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Biochemical approach for the study of WAKAN-YAKU

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Abstract

As a biochemist, I have been studying lipolytic and lipogenic pathways in fat cells since 1963. In 1966, I proposed "hormone-sensitive substrate theory" in which catecholamine might not act on lipase but on substrate during its lipolytic process.[1] In 1994, I succeeded in isolation of lipid droplets from rat fat cells without destroying their structure and proved that the lipid droplets, especially their surface character, played a key role in catecholamine-induced lipolysis in fat cells.[2] On the lipogenic pathway in fat cells and glucose uptake into skeletal muscle, I suggested involvement of Na^+/H^+ antiport in the action of insulin in 1989.[3] Based on these biochemical evidences, I tried to apply these experimental results to the study of WAKAN-YAKU and develop its scientific approach.

Key words Lipolysis, Lipogenesis, Extracellular fluid, Fat cells.

Insulin-like substances in Korean red ginseng

Insulin is known to stimulate lipogenesis from glucose and inhibit catecholamine-induced lipolysis in fat cells. On the other hand, catecholamine inhibited insulin-mediated lipogenesis from glucose and stimulated lipolysis in fat cells. An important point on the actions of insulin and catecholamines, is that these hormonnes discriminate lipogenesis and lipolysis in fat cells and regulate each metabolic pathway in the opposite direction.

Experiments were designed to identify such hormone-like substances in medicinal plants. *Panax ginseng* is a medicinal plant long used in the treatment of various pathological states including general complaints such as head ache, shoulder ache, chilly constitution and anorexia, and diabetes. There have been many pharmacological studies on *Panax ginseng* roots. It was reported that oral administration of an aqueous alcoholic extract of ginseng roots decreased the blood sugar level of rabbits and that *Panax ginseng* suppressed hyperglycemia induced by epinephrine and high carbohydrate diets. These findings suggest that *Panax ginseng* roots contain insulin-like substances. Insulin is well known to inhibit epinephrine-induced lipolysis and stimulate lipogenesis from glucose in fat cells.

One hundred grams of Korean red ginseng powder were mixed with water. The water extract of the red ginseng was subjected to dialysis against water. The outer dialysate was then subjected to gel-filtration on Bio-Gel P-2 column as shown in Fig.1. Anti-lipolytic

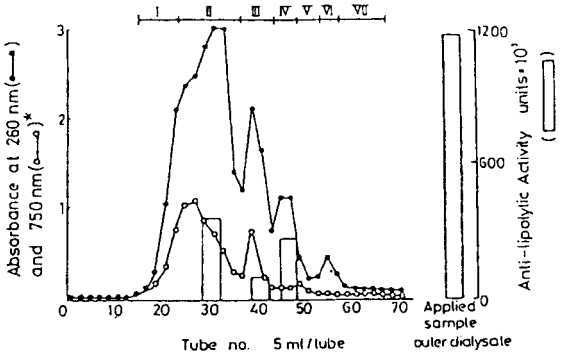

Fig. 1 Gel filtration of the outer dialysate on a Bio Gel P-2 column. Column size, 2.2×43 cm. Elution was carried out with water. *Absorbance of protein in the method of Lowry *et al.*

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activity was eluted mainly in fractions II and IV. Fraction IV was determined to be adenosine.[4] Adenosine inhibited norepinephrine-induced lipolysis in rat fat cells and stimulated lipogenesis from glucose both in the presence and absence of insulin as shown in Fig.2 and 3.

Fraction II was then applied to a Dowex-2×8[Cl-form] column (2.2×15 cm), washed with water and 0.01N HCl, and then eluted with 0.5N HCl. The eluate was subjected to dialysis with dialysis membrane to remove larger molecules with Mr greater than 1,000 dalton and outer dialysate was concentrated. The concentrated material was then applied to reverse phase chromatography as shown in Fig. 4. Each fraction was hydrolyzed (6N HCl, 100°C, 24h), and subjected to the ninhydrine reaction. Peaks at around tube

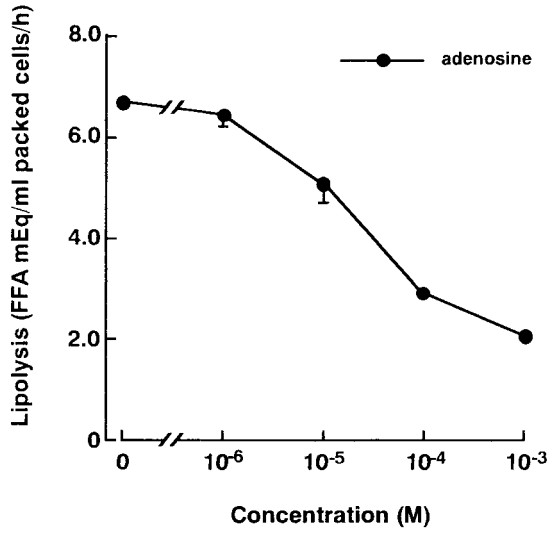


Fig. 2 Effect of adenosine on norepinephrine-induced lipolysis in rat fat cells.

