Ketone Body Utilization and Its Metabolic Effect in Resting Muscles of Normal and Streptozotocin-Diabetic Rats

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Abstract. By using an *in situ* rat hindquarter perfusion, we evaluated ketone body utilization and its metabolic effects in the resting muscle of 24 h fasted normal and streptozotocin (STZ)-diabetic rats. Under the perfusion with ketone body-supplementation (1 mM each of acetoacetic acid (AcAc) and 3-hydroxybutyric acid (3-OHB)), the AcAc and 3-OHB uptake of STZ-diabetic rats was significantly ($P<0.05$) smaller than that of normal rats. This might be explained by the low enzyme activity of 3-oxoacid CoA transferase demonstrated in the hindlimb muscles of STZ-diabetic rats and this reduced ketone body uptake would be one of the causes of the development of diabetic ketoacidosis. The glucose uptake and the phosphofructokinase (PFK) activity of normal rats were significantly ($P<0.05$) higher than those of STZ-diabetic rats. In both normal and STZ-diabetic rats, the glucose utilization and PFK activity of the muscles in the ketone body-supplemented condition were significantly ($P<0.05$) lower than those in the non-supplemented condition. This inhibition of glucose utilization by ketone bodies should be due to the mechanism by which the oxidation of ketone bodies inhibits PFK in the muscle.

Key words: Ketone body, Rat hindquarter perfusion, Streptozotocin diabetic rats, 3-Oxoacid CoA transferase, Phosphofructokinase.

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KETONE bodies are oxidized in extra-hepatic tissues such as skeletal muscles, cardiac muscle, brain and kidney, and are a very important fuel in the prolonged fasting state [1]. However, there are few reports which have dealt with ketone body metabolism in peripheral tissues such as skeletal muscles. Previous studies concerning ketone body metabolism in muscles have been performed by using an isolated diaphragm [2], or soleus muscle [3]. However, more physiological and serious studies are needed to clarify ketone body metabolism in normal and diabetic muscles. We therefore evaluated the resting muscle's ketone body and

glucose utilization by means of an *in situ* hindquarter perfusion technique in both normal and STZ-diabetic rats.

Materials and Methods

Animals

Male Wistar rats were obtained from Dōken Experimental Animal Co. (Ibaraki, Japan). A mild diabetic state was induced at 6 weeks of age by injecting STZ (25 mg/kg, Upjohn Co., USA), dissolved in 300 μ l of citrate buffer (pH 4.5) into a tail vein of 24 h fasted rats.

Hindquarter perfusion

Both normal and STZ-diabetic rats were used

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for this experiment under pentobarbital anesthesia (50 mg/kg, i.p.). Perfusion of the isolated rat hindquarter was carried out according to the method described by Chan and Dehaye [4]. The standard medium used for perfusion was composed of a Krebs-Henseleit bicarbonate buffer (KHB buffer, pH 7.4) oxygenated with 95% O₂-5% CO₂ containing 3% Dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden), human erythrocytes washed with 0.9% NaCl solution and KHB buffer, and 5.5 mM glucose. After the hindlimb had been washed out with 50 ml of oxygenated KHB buffer, the rat was placed in the perfusion apparatus, based on a modification of the Miller-Mortimore model [5], and the hindlimb was perfused at a flow rate of 4 ml/min with 85 ml of recirculating perfusate. After pre-perfusion with the perfusate described above for 20 min at 37°C, the tissue was then perfused either with the KHB buffer added 1 mM AcAc, 1 mM 3-OHB and 10 μ U/ml regular insulin (ketone body-supplemented condition, each value being the final concentration) or with KHB buffer added only 10 μ U/ml regular insulin (ketone body non-supplemented condition). After pre-perfusion for a further 5 min, 0.4 ml aliquots of perfusate were collected at 10 min intervals for 100 min. The samples were placed in chilled tubes and the supernatant obtained by centrifugation at 3000 rpm for 15 min at 4°C was stored at -80°C until assayed. For the assay of lactate, 0.3 ml aliquots of perfusate were also collected at 0 min, 40 min, 60 min and 90 min in chilled tubes containing 0.6 M HClO₄. After the perfusion, the perfused hindlimb muscles were weighed and the glucose and ketone body uptake was expressed as values per muscle weight. In order to evaluate the uptake of ketone bodies and glucose by erythrocytes in the perfusion medium, perfusate containing 1 mM AcAc, 1 mM 3-OHB, 5.5 mM glucose and 10 μ U/ml regular insulin was circulated in the perfusion apparatus without passing the hindlimb.

