Norepinephrine-Augmenting Lipolytic Effectors from *Astilbe thunbergii* Rhizomes

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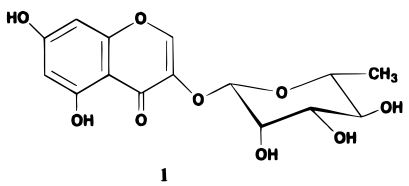
An EtOAc-soluble fraction from a 80% Me_2CO extract of the rhizomes of *Astilbe thunbergii* enhanced norepinephrine-induced lipolysis in rat fat cells, while an EtOAc-insoluble fraction had no effect. The active substances isolated from the EtOAc-soluble fraction of the rhizomes were identified as eucryphin (**1**), bergenin (**2**), and astilbin (**3**), which enhanced norepinephrine-induced lipolysis at concentrations of 10−1000 μg/mL, while they themselves did not cause lipolysis. Furthermore, these compounds slightly stimulated adrenocorticotrophic hormone-induced lipolysis and inhibited insulin-induced lipogenesis from glucose.

The dried rhizomes of species such as *Astilbe chinensis* (Maxim.) Franch. et Savat., *A. rivularis* Buch.-Ham. ex D. Don var. *rivularis* Buch.-Ham. ex D. Don, *A. japaonica* (Morr. et Decne.) A. Gray, and *A. thunbergii* (Sieb. et Zucc.) Miq., known as " Hong Shengma" (Chinese name) and "Aka-Shouma" (Japanese name), are used as substitute drugs for " Shengma".[1] The latter drug is extracted from the rhizomes of *Cimicifuga* species [2,3] such as *C. heracleifolia* Komarov, *C. dahurica* (Turxz.) Max., and *C. foetida* L. in the People's Republic of China and Japan. The isolation of bergenin (**2**) and astilbin (**3**) from the rhizomes of *A. thunbergii* has been reported.[4,5] The physiological action of rhizomes of *A. thunbergii*, however, is not yet well undersood. In the present work, it has been determined that an 80% acetone extract of the rhizomes of *A. thunbergii* caused an increase in lipolysis in the presence of norepinephrine in rat fat cells. Therefore, an investigation was performed to isolate and identify the active substances of this natural drug using a norepinephrine-induced lipolytic assay to guide fractionation. In addition, we investigated the effects of these active substances isolated from the rhizomes of *A. thunbergii* on adrenocorticotrophic hormone (ACTH)-induced lipolysis and insulin-induced lipogenesis from glucose in fat cells and norephinephrine-induced lipolysis in endogenous lipid droplets (a cell-free system).



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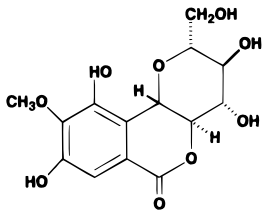

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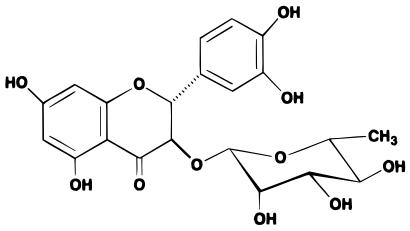

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Results and Discussion

As shown in Table 1, the 80% acetone extract of the rhizomes of *A. thunbergii* enhanced norepinephrine-induced lipolysis at the concentration range of 1−1000 μg/mL, dose dependently, while it did not cause lipolysis in the absence of norepinephrine. The EtOAc-soluble fraction separated from the 80% acetone extract also enhanced norepinephrine-induced lipolysis at the concentrations of 1−1000 μg/mL in a dose-dependent fashion, but the EtOAc-insoluble fraction had no effect on norepinephrine-induced lipolysis. The EtOAc-soluble fraction (25 g) was chromatographed over Si gel (1 kg) with $CHCl_3$−MeOH (5:1) as eluent, to afford three active substances (**1**−**3**) that enhanced norepinephrine-induced lipolysis.