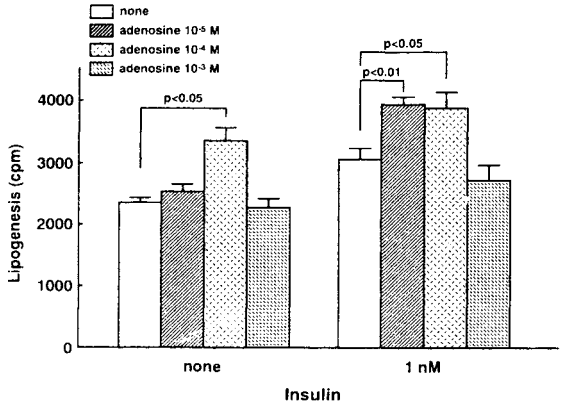


Fig. 3 Effect of adenosine on insulin-induced lipogenesis in rat fat cells.

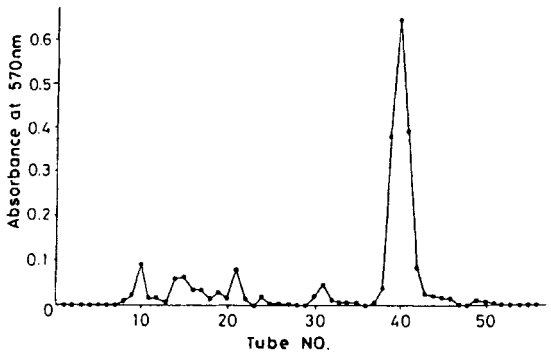
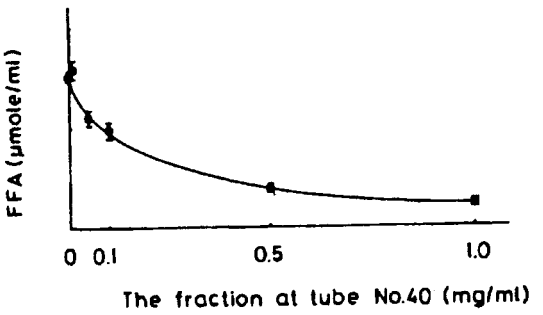


Fig. 4 Reverse-phase chromatography of the eluate from Dowex-2 column. Reverse-phase HPLC was done on a TSK gel ODS-80OTM column (TOSOH 4.6 mm ID×25 cm). Elution was carried out with 0.1 % TFA in water. Each fraction was hydrolyzed (6N HCL, 100°C 24h) and subjected to ninhydrin reaction



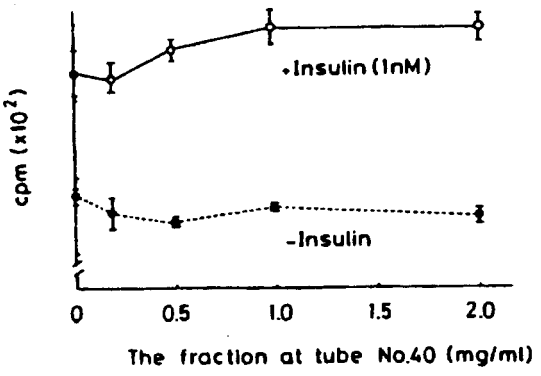


Fig. 5 Effect of the fraction at tube No.40 on epinephrine-induced lipolysis and lipogenesis from glucose in fat cells. Lipogenesis was examined in the presence (○) and absence(●) of insulin (1 nM).

No.40 showed high ninhydrin reactions. The peaks at around tube No.40 (the fraction at tube No.40) did not contain any amino acids. On the other hand, only glutamic acid was demonstrated after acid hydrolysis, suggesting that the active principle may be a derivative of glutamic acid. The fraction at tube No.40 was found to inhibit epinephrine-induced lipolysis in fat cells as shown in Fig. 5. In addition to anti-lipolytic activity, the fraction at peak No.40 stimulated lipogenesis from glucose in the presence of insulin (Fig. 5).

Therefore, we concluded that this fraction is the insulin-like substance. The chemical structure of this substance was determined to be pyroglutamic acid. The yields of pyroglutamic acid and adenosine are 0.3 % and 0.03 % from Korean red ginseng powder, respectively.

