Enhancement of Lipolytic Responsiveness of Adipocytes by Novel Plant Extract in Rat

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Subcutaneous adipocytes accumulate excess energy as triglycerides, but lipolytic response is less sensitive to catecholamines than visceral adipocytes. Obesity also induces catecholamine resistance of adipocytes. We have searched for crude drugs that could enhance the lipolytic response to noradrenalin. In this study, the lipolysis-promoting activities and action mechanisms of a novel plant extract from *Hemerocallis fulva* (HE) were investigated in isolated adipocytes from rat subcutaneous fat. HE exhibited no lipolysis-promoting activity alone but markedly promoted lipolysis when combined with noradrenaline; however, this synergistic activity was accompanied by no increase of intracellular cAMP production. This activity of HE was also observed when combined with cAMP analogue and was further enhanced by phosphodiesterase inhibitor. PKA inhibitor could reduce these activities of HE. These results indicate that HE is a novel lipolysis-promoting material that can sensitize the lipolytic response of adipocytes to catecholamine and suggest that HE can amplify the intracellular signaling pathway related to PKA or modify the other mechanism-regulating lipase activity. This characteristic material could contribute to improvement of adipose mobility in obesity-related disorder or in subcutaneous adiposity and to suppression of body fat accumulation. Exp Biol Med 234:1445–1449, 2009

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Introduction

When energy intake surpasses energy consumption, excess energy is stored as body fat in adipose tissue in various areas of the body. Excessive accumulation of intra-abdominal (visceral) fat causes problems particularly in terms of health, such as insulin resistance, while that of subcutaneous fat also causes problems in terms of esthetics. A representative measure against excessive body fat accumulation is promotion of the lipolysis of neutral lipid (triglyceride) accumulated in adipocytes, reducing adipocyte size.

The lipolytic reaction in adipocytes is mainly explained by the intracellular signaling mechanism, using cAMP as a second messenger. That is, adrenaline and noradrenaline stimulate β adrenergic receptor (βAR) on the adipocyte cell surface and sequentially induce the activation of stimulatory G protein (Gs), increase cAMP due to the activation of adenylate cyclase, activate cAMP-dependent protein kinase A (PKA) and activate hormone-sensitive lipase (HSL), resulting in the hydrolysis of triglycerides in intracellular lipid droplets. There are at least three subtypes of βAR involved in lipolysis—β1, β2 and β3 (1–3).

β adrenergic agonists, adenylate cyclase activators, and phosphodiesterase (PDE) inhibitors can promote lipolysis; however, subcutaneous fat shows lower lipolytic responses to hormones than visceral fat, and the above drugs with effects on visceral fat do not always have similar, adequate effects on subcutaneous tissue (4, 5). And catecholamine resistance is known to be induced by obesity, showing low lipolytic response and low thermogenesis (6–8). The precise causes of these regional differences and obesity-related alteration remain elusive, and effective agents for mobilization of these low-responding adipocytes are needed.

Therefore, our strategy was to search for novel materials that could improve the responsiveness of adipocytes to catecholamines. We have found several plant

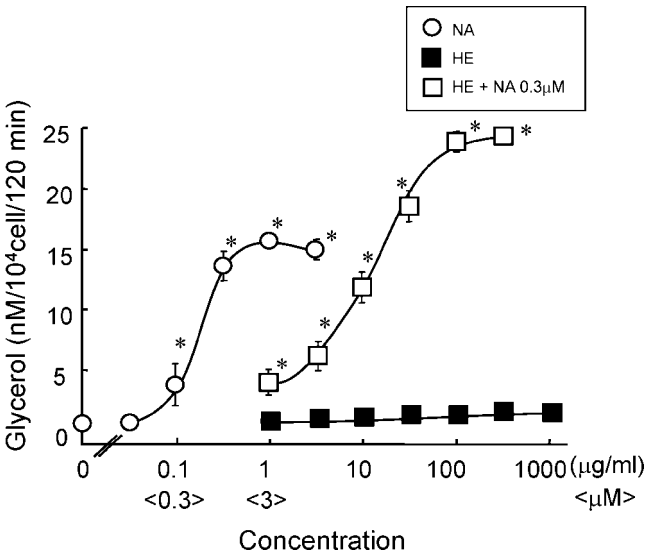

Figure 1. Promotion of lipolysis by NA or HE. Adipocytes were isolated from subcutaneous fat pad and treated with NA and/or HE for 120 min, and released glycerol was measured. Each value represents the mean ± SEM of three incubation tubes for each condition. *$P < 0.05$ compared with the value of the control group (incubation without any samples). < > represents concentration of NA.

extracts with lipolysis-promoting activity by *in vitro* screening, and we selected *Hemerocallis fulva* as a natural material with strong activity to use for the present study. In this study, we investigated the lipolysis-promoting activity and its mechanism of a novel plant extract on subcutaneous adipocytes isolated from rats, and we found unique characteristics of this material.