In the present study, we found first that **1**−**3** enhanced norepinephrine-induced lipolysis in fat cells (Figure 1). These three compounds did not stimulate lipolysis in the absence of norepinephrine. Compounds **1**, **2**, and **3** were identified as eucryphin, bergenin, and

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Table 1. Effects of Various Extracts of the Rhizomes of *A. thunbergii* on Norepinephrine-Induced Lipolysis in Fat Cells[a]

addition (/mL reaction mixture)	lipolysis (FFA μmol/mL packed fat cells/h) mean ± S.E.	% of control
none	0.0 ± 0.0	
norepinephrine (0.05 μg)	6.37 ± 0.22	100
norepinephrine + 80% Me_2CO extract		
(1 μg)	7.55 ± 0.10[b]	118.5
(10 μg)	8.02 ± 0.10[c]	125.9
(100 μg)	9.25 ± 0.07[c]	145.2
(1000 μg)	9.52 ± 0.13[c]	149.5
none	0.0 ± 0.0	
norepinephrine (0.05 μg)	5.50 ± 0.03	100
norepinephrine + EtOAc-soluble fraction		
(1 μg)	6.88 ± 0.68[b]	125.1
(10 μg)	6.99 ± 0.50[b]	127.1
(100 μg)	7.36 ± 0.41[c]	133.8
(1000 μg)	10.1 ± 0.50[c]	183.6
none	0.0 ± 0.0	
norepinephrine (0.05 μg)	7.34 ± 0.10	100
norepinephrine + EtOAc-insoluble fraction		
(1 μg)	7.41 ± 0.20	101.0
(10 μg)	7.97 ± 0.49	108.6
(100 μg)	8.21 ± 0.13	111.9
(1000 μg)	7.26 ± 0.80	98.9

[a] Results are expressed as mean ± S.E. of three experiments. Significantly different from norepinephrine alone. [b] $p < 0.05$. [c] $p < 0.01$.

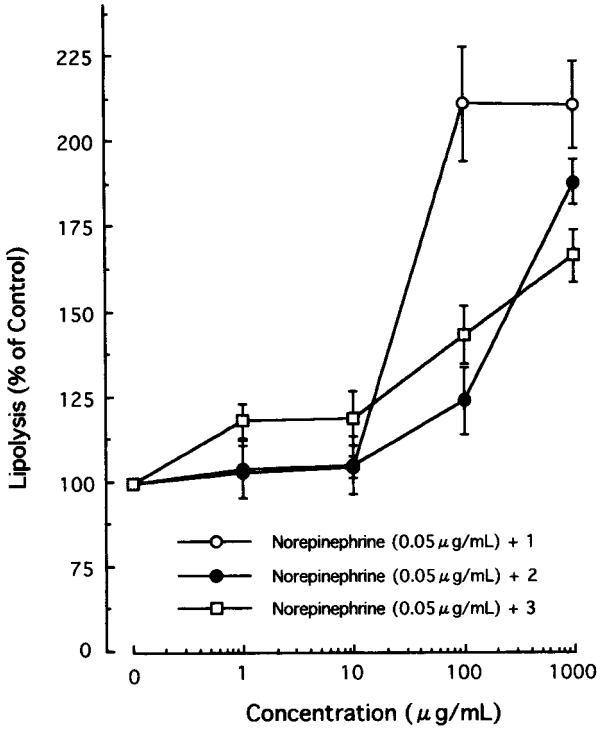

Figure 1. Effects of **1**−**3** isolated from the rhizomes of *A. thunbergii* on norepinephrine-induced lipolysis in fat cells. (Values are expressed as the mean ± S.E. of three experiments. The activity of norepinephrine-induced lipolysis is expressed as 100%).

astilbin, respectively, based on comparison of their spectral data with literature data.[4−6] This is the first report of the isolation of eucryphin (**1**) from the rhizomes of *A. thunbergii*.

