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Note

Amino Acid Mixture Identical to Vespa Larval Saliva Increases both Leptin Secretion and Basal Lipolysis in Rat Adipocytes

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The effect of vespa amino acid mixture solution (VAAM), a unique amino acid mixture identified in the saliva of the larva of a species of hornet endemic to Japan on adipocyte lipolysis was investigated in order to verify the possible mechanisms underlying the regulatory effect of VAAM on *in vivo* fat metabolism. VAAM significantly increased basal leptin secretion and lipolysis without an increase in cAMP production. However, VAAM failed to amplify either isoproterenol or dibutylyl cAMP-stimulated lipolysis, which is increased intracellular cAMP productions, accompanied by a significant decrease in leptin secretion. These results suggest that VAAM may enhance basal lipolysis independent of cAMP productions, but is dependant on leptin secretion from adipocytes themselves, at least in part.

Keywords: vespa amino acid mixture, adipocyte, leptin, lipolysis

Introduction

Adipocyte lipolysis has been well known to be mediated by increases in intracellular levels of cAMP leading to activation of hormone-sensitive lipase via β-adrenergic stimulation (Izawa and Nomura, 2002). On the other hand, there is growing evidence that adipokines, such as tumor necrosis factor-α (Gasic *et al.*, 1999, Green *et al.*, 1994, Rahn *et al.*, 2000, Zhang *et al.*, 2002), inter leukin-6 (Path *et al.*, 2001, vanHall *et al.*, 2003), and leptin (Fruhbeck *et al.*, 1998, Kawaji *et al.*, 2001, Rodruguez *et al.*, 2003, Siegrist-Kaiser *et al.*, 1997), have direct effects on the lipolytic activation in adipocytes. Interestingly, recent studies showed that in adipocytes, amino acid precursors of citric acid cycle intermediates strongly stimulate *per se* basal secretion of leptin, and in turn, basal lipolysis is enhanced by leptin in a dose-independent manner (Fruhbeck *et al.*, 1998, Cammisotto *et al.*, 2005). Therefore, amino acid-induced secretion of leptin would be capable of regulating basal lipolysis in mammalian fat cells.

Vespa amino acid mixture solution (VAAM) is a unique amino acid mixture which mainly consists of free amino acids identified in the saliva of larva of a species of hornet endemic to Japan (Abe *et al.*, 1991). VAAM, which has been offered commercially as a sports drink in Japan, comprises 17 free amino acids, and high amounts of L-leucine, L-valine, L-phenylalanine and L-aspartate (Abe *et al.*, 1991). In a previous study, VAAM has been shown to regulate fat metabolism in mice (Abe *et al.*, 1997), and its oral administration induces a significant increase in the plasma levels of free fatty acids (FFA), ketone bodies and catecholamines (Abe *et al.*, 1997). VAAM is thus expected as one of supplement regulating fat metabolism. However, to date, no evidence has been obtained as to whether VAAM has a direct effect on adipocyte lipolysis following alteration in leptin secretion from adipocytes themselves.

Here, we firstly show that VAAM increased basal lipolysis and leptin secretion but did not amplify agonist-stimulated lipolysis in adipocytes isolated from rat.

Material and Methods

Animals Male wistar rats (SLC, Japan), with initial body weights of 300–350 g, were housed three or four days in a cage in a temperature-controlled room at 23 ºC with a

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12:12-h light-dark cycle. Treatment of the animals was approved by the animal care committee of the Institute of Physical and Chemical Research (RIKEN) following National Institution of Health (NIH, USA) guidelines. Food and water were available *ad libitum*, but rats were fasted for 12 hours before the experiments. The rats were lightly anesthetized with pentobarbital (50 mg/kg body weight) and were sacrificed. Then, the epididymal adipose fat pads were removed and used for adipocyte isolation.

Analytical procedures Adipocytes were isolated by modification of the method of Rodbell (1964). Isolated adipocytes (approx. 10^5 cells) were incubated in plastic vials in buffer (Krebs-Ringer bicarbonate solution buffer with 10 mM HEPES, pH 7.4, containing 5.5 mM glucose and 2% (w/v) fatty acid-free bovine serum albumin with 0.05 mg/ml adenosine deaminase) with or without VAAM for 2 hours, and further incubated for 30 min with indicated agonists.

Thereafter, a cell-free incubation medium was assayed for glycerol and leptin. Leptin concentrations were determined by the EIA kit (Immuno-Biological Laboratories, Japan) according to the manufacturer's protocol. The number of adipocytes and glycerol were determined according to the previous study (Izawa *et al.*, 1991). The lysis buffer included in the cAMP assay kit was added to the tubes containing the resultant cells, and then cAMP was assayed for total cellular cAMP by using a cAMP EIA system kit (Amersham-Pharmacia Biotech, UK).

