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## Abstract

We studied the effects of insulin and glucagon on energy and carbohydrate metabolism of rat hepatocytes in primary culture. The aim of this study is to elucidate the mechanism of the synergistic action of insulin and glucagon and to evaluate the combined effects of these hormones on liver injury. Insulin increased the level of adenosine triphosphate in hepatocytes in the presence of glucagon. Insulin increased the activities of glucokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11), pyruvate kinase (EC 2.7.1.40) type L and glucose 6-phosphate dehydrogenase (EC 1.1.1.49). Glucagon had no antagonistic effect on these increases. Glucagon increased the activity of glucose 6-phosphate (EC 3.1.3.9) (G6Pase) in the presence or absence of insulin, while insulin had no effects on the levels of G6Pase and fructose 1,6-bisphosphatase (EC 3.1.3.11) in the presence or absence of glucagon. Metabolite analysis of cultured hepatocytes indicated that insulin and glucagon have antagonistic effects on the glycolytic activity of hepatocytes. These combined effects of insulin and glucagon may partially explain the preventive effects of these hormones on liver injury.

**KEYWORDS:** cultured rat hepatocytes, energy metabolism, carbohydrate metabolism, insulin, glucagon

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We studied the effects of insulin and glucagon on energy and carbohydrate metabolism of rat hepatocytes in primary culture. The aim of this study is to elucidate the mechanism of the synergistic action of insulin and glucagon and to evaluate the combined effects of these hormones on liver injury. Insulin increased the level of adenosine triphosphate in hepatocytes in the presence of glucagon. Insulin increased the activities of glucokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11), pyruvate kinase (EC 2.7.1.40) type L and glucose 6-phosphate dehydrogenase (EC 1.1.1.49). Glucagon had no antagonistic effect on these increases. Glucagon increased the activity of glucose 6-phosphatase (EC 3.1.3.9) (G6Pase) in the presence or absence of insulin, while insulin had no effects on the levels of G6Pase and fructose 1,6-bisphosphatase (EC 3.1.3.11) in the presence or absence of glucagon. Metabolite analysis of cultured hepatocytes indicated that insulin and glucagon have antagonistic effects on the glycolytic activity of hepatocytes. These combined effects of insulin and glucagon may partially explain the preventive effects of these hormones on liver injury.

*Key words* : cultured rat hepatocytes, energy metabolism, carbohydrate metabolism, insulin, glucagon

It is now well established that insulin and glucagon are important to the regulation of the blood glucose level through their antagonistic effects on hepatic carbohydrate metabolism. On the other hand, these hormones have a synergistic effect on liver regeneration (1, 2). In fact, it has been suggested that the administration of both insulin and glucagon improves the survival rate of patients or animals with severe liver injury (3, 4). Recently, Okita *et al.* (5)

demonstrated the usefulness of glucagon and insulin therapy for human fulminant hepatitis. Experimental *in vivo* systems for the elucidation of the synergistic action of insulin and glucagon may not be applicable to the study of apparently opposing effects of these hormones because of the complexity of the hormonal actions under pathological conditions. Accordingly, we adopted primary cultured rat hepatocytes as an experimental system to study the effects of insulin and glucagon on energy and carbohydrate metabolism of hepatocytes. We compared

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the results with those obtained *in vivo* to delineate further the mechanism of the synergistic action of these hormones in carbohydrate metabolism.

## Materials and Methods

**Materials.** Collagenase Type 1 (EC 3.4.24.3) was obtained from Cooper Biomedical, Cout Malvern, USA. Bovine insulin, glucagon, fructose 6-phosphate kinase (pyrophosphate dependent) (EC 2.7.1.90) and reagents used as substrates or cofactors were from Sigma Chemical Co., St. Louis, MO, USA. Purified enzyme preparations and calf thymus DNA were from Boehringer Mannheim, GmbH, West Germany. Other reagents were of the highest grade available commercially.

**Isolation of hepatocytes.** Male Sprague-Dawley rats (150–200 g), obtained from CLEA Japan, Inc., Tokyo, Japan, were kept in an animal room maintained at about 23°C with alternating light-dark periods of 12 h. The animals were fed Oriental Laboratory chow MF from Oriental Yeast Co., Ltd., Tokyo, Japan, and given water *ad libitum*. Rats were sacrificed 3–4 h after the end of the feeding dark period. Hepatocytes were isolated by a two-step procedure essentially as described by Seglen (6) with modifications using Krebs-Ringer bicarbonate buffer for perfusion and direct bubbling of an O<sub>2</sub>/CO<sub>2</sub> (19 : 1) mixture in the perfusate. The viability of the cells was assayed by the trypan blue exclusion test, and cells were not used if the value did not exceed 85%. The number of the cells was adjusted to  $0.5 \times 10^6$  cells/ml in culture medium.

**Culture medium.** L-leucine (52 mg/l), L-methionine (15 mg/l), L-phenylalanine (32 mg/l) and L-ornithine (68 mg/l) were added to Eagle's minimal essential medium lacking L-arginine, L-leucine, L-methionine and L-phenylalanine (Eagle's MEM Nissui 5, Nissui Seiyaku Co., Ltd., Tokyo, Japan) to prepare a basal culture medium. The medium contained glucose (100 mg/dl), but did not contain gluconeogenic substrates.

