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Mechanism of Action of Rhatannin on Plasma Amino Acid Levels in Rats

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An intraperitoneal (*i.p.*) injection of rhatannin (12.5 mg/kg body weight, condensed tannin purified from Rhei Rhizoma) decreased the concentration of plasma amino acids in rats. The early effect of rhatannin on the levels of amino acids was compared with those of selected hormones. Although glucagon (0.5 mg/kg), adrenaline (0.5 mg/kg), and corticosterone (0.5 mg/kg) exerted similar decreasing effects on amino acids, the effects of rhatannin and glucagon were different from those of the others in that only these two substances caused a decrease in lysine concentration. Rhatannin also increased the levels of plasma glucose and urea 2 h after *i.p.* administration in rats starved for 24 h. The effects of rhatannin observed in the present study cannot be ascribable to its effect on the levels of glucagon and insulin, since rhatannin did not affect the physiological levels of these hormones.

Glucose release from liver cells in the presence of glucagon was synergistically enhanced by rhatannin, although rhatannin itself had no effect on basal glucose release from liver cells. When this synergistic effect of rhatannin was examined at 10 nM glucagon, glucose release increased with an increase in the rhatannin concentration. The maximal level of enhancement was achieved at rhatannin concentrations ranging from 5 to 60 μg/ml. On the other hand, glucose release induced by adrenaline or dibutyryl adenosine 3′,5′-cyclic monophosphate (dibutyryl c-AMP) was not affected by rhatannin. Thus, it was suggested that the effects of rhatannin on plasma amino acids, glucose, and urea observed *in vivo* might be mediated by rhatannin's primary effect on the glucagon regulatory pathway.

Keywords——rhubarb; tannin; plasma amino acid; glucagon; hepatocyte; glucose

It has been generally accepted that steady-state concentrations of free amino acids in plasma are maintained by the net balance between release from endogeneous protein stores and utilization by various tissues. In the postabsorptive state, the liver and muscle play a major role in determining the circulation levels and turnover of amino acids.[1] The release and the utilization of amino acids in these organs are under hormonal control.[2]

It was shown previously[3] that the concentrations of rat plasma amino acids were decreased two to eight hours after an intraperitoneal (*i.p.*) injection of rhatannin[4] (condensed tannin purified from Rhei Rhizoma). In the present study, changes in the levels of plasma amino acids within two hours after an *i.p.* injection of rhatannin were compared with those induced by hormones, such as glucagon, adrenaline, corticosterone, and insulin. Rhatannin exerted a decreasing effect on free amino acid concentrations and simultaneously increased the levels of glucose and urea in plasma. The mechanisms responsible for the *in vivo* effect of rhatannin were analyzed by determining glucose release from isolated rat liver cells which had been shown to retain many liver functions involving ureogenesis, gluconeogenesis and responsiveness to hormones.[5] The results suggested that the effect of rhatannin on the levels of amino acids, glucose, and urea might be mediated through its action on the glucagon regulatory pathway.

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Materials and Methods

Materials——Rhatannin was isolated from rhubarb (Rhei Rhizoma) by the method described previously.[4] The following compounds were purchased: insulin (Shimizu Pharmaceutical, Japan); glucagon (Novo Pharmaceutical Ind., Denmark); adrenaline, corticosterone, N^2, O^2-dibutyryl adenosine 3′,5′-cyclic monophosphate (dibutyryl c-AMP), trypsin inhibitor Type II-s (Sigma Chemical Co., U.S.A.); collagenase Class II (Worthington Diagnostic Systems Inc., U.S.A.); *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid (HEPES), ethyleneglycol-bis-(β-amino-ethylether) *N*, *N*′-tetraacetic acid (EGTA) (Dojin Laboratories, Japan).