Isolated fat cells are well known to possess opposite pathways of lipid metabolism; lipolysis and lipogenesis which will be postulated to be negative and positive (陰と陽) metabolic pathways in traditional medicine. Lipolysis is stimulated by catecholamines and lipogenesis is activated by insulin. In various pathological conditions, the balance between lipolysis and lipogenesis is often broken. For example, lipolysis is accelerated in diabetes and lipogenesis is enhanced in obesity.

From ancient times, *Panax ginseng* is believed to improve pathological conditions of diabetes mellitus. If so, *Panax ginseng* should contain inhibitions toward lipolysis and stimulators toward lipogenesis, because lipolysis is accelerated and lipogenesis is inhibited in diabetes.

It is well known that propranolol, a β-blocker, inhibits epinephrine-mediated lipolysis in fat cells. In addition to the anti-lipolytic activity, propranolol also inhibits insulin-stimulated lipogenesis in fat cells (unpublished data). In contrast to propranolol, pyroglutamic acid and adenosine selectively inhibit the epinephrine-induced lipolysis and stimulate lipogenesis.

Based on these experimental results, we suggest that pyroglutamic acid and adenosine should be called selective modulators to discriminate negative and positive metabolic pathways.

Catecholamine-like substances in *Astilbe thunbergii*

The dried rhizomes of species such as *Astilbe chinensis* (Maxim.) Franch. et Savat., *A. revularis* 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". The latter drug is extracted from the rhizomes of *Cimicifuga* species such as *C. heracleifolia* Komarov, *C. dahurica* (Turxz.) Max., and *C.foetida* L. in the People's Republic of China and Japan. The rhizomes of *A. thubergii* are known to contain eucryphin, bergenin and astilbin as shown in Fig. 6.

We first found that these compounds (1-3 in Fig.

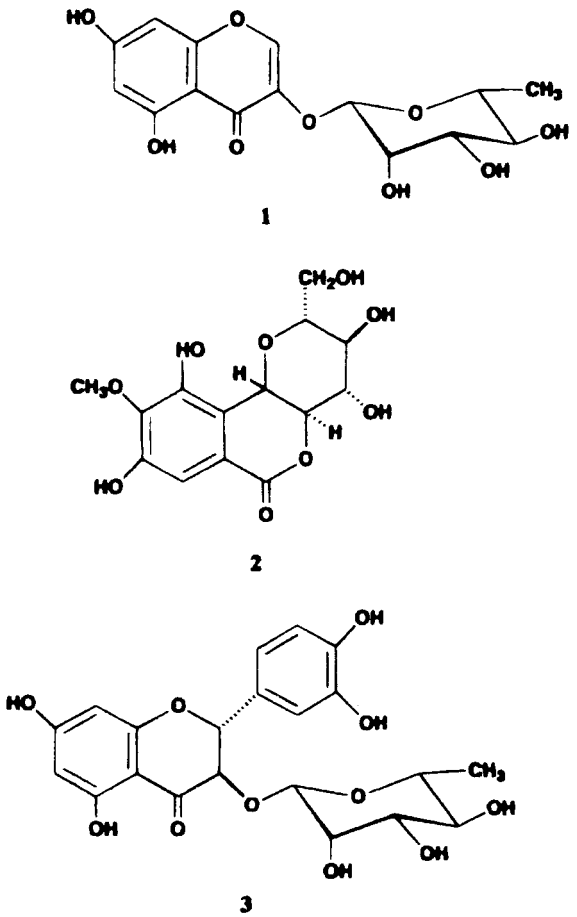


Fig. 6 The chemical structures of the compounds found in the rhizomes of *A. thunbergii*.

6) enhanced norepinephrine-induced lipolysis in fat cells, whereas they did not stimulate lipolysis in the absence of the hormone (Fig. 7).

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. 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.[5] However, Okuda *et al.* found that cAMP-dependent activation of HSL stimulated lipolysis of [^{3}H] triolein emulsified with gum arabic, but not that of endogenous lipid droplets prepared from fat cells.[6] The endogenous

