Muscle Damage Induced by Experimental Hypoglycemia

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To determine organ damage due to hypoglycemia, we studied the effects of insulin dose and hypoglycemia duration on serum enzyme activity in rabbits. Thirty rabbits were randomly divided into five groups according to hypoglycemia duration and insulin dose: A_2, hypoglycemia for 30 minutes with 2 U/kg insulin; A_{10}, hypoglycemia for 30 minutes with 10 U/kg insulin; B_2, hypoglycemia for 60 minutes with 2 U/kg insulin; B_{10}, hypoglycemia for 60 minutes with 10 U/kg insulin; and C, no hypoglycemia with 10 U/kg insulin and 50% glucose. Insulin-induced hypoglycemia was reversed by intravenous injection of glucose. Alterations in serum enzyme activity and creatine kinase (CK) isoenzyme distribution were determined before and after insulin injection. Serum CK activity increased significantly in all hypoglycemic groups compared with preinjection values, and tended to remain high for 24 hours in both groups A_{10} and B_{10}. Serum activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) increased only in group B_{10}. In addition, the level of band 4 of serum CK isoenzymes, which exists predominantly in skeletal muscle and myocardium, increased significantly in group B_{10}. These results suggest that the increase in both serum enzyme and CK band 4 isoenzyme activities during hypoglycemia is primarily due to damage in muscle rather than liver, and that the hypoglycemia duration and insulin dosage may influence the extent of organ damage.

WHETHER ACUTE HYPOGLYCEMIA in diabetic patients causes hepatic damage as detected by an immediate and significant increase in serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activity remains controversial.[1-4] In our previous study,[5] the plasma activity of ALT, AST, and lactate dehydrogenase (LDH) increased significantly in rabbits with insulin-induced hypoglycemia. At the same time, we demonstrated a remarkable increase in the plasma activity of creatine kinase (CK), which suggested that changes in these plasma enzyme activities by hypoglycemia are primarily due to myocardium and/or skeletal muscle damage rather than hepatic damage. In the present study, we examined the effects of insulin dosage and hypoglycemia duration on the serum activity of ALT, AST, LDH, and CK. In addition, by analyzing the distribution of CK isoenzymes in different tissues, we attempted to identify the organ most likely to be damaged by insulin-induced hypoglycemia.

MATERIALS AND METHODS

Animals

Thirty-six male Japanese white rabbits (age, 20 weeks; body weight, 2.9 to 3.3 kg) were purchased from Funabashi Farm (Chiba, Japan) and acclimated for 2 weeks before the experiment. The rabbits were housed in individual stainless steel cages in a temperature- and humidity-controlled (23° ± 3°C and 50% ± 5%, respectively) room and fed commercial rabbit chow (RC-4; Oriental Yeast, Chiba, Japan). Body weight was measured before the experiment.

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Experimental Design

Thirty of 36 rabbits were randomly divided into five groups: A_2, hypoglycemia for 30 minutes with 2 U/kg insulin (n = 6); A_{10}, hypoglycemia for 30 minutes with 10 U/kg insulin (n = 6); B_2, hypoglycemia for 60 minutes with 2 U/kg insulin (n = 6); B_{10}, hypoglycemia for 60 minutes with 10 U/kg insulin (n = 6); and C, no hypoglycemia with 10 U/kg insulin and 50% glucose (n = 6). Acute hypoglycemia was induced by intravenous injection of soluble human insulin (Novolin R 40; Novo Nordisk, Copenhagen, Denmark) and reversed by multiple glucose injection (Fig 1). The remaining six rabbits did not receive treatment of any kind, to obtain the normal distribution of CK isoenzymes in different tissues.

Blood Sampling

To measure the blood glucose concentration in the hypoglycemic and recovery periods, 0.2 mL blood was drawn into tubes containing sodium fluoride before and at 15, 30, 60, 90, 120, 240, and 360 minutes after insulin injection in groups A_2 and A_{10}, and before and at 30, 60, 90, 120, 150, 240, and 360 minutes after insulin injection in groups B_2, B_{10}, and C. Before and at 6, 24, and 48 hours after insulin injection, 1.5 mL blood was collected into Bunrimate MF tubes (Ono, Tokyo, Japan) for determination of Na^+, K^+, Cl^-, and hematocrit levels and ALT, AST, LDH, and CK isoenzyme activity.