Animals——Male Wistar rats weighing 170—200 g were employed throughout the experiment. They were housed in air-conditioned quarters at 25 °C and 60% relative humidity. Animals were fed on laboratory pellet chow (CLEA Japan Inc., Japan) and tap water freely. They were fasted for 24 h before the experiments, unless otherwise stated. Rats were injected with rhatannin (12.5 mg/kg body weight), glucagon (0.5 mg/kg), adrenaline (0.5 mg/kg), corticosterone (0.5 mg/kg), or insulin (75 mIU/kg) intraperitoneally. They were routinely killed by decapitation.

Preparation and Incubation of Hepatocytes——Parenchymal hepatocytes were isolated from fed or 24 h-starved male Wistar rats weighing 200—250 g by perfusion of the liver *in situ* with collagenase, as described by Seglen.[6] The proportion of viable cells was estimated by mixing the cell suspension with an equal volume of freshly prepared Trypan blue (0.16% in 0.34% NaCl solution), after which a minimum of 100 cells were examined to determine the Trypan blue exclusion ratio. The viability of the isolated liver cells was always 85—95%.

The purified hepatocytes were suspended in glucose-free Hanks salt solution containing 10 mM HEPES or glucose-free Hanks salt solution containing 10 mM HEPES and 5 mM sodium lactate, 5 mM alanine, or 5 mM serine. The reaction mixture containing 4×10^6 or 8×10^6 cells/plastic dish (60 × 15 mm) was incubated in a humidified incubator at 37 °C under 5% CO_2 in air for 1 or 2 h. Reactions were stopped by cooling the cell suspensions to 0 °C with ice-water and cells were removed by centrifugation (1000 *g* × 10 min). The glucose concentration of the supernatants was estimated.

Assays——The concentrations of amino acids in plasma were determined by using a Hitachi model 835 amino acid analyzer (Hitachi Ind., Japan). The deproteinization of plasma was carried out with an equal volume of 2% sulfosalicylic acid. Estimations of urea-nitrogen, protein, and glucose were carried out with commercial reagents, "BUN EIKEN" (Eiken Chemical Co., Ltd., Japan), biuret solution and Glucose B-test Wako (Wako Chemical Ind., Japan), respectively, using a DSA-560 discrete sample analyzer (Beckman Instrument Inc., U.S.A.).

Estimations of glucagon and insulin in plasma were carried out by radio-immunoassay by Otsuka Assay Laboratory (Tokushima, Japan).

Results

Characterization of Effects of Rhatannin on Levels of Plasma Amino Acid, Glucose, Urea, and Proteins

In order to analyze the mechanism by which rhatannin decreases the levels of amino acids,[3] the changes in the concentrations of the individual amino acids in fed rats treated with rhatannin (12.5 mg/kg body weight) or hormones, *i.e.*, glucagon (0.5 mg/kg), adrenaline (0.5 mg/kg), corticosterone (0.5 mg/kg), and insulin (75 mIU/kg), were compared. The results are illustrated in Fig. 1. Rhatannin decreased the levels of all amino acids 1 h after treatment and the decrease was more profound after 2 h. The reductions in amino acids observed 1 h after glucagon administration were similar to the data as reported by Bromer and Chance.[7] Of the amino acids, Leu, Phe, Val, and Ile returned to the control levels after 2 h. One hour after treatment, adrenaline had decreased the levels of all amino acids except Lys. Several amino acids returned to the control levels after 2 h. Corticosterone did not decrease the levels of Lys, Glx, His, Ser, and Gly 1 h after treatment, and Ala, His, Ser, and Gly were increased 2 h after treatment. Insulin decreased the level of Tyr 1 h after treatment and the level of Gly 2 h after treatment. Glucagon among the hormones tested and rhatannin shared a common effect specific to Lys, the level of which has been demonstrated to be controlled by glucagon.[8,9] The decreasing effect of rhatannin on levels of Lys might be mediated by either levels in the blood or regulatory pathways of glucagon or insulin, since glucagon and insulin act upon the liver in opposite ways, suggesting that the relative concentrations in the blood of these two hormones may determine the net amino acid balance. Therefore, the levels of these hormones found in the peripheral blood of the control and rhatannin-treated groups of rats