Materials and Methods

Preparation of Plant Extract. The flower portion of *Hemerocallis fulva* within *Liliaceae*, a type of daylily purchased from Showa Bussan Corporation (Osaka, Japan), was subjected to extraction with a 50% ethanol aqueous solution at room temperature, followed by heating and concentration, and an extract (HE) was obtained. A part of the extract was frozen and dried, and the concentration of the dry matter of the extract was obtained. In this experiment, the extract concentration was expressed as the extracted dry matter concentration. The dried extract was soluble in water and Hank's buffer (at least 10%).

Isolation of Adipocytes. Isolated adipocytes were prepared from the abdominal-inguinal subcutaneous fat pads of three Wistar male rats aged 9 weeks and weighing about 180–220 grams, according to the method reported by Rodbell (9). Briefly, the excised adipose tissue was cut into small pieces using ophthalmologic scissors, suspended and washed with Hank's buffer containing 2% bovine serum albumin fraction and digested with collagenase in the presence of 1 mg/ml glucose in Hank's buffer for about 60 minutes at 37°C. The digested tissue was filtered through a nylon mesh, and floating cells were collected and gently rinsed with Hank's buffer and suspended in the same buffer.

Measurement of Lipolysis and cAMP. The isolated adipocyte suspension was incubated with various concentrations of noradrenaline (NA), HE, dibutyryl cAMP (dbcAMP) as a cAMP analogue, caffeine (Caf) as a PDE inhibitor and H89 as a PKA inhibitor in Hank's buffer at 37°C for 2 hours. As an indicator of lipolysis, the glycerol released into the buffer after incubation was measured using the enzyme method (10). Briefly, the buffer after stimulation was mixed with glycerol-detecting solution, and absorbance was measured at 480 nm. The composition of the glycerol-detecting solution was 2.7 mmol/l p-chlorophenol, 0.04% Triton X-100, 2 mmol/l magnesium sulfate, 2 mmol/l ATP, 0.05 mmol/l 4-amino antipiline, 1 mmol/l EDTA, 0.5 U glycerokinase, 4 U glycerol-3-phosphate oxidase and 2 U peroxidase. The quantified glycerol concentration was normalized by the number of isolated adipocytes, calculated using a hematocytometer.

Concentration of intracellular cAMP was measured by cAMP Biotrak Enzymeimmunoassay according to the manufacturer's instruction. Briefly, adipocytes incubated with isobutylmethylxanthine (IBMX) for 30 minutes similar to lipolysis tests were washed by phosphate-buffered saline and dissolved by lysis reagent. These samples were carefully applied on the plate coated by cAMP-peroxidase conjugate, followed by addition of enzyme substrate and colorimetrical measuring at 630 nm.

All determined data are presented as the mean ± SEM of triplicate incubation tubes for each condition. The data were statistically analyzed by ANOVA, followed by the Bonferroni method for multiple comparisons between pairs. Each experiment was repeated three times with similar results, and typical results of one experiment were shown in figures.

Chemicals. Bovine serum albumin fraction was purchased from Sigma (MO, USA). Collagenase Type I was from Worthington Biochemical (NJ, USA); NA and Caf were from Wako Pure Chemical Industries (Osaka, Japan); dbcAMP and IBMX were from Sigma-Aldrich Japan (Tokyo, Japan); H89 was from Funakoshi Corporation (Tokyo, Japan); and cAMP was from Biotrak Enzymeimmunoassay from GE Healthcare UK (Buckinghamshire, England).

Statement of Ethics. We were certain that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. All animal experiments were conducted in the Experimental Animal Facility of Kao Tochigi Institute. The Animal Care Committee of Kao Tochigi Institute approved the present study. All experiments strictly followed the guidelines of that committee.