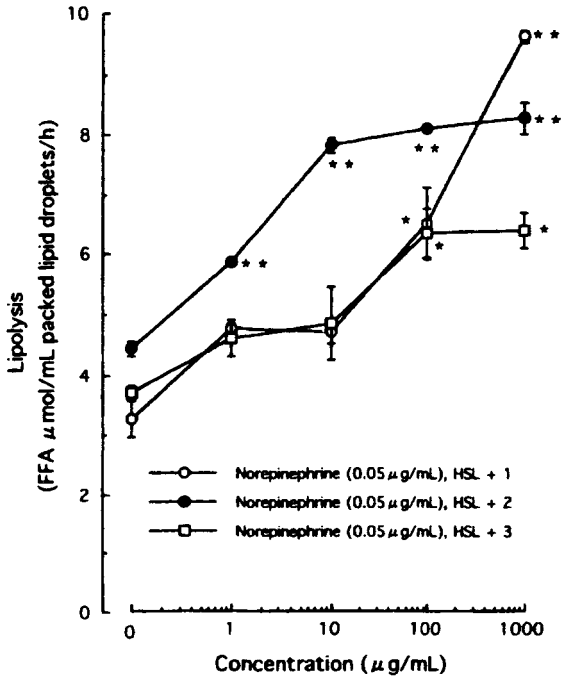


Fig. 8 Effects of 1-3 on norepinephrine-induced lipolysis in a cell-free system consisting of intact lipid droplets and HSL solution. (Values are expressed as the mean±S. E. of three experiments. Significantly different from norepinephrine alone. $*p<0.05$, $**p<0.01$).

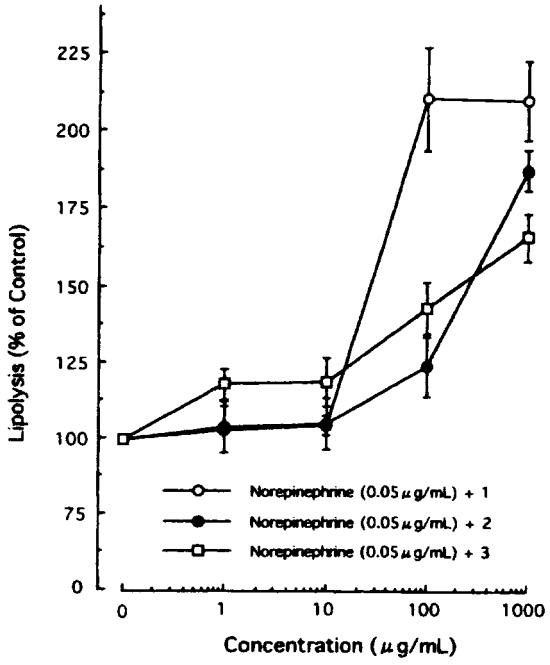


Fig. 7 Effects of 1-3 isolated from the rhizomes of *A. thunbergii* on norepinephrine-induced lipolysis in fat cells. (Values are expressed as the mean±S. E. of three experiments. The activity of norepinephrine-induced lipolysis is expressed as 100 %).

lipid droplets were found to show lipolysis in response to catecholamines, theophylline, and p-aminophenol in the presence of HSL.[7,9] Previously, we suggested that phospholipids in the endogenous lipid droplets were important in catecholamine-mediated lipolysis.[2]

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 (Fig. 7 and 8, Table I), indicating that the site of the stimulatory actions of these substances was not HSL but the endogenous lipid droplets. 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 (100 μg/mL) stimulated ACTH-induced lipolysis. Moreover, they inhibited insulin-induced lipogenesis from glucose.[10]

Therefore, encryphin, bergenin and astilbin were identified to be catecholamine-like substances which stimulated lipolysis and inhibited lipogenesis in fat

Table I 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. Results are expressed as mean±S.E. of three experiments.

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 (1000μg)	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

cells. In other words, these substances are selective modulators which discriminate negative and positive (陰と陽) metabolic pathways.

A new hypothesis on insulin action

In traditional medicine, body water (sui '水' in Japanese) is supposed to be an important factor which maintains good health. A problem is what is the body water. In 1990, we found that insulin-mediated 2-deoxy-D-glucose (2-DG) uptake by rat soleus muscle was inhibited by reduction in the pH of the medium from 7.4 to 6.8[11] (Fig. 9).