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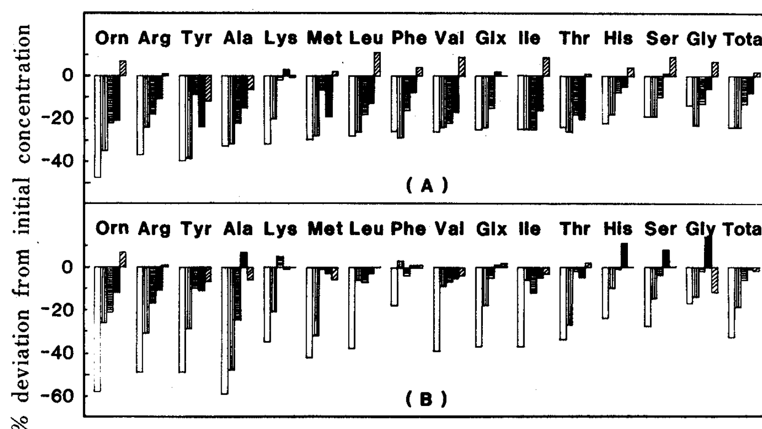

Fig. 1. Comparison of Effects on Levels of Individual Amino Acids by Rhatannin, Glucagon, Adrenaline, Corticosterone, and Insulin

(A), 1 h after treatment; (B), 2 h after treatment.
The data are presented as percent deviations from the initial concentrations of plasma amino acids.
The initial concentrations of amino acids (μmol/100 ml plasma; mean value $\pm$ S.D., 8 animals) were as follows: Orn, 7.7 $\pm$ 1.3; Arg, 27.0 $\pm$ 2.8; Tyr, 11.3 $\pm$ 1.9; Ala, 50.7 $\pm$ 3.5; Lys, 47.6 $\pm$ 3.7; Met, 7.5 $\pm$ 0.5; Leu, 20.5 $\pm$ 2.0; Phe, 7.9 $\pm$ 0.7; Val, 29.1 $\pm$ 3.0; Glx, 71.6 $\pm$ 6.2; Ile, 12.9 $\pm$ 1.1; Thr, 39.2 $\pm$ 7.0; His, 11.2 $\pm$ 0.8; Ser, 25.1 $\pm$ 3.3; Gly, 43.7 $\pm$ 5.1; total amino acids, 413.0.
Rhatannin, ▭ ; glucagon, ▤ ; adrenaline, ▥ ; corticosterone, ■; insulin, ▨ .

TABLE I. Time Course of the Effect of Rhatannin on Plasma Glucose, Urea-Nitrogen, and Proteins in Rats Starved for 24 h

Time after treatment (h)	No. of rats	Glucose		Urea-nitrogen		Total proteins	
		mg/100 ml	(%)	mg/100 ml	(%)	g/100 ml	(%)
Control	12	81 $\pm$ 9	(100)	12.3 $\pm$ 1.2	(100)	5.65 $\pm$ 0.21	(100)
1	9	91 $\pm$ 13[a]	(112)	13.4 $\pm$ 3.7	(109)	5.43 $\pm$ 0.12	(97)
2	9	105 $\pm$ 14[b]	(130)	17.4 $\pm$ 2.4[b]	(141)	5.24 $\pm$ 0.18[b]	(93)

a) $p<0.05$. *b)* $p<0.001$. The values are each the mean $\pm$ S.D.

starved for 24 h were compared. No significant difference in the levels of these hormones was found. The concentrations of glucagon were 56 $\pm$ 14 pg/ml in the control, and 64 $\pm$ 15 pg/ml and 64 $\pm$ 10 pg/ml, 1 and 2 h after treatment, respectively. The concentrations of insulin were 12.3 $\pm$ 2.2 μU/ml in the control, and 16.0 $\pm$ 3.9 μU/ml and 14.3 $\pm$ 2.6 μU/ml, 1 and 2 h after treatment, respectively. These results suggested that the reduction in lysine levels induced by rhatannin might involve an effect on the regulatory pathways of glucagon or insulin without affecting the levels of these hormones.