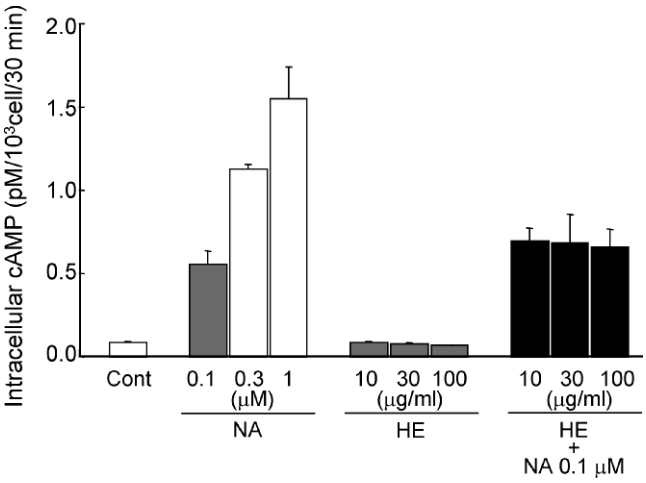

Figure 2. Promotion of intracellular cAMP production by NA and HE. Adipocytes were isolated from subcutaneous fat pad and treated with various concentration of NA and HE with IBMX for 30 min, and intercellular cAMP was measured. Each value represents the mean ± SEM of three incubation tubes for each condition.

Results

Lipolysis-Promoting Activity in Adipocytes. The lipolysis-promoting activity of NA and HE in isolated subcutaneous adipocytes was compared. As shown in Figure 1, NA showed marked lipolysis-promoting activity, and its EC_{50} was about 0.6 μM. However, HE showed no lipolysis-promoting activity. The lipolysis-promoting activity of HE occurred when it was used in combination with NA at 0.3 μM, the concentration that increased lipolysis to a modest level. The maximum response to this combination was higher than that to NA (3 μM), and EC_{50} was about 20 μg/ml, showing that HE could sensitize responsiveness of subcutaneous adipocytes to NA and induced amplification of NA-induced lipolysis. HE (10 μg/ml) combined with various concentration of NA also exhibited a marked promotion of NA-induced lipolysis, showing increase of maximum response level (data not shown).

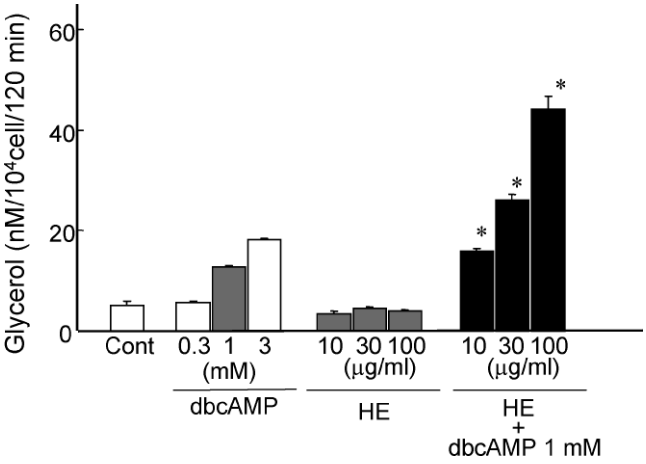

Figure 3. Promotion of lipolysis by HE combined with cAMP analogue. Adipocytes were isolated from subcutaneous fat pad and treated with HE and dbcAMP for 120 min, and released glycerol was measured. Each value represents the mean ± SEM of three incubation tubes for each condition. *$P < 0.05$ compared with the value of 1mM dbcAMP.

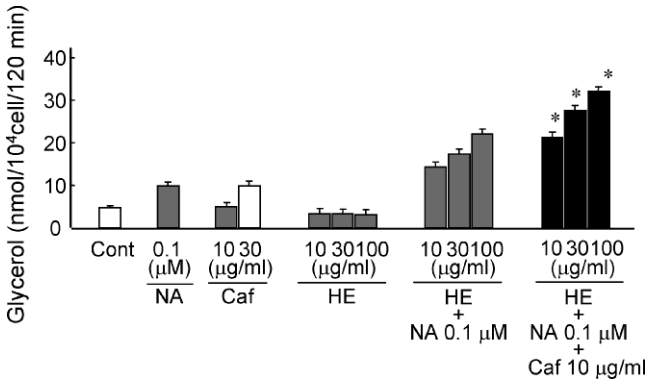

Figure 4. Promotion of lipolysis by HE combined with NA and PDE inhibitor. Adipocytes were isolated from subcutaneous fat pad and treated with HE, NA, and Caf for 120 min, and released glycerol was measured. Each value represents the mean ± SEM of three incubation tubes for each condition. *$P < 0.05$ compared with the value of each HE + NA.