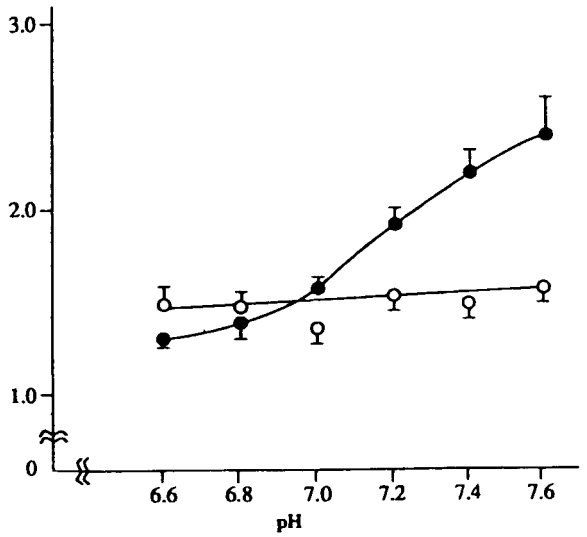


Fig. 9 pH-dependence of basal and insulin-stimulated 2-deoxy-D-glucose uptakes by rat soleus muscle. Rat soleus was incubated in *Hanks* buffer solution at different pH values in the absence(○)or presence(●)of 10 nM insulin for 15 min. Then 2-deoxy-D glucose uptake was intiated by adding radioactive tracer. Bars show standard errors of means (n=6-10).

Klip, Ramlal and Cragoe reported that in addition to increase in 2-DG uptake, activation of Na^+/H^+ antiport is one of the earliest responses of muscle cells to insulin.[12] To confirm this activation of Na^+/H^+ antiport, we examined amiloride-sensitive ^{22}Na uptake into rat soleus muscles. Amiloride is known to be an inhibitor of Na^+/H^+ antiport. Amiloride-sensitive ^{22}Na uptake was 8.50 nmole mg^{-1} tissue in the absence of insulin and 48.13 nmol mg^{-1} tissue in its presence, suggesting that insulin stimulate Na^+/H^+ antiport in the muscles. When medium Na^+ was replaced by other ions such as K^+, Rb^+ and $choline^+$, insulin failed to stimulate 2-DG uptake into the soleus muscles. On the other hand, in medium with L_i^+ in place of Na^+, 2-DG uptake was 1.79 pmol mg^{-1} tissue in the absence of insulin and 3.66 pmol mg^{-1} tissue in its presence. Thus insulin increased 2-DG uptake in the presence of Na^+ or L_i^+, which are known to be substrates of Na^+/H^+ antiport.[13] All these results suggest that Na^+/H^+ antiport is closedly related to the stimulatory action of insulin on 2-DG uptake.

Stimulation of Na^+/H^+ antiport by insulin causes alkaline shift of cytoplasmic pH which may accelerate translocation of glucose transporter type 4 from microsomal fraction to plasma membrane possibly through increase in free Ca^{++} of cytoplasm.[14] The increase in cytoplasmic pH is sufficient to activate glycolytic enzyme including phosphofructokinase.[14] Therefore, it seems likely that the increase in cyto-

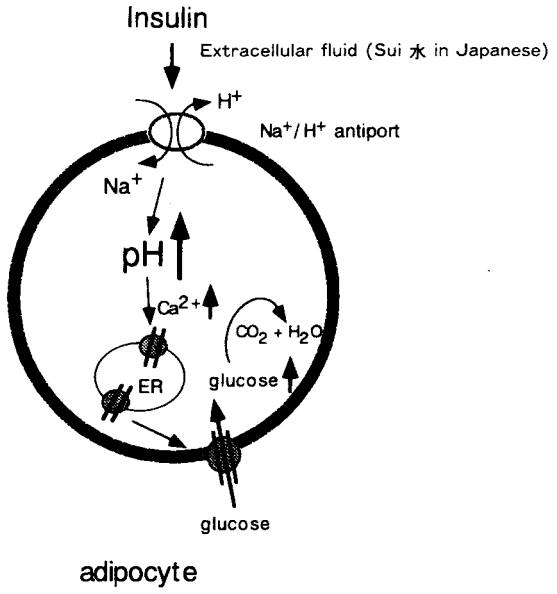


Fig. 10 Hypothetical scheme on insulin-stimulated glucose metabolism.

plasmic pH may be a messenger of insulin actions on glucose uptake and post receptor glucose metabolism (Fig. 10).

The effect of medium pH on insulin-stimulated 2-DG uptake may be explained by assuming that Na^+/H^+ antiport is involved in insulin-mediated glucose uptake. Reduction of the extracellular pH implies increase in the H^+ content of extracellular medium, which would reduce efflux of intracellular H^+ and thus inhibit Na^+/H^+ antiport with consequent decrease of insulin-mediated 2-DG uptake. The medium used in the experiment of Fig. 9 corresponds to extracellular fluid *in vivo*. We found that pH value of extracellular fluid surrounding rat skeletal muscle was easily reduced by hemrrhage, endotoxin shock, interception of blood flow and / or CO_2 inhalation.[15] Based on these results, I suggests that body water (sui '水' in Japanese) in traditional medicine may involve extracellular fluid and the reduction of its pH may cause insulin resistance corresponding to water toxicity (suidoku '水毒' in Japanese) in traditional medicine.

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