**Hepatocyte culture.** The hepatocytes in 10 ml of the basal medium supplemented with 10% fetal calf serum, 10 nM dexamethasone-21 disodium phosphate (dexamethasone, Nippon Merk Banyu Co., Tokyo, Japan) and 0.5 nM insulin were plated

into 100 mm Falcon plastic dishes and allowed to attach for 4 h at 37°C in an atmosphere of humidified air containing 5% CO<sub>2</sub>. The medium was then replaced with a serum-free medium containing 10 nM dexamethasone and 0.5 nM insulin, and the cells were incubated for 20 h. Thereafter, the medium was changed every 24 h for 74 h with basal media to which various hormones were added: group D, 10 nM dexamethasone; group D + I, 10 nM dexamethasone + 10 nM insulin; group D + G, 10 nM dexamethasone + 10 nM glucagon and group D + I + G, 10 nM dexamethasone + 10 nM insulin + 10 nM glucagon. These concentrations of hormones were chosen to give maximal effects (7).

**Assay for metabolites and enzyme activities.** Two hours after the last medium change, cultured cells were processed as follows. For the assay of adenine nucleotides, glycogen and glycolytic intermediates, the cells were scraped from 10 dishes with 3 ml of 6% perchloric acid (PCA) after complete removal of culture medium. The concentrations of acid-soluble metabolites were corrected by using a mean recovery of 82%, which was obtained by adding 0.1 ml of 2.5 mM isocitrate in 15% PCA to every dish. Cell suspensions were homogenized with a Polytron homogenizer at a speed of 75% of the maximum for 30 sec. One ml of the homogenate was used for the glycogen assay (8, 9), and the remainder was centrifuged for 15 min at  $1,500 \times g$  at 4°C. The supernatant was neutralized with 30% potassium hydroxide (KOH) and used for the assay of adenine nucleotides, glycolytic intermediates and isocitrate by enzymatic methods (10–15). The pellets obtained by the centrifugation were used for the assay of DNA (16). Concentrations of glucose, pyruvate, lactate and cyclic adenosine 3', 5'-monophosphate (cAMP) in the medium were determined after deproteinization by the PCA treatment followed by neutralization as described above. The cAMP concentration was assayed with a Yamasa cAMP assay kit (Yamasa Shoyu Co., Ltd., Chiba, Japan), and pyruvate and lactate were analyzed by enzymatic methods (17, 18). Glucose was determined by the glucose oxidase method using a Unikit glucose-E (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan). Fructose 2, 6-bisphosphate (F2, 6-P<sub>2</sub>) was assayed by the method of Van Schaftingen (19) using cell extracts made from two dishes each extracted twice

with 2 ml of 0.3 N KOH. The mean recovery of F2, 6-P<sub>2</sub> was 98% after correction for isocitrate which was added as a marker.

For the determination of key enzyme activities, cells were washed twice with a homogenization buffer containing 50 mM Tris-HCl, 100 mM KCl and 1 mM EDTA (pH 7.5), harvested with a rubber policeman from 5 dishes with 1 ml of the same buffer, and homogenized with a Polytron homogenizer at a speed of 50% of the maximum for 20 sec. A portion of each homogenate was used for determination of G6Pase activity (20) and the protein concentration (21). The remaining portion of each homogenate was centrifuged at 100,000 ×g for 30 min at 4°C, and the resultant supernatant was assayed for glucokinase (hexokinase type IV) (EC 2.7.1.1) (GK) and hexokinase (EC 2.7.1.1) (HK) (22, 23), phosphofructokinase (EC 2.7.1.11) (PFK) (24), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (G6PD) (25), fructose 1,6-bisphosphatase (EC 3.1.3.11) (FBPase) (26), and pyruvate kinase (EC 2.7.1.40) (PK) (27) activities. Isozymes of PK were separated electrophoretically (28).

Liver tissues from fed and 48 h-fasted rats

were clamped rapidly with a stainless steel tong previously cooled in liquid N<sub>2</sub>. The frozen samples were powdered in liquid N<sub>2</sub> with 6% PCA for the assay of adenine nucleotides, glycolytic intermediates and glycogen and with 0.3 N KOH for the assay of F2, 6-P<sub>2</sub>. For the assay of key enzyme activities, liver tissues were homogenized and centrifuged by the same method as hepatocytes.

*Statistical analysis.* Results were expressed on the basis of μgDNA for carbohydrate metabolites and μmol/min/g of protein (U/g of protein) for enzyme activities. Data were given as the mean ± standard deviation, and statistical evaluation was made by Student's *t* test.

## Results

*Adenine nucleotide levels.* Total adenine nucleotide levels of insulin-treated cultured hepatocytes (Groups D+I and D+I+G) were significantly higher than those of hepatocytes not treated with insulin (Groups