Tissue Sampling

Six rabbits were anesthetized with intravenous injection of sodium pentobarbital (20 mg/kg). Tissue samples of the heart, quadriceps, liver, and brain were rapidly excised, rinsed with physiological saline, immersed in liquid nitrogen, and stored at −80°C. Within 2 weeks, samples were minced with dry ice using a hardmill, placed in saline, and homogenized at 4°C. Each sample was then centrifuged at 3,000 × *g* for 30 minutes at 4°C, and the supernatants were stored at −30°C until assayed for CK isoenzymes.

Analytical Methods

Blood glucose was determined immediately after each test by the glucose oxidase method using a Fuji Drichem System (Fuji, Tokyo, Japan). All other blood samples were centrifuged at 3,000 × *g* for 10 minutes, and the serum was separated for determination of ALT, AST, LDH, and CK activity and stored at −20°C until assay using conventional standard methods with a COBAS MIRA analyzer (Roche, Tokyo, Japan). Immediately after blood collection, serum concentrations of Na^+, K^+, and Cl^- were measured using an EA03 automated electrolyte analyzer (A&T, Tokyo, Japan). The hematocrit was determined in

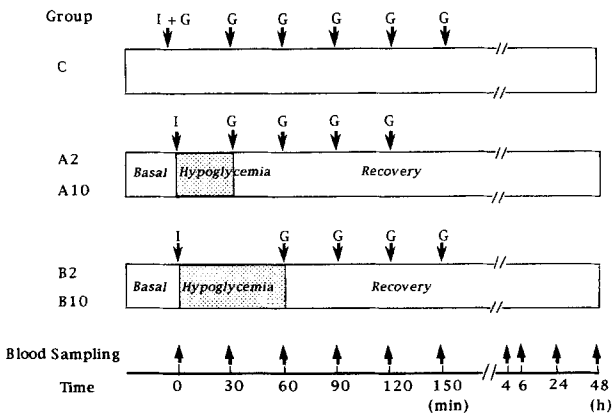

Fig 1. Experimental protocol. Acute hypoglycemia was induced for 30 minutes in groups A_2 and A_{10} and for 60 minutes in groups B_2 and B_{10} by intravenous injection of insulin and reversed by intravenous injection of glucose solution. Experiments were performed with insulin doses of 2 U/kg (groups A_2 and B_2) to 10 U/kg (groups A_{10} and B_{10}). In group C, glucose was injected at 0, 30, 60, 90, 120, and 150 minutes after 10 U/kg insulin injection to prevent hypoglycemia. I, injection of insulin (2 or 10 U/kg body weight); G, injection of 50% glucose solution (5 mL).

heparinized capillary tubes for each sample. CK isoenzymes in the tissues and serum were separated by agarose gel electrophoresis and analyzed using a densitometer as previously described.[6,7]

Statistical Analysis

The data are presented as the mean ± SD. The statistical significance of differences was assessed by Wilcoxon's signed-rank test. A P value less than .05 was considered statistically significant.

RESULTS

Blood Glucose Concentration

Blood glucose levels in all hypoglycemic rabbits declined significantly to about 50 mg/dL after insulin injection. Hypoglycemia was reversed after 30 or 60 minutes by multiple glucose injections. Blood glucose increased temporarily to greater than 140 mg/dL at 90 minutes and returned to the initial levels by 360 minutes in all hypoglycemic groups (Fig 2). In group C, blood glucose concentrations remained above 70 mg/dL during the experiment.

Hematocrit and Plasma Electrolytes

The hematocrit and plasma concentrations of Na^+, K^+, and Cl^- did not change significantly in any of the groups (data not shown).

Serum Enzyme Activity

Serum CK activity increased significantly after insulin injection in all hypoglycemic groups (Fig 3). Serum CK activity in groups A_{10} and B_{10} remained significantly high until at least 24 hours after insulin injection. Furthermore, significant increases in serum AST, ALT, and LDH activity were observed only in group B_{10} (Table 1). However, no remarkable changes in serum enzyme activity were found in group C.