It has been shown that glucagon and adrenaline increase gluconeogenesis from alanine.[5] Rhatannin, glucagon, adrenaline, and corticosterone shared a reducing effect on alanine levels, although the time courses of the effects were not the same. To see if this decrease in alanine induced by rhatannin resulted in an increase in plasma glucose, the effects of rhatannin on the concentrations of plasma glucose, urea-nitrogen, and proteins were examined in rats starved for 24 h. The results are shown in Table I. Plasma glucose was significantly increased after rhatannin injection. The concentration of urea-nitrogen was also increased 2 h after treatment. The concentration of plasma proteins was decreased 2 h after treatment. These results suggested that a part of the increase of plasma glucose might be due

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to gluconeogenesis from alanine.

The effects of rhatannin on amino acid metabolism were studied further by the use of isolated liver cells.

Effect of Rhatannin on Glucose Release from Hepatocytes Isolated from Fed Rats

Isolated rat liver cells provide a means to analyze the potency of rhatannin itself on glucose release. The effects of rhatannin on glucose release were compared with the effects of glucagon and adrenaline, or that of dibutyryl c-AMP, which is an analogue of the intracellular messenger of glucagon. The experimental conditions were selected on the basis of preliminary experiments. The results are shown in Table II. Glucagon, adrenaline, and dibutyryl c-AMP increased glucose release in a dose-dependent fashion, mainly due to enhancement of glycogenolysis, as reported by Garrison and Haynes.[10] As each control value in Table II shows, the addition of rhatannin (15 μg/ml) did not alter the levels of basal glucose release from control liver cells. However, rhatannin enhanced glucose release in the presence of glucagon at concentrations ranging from 1 nM to 1 μM. Further decreasing the glucagon concentration to 0.1 nM in the presence of rhatannin resulted in a loss of observable glucagon action. On the other hand, rhatannin did not enhance the levels of the glucose release elevated by adrenaline or dibutyryl c-AMP at any concentration. The results indicated that the presence of glucagon was required for the expression of the enhancing action of rhatannin

TABLE II. Effect of Rhatannin on Glucose Release from Hepatocytes from Fed Rats, in the Presence of Various Concentrations of Glucagon, Adrenaline, and Dibutyryl c-AMP

		Glucose liberation μg/h/4 $\times$ 10^6 cells	
		Rhatannin (15 μg/ml)	
		Without	With
Exp. I			
Control		293 $\pm$ 11	289 $\pm$ 11
Glucagon	0.1 nM	370 $\pm$ 22	311 $\pm$ 34
	1 nM	419 $\pm$ 25	459 $\pm$ 26
	10 nM	468 $\pm$ 15	626 $\pm$ 28
	100 nM	513 $\pm$ 33	641 $\pm$ 22
	1 μM	669 $\pm$ 28	730 $\pm$ 23
Exp. II			
Control		239 $\pm$ 9	235 $\pm$ 13
Glucagon	10 nM	313 $\pm$ 11	414 $\pm$ 13
Adrenaline	0.1 nM	251 $\pm$ 12	251 $\pm$ 6
	1 nM	261 $\pm$ 11	263 $\pm$ 2
	10 nM	311 $\pm$ 7	306 $\pm$ 14
	100 nM	440 $\pm$ 14	424 $\pm$ 11
	1 μM	474 $\pm$ 6	469 $\pm$ 6
Exp. III			
Control		234 $\pm$ 6	231 $\pm$ 6
Glucagon	10 nM	314 $\pm$ 11	364 $\pm$ 16
Dibutyryl c-AMP	1 nM	253 $\pm$ 6	244 $\pm$ 19
	10 nM	307 $\pm$ 26	313 $\pm$ 11
	100 nM	374 $\pm$ 17	371 $\pm$ 14
	1 μM	413 $\pm$ 8	412 $\pm$ 7
	10 μM	422 $\pm$ 5	419 $\pm$ 5

Isolated liver cells from fed rats (4 $\times$ 10^6 cells/2 ml buffer/dish) were incubated for 1 h at 37 °C. The values are each the mean $\pm$ S.D. of five dishes.