Generally, lipolytic action in fat cells plays an important role in energy metabolism in animals. It is well known that lipolytic action in fat cells is stimulated by various pharmacological lipolytic hormones, such as epinephrine, norepinephrine, ACTH, and growth hormone. It is postulated that cyclic AMP (cAMP) plays a key role in the lipolysis stimulated by the above lipolytic hormones. Catecholamines such as epinephrine and norepinephrine are thought to stimulate adenylate cyclase in the membranes of fat cells and to increase the cAMP level of the cells.[7] This increased level of cAMP stimulates protein kinase A activity, which in turn activates hormone-sensitive lipase (HSL), and the activated HSL catalyses the hydrolysis of triglyceride in fat cells.[8,9] However, Okuda et al. found that cAMP-dependent activation of HSL stimulated lipolysis of [^{3}H]triolein emulsified with gum arabic, but not of endogenous lipid droplets prepared from fat cells.[10,11] The endogenous lipid droplets were found to show lipolysis in response to catecholamines, theophylline, and *p*-aminophenol.[12−15] Previously, we suggested that phospholipids in the endogenous lipid droplets were important in catecholamine-mediated lipolysis.[16]

In the present experiments, compounds **1**−**3** enhanced norepinephrine-induced lipolysis in both fat cells and a cell-free system consisting of HSL and endogenous lipid droplets, but not in the sonicated lipid droplets and HSL (Figures 1 and 2, Table 2). It is suggested that **1**−**3** stimulate the binding to the phospholipids of norepinephrine and, consequently, elicit a greater degree of lipolysis than norepinephrine alone. In addition, **1**−**3** at a higher concentration (1000 μg/mL) stimulated ACTH-induced lipolysis (Table 3). Moreover, they inhibited insulin-induced lipogenesis from glucose (Figure 3). Therefore, the crude drug "Hong Shengma" ("Aka-Shouma", Japanese name) may activate the actions of lipolytic hormones and selectively inhibit the effect of antilipolytic hormones such as insulin. Compounds, **1**−**3** are more selective modulators of lipolytic and antilipolytic hormones than a β-blocker, which inhibits both the effects of lipolytic hormones, such as epinephrine and ACTH, and of insulin, such as the hormone-induced lipogenesis in fat cells.[17,18] In a previous study, we found that various furocoumarins isolated from the roots of *Angelica dahurica* and *Angelica shikokiana* enhanced epinephrine-induced lipolysis and

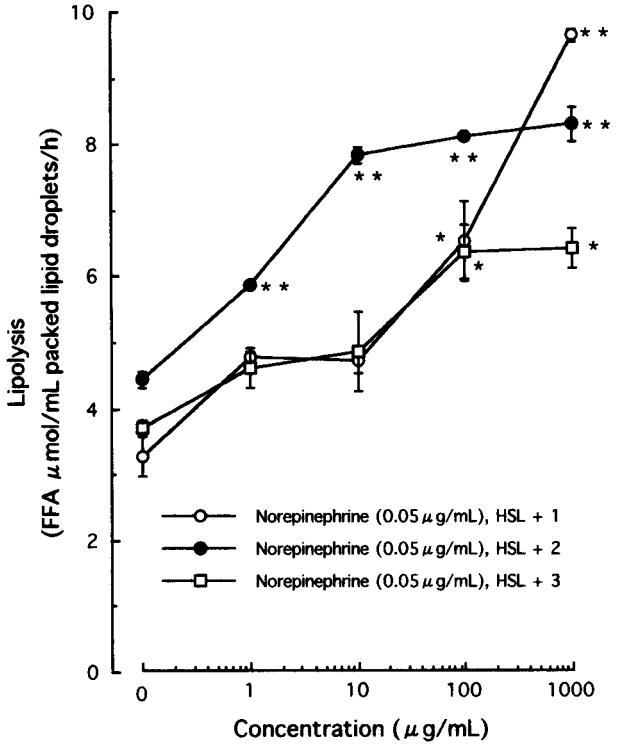

Figure 2. Effects of **1**−**3** on norepinephrine-induced lipolysis in a cell-free system consisting of intact lipid droplets and HSL solution. (Values are expressed as the mean ± S.E. of three experiments. Significantly different from norepinephrine alone. * $p < 0.05$; ** $p < 0.01$).