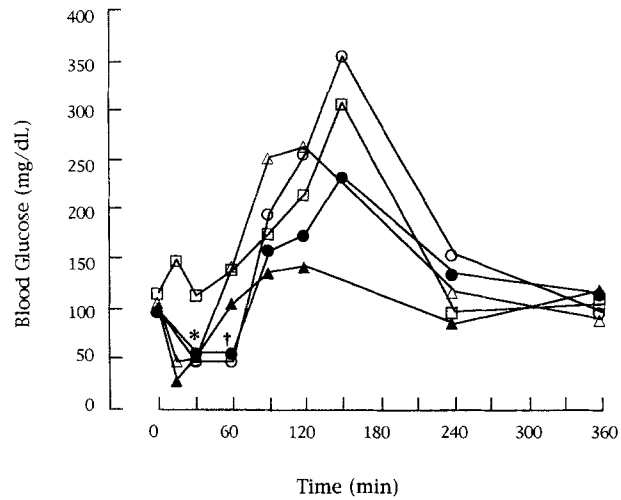

Fig 2. Changes in blood glucose concentration after insulin injection. In group C (□), insulin 10 U/kg body weight was injected followed by injection of glucose solution after 0, 30, 60, 90, 120, and 150 minutes. In group A_2 (△), insulin 2 U/kg body weight was injected followed by injection of glucose solution after 30, 60, 90, and 120 minutes. In group A_{10} (▲), insulin 10 U/kg body weight was injected followed by injection of glucose solution after 30, 60, 90, and 120 minutes. In group B_2 (○), insulin 2 U/kg body weight was injected followed by injection of glucose solution after 60, 90, 120, and 150 minutes. In group B_{10} (●), insulin 10 U/kg body weight was injected followed by injection of glucose solution after 60, 90, 120, and 150 minutes. *Groups A_2 and A_{10} and groups B_2 and B_{10} significantly different from group C at 30 minutes after insulin injection ($P < .01$). †Groups B_2 and B_{10} significantly different from group C at 60 minutes after insulin injection ($P < .01$).

CK Isoenzyme Distribution in Different Tissues and Serum

Four CK isoenzyme bands (1, 2, 3, and 4) were found in rabbit tissue (Fig 4). In myocardium, bands 4 and 2 comprised 59.8% and 24.5%, respectively, of the CK isoenzymes. The quadriceps and brain contained 96.7% of band 4 and 94.1% of

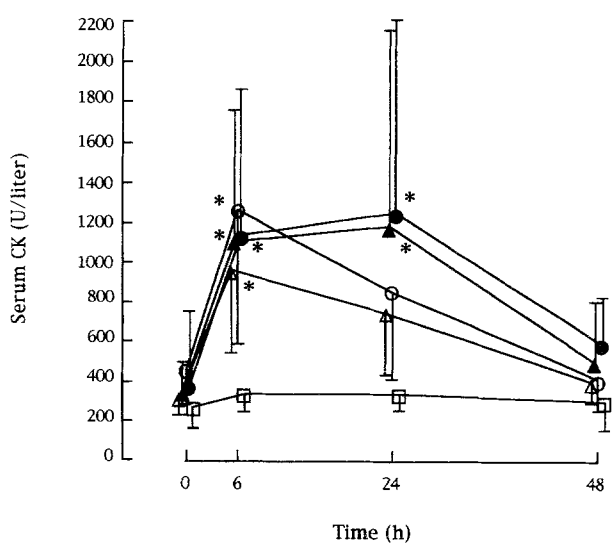

Fig 3. Changes in serum CK activity after insulin injection. □, group C; △, group A_2; ▲, group A_{10}; ○, group B_2; ●, group B_{10}. *$P < .05$ v values before insulin injection.