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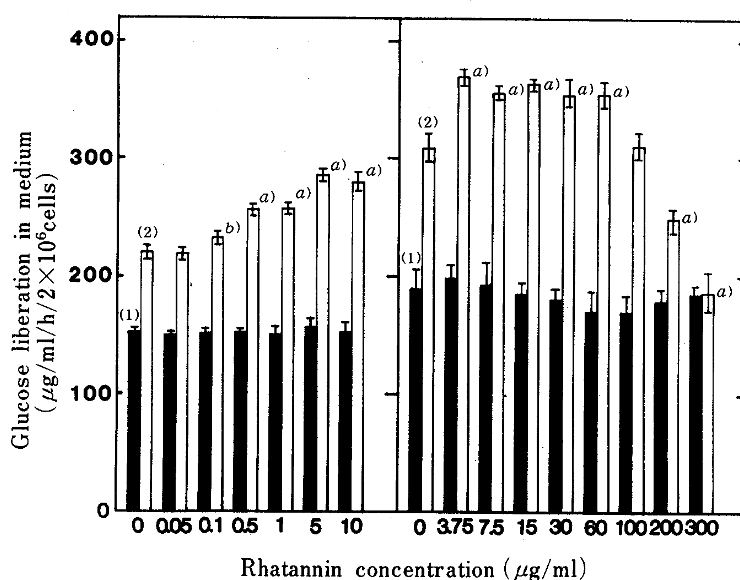

Fig. 2. Effect of Graded Doses of Rhatannin on Glucagon-Induced Glucose Release from Rat Liver Cells

Isolated hepatocytes from fed rats (4×10^6 cells/2 ml buffer/dish) were incubated for 1 h at 37 °C. The values are each the mean ± S.D. of 5 paired dishes. (1) *vs.* (2), $p<0.001$; *a*) *vs.* (2), $p<0.001$; *b*) *vs.* (2), $p<0.01$.

Without glucagon, ■; with 10 nM glucagon, □.

TABLE III. Effect of Rhatannin on Glucose Release due to Gluconeogenesis from Sodium Lactate, Alanine, or Serine in the Presence of 1 μM Glucagon

Glucose precursors		Glucagon	Glucose liberation μg/2 h/4 × 10⁶ cells Rhatannin (15 μg/ml) Without	With
None		0	19.1 ± 0.6	17.8 ± 1.5
		1 μM	19.4 ± 0.4	N.D.
Sodium lactate	5 mM	0	108.3 ± 4.3	108.7 ± 4.4
		1 μM	119.3 ± 6.3	126.9 ± 4.3[a]
Alanine	5 mM	0	52.1 ± 1.9	50.2 ± 3.2
		1 μM	54.1 ± 3.5	60.6 ± 2.5[b]
Serine	5 mM	0	39.2 ± 0.8	36.7 ± 1.4
		1 μM	42.0 ± 0.4	49.4 ± 2.1[b]

Isolated liver cells from rats starved for 24 h (8×10^6 cells/2 ml buffer/dish) were incubated for 2 h at 37 °C. The values are each the mean ± S.D. of five dishes. *a*) $p<0.05$. *b*) $p<0.001$. N.D., not determined.

on glucose release from liver cells. This enhancing action of rhatannin was tested at graded doses and the results are illustrated in Fig. 2. Irrespective of the experimental conditions, no dose of rhatannin affected basal glucose release from liver cells. Glucose release induced by glucagon was enhanced dose-dependently by rhatannin. The maximal level of enhancement was observed at concentrations ranging from 5 to 60 μg/ml. Rhatannin at a concentration of 100 μg/ml did not enhance the glucose release from cells by glucagon. A further increase of rhatannin to 300 μg/ml resulted in the loss of observable glucagon action.