**Table 1** Adenine nucleotide and energy charge levels of cultured hepatocytes and liver tissues of rats<sup>a</sup>

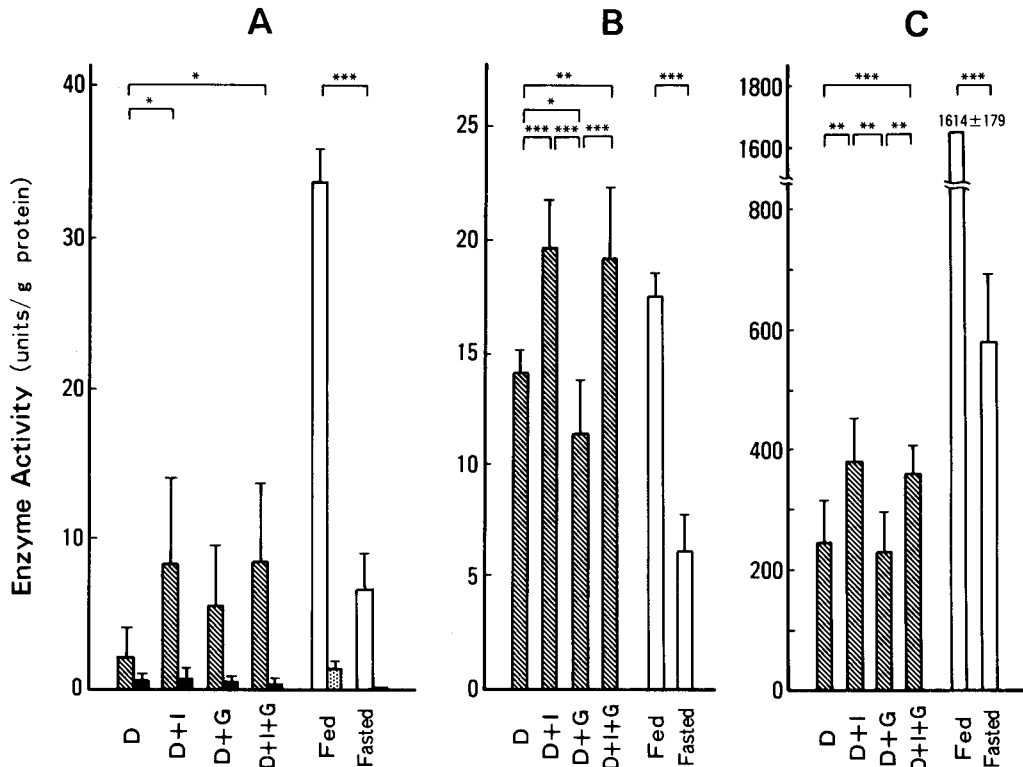
Experimental conditions	ATP	ADP	AMP	Total adenine nucleotides	Energy <sup>b</sup> charge
(A) Cultured hepatocytes <sup>c</sup>					
D (5)	862 ± 130	275 ± 33	16 ± 36	1140 ± 127	0.87 ± 0.03
D+I (5)	1180 ± 103	356 ± 159	24 ± 29	1570 ± 212	0.88 ± 0.03
D+G (5)	700 ± 162	326 ± 161	17 ± 15	1043 ± 236	0.83 ± 0.03
D+I+G (5)	1390 ± 123	353 ± 126	28 ± 35	1755 ± 193	0.90 ± 0.03
(B) Liver tissues <sup>a</sup>					
Fed (5)	1910 ± 146	374 ± 44	111 ± 39	2390 ± 119	0.88 ± 0.02
Fasted (5)	1160 ± 151	318 ± 27	138 ± 18	1620 ± 176	0.81 ± 0.02

a: Results (pmol/μgDNA) are expressed as the mean ± standard deviation. The numbers of experiments or animals are given in parentheses. Experimental details are described under Materials and Methods. Statistical analysis was performed by Student's *t* test. \*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001.

b: Energy charge was calculated by the equation (ATP + 1/2ADP)/(ATP + ADP + AMP). Nucleotides were expressed as molar concentrations.

c: Insulin (I) and/or glucagon (G) were added to the medium of the primary culture of rat hepatocytes in the presence of dexamethasone (D).

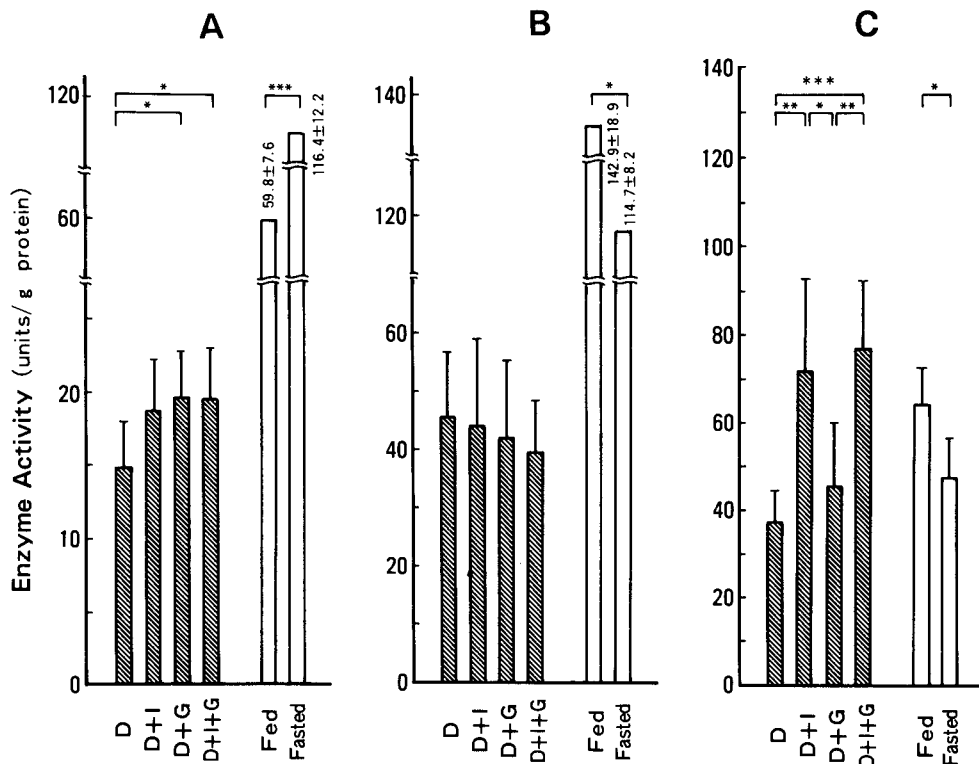
d: Liver tissues from fed or 48 h-fasted rats were analyzed.