Table 2. Effects of Compounds **1**−**3** on Lipolysis in a Cell-free System Consisting of Sonicated Lipid Droplets and HSL in the Presence or Absence of Norepinephrine[a]

addition (/mL reaction mixture)	lipolysis (FFA μmol/mL packed sonicated lipid droplets/h) mean ± S.E.	% of control
none	6.523 ± 0.04	100
1 (1000 μg)	6.432 ± 0.014	98.5
2 (1000 μg)	6.429 ± 0.18	98.6
3 (1000 μg)	6.245 ± 0.14	95.7
norepinephrine (0.05 μg)	6.696 ± 0.01	102.7
norepinephrine + **1** (1000 μg)	6.520 ± 0.08	100.0
norepinephrine + **2** (1000 μg)	6.276 ± 0.08	96.2
norepinephrine + **3** (1000 μg)	6.423 ± 0.03	98.5

[a] Results are expressed as mean ± S.E. of three experiments.

Table 3. Effects of **1**−**3** on ACTH-Induced Lipolysis in Fat Cells[a]

addition (/mL reaction mixture)	lipolysis (FFA μmol/mL packed fat cells/h) mean ± S.E.	% of control
none	0 ± 0	
ACTH (0.5 μg)	2.04 ± 0.03	100
ACTH + **1** (100 μg)	1.62 ± 0.03	79.4
(1000 μg)	6.79 ± 0.30[b]	332.8
ACTH + **2** (100 μg)	2.16 ± 0.20	105.9
(1000 μg)	7.28 ± 0.40[b]	356.9
ACTH + **3** (100 μg)	3.99 ± 0.05[b]	195.6
(1000 μg)	5.68 ± 0.50[b]	278.4

[a] Results are expressed as mean ± S.E. of three experiments. Significantly different from ACTH alone. [b] $p < 0.01$.

inhibited insulin-induced lipogenesis from glucose in fat cells.[19,20] In the present study, it is thus of great interest that a chromone glycoside (**1**), an isocoumarin (**2**), and a flavanone glycoside (**3**) have the same enhancing effects on norepinephrine-induced lipolysis and inhibitory effects on insulin-induced lipogenesis as the furocoumarin derivatives. From these results, it seems likely that **1**−**3** isolated from the rhizomes of *A. thunbergii* may be effective in formulations for the treatment of obesity. Further experiments are needed to examine their actions in vivo.

Experimental Section

General Experimental Procedures. Melting points, determined on a Yamato MO-21 capillary apparatus, are uncorrected. IR and ORD spectra were measured on a Shimadzu IR-400 spectrometer and a JASCO ORD/UV-5 spectrometer, respectively. ^{1}H NMR (499.83 MHz) and ^{13}C NMR (125.68 Hz) spectra were recorded in DMSO-d_6 and solvent + D_2O on a Varian Unity Inova 500 spectrometer. MS were measured on a Hitachi M-4000H spectrometer. Column chromatography was performed using Si gel 60 (70−230 mesh, ASTM, Merck, Darmstadt, Germany) as adsorbent.

Materials. Collagenase (type IV) was purchased from Worthington Biochemical Co. (Freehold, NJ). Norepinephrine, ACTH, and insulin were obtained from Sankyou Co. Ltd. (Tokyo, Japan), Daiichi Pharmacy Co. Ltd. (Tokyo, Japan), and Sigma Chemical Co. (St. Louis, MO), respectively. Bovine serum albumin (BSA) was purchased from Wako Pure Chemical Industry (Osaka, Japan) and was extracted by the method of Chen [21] to remove free fatty acids. Other chemicals were of reagent grade. Test compounds were dissolved or suspended in Hanks balanced solution (pH 7.4).

Plant Material. The crude drug " Hong-Shengma", ("Aka-Shouma" Japanese name), which is the dried rhizomes of *A. thunbergii* (Saxifragaceae), was purchased from Tochimoto Tenkaido Co. (Osaka, Japan) in August 1997, and voucher samples are stored at the Second Department of Pharmacognosy, Osaka University of Pharmaceutical Sciences.

Animals. Young male Wistar rats (5 weeks old) were obtained from Clea Co. (Osaka, Japan). Rats weighing 150−160 g (6 weeks old) were given a standard laboratory diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. They were killed by cervical dislocation, and their epididymal adipose tissue was quickly removed.