Table 1. Changes in Serum Activity of ALT, AST, and LDH After Insulin-Induced Hypoglycemia in Rabbits

	Enzyme Level (U/L)			
		After Insulin Injection (h)		
Group (n = 6)	Initial	6	24	48
C				
ALT	60 ± 25	60 ± 20	68 ± 30	62 ± 29
AST	45 ± 24	42 ± 27	68 ± 49	49 ± 35
LDH	141 ± 35	171 ± 33	158 ± 45	156 ± 78
A_2				
ALT	50 ± 16	55 ± 21	55 ± 21	51 ± 21
AST	28 ± 8	37 ± 14	28 ± 13	23 ± 10
LDH	156 ± 44	143 ± 57	102 ± 22	104 ± 57
A_{10}				
ALT	66 ± 17	76 ± 19	68 ± 17	73 ± 13
AST	35 ± 4	48 ± 17	29 ± 4	38 ± 15
LDH	148 ± 39	196 ± 66	131 ± 43	166 ± 148
B_2				
ALT	59 ± 20	66 ± 18	66 ± 14	68 ± 15
AST	44 ± 19	30 ± 11	25 ± 7	27 ± 8
LDH	152 ± 43	164 ± 48	95 ± 2	115 ± 33
B_{10}				
ALT	54 ± 15	62 ± 15*	64 ± 13*	58 ± 13
AST	30 ± 6	47 ± 13*	29 ± 6	24 ± 11
LDH	110 ± 19	248 ± 98*	155 ± 61	151 ± 95

*$P < .05$ *v* initial value (Wilcoxon signed-rank test).

band 1, respectively, but none of band 1 and band 3, respectively. Band 1 (21%) and band 3 (57.1%) were found in the liver. Band 4, in accordance with the marked increase in serum CK in group B_{10}, increased significantly from 78.5% at basal to 90.1% at 6 hours after insulin injection in this group, whereas none of the other bands increased (Fig 5). In group C, band 4 showed a slight but statistically insignificant increase from 73.7% to 78%.

DISCUSSION

Hypoglycemia is a common side effect of insulin treatment in diabetic patients. The Diabetes Control and Complications Trial Research Group reported that in a total of 1,441 patients evaluated for a mean period of 6.5 years, there were 3,788 episodes of severe hypoglycemia (requiring assistance), of which 1,027 were associated with coma and/or seizure.[8] Severe hypoglycemia may be accompanied by coma and other rare complications such as cerebral infarction,[9,10] myocardial infarction,[11-14] and deterioration of diabetic retinopathy.[15] Soler and Khardori[1] reported three patients with markedly elevated serum transaminases following hypoglycemic coma induced by insulin. They did not measure CK activity. However, an increase in the plasma activity of hepatic enzymes has been reported as an uncommon consequence of hypoglycemia. Jones et al[2] could not demonstrate abnormal plasma enzyme activity following controlled insulin-induced hypoglycemia in eight cases of insulin-dependent diabetes mellitus. Patrick et al[3] reported that ALT and AST activities were unchanged in diabetics after controlled insulin-induced hypoglycemia, and that gamma-glutamyl transferase activity increased during hypoglycemia in three diabetics who were receiving phenytoin therapy or had a history of excessive alcohol consumption.

In cases reported by Soler and Khardori,[1] a pronounced increase in ALT and AST activity was observed following hypoglycemic coma that lasted for more than 1 hour. Controlled insulin-induced hypoglycemia lasted only 20 minutes in the other studies.[2-4] Differences in the duration and severity of hypoglycemia may partly contribute to the apparent discrepancies among these reports. In a previous study,[5] we induced hypoglycemia in rabbits by intravenous injection of insulin at a dose of 10 U/kg body weight, which was reversed by intravenous injection of glucose solution at 30, 60, and 90 minutes after insulin injection. Blood glucose declined to about 50 mg/dL at 20, 30, and 120 minutes. As the total duration of hypoglycemia was more than 30 minutes, a significant increase in the plasma activity of ALT, AST, LDH, and CK was observed in hypoglycemic rabbits.[5]