**Fig. 1** Effects of insulin (I) and glucagon (G) on key glycolytic enzyme activities in cultured hepatocytes in the presence of dexamethasone (D) and in liver tissues. Results are expressed as the mean  $\pm$  standard deviation of 6 experiments for hepatocytes (D) and 5 animals for fed and 48 h-fasted liver tissues. Experimental details are described under Materials and Methods. Statistical analysis was made by Student's *t* test. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Fig. 1A, Glucokinase (GK)+hexokinase (HK); B, phosphofructokinase; C, pyruvate kinase. Hepatocytes, and ; liver tissues, and . In Fig. 1A, and , GK; and , HK.

D and D+G) (Table 1). The nucleotide levels in the cultured cells were lower than that of the liver tissue from fed rats and close to that of 48 h-fasted rat liver tissue. ATP levels of cultured cells in the insulin-treated groups (D+I and D+I+G) were higher than those in the groups without insulin treatment (D and D+G). The highest ATP level was noted in the D+I+G group, to which both insulin and glucagon were added. The ATP levels of cultured cells were considerably lower than that of the liver tissue from fed rats. Energy charge levels  $[(\text{ATP} + 1/2 \text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})]$  in hepatocytes were no less than the *in vivo* level.

*Key enzyme activities of glycolysis, gluconeogenesis and pentose phosphate pathway.* Activities of key glycolytic enzymes of hepatocytes under different hormonal conditions are shown in Fig. 1 and compared with those of the liver tissues. The GK activities in the insulin-treated groups (D+I and D+I+G) were approximately 4 times higher than the level in the Group D. Thus, glucagon did not counteract the insulin effect, and in fact, it doubled the GK activity in the absence of insulin (D vs. D+G) (statistically not significant). The hepatocyte GK activity raised by the addition of insulin was roughly 1/4 of the tissue activity of fed rats and was on the same level as that



**Fig. 2** Effects of insulin and glucagon on gluconeogenic enzyme and G6PD activities in cultured hepatocytes (▨) and liver tissues (□). Results are expressed as the mean ± standard deviation of 6 experiments for hepatocytes and 5 animals for fed and 48 h-fasted liver tissues. Symbols, see the legend to Fig. 1. Fig. 2A, Glucose 6-phosphatase; B, fructose 1,6-bisphosphatase; C, glucose 6-phosphate dehydrogenase.

of the fasted rats. Hexokinase (HK) activities of cultured cells were less than half the tissue activity of fed rats and were not altered by the addition of hormones.

Addition of insulin increased the hepatocyte activity of PFK. Glucagon did not suppress the insulin-dependent increase in PFK activity; however, glucagon alone decreased PFK activity to some extent. Unlike GK, PFK of the cultured hepatocytes increased upon addition of insulin to levels comparable to that of the liver tissue of fed rats.

The PK activities in the insulin-treated D+I and D+I+G groups were higher than that in the D group. Glucagon showed no inhibitory effects on PK activity in cultured cells in either the presence or absence of

insulin. As in the case of GK, the hepatocyte PK activity, which was increased by insulin treatment, was 1/4 that of the tissue level of fed rats, and lower than that of 48 h-fasted rats. The electrophoretic study showed that most of the PK activity of the cultured hepatocytes was of the L type, and that the M<sub>2</sub> type was almost absent.

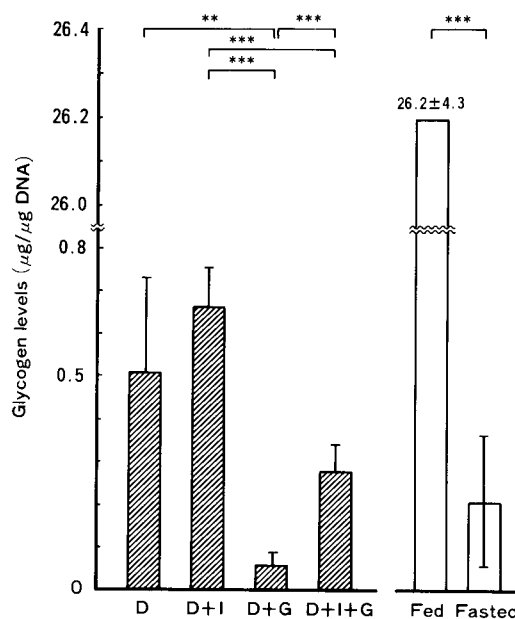
Key enzyme activities of gluconeogenesis and pentose phosphate pathway under varied hormonal conditions are shown in Fig. 2 and compared with those of liver tissues. G6Pase activity was higher in the glucagon-treated groups (D+G and D+I+G) than in the control D group. Insulin had no inhibitory effect, whether with or without glucagon. FBPase activity did not change upon

the addition of the hormones. These gluconeogenic enzymes had less than half the activities in the cultured hepatocytes as in the liver tissue of fasted rats.

G6PD activity increased approximately twice upon insulin treatment (Groups D+I and D+I+G) as compared with the control activity (Group D). Glucagon did not counteract the insulin effect, nor did it decrease the activity when it was added alone. The hepatocyte G6PD activity which was increased by the addition of insulin was higher than that in the liver tissue of fed rats.