Measurement of Norepinephrine- and ACTH-Induced Lipolysis in Fat Cells. Isolated fat cells were obtained from rat epididymal adipose tissues by the method of Rodbell.[22] An aliquot of the fat cell fraction (50 μL packed volume) was incubated for 1 h at 37 °C in 200 μL of Hanks balanced solution (pH 7.4) supplemented with 2.5% BSA, norepinephrine (25 μL, final concentration: 0.05 μg/mL) or ACTH (25 μL, final concentration: 0.5 μg/mL), and the indicated amounts of test compounds (25 μL). The release of free fatty acid (FFA) was measured as described previously.[19] Briefly, the incubation mixture (250 μL) was mixed with 3 mL of $CHCl_3$−*n*-heptane (1:1) containing 2% MeOH and extracted by shaking the tube horizontally for 10 min in a shaker. The mixture was centrifuged at 2000 × *g* at 25 °C for 5 min, and the upper aqueous phase was removed by suction. Copper reagent (1 mL) was then added to the lower organic phase. The tube was shaken for 10 min, the mixture was centrifuged at 2000 × *g* at 25 °C for 10 min, and 0.5 mL of the upper organic phase,

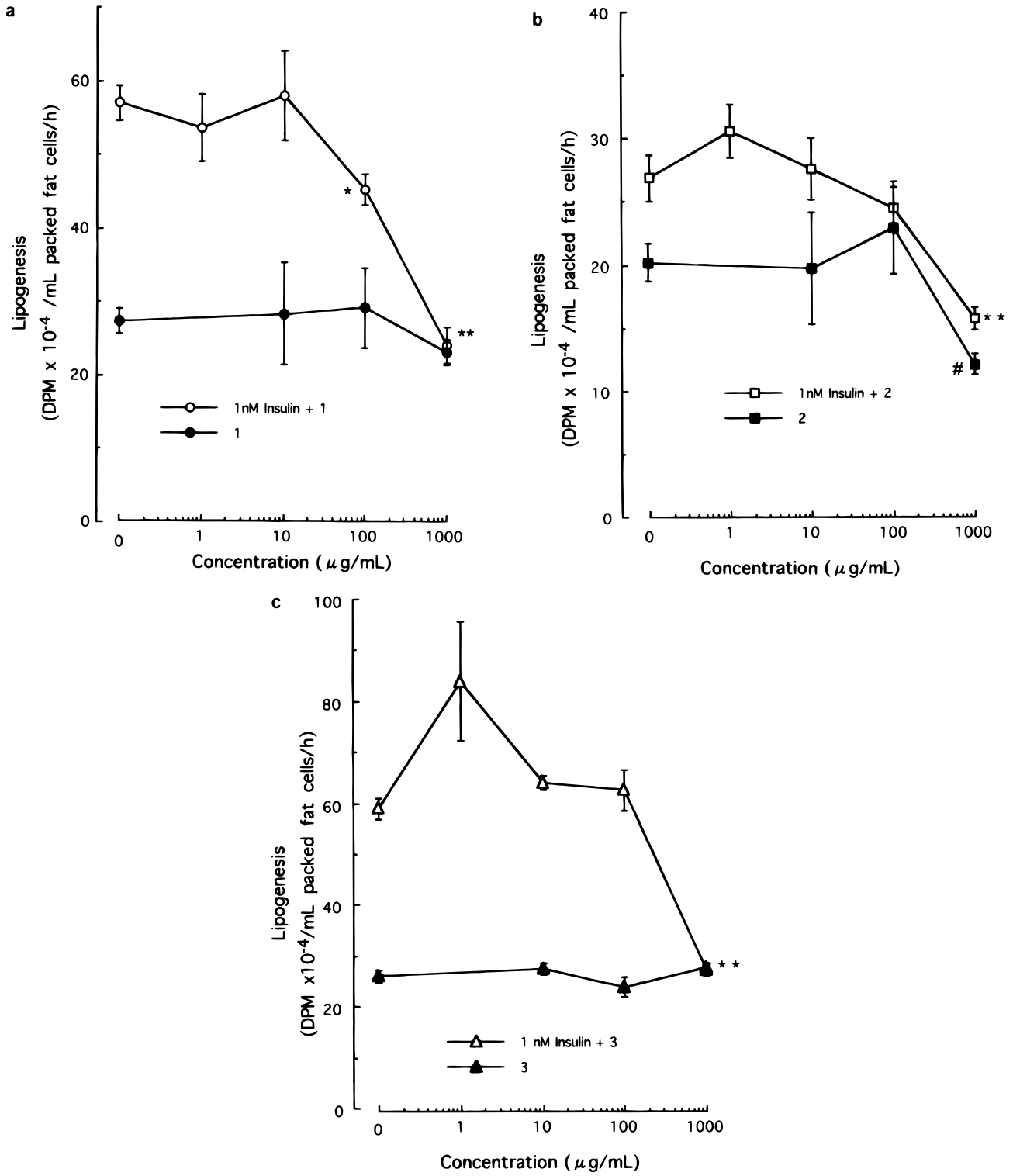

Figure 3. Effects of **1**−**3** on lipogenesis and insulin-induced lipogenesis from [U−^{14}C]glucose in fat cells. (Values are expressed as the mean ± S.E. of 3−6 experiments. Significantly different from no addition: # $p < 0.01$. Significantly different from insulin alone. *$p < 0.05$; **$p < 0.01$).

which contained the copper salts of the extracted fatty acid, was treated with 0.5 mL of 0.1% (w/v) bathocuproine in $CHCl_3$ containing 0.05% 3-(2)-*tert*-butyl-4-hydroxyanisole. Absorbance was then measured at 480 nm. Lipolysis was expressed as μmol of FFA released per mL of packed fat cells per 1 h.

Preparation of HSL Solution. Rat epididymal adipose tissue (0.7 g) was cut into small pieces with scissors and homogenized in 1 mL of Hanks balanced solution (pH 7.4) in a Potter−Elvehjem homogenizer by five strokes of a Teflon pestle. The homogenate was centrifuged for 15 min at 2500 × *g* at 10 °C, and the supernatant was applied to a heparin−Sepharose column (5 × 20 mm), equilibrated with Hanks balanced solution, to remove lipoprotein lipase. The unadsorbed fraction was used as the HSL solution. HSL activity in this fraction was not reduced by 1 M NaCl or antiserum to bovine lipoprotein lipase, indicating that