Other determinations and statistical analysis Preparation of nutrients was carried out as described previously (Abe *et al.*, 1995, 1997, Tsuchita *et al.*, 1997). Briefly, each amino acid was dissolved in distilled water according to larval saliva of *Vespa mandarinia*-derived amino acid composition distribution. Then, high-performance liquid chromatography analysis was performed to confirm and check the preparations. Prepared solutions were frozen at -80°C until used for experiments. Composition of the amino acid mixture was as follows (μ mol/ml): Asp, 0.12; Thr, 5.4; Ser, 1.86; Glu, 2.4; Pro, 13.55; Gly, 14.37; Ala, 4.54; Val, 4.4; Met, 0.4; Ile, 3.4; Leu, 4.62; Tyr, 4.48; Phe, 2.89; Lys, 6.48; Trp, 1.65; His, 1.94; and Arg, 2.64.

The glycerol release from adipocytes showed a linear time-dependent relationship with VAAM. It significantly increased over 120-150 minutes, compared to the VAAM-untreated control group. Maximal dose of both isoproterenol (ISO) and dibutylyl cAMP (db-cAMP) on lipolysis were used as previously reported (Izawa *et al.*, 2000, Rodriges *et al.*, 2003). Values represent the mean±SD. Significant differences between means were assessed Scheffe's test after the analysis of variance had been performed to establish that there were significant differences between the groups. A value of $P<0.05$ was regarded as significant.

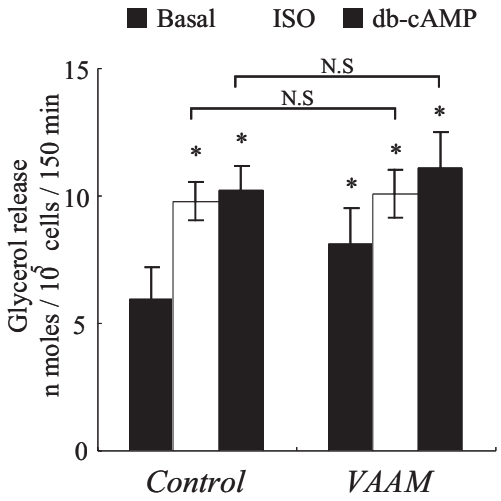

Fig. 1. Effect of treatment with isoproterenol (ISO), dybtilyl-cAMP (db-cAMP) and VAAM on the rate of lipolysis in adipocyte (n=9). After the adipocytes were preincubated with or without VAAM for 2 hours, they were incubated with ISO and db-cAMP. Bars and vertical lines indicate mean±S.D. *P<0.05 vs. basal value of control. N.S; not significant.

Results and Discussion

As shown in Fig. 1, VAAM increased basal lipolysis, but amplified neither ISO- nor db-cAMP-stimulated lipolysis. Although it is generally accepted that adipocyte lipolysis is mediated by increases in cAMP production, VAAM did not have any effect on production of basal and ISO-stimulated cAMP (Fig. 2). On the other hand, VAAM stimulated basal leptin secretion, but activation of protein kinase A by ISO or db-cAMP reduced leptin secretion did not (Fig. 3). These data suggest that one of the mechanisms underlying VAAM-enhanced basal lipolysis might involve a leptin-mediated but not cAMP-dependent pathway. Indeed, several lines of study have reported that leptin is capable of increasing in lipolysis under basal conditions in rat adipocytes (Fruhbeck *et al.*, 1998, Wang *et al.*, 1999). Moreover, because leptin secretion is reduced by increases in cAMP production and protein kinase A activation (Szkudelski *et al.*, 2005), it will follow that VAAM did not amplify agonist-induced lipolysis under those conditions.