*Levels of carbohydrate metabolites.* Glucose concentrations in 2 h-incubated media after the last medium change were  $98.8 \pm 4.6$  mg/dl in the D group,  $97.1 \pm 1.8$  mg/dl in the D+I group,  $100.0 \pm 3.2$  mg/dl in the D+G group and  $97.4 \pm 4.4$  mg/dl in the D+I+G group. There was no significant difference among them. The cAMP level in the medium of the glucagon-treated D+G group was  $2.8 \pm 1.1$  pmol/ $\mu$ g DNA, and that of the glucagon and insulin-treated D+I+G group was  $2.1 \pm 1.5$  pmol/ $\mu$ g DNA. These levels were appreciably higher than those of the glucagon-untreated D and D+I groups ( $< 0.2$  pmol/ $\mu$ g DNA). The glycogen contents of the cultured hepatocytes and liver tissues are shown in Fig. 3. The glycogen content of hepatocytes was increased by the addition of insulin (D vs. D+I, and D+G vs. D+I+G), although the difference between the D and D+I groups was not statistically significant. The addition of glucagon significantly reduced the level of glycogen (D vs. D+G, and D+I vs. D+I+G). The glycogen content of the cultured hepatocytes under varied hormonal conditions was much lower than that in the liver tissue of fed rats. However, it was approximately equal to or greater than that of liver tissue of fasted rats, except for the D+G group.

The glycolytic intermediates of cultured



**Fig. 3** Effects of insulin and glucagon on glycogen levels of cultured hepatocytes (▨) and liver tissues (□). Results are expressed as the mean  $\pm$  standard deviation of 5 experiments for hepatocytes and 5 animals for fed and 48 h-fasted liver tissues. Symbols, see the legend to Fig. 1.

hepatocytes are shown in Table 2 and compared with those of liver tissue. The levels of glucose 6-phosphate (G6P) of cultured hepatocytes showed marked increases in the glucagon-treated groups (D vs. D+G, and D+I vs. D+I+G). The levels of fructose 6-phosphate showed a change similar to those of G6P except that there was no difference between the D+I and D+I+G groups. Although the fructose 1,6-bisphosphate (FBP) levels of the D+I and D+I+G groups did not differ from that of the D group, the FBP level was significantly reduced in the D+G group. Other glycolytic intermediates leading to lactate in the D+G group were reduced compared with those of the control D group. The addition of glucagon significantly reduced the level of pyruvate (D vs. D+G, and D+I vs. D+I+G). Lactate levels in the insulin-treated D+I and D+I+G groups increased signif-



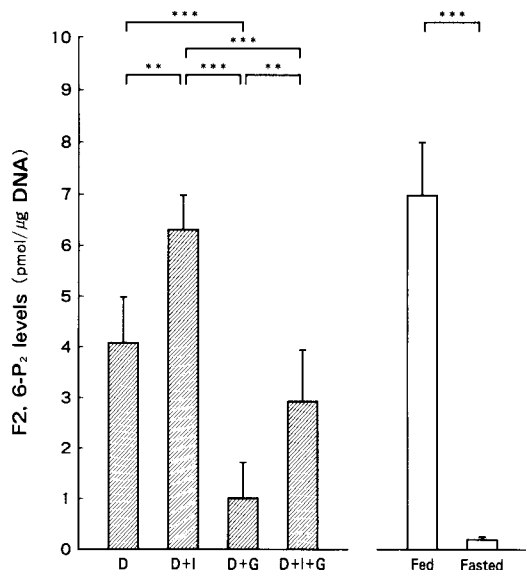
Table 2 Levels of glycolytic intermediates in cultured hepatocytes and liver tissues of rats<sup>a</sup>

Experimental conditions	G6P	F6P	FBP	DAP/GAP	PEP	Pyruvate	Lactate
(A) Hepatocyte culture <sup>b</sup>							
D (5)	10.2 ± 7.7	7.6 ± 4.1	15.6 ± 2.4	62.5 ± 21.2	55.4 ± 8.9	12.6 ± 1.9	15.2 ± 3.5
D+I (5)	9.2 ± 3.3	9.0 ± 3.8	14.3 ± 2.7	59.2 ± 14.2	73.1 ± 23.1	16.5 ± 3.4	24.7 ± 3.5
D+G (5)	50.3 ± 6.7	16.7 ± 3.1	5.1 ± 1.8	33.6 ± 10.3	38.3 ± 9.6	3.3 ± 1.2	9.8 ± 1.5
D+I+G (5)	40.8 ± 12.4	13.8 ± 3.9	15.3 ± 3.7	59.0 ± 7.5	89.4 ± 13.3	11.8 ± 1.4	23.4 ± 3.8
(B) Liver tissues <sup>b</sup>							
Fed (5)	212.1 ± 57.3	61.8 ± 9.6	6.8 ± 1.8	21.1 ± 5.3	19.4 ± 3.7	93.9 ± 23.8	734.1 ± 390.0
Fasted (5)	33.4 ± 14.7	10.5 ± 1.5	3.0 ± 1.5	7.2 ± 3.3	9.4 ± 4.2	51.2 ± 13.2	254.2 ± 159.0

a: Results (pmol/μgDNA) are expressed as the mean ± standard deviation. The numbers of experiments or animals are given in parentheses. Pyruvate and lactate in the medium (A) represent the amounts of these metabolites accumulated in 2 h of the culture. Statistical analysis was performed by Student's *t* test. \*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001.

G6P: glucose 6-phosphate, F6P: fructose 6-phosphate, FBP: fructose 1, 6-bisphosphate, DAP/GAP: dihydroxyacetone phosphate + glyceraldehyde 3-phosphate, PEP: phosphoenolpyruvate.

b: For experimental conditions and other symbols, see legends to Table 1.