lipolytic activity due to lipoprotein lipase was minimal in the preparation.

Preparation of Endogenous Lipid Droplets. Isolated fat cells were obtained from epididymal adipose tissues by the method of Rodbell.[22] A 1-mL packed volume of cells was suspended in 4 mL of 5 mM Tris−HCl buffer (pH 7.4). The suspension was mixed by slowly inverting the centrifuge three times and then was centrifuged at 200 × *g* at 25 °C for 3 min. The fat layer was mixed with 4 mL of 5 mM Tris-HCl buffer (pH 7.4) containing 0.025% Triton X-100 by slowly swinging the tube three times, and the mixture was centrifuged at 200 × *g* at 25 °C for 3 min. The fat layer was washed once with Hanks balanced solution (pH 7.4) and suspended in the same buffer. This suspension was named "endogenous lipid droplets" [23] and used in this study.

Measurement of Norepinephrine-Induced Lipolysis in a Cell-Free System Consisting of HSL and Endogenous Lipid Droplets. An aliquot of endogenous lipid droplets (25 μL packed volume) was incubated for 1 h at 37 °C in 100 μL of Hanks balanced solution (pH 7.4) supplemented with 2.5% BSA, norepinephrine (25 μL, final concentration: 0.05 μg/mL), HSL fraction (100 μL), and the indicated amounts of test compounds (25 μL). Another experiment was performed as follows. An aliquot (25 μL) of endogenous lipid droplets was mixed with 125 μL of Hanks balanced solution containing 2.5% BSA and 1.017 mg of gum arabic, and the mixture was sonicated for 5 min. The sonicate (75 μL) was incubated with the HSL fraction (100 μL), Hanks balanced solution containing 2.5% BSA (50 μL), norepinephrine (25 μL, final concentration: 0.05 μg/mL), and the indicated amount of test compound (25 μL) for 1 h at 37 °C. The release of FFA was measured by the method described above. Lipolysis was expressed as μmol of FFA released per mL of packed lipid droplets per 1 h.