In the present study, the results suggest that insulin-induced hypoglycemia may lead to increased serum CK activity. The increased activity was sustained for 24 hours in groups that received the high dose of insulin. At the same time, the serum activity of ALT, AST, and LDH increased in the group with hypoglycemia for 60 minutes induced by high-dose insulin, but not in the other groups. These results suggest that the hypoglycemia duration and insulin dosage have a profound influence on the increased activity of serum enzymes observed after insulin-induced hypoglycemia. CK is known to be a muscle-localized enzyme, and thus increased serum CK activity may serve as an indicator of muscular irritability or muscular damage. Plasma CK activity has been reported to increase during occlusion-reperfusion of a coronary artery[16] and also in simvastatin (hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitor)-treated rabbits with skeletal muscle damage.[17] The increases in serum ALT, AST, LDH, and CK activity by hypoglycemia in the present study suggest that the damage was located in the muscle rather than the liver and that the organ damage tends to increase in severity as the injected insulin dose increases.

Four CK isoenzyme bands were detected in the various rabbit tissues, with different organs showing different distribution of each. Evidently, electrophoretic bands 4 and 2 represent CK-MM and CK-MB isoenzymes, respectively, with band 1 indicating CK-BB isoenzyme. Band 3 is assumed to be one of the anodic variant isoenzymes, as its mobility lies between that of CK-MM and CK-MB.[18,19] The formation of band 3 has been variously

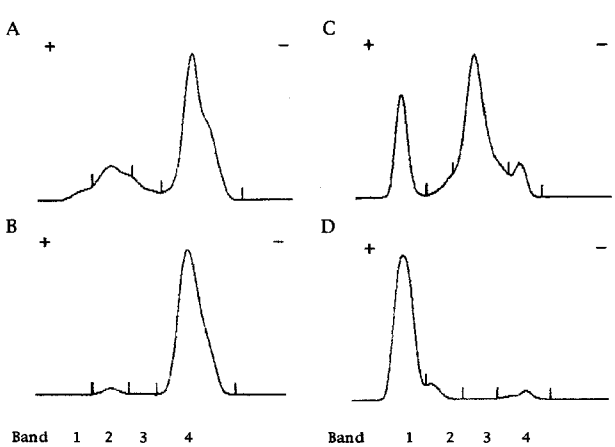

Fig 4. CK isoenzyme bands in rabbit tissue. Bands 1, 2, 3, and 4 of CK isoenzyme are successively shown from anode to cathode in myocardium (A), quadriceps (B), liver (C), and brain (D).

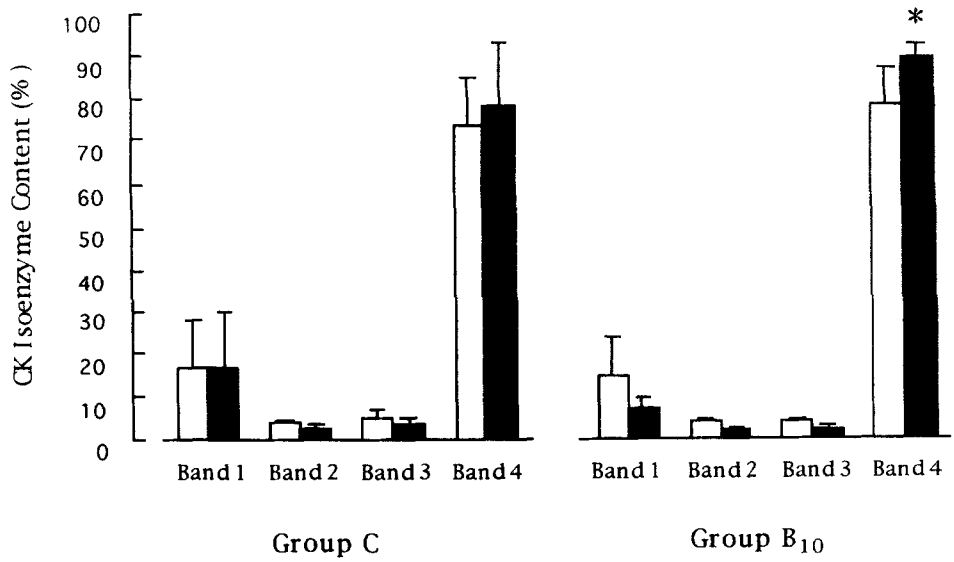

Fig 5. Changes in serum CK isoenzymes in groups B_{10} and C. □, before insulin injection; ■, 6 hours after insulin injection. *$P <$ .05 *v* values before insulin injection.