**Fig. 4** Effects of insulin and glucagon on F<sub>2,6</sub>-P<sub>2</sub> levels of cultured hepatocytes (▨) and liver tissues (□). Results are expressed as the mean ± standard deviation of 5 experiments for hepatocytes and 5 animals for fed and 48 h-fasted liver tissues. Symbols, see the legend to Fig. 1.

icantly, as compared with the D group, although there was no difference between the insulin-treated groups. The hexose 6-phosphate levels of cultured hepatocytes were considerably lower than those of the liver tissue of fed rats. However, hexose 6-phosphate levels of the glucagon-treated D+G and D+I+G groups were higher than the liver tissue level of fasted rats. Levels of FBP, dihydroxyacetone phosphate+glyceraldehyde 3-phosphate (DAP/GAP) and phosphoenolpyruvate (PEP) in cultured hepatocytes were higher than those *in vivo* except for the FBP level of the D+G group. The concentrations of pyruvate and lactate in the media after 2 h of incubation were considerably lower than those in the liver tissues. It was difficult to accurately measure the concentrations of pyruvate and lactate in cultured hepatocytes in the presence of their high concentrations in the medium.

F<sub>2,6</sub>-P<sub>2</sub> levels in the cultured hepatocytes under the varied hormonal conditions are shown in Fig. 4 in comparison with those in the liver tissue of fed and fasted rats. The F<sub>2,6</sub>-P<sub>2</sub> levels in the insulin-treated groups were significantly higher than those in the respective control groups (D vs. D+I, and D+G vs. D+I+G). The group of hepatocytes given glucagon alone showed the lowest level of F<sub>2,6</sub>-P<sub>2</sub>, and the group of hepatocytes given insulin alone showed the highest level, which was comparable to the *in vivo* level of fed rats. The fasted level of F<sub>2,6</sub>-P<sub>2</sub> in liver tissue was lower than that in cultured hepatocytes with glucagon alone.

## Discussion

By adopting a primary culture system of rat hepatocytes, we could study the metabolic regulation of parenchymal liver cells without contamination by non-parenchymal cells and without the effects of other organs or tissues. We used a dexamethasone-supplemented arginine free medium without serum to enhance parenchymal cell function (29) and to inhibit the proliferation of non-parenchymal cells (30), which might cause a pattern of gene expression similar to that of fetal hepatocytes (31). Under the present conditions, contamination by and proliferation of non-parenchymal cells were negligible as indicated by the cultured hepatocytes having mostly PK-L, a useful marker of parenchymal cells, and having almost no PK-M<sub>2</sub>, a marker of non-parenchymal cells (32).

It has been reported that ATP levels decline to one half the *in vivo* level immediately after cell preparation, but recover to the *in vivo* level after 24 h (33). In our experiment, ATP levels 74 h after incubation varied depending on hormonal condi-

tions, the highest ATP level per DNA being obtained when both insulin and glucagon were added. However, this level was still below the *in vivo* level of fed animals. Insulin alone increased the ATP level slightly, but glucagon alone did not. Thus, the effect of insulin was augmented by the presence of glucagon. These results indicated that insulin and glucagon had a synergistic effect on energy metabolism of hepatocytes. On the other hand, the energy charge of cultured hepatocytes was kept at the *in vivo* level irrespective of the hormonal conditions, indicating that the energy charge level is a minimal requirement for the maintenance and survival of cultured cells.

In the present study, the GK, PFK, PK-L and G6PD activities were raised by insulin, and the G6Pase activity by glucagon. These hormones did not have antagonizing effects in each other. These results suggested that insulin and glucagon acted independently on key enzyme activities of carbohydrate metabolism in hepatocytes.

When the key enzyme activities of carbohydrate metabolism in cultured cells are compared with those *in vivo*, the activities of liver-specific GK, PK-L, G6Pase and FBPase after 3 days of culture were either similar to or lower than those of the 48 h-fasted rat liver tissue, even when insulin and glucagon were added together to the culture. The activities of PFK and G6PD, enzymes not specific to the liver, were maintained at about the level of the liver tissue of fed rats when insulin was added. The above results are supported by recent findings that cultured hepatocytes dedifferentiate with a rapid loss of liver-specific mRNA, while the transcription of mRNA of a common gene remains at *in vivo* levels (34). Although the cultured hepatocytes were partially dedifferentiated as mentioned above, fetal type liver enzymes (35) were practically absent in the hepatocytes em-

ployed in the present experiments.

Unlike the hormonal effects on energy metabolism and key enzyme activities of carbohydrate metabolism in cultured hepatocytes, insulin and glucagon showed antagonistic effects on levels of carbohydrate metabolites. The cAMP concentration in the culture medium increased when glucagon was added and decreased upon treatment with insulin, in the same manner as in perfused rat livers (36).

Glycogen and F2,6-P<sub>2</sub> levels are regulated by cAMP (37). The elevation of hexose 6-phosphate levels in cultured hepatocytes upon addition of glucagon can be explained by the decline of F2,6-P<sub>2</sub> which decreased the flux from F6P to FBP and, in addition, by the decomposition of glycogen. On the other hand, the decrease in pyruvate caused by addition of glucagon is probably attributable to the inhibited flux from PEP to pyruvate, which is regulated by cAMP (37). Insulin increased the concentrations of pyruvate and lactate in the medium, and glucagon inhibited the insulin effect. Thus, the actions of insulin and glucagon on the glycolytic metabolite levels seemed to be antagonistic.