The change of intracellular cAMP concentration by HE treatment was compared to NA. As shown in Figure 2, unexpectedly, cAMP was not significantly affected by HE treatment.

Influence of cAMP Analogue and PDE Inhibitor. Since HE activity on NA-induced lipolysis was not caused by cAMP production, combination with dbcAMP was evaluated. As shown in Figure 3, dbcAMP promoted lipolysis and was enhanced by HE addition in dose-dependent manner, showing synergistic effect of HE with cAMP-dependent signaling. Various concentration of dbcAMP-induced lipolysis was enhanced by HE addition (data not shown).

Influence of Caf on HE activity was shown in Figure 4. NA-induced lipolysis enhanced by HE was further amplified by Caf co-treatment.

Relation of HE Activity to PKA. Relationship of PKA activation to HE activity was investigated. The influence of PKA inhibitor on HE-enhanced lipolysis was shown in Figure 5. H89 (50 μM) diminished the NA-induced lipolysis as well as its enhancement by HE, showing that the PKA signaling pathway was essential for the linkage of HE activity and lipolysis.

Discussion

We have found HE as a plant extract that could modify lipolytic responsiveness in low-responding adipocytes, and we evaluated their activity and its action mechanism in isolated subcutaneous adipocytes in this study. Interestingly, HE alone did not exert lipolysis-promoting effects, but in combination with NA it showed marked potentiation. In cases of combination of two compounds with different pharmacological activity, for instance the combination of a β agonist and a PDE inhibitor, their co-treatment often

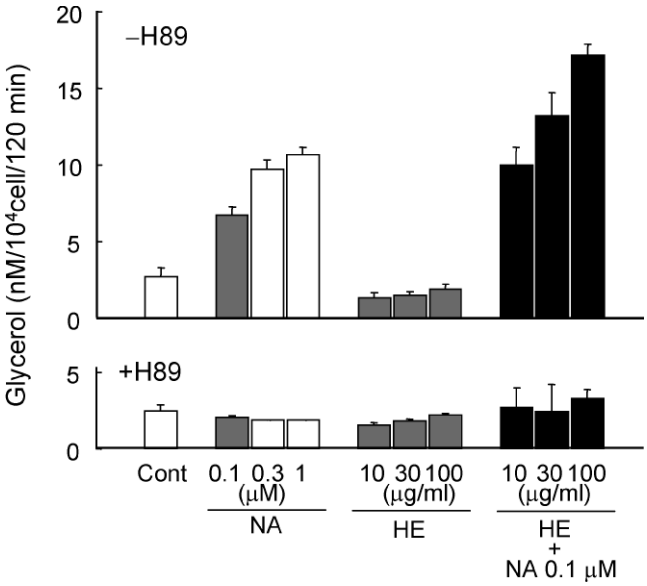

Figure 5. Affect of PKA inhibitor on lipolysis-promotion by HE. Adipocytes were isolated from subcutaneous fat pad and treated with HE, NA, and H89 (50 μM) for 120 min, and released glycerol was measured. Each value represents the mean ± SEM of three incubation tubes for each condition.

produces positive regulation of co-factor, inducing a synergistic effect. Unexpectedly, HE did not affect cAMP production; however, HE drastically amplified the cAMP signal of cAMP analog or PDE inhibitor. The results of combination treatments with NA, dbcAMP or caffeine showed that, distinct from catecholamines and PDE inhibitors, HE could not affect βAR, adenylate cyclase and PDE but could amplify cAMP-mediated intracellular signal transduction. Since PKA inhibitor could inhibit the lipolysis-promotion by HE, at least PKA activation was essential to the linkage of HE activity and lipolysis.

There have been many reports showing regional differences in adipose metabolism (11–13). Subcutaneous adipocytes have common function to visceral adipocytes, such as energy storage and adipocytokines secretion, but have low responsiveness to dietary treatments or exercise (14) and lipolysis-promoting hormones, such as catecholamines, compared with visceral adipocytes. Expression level, receptor-binding number and enzymatic activity in βAR and HSL have been discussed. In addition to stimulatory signal transduction, there are anti-lipolytic pathways, such as α2AR, PDE and insulin signal. We have confirmed rapid desensitization of lipolysis and found regional differences in responses to various β-agonistic stimulations (15); however, there has not been any critical evidence showing the precise cause of these regional differences. Obesity and obesity-related diabetes were accompanied by catecholamine resistance, showing low metabolic responsiveness (6–8). In recent years, regulatory mechanism of HSL activity or location implicating perilipin, novel adipose triglyceride lipase and other cofactors have been suggested (16). It is necessary to confirm whether HE can affect PKA activity, lipase activity and interaction between peri-lipid droplet proteins, such as perilipin and CGI-58, in further study.

