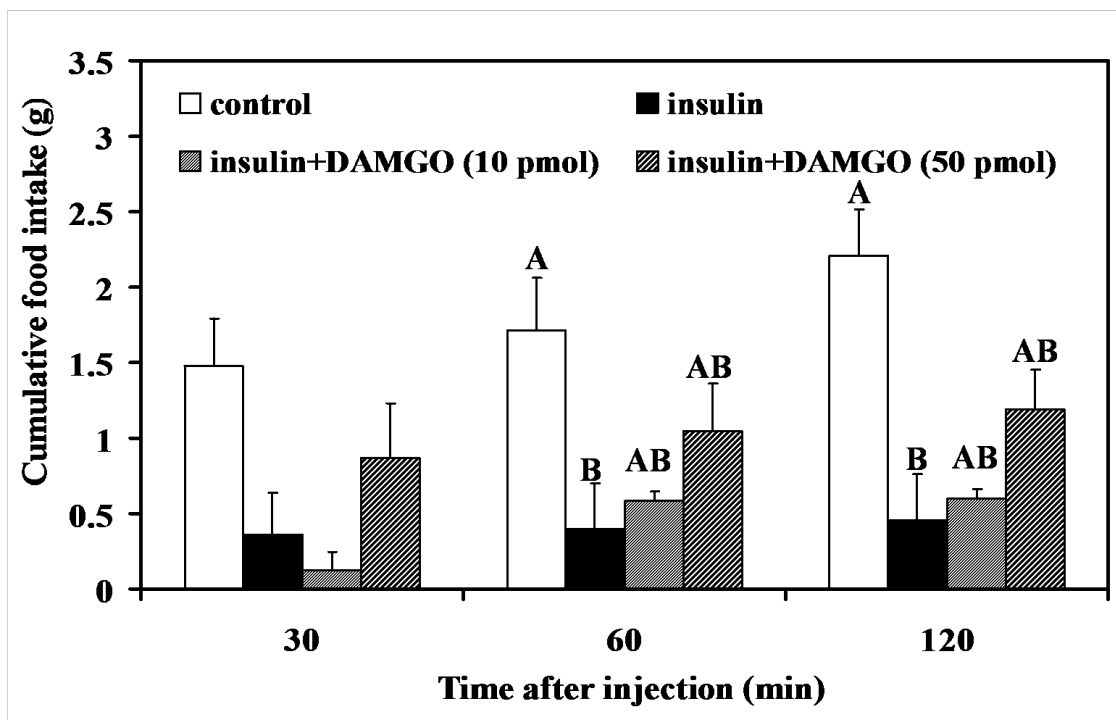
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**Fig. 1**



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**Fig. 2**



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**Fig. 3**

### *Legends*

- Fig. 1. Effect of central insulin or insulin co-injected with  $\beta$ -endorphin (50 pmol) on POMC mRNA expression in chick diencephalon as determined by quantitative RT-PCR. The number of chicks used were as follows: 15 min control, 7; 15 min insulin, 5; 15 min insulin+ $\beta$ -endorphin, 6; 30 min control, 4; 30 min insulin, 4; 30 min insulin+ $\beta$ -endorphin, 4; 60 min control, 6; 60 min insulin, 4; 60 min insulin+ $\beta$ -endorphin, 4, respectively. The values represent means  $\pm$  S.E.M. Means with different letters are significantly different at  $P<0.05$ .
- Fig. 2. Cumulative food intake of fasted chicks injected ICV with saline, insulin (100 ng) or insulin co-injected with two doses of  $\beta$ -endorphin (25 or 50 pmol). The values are means  $\pm$  SEM. The number of chicks used were: control, 6; insulin, 6; insulin+ $\beta$ -endorphin (25 pmol), 7; insulin+ $\beta$ -endorphin (50 pmol), 7. Means with different letters are significantly different at  $P<0.05$ .
- Fig. 3. Cumulative food intake of fasted chicks injected ICV with saline, insulin (100 ng) or insulin co-injected with two doses of DAMGO,  $\mu$ -opioid receptor agonist (10 or 50 pmol). The values are means  $\pm$  S.E.M. The number of chicks used were: control, 7; insulin, 5; insulin+DAMGO (10 pmol), 4; insulin+DAMGO (50 pmol), 5. Means with different letters are significantly different at  $P<0.05$ .