When the levels of glycolytic intermediates of the cultured hepatocytes were compared with those *in vivo*, reduced levels of hexose 6-phosphates in the hepatocytes could be explained by decreased activity of GK and relatively high activity of PFK. The excessive accumulation of glycolytic intermediates from FBP to PEP in the hepatocytes might be caused by the lower PK activity of the hepatocytes. Thus, the glycolytic activity of cultured hepatocytes appeared to be rather low, as further evidenced by the rate of lactate production in cultured hepatocytes being less than 1/1000 the rate in perfused rat liver, with 5 mM glucose as a substrate (38).

Carbohydrate metabolism in cultured cells

was found to appreciably differ from that *in vivo*, indicating that additional factors such as cell-cell contact may be required for the maintenance of differentiated functions under culture conditions (39). Since the response of key enzymes to hormonal regulation was insufficient, resulting in an undifferentiated pattern of the liver-specific enzymes, hepatocytes in primary culture are similar to those in an experimental system of liver injury (40). Cultured hepatocytes may degenerate, because they die in a week when they are cultured in this experimental system. The degenerative change may cause the dedifferentiated pattern of key enzymes as Taketa *et al.* showed in acute carbon tetrachloride intoxication of rats (40). In the present study, insulin and glucagon increased the activities of liver-specific enzymes and adenine nucleotide levels of cultured hepatocytes. The preventive effect of insulin and glucagon on liver injury, and the restitution of injured hepatocytes by these hormones may be partially accounted for by the observed synergistic effects of insulin and glucagon in the present study.

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## References

1. Bucher NLR and Swaffield MN: Regulation of hepatic regeneration in rats by synergistic action of insulin and glucagon. *Proc Natl Acad Sci USA* (1975) **72**, 1157-1160.
2. Starzl TE, Watanabe K, Porter KA and Putnam CW: Effects of insulin, glucagon and insulin/glucagon infusions on liver morphology and cell division after complete portacaval shunt in dogs. *Lancet* (1976) **I**, 821-825.
3. Farivar M, Wands JR, Isselbacher KJ and Bucher NLR: Effect of insulin and glucagon on fulminant murine hepatitis. *N Engl J Med* (1976) **259**, 1517-1519.
4. Baker AL, Jaspan JB, Haines NW, Hatfield GE, Krager PS and Schneider JF: A randomized clinical trial of insulin and glucagon infusion for treatment of alcoholic hepatitis: Progress report in 50 patients. *Gastroenterology* (1981) **80**, 1410-1414.
5. Okita K, Matsuda S, Hanta T, Yasunaga M, Sanuki K and Takemoto T: Clinical evaluation of glucagon and insulin in therapy of fulminant hepatitis. *Gastroenterol Jpn* (1987) **22**, 605-613.
6. Seglen PO: Preparation of isolated rat liver cells. *Methods Cell Biol* (1976) **13**, 29-83.
7. Probst I, Schwartz P and Jungermann K: Induction in primary culture of 'gluconeogenic' and 'glycolytic' hepatocytes resembling peripotal and perivenous cells. *Eur J Biochem* (1982) **126**, 271-278.
8. Keppler D and Decker K: Glycogen; in *Methods of Enzymatic Analysis*, Bergmeyer, Bergmeyer and Grassl eds, 3rd Ed, Vol 6, Verlag Chemie, Weinheim (1983) pp 11-18.
9. Lust WD, Passonneau JV and Crites SK: The measurement of glycogen in tissues by amylo- $\alpha$ -1,4- $\alpha$ -1,6-glucosidase after the destruction of preexisting glucose. *Anal Biochem* (1975) **68**, 328-331.
10. Trautchohl I, Lamprecht W and Schweitzer G: Adenosine 5'-triphosphate, UV-method with hexokinase and glucose 6-phosphate dehydrogenase; in *Methods of Enzymatic Analysis*, Bergmeyer, Bergmeyer and Grassl eds, 3rd Ed, Vol 7, Verlag Chemie, Weinheim (1983) pp 346-357.
11. Jaworek D and Welsch J: Adenosine 5'-diphosphate and adenosine 5'-monophosphate, UV-method; in *Methods of Enzymatic Analysis*, Bergmeyer, Bergmeyer and Grassl eds, 3rd Ed, Vol 7, Verlag Chemie, Weinheim (1983) pp 365-370.
12. Michal G: D-glucose 6-phosphate and D-fructose 6-phosphate; in *Methods of Enzymatic Analysis*, Bergmeyer, Bergmeyer and Grassl eds, 3rd Ed, Vol 6, Verlag Chemie, Weinheim (1983) pp 191-198.
13. Michal G: D-fructose 1,6-bisphosphate, dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate; in *Methods of Enzymatic Analysis*, Bergmeyer, Bergmeyer and Grassl eds, 3rd Ed, Vol 6, Verlag Chemie, Weinheim (1983) pp 342-350.
14. Lamprecht W and Heinz F: D-glycerate 2-phosphate and phosphoenolpyruvate; in *Methods of Enzymatic Analysis*, Bergmeyer, Bergmeyer and Grassl eds, 3rd Ed, Vol 6, Verlag Chemie, Weinheim (1983) pp 555-561.
15. Goldberg DM and Ellis G: Isocitrate Dehydrogenase; in *Methods of Enzymatic Analysis*, Bergmeyer, Bergmeyer and Grassl eds, 3rd Ed, Vol 3, Verlag Chemie, Weinheim (1983) pp 183-190.
16. Burton K: A study of conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* (1955) **62**, 315-322.
17. Lamprecht W and Heinz F: Pyruvate; in *Methods*