attributed to polymeric aggregation of CK monomers or to complexing of CK isoenzymes with immunoglobulins, lipids, lipoproteins, or unknown serum components.[20] Further characterization of this variant band is clearly required. Band 4 exists predominantly in skeletal muscle (96.7%) and myocardium (59.8%). During hypoglycemia, serum CK band 4 was remarkably more abundant at 6 hours than before injection compared with the control group. These data indicate that insulin-induced hypoglycemia primarily damages muscle.

We also measured CK concentrations in various rabbit tissues and compared the results with findings in human tissues (Table 2).[21,22] In skeletal muscle, the CK concentration was twofold higher in rabbit versus human tissues, and there is some variation in the CK concentration among different human muscles. Myocardial CK levels were equivalent in rabbit and human tissues. Lower brain CK levels and slightly higher liver CK levels were observed in rabbits. The blood volume of rabbits is estimated to be 5.56% of body weight using the Welcker method.[23] As the net increase in serum CK induced by hypoglycemia was between 800 and 1,000 U/L, a small amount of tissue necrosis could account for the increase in serum CK levels in the present study, aside from the metabolic rate of elimination of CK in the serum. However, as damage due to hypoglycemia may involve entire muscles (including skeletal and heart muscle), the increase in serum enzyme activity is most likely primarily due to a leakage of enzymes induced by injury of the musculature membrane integrity rather than a "bolus-like" release of tissue enzymes from muscle infarction or necrosis.

Table 2. Comparison of CK Concentrations Between Rabbit and Human Tissues

	CK Concentration (U/g wet tissue)				
	Rabbit		Human*	Human†	
Tissue	Mean	Range	Mean	Mean	Range
Skeletal muscle					
Quadriceps	10,172	8,625-10,996	—		—
Psoas major		—	5,233	1,800	700-2,400
Pectoralis major		—	3,665	2,400	2,200-2,500
Abdominis		—	4,845	2,300	2,100-2,500
Myocardium	706	437-955		400	200-800
Atrium		—	632		—
Ventricle		—	1,026		—
Brain	4	2-8	192	70	0-150
Liver	6	2-12	4	2	1-3

*Yasui and Ishii.[21]
†Smith.[22]

None of the hypoglycemic rabbits experienced convulsion in the present study, which suggests that the mechanism of muscle damage in hypoglycemia involves a metabolic disorder. Prolonged insulin-induced hypoglycemia may decrease the substrate supply for energy metabolism, thereby suppressing both gluconeogenesis and lipolysis.[24] A change in oxidation-reduction in muscles resulting from depletion of intracellular adenosine triphosphate during hypoglycemia is one possible explanation for the organ damage observed in the present study. This possibility needs to be further investigated.

In the present study, endogenous CK fluctuations were observed in rabbits, consistent with results reported in the literature.[23,25] Serum CK and CK band 4 isoenzyme activity increased remarkably in the hypoglycemic group but not in the control group, which suggests that endogenous CK fluctuations did not significantly affect the results obtained in this study.

Anemia due to excess blood sampling and electrolyte imbalance induced by administration of large doses of insulin and glucose may affect muscle metabolism. However, the hematocrit and serum electrolytes remained unchanged throughout the study. The changes in the serum activity of ALT, AST, LDH, and CK may be due to metabolic disturbances in muscle following hypoglycemia induced by insulin.

In conclusion, this study demonstrates that serum CK activity increases remarkably during insulin-induced hypoglycemia, accompanying an elevation in serum ALT, AST, and LDH activity in prolonged hypoglycemia induced by high-dose insulin. In addition, serum CK band 4 isoenzyme, which exists predominantly in the skeletal muscle and myocardium, also increases significantly during hypoglycemia. These results indicate that prolonged hypoglycemia may cause damage to the skeletal and heart muscle rather than the liver.

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