Measurement of Insulin-Induced Lipogenesis from [U−^{14}C] Glucose in Fat Cells. An aliquot of the fat cell fraction (50 μL packed volume) was incubated for 1 h at 37 °C in 200 μL of Hanks balanced solution (pH 7.4) supplemented with 2.5% BSA, [U−^{14}C] glucose (25 μL, final concentration 0.5 μCi = 18.5 KBq/mL), insulin (25 μL, final concentration: 1 nM), and the indicated amount of test compound (25 μL). The reaction was stopped by adding 5 mL of Dole's extraction mixture.[24] The test tube was shaken vigorously for 5 min. Then, 3 mL of heptane and 2 mL of H_2O were added, and the mixture was shaken vigorously for 5 min. A 3-mL aliquot of the upper phase (heptane layer) was transferred to a stoppered glass test tube and shaken vigorously with an equal volume of alkaline EtOH (0.05 M NaOH in 50% EtOH) to remove FFA. A 1-mL aliquot of the heptane layer was placed in a counting vial containing 10 mL of scintillation fluid (ACS II, Amersham Co., Tokyo, Japan), and radioactivity was estimated with a Packard liquid scintillation counter. Lipogenic activity was expressed as DPM per mL packed fat cells per 1 h.

Data and Statistical Analysis. Values are expressed as mean ± standard error of the mean. Statistical analysis was performed with Student's *t*-test.

Extraction and Isolation. The dried, crushed rhizomes (1 kg) of *A. thunbergii* were extracted with 80% Me_2CO (1 L × 3 times) for 3 h under reflux. The combined 80% Me_2CO extracts were concentrated in vacuo to give a dark reddish brown extract (63.5 g). A portion (50 g) of the 80% Me_2CO extract was suspended in distilled H_2O (1 L), extracted with EtOAc (300 mL × 6 times), and divided into EtOAc-soluble and -insoluble fractions. The EtOAc-soluble and -insoluble fractions were concentrated in vacuo to give 26.4 and 21.3 g residues, respectively. The EtOAc-soluble fraction (25 g) was chromatographed over Si gel (1 kg) with $CHCl_3$−MeOH (5:1) as eluent, to afford three active active substances(**1**−**3**) that enhanced norepinephrine-induced lipolysis.

Eucryphin (1): colorless crystalline powder obtained from MeOH; mp 231−233° [lit. mp 231−233°];[6] $[\alpha]^{20}_D$ −185.5° (*c* 0.51, MeOH) [lit. $[\alpha]^{20}_D$ −187.5° (*c* 0.47, MeOH)].[6] This compound exhibited closely comparable spectral data (UV, IR, ^{1}H NMR, ^{13}C NMR, MS) to literature values;[6] yield 1.25 g.

Bergenin (2): colorless crystalline powder obtained from MeOH; mp 236−238° [lit. mp 238°];[4] $[\alpha]^{20}_D$ −38.5° (*c* 2.01, MeOH) [lit. $[\alpha]^{18}_D$ −37.7° (*c* 1.96, EtOH)].[4] Compound **2** was identified as bergenin by comparison with an authentic sample isolated from the cortex of *Mallotus japanica* (Thunb.) Muell. Arg. (Euphorbiaceae);[4,5] yield 3.7 g.

Astilbin (3): colorless crystalline powder obtained from a mixture of MeOH and distilled H_2O; mp 189−191° [lit. mp 190−192°];[25] $[\alpha]^{20}_D$ −12.7° (*c* 1.02, MeOH) [lit. $[\alpha]^{25}_D$ −14.6° (*c* 0.52, EtOH)].[25] Compound **3** was identified as astilbin by comparison of UV, IR, MS, ^{1}H NMR, and ^{13}C NMR spectral data with reference values;[6,25] yield 5.6 g.

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