Hemerocallis fulva is a species of *Hemerocallis*, native to Asia from the Caucasus. It has never been known if these plants have some pharmacological effects or compounds concerning fat metabolism or adipocyte function. We identified several water-soluble active compounds, which were included within HE (<10 %), but further investigation is necessary to identify precise active substances and their capability to bind or to cross the adipocyte plasma membrane.

This study indicated that a novel natural product, HE, promotes lipolysis via a unique mechanism involving PKA-related signal transduction. It was suggested that this product can amplify cAMP signaling without promotion of cAMP production and possibly act at the post-PKA level, such as on co-factors modifying lipase activity or contact to HSL. Effective use of the unique plant extracts with lipolysis-promoting activity may be beneficial for improving the subcutaneous fat sensitivity to hormones and for control of body fat metabolism. Further studies are necessary to elucidate the active compounds and action mechanisms of this natural product and to evaluate their possible application to the health promotion and esthetics.

1. Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, Strosberg AD. Molecular characterization of the human β3-adrenergic receptor. Science 245:1118–1121, 1989.
2. Arner P. Regulation of lipolysis in fat cells. Diabetes Rev 4:450–463, 1996.
3. Hoffstedt J, Arner P, Hellers G, Lonnqvist F. Variation in adrenergic regulation of lipolysis between omental and subcutaneous adipocytes from obese and non-obese men. J Lipid Res 38:795–804, 1997.
4. Hellmer J, Marcus C, Sonnenfeld T, Arner P. Mechanisms for differences in lipolysis between human subcutaneous and omental fat cells. J Clin Endocrinol Metab 75:15–20, 1992.
5. Tavernier G, Galitzky J, Valet P, Remaury A, Bouloumie A, Lafontan M, Langin D. Molecular mechanisms underlying regional variations of catecholamine-induced lipolysis in rat adipocytes. Am J Physiol 268: E1135–E1142, 1995.
6. Mauriège P, Prud'homme D, Lemieux S, Tremblay A, Després JP. Regional differences in adipose tissue lipolysis from lean and obese women: existence of postreceptor alterations. Am J Physiol 269:E341–E350, 1995.
7. Bairras C, Mauriege P, Bukowiecki L, Atgie C. Regulation of lypolysis in white adipose tissues of lean and obese Zucker rats. J Physiol Biochem 63:287–296, 2007.
8. Böttcher H, Fürst P. Decreased white fat cell thermogenesis in obese individuals. Int J Obes Relat Metab Disord 21:439–444, 1997.
9. Rodbell M. Metabolism of isolated fat cells I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 239:375–380, 1964.
10. Kawano-Takahashi Y, Ohminaji H, Ubagai E, Okuda H. Mechanisms of action of mazindol in preventing onset and development of obesity induced by gold thioglucose injection. Int J Obes 8:655–664, 1984.
11. Hellmer J, Marcus C, Sonnenfeld T, Arner P. Mechanisms for differences in lipolysis between human subcutaneous and omental fat cells. J Clin Endocrinol Metab 75:15–20, 1992.
12. Tavernier G, Galitzky J, Valet P, Remaury A, Bouloumie A, Lafontan

M, Langin D. Molecular mechanisms underlying regional variations of catecholamine-induced lipolysis in rat adipocytes. Am J Physiol 268: E1135–E1142, 1995.

13. Vidal-Puig A. Gene expression in visceral and subcutaneous adipose tissue. Ann Med 33:547–555, 2001.
14. Doucet E, St-Pierre S, Alméras N, Imbeault P, Mauriège P, Pascot A, Després JP, Tremblay A. Reduction of visceral adipose tissue during weight loss. Eur J Clin Nutr. 56:297–304, 2002.
15. Mori S, Nojiri H, Yoshizuka N, Takema Y. Rapid desensitization of lipolysis in the visceral and subcutaneous adipocytes of rats. Lipids 42: 307–314, 2007.
16. Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 306:1383–1386, 2004.
17. Marcinkiewicz A, Gauthier D, Garcia A, Brasaemle DL. The phosphorylation of serine 492 of perilipin a directs lipid droplet fragmentation and dispersion. J Biol Chem 281:11901–11909, 2006.