- of Enzymatic Analysis, Bergmeyer, Bergmeyer and Grassl eds, 3rd Ed, Vol 6, Verlag Chemie, Weinheim (1983) pp 570-577.
18. Noll F: L-(+)-lactate; in Methods of Enzymatic Analysis, Bergmeyer, Bergmeyer and Grassl eds, 3rd Ed, Vol 6, Verlag Chemie, Weinheim (1983) pp 582-588.
  19. Van Schaftingen E, Lederer B, Bartrons R and Hers HG: A kinetic study of pyrophosphate:Fructose-6-phosphate phosphotransferase from potato tubers. Application to a microassay of fructose 2, 6-bisphosphate. *Eur J Biochem* (1982) **129**, 191-195.
  20. Koide H and Oda T: Pathological occurrence of glucose-6-phosphatase in serum in liver disease. *Clin Chim Acta* (1959) **4**, 554-561.
  21. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* (1951) **193**, 265-275.
  22. Newgard CB, Hirsch LJ, Foster DW and McGarry JD: Studies on the mechanism by which exogenous glucose is converted into liver glycogen in the rat. A direct or an indirect pathway? *J Biol Chem* (1983) **258**, 8046-8052.
  23. Venüela E, Salas M and Sols A: Glucokinase and hexokinase in liver in relation to glycogen synthesis. *J Biol Chem* (1963) **238**, 1175-1177.
  24. Pogell BM, Tanaka A and Siddons RC: Natural activators for liver fructose 1,6-diphosphatase and the reversal of adenosine 5'-monophosphate inhibition by muscle phosphofructokinase. *J Biol Chem* (1968) **243**, 1356-1367.
  25. Taketa K, Kaneshige Y, Tanaka A and Kosaka K: Differential response of glucose 6-phosphate dehydrogenase and lipogenesis to fat-free diets. *Biochem Med* (1970) **4**, 531-545.
  26. Taketa K and Pogell BM: Allosteric inhibition of rat liver fructose 1,6-diphosphatase by adenosine 5' monophosphate. *J Biol Chem* (1965) **240**, 651-662.
  27. Tanaka T, Harano Y, Soe F and Morimura H: Crystallization, characterization and metabolic regulation of two types of pyruvate kinase isolated from rat tissues. *J Biochem* (1967) **62**, 71-91.
  28. Susor WA and Rutter WJ: Some distinctive properties of pyruvate kinase purified from rat liver. *Biochem Biophys Res Commun* (1968) **30**, 14-20.
  29. Enat R, Jefferson DM, Ruizo-Opazo N, Gatmaitan Z, Leinwand LA and Reid LM: Hepatocyte proliferation *in vivo*. Its dependence on the use of serum-free hormonally defined medium and substra of extracellular matrix. *Proc Natl Acad Sci USA* (1984) **81**, 1411-1415.
  30. Macgowan JA, Strain AJ and Bucher NLR: DNA synthesis in primary cultures of adult rat hepatocytes in a defined medium: Effects of epidermal growth factor, insulin, glucagon, and cyclic-AMP. *J Cell Physiol* (1981) **108**, 353-363.
  31. Guguen-Guillouzo C and Guillozo A: Modulation of functional activities in cultured rat hepatocytes. *Mol Cell Biochem* (1983) **53/54**, 35-56.
  32. Reyes A and Cárdenas ML: All hexokinase isozymes coexist in rat hepatocytes. *Biochem J* (1984) **221**, 303-309.
  33. Bissell DM, Hammaker LE and Meyer UA: Parenchymal cells from adult rat liver in nonproliferating monolayer culture. I. Functional studies. *J Cell Biol* (1973) **59**, 722-734.
  34. Clayton DF and Darnell JE: Changes in liver-specific compared to common gene transcription during primary culture of mouse hepatocytes. *Mol Cell Biol* (1983) **3**, 1552-1561.
  35. Taketa K, Watanabe A, Shimamura A, Takesue A, Ueda M, Masuji H and Sato J: Undifferentiated patterns of key glycolytic and gluconeogenic enzymes in epithelial cell lines derived from rat liver. *Acta Med Okayama* (1975) **29**, 151-159.
  36. Exton JH, Lewis SB, Ho RJ, Robison GA and Park CR: The role of cyclic AMP in the interaction of glucagon and insulin in the control of liver metabolism. *Ann NY Acad Sci* (1971) **185**, 85-100.
  37. Pilkis SJ, Fox E, Wolfe L, Rothbarth L, Colosia A, Stewart HB and EL-Maghrabi MR: Hormonal modulation of key hepatic regulatory enzymes in the gluconeogenic/glycolytic pathway. *Ann NY Acad Sci* (1987) **478**, 1-19.
  38. Woods HF and Krebs HA: Lactate production in the perfused rat liver. *Biochem J* (1971) **125**, 129-139.
  39. Nakamura T, Yoshimoto K, Nakayama Y, Tomita Y and Ichihara A: Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell-cell contact and cell membranes. *Proc Natl Acad Sci USA* (1983) **80**, 7229-7233.
  40. Taketa K, Tanaka A, Watanabe A, Takesue A, Aoe H and Kosaka K: Undifferentiated patterns of key carbohydrate-metabolizing enzymes in injured livers. I. Acute carbon-tetrachloride intoxication of rat. *Enzyme* (1976) **21**, 158-173.

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