Effect of Rhatannin on Glucose Release from Hepatocytes Isolated from Starved Rats

Preliminary experiments with isolated liver cells from starved rats confirmed the

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observations of Garrison and Haynes[10] that glucagon, adrenaline, and dibutyryl c-AMP increased glucose release in the presence of sodium lactate in a dose-dependent fashion. Rhatannin was tested for enhancement of glucose release induced by 1 μM glucagon. The data are shown in Table III. Rhatannin (15 μg/ml) enhanced glucose release induced by 1 μM glucagon in the presence of glucose precursors, such as sodium lactate, alanine, and serine. The increments of glucose release induced by rhatannin were 6, 12, and 18% using sodium lactate, alanine, and serine, respectively, as glucose precursors. The enhancing effect of rhatannin on glucose release from liver cells was shared by glucagon. Rhatannin also exerted similar effects on liver cells from starved rats.

Discussion

It has been shown that the most important aspect of glucagon's interaction with amino acid metabolism is its effect on hepatic gluconeogenesis and ureogenesis.[11] Moreover, the relative concentrations of glucagon and insulin determine whether amino acids are conserved for protein synthesis or employed for gluconeogenesis and whether glucose is added to or removed from the circulating blood.[12] When the concentration of glucagon is high relative to that of insulin, the use of amino acids in gluconeogenesis and ureogenesis will be increased. In the present *in vivo* study, it was shown that rhatannin decreased the levels of plasma amino acids and simultaneously increased the plasma glucose and urea levels in rats starved for 24 h. No significant difference in levels of glucagon and insulin was found between rhatannin-treated and control rats. The results indicated that the observed effects of rhatannin were not ascribable to its effect on the levels in blood of these hormones.

It has been demonstrated that glucagon and adrenaline stimulate glucose release from isolated rat liver cells, reflecting gluconeogenesis and glycogenolysis.[5,10,13] The action of glucagon in gluconeogenesis and glycogenolysis is known to be initiated by interaction with its receptors on liver cells,[14] resulting in an increase in the concentration of intracellular c-AMP.[15] On the other hand, the action of adrenaline in adult rat liver cells is mediated mainly through alpha-1 adrenergic receptors, although this is not the case in other rat tissues or in the livers of other animals.[16] The alpha-1 adrenergic effect in gluconeogenesis and glycogenolysis is known to be mediated *via* phosphatidyl-inositol turnover,[17] the release of bound intracellular Ca^{2+} and the entry of extracellular Ca^{2+}.[18]

In the present study using rat liver cells, it was demonstrated that glucose release induced by glucagon was synergistically enhanced by rhatannin. Furthermore, rhatannin itself had no effect on the basal glucose release (Tables II and III). In contrast, rhatannin did not enhance glucose release induced by adrenaline and dibutyryl c-AMP (Table II). These results suggest that the synergistic effect of rhatannin and glucagon on glucose release from liver cells might be parallel to the synergistic increase in the c-AMP level. For example, a similar synergistic effect of prostaglandin E_2 and either theophilline or quercetin has been reported by Graziani *et al.* at the concentration of c-AMP in Ehrlich acites tumor cells, although theophilline and quercetin themselves had no effect on the levels of c-AMP in these cells.[19] The positive effect of quercetin and theophilline on the elevated level of c-AMP induced by prostaglandin E_2 has been shown to be accounted for by inactivation of cyclic AMP phosphodiesterase. A similar mechanism might be involved in the synergistic effect of rhatannin and glucagon on the c-AMP level.

The question arises whether a single pathway can account for the various effects of rhatannin *in vivo* observed in the present experiment. Rhatannin and several hormones were compared for capacity to reduce concentrations of free amino acids in plasma. It was noted, in common with glucagon, that levels of all amino acids in plasma were decreased by rhatannin. As discussed above, rhatannin action on glucose release from liver cells might be mediated by

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its effect on the glucagon regulatory pathway. If this is the case, the changes in plasma glucose, urea, and amino acids might be explained in terms of regulatory mechanisms of glucagon. However, the possibility that adrenaline and glucocorticoid participate in the effects of rhatannin on these parameters cannot be excluded, because the levels of these hormones have not yet been determined.

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