The mechanism(s) by which VAAM increases basal leptin secretion is unclear at present. However, it is rational to speculate that several amino acids, which comprise VAAM, play a role in enhanced leptin secretion by VAAM. Of 17 varieties of free amino acids, L-leucine stimulates secretion of leptin in adipocytes (Lynch, 2001). This action of L-leucine is essential in the presence of glucose (Cammisotto *et al.*, 2005), and the incubation medium we used included

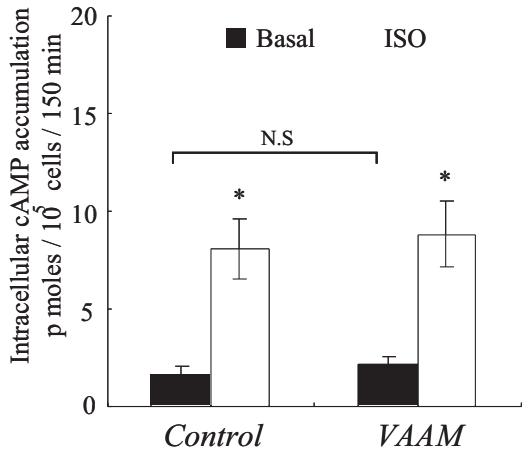

Fig. 2. Intracellular cAMP accumulations were measured with or without VAAM in adipocytes. Values represent the mean±S.D of three independent experiments (12 rats per group). *P<0.05 vs. basal value in each condition. N.S; not significant.

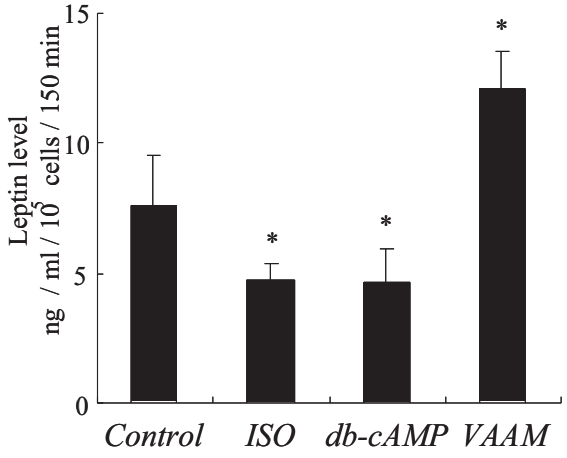

Fig. 3. Adipocytes-derived leptin levels were measured with or without VAAM in adipocyte. Values represent the mean±S.D of three independent experiments (12 rats per group). *P<0.05 vs. basal value of control. N.S; not significant.

5.5 mM glucose. Moreover, L-valine, L-methionine and L-phenylalanine, which are the other amino acids of which VAAM is composed, also mimic and promote glucose action (Cammisotto *et al.*, 2005). Interestingly, a recent study shows that amino acid precursors of citric acid cycle intermediates potently stimulate *per se* basal leptin secretion from adipocytes (Cammisotto *et al.*, 2005). Thus, VAAM-induced leptin secretion might be due to the stimulatory effect of these amino acids which comprise VAAM on leptin secretions. In addition, despite the fact that leptin has been shown to enhance adipocyte lipolysis in previous studies (Fruhbeck *et al.*, 1998, Kawaji *et al.*, 2001, Rodriguez *et al.*, 2003, Siegrist-Kaiser *et al.*, 1997), we lack adequate experimental data and explanations on whether addition of leptin amplifies adipocyte lipolysis with VAAM, at present. Further studies are required to elucidate and clarify these matters.

On the other hand, it has been reported that cAMP is inhibitory toward leptin secretion (Szkudelski *et al.*, 2005; Cammisotto and Bukowiecki, 2002). Moreover, Szkudelski *et al.* (2005) showed that in isolated adipocytes, L-alanine- and L-leucine-induced leptin secretion was decreased in the incubation with db-cAMP, suggesting that the inhibition of PKA activity was of major importance for potentiation of leptin secretion. Thus, even if VAAM enhances leptin secretion from adipocytes, it by no means amplifies adipocyte lipolysis under the condition where either cAMP production or PKA activation increases because VAAM-induced secretion of leptin is blunted under such conditions as found in this study (Fig. 3).

In this report, VAAM was shown to enhance basal adipocyte lipolysis with an increase in leptin secretion. This finding may indicate the possible role of VAAM in preventing or treating obesity. However, enhancement of adipocyte lipolysis by the supplementation of VAAM could not be expected during exercise, because physical exercise increases plasma catecholamine levels, and in turn, results in the increase in intracellular cAMP production via β-adrenergic receptor activation in adipocytes. Consequently, the previous findings that significant increases in plasma FFA and ketone bodies were observed in *in vivo* studies (Abe *et al.*, 1995, 1997) might be due to other effect of VAAM on lipid metabolism in targeted tissues other than for adipocytes. Further studies are required to resolve this issue.

In conclusion, VAAM has stimulatory effects on basal lipolysis through the enhanced secretion of leptin. This action was obstructed by the increase in either intracellular cAMP levels or PKA activation. Thus, VAAM has the stimulatory effects of lipolysis in adipocytes without any stimulus, at least in part, via the leptin-dependent lipolytic pathway.

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