Preparation of tissue extracts

After perfusion, red portions of gastrocnemius muscle were obtained by the method of Hagg *et al.* [6] and stored for 1-3 days in liquid N₂. Thereafter, the muscle was pulverized in a mortar under liquid N₂ and used for determination of enzyme activities of 3-oxoacid CoA transferase and phosphofructokinase (PFK).

Sample analysis

The plasma and perfusate glucose concentration was measured by a glucose oxidase method (glucose analyzer 2, Beckman Instruments, Fullerton, CA, USA). Plasma and perfusate AcAc, 3-OHB and total ketone body (TKB) concentrations were determined by the method of Harano *et al.* [7] (Ketone test[®], Sanwa Kagaku Co., Nagoya, Japan). 3-OHB was calculated by subtracting the AcAc value from the TKB value. The lactate concentration in the perfusate was determined with a commercial kit (Lactate-UV-test[®], Boehringer-Mannheim, Mannheim, Germany). The plasma immunoreactive insulin (IRI) concentration was determined by a radioimmunoassay (RIA) according to the method of Herbert *et al.* [8].

Determination of enzyme activities

After small slices were removed from the frozen tissue, the tissue (10 mg) was weighed rapidly, and homogenization was begun on the still frozen tissue, so that thawing occurred during tissue disintegration. In both cases, 1 ml chilled homogenization medium [0.25 M sucrose, 1 mM 2-mercaptoethanol, 10 mM Tris-HCl buffer (pH 7.4) for 3-oxoacid CoA transferase; 150 mM KCl, 50 mM KHCO₃, 6 mM EDTA for PFK] was used. The tissues were homogenized with a glass pestle driven by a low-speed motor, and then centrifuged for 20 min at 30000 g. Enzyme determinations were made on this supernatant fluid (enzyme sample). 3-Oxoacid CoA transferase activity was determined as the rate of acetoacetyl-CoA formation from succinyl-CoA and acetoacetate by the method of Williamson *et al.* [9]. Namely, the silica cuvettes (1 cm) contained in a final volume of 2 ml: Tris-HCl buffer, pH 8.5 (100 μ mol), MgCl₂ (10 μ mol), iodoacetamide (10 μ mol) (to inhibit acetoacetyl CoA thiolase and enzymes of the hydroxymethylglutaryl-CoA pathway) and succinyl-CoA (0.2 μ mol). The enzyme sample (0.1 ml) was added and the rate of increase in extinction at 313 nm was measured for 2 min at 25°C. Under these assay conditions the millimolar extinction coefficient of the acetoacetyl-CoA-Mg²⁺ complex was found to be 12 (313 nm). PFK activity was determined by the method of Shonk and Boxer [10]. Namely, the silica cuvettes (1 cm) contained in a final volume of 3.54 ml: 3.0 ml buffer [Triethanolamine (50 mM), EDTA (5 mM), pH 7.6], MgCl₂ (10 mM), Aldolase (1.0 U), α -glycerolphosphate dehydrogenase (7.1

U)-triosephosphate isomerase (18.3 U) mixture, NADH (0.3 mM) and ATP (2.8 mM).

After the enzyme sample (0.02 ml) was added, the absorbance at 340 nm was briefly recorded, the enzyme reaction was initiated by the addition of the substrate, fructose-6-phosphate (2.8 mM). The rate of increase in extinction at 340 nm was measured for 2 min at 25°C. Under these assay conditions the millimolar extinction coefficient was found to be 6.3 (340 nm). All chemicals were of reagent grade and obtained from local suppliers.

Statistics

Data were analyzed by analysis of variance followed by group comparisons (Student's *t* test) when a significant *F*-value was obtained, and $p < 0.05$ was considered to be significant. All data in this paper are expressed as the mean $\pm$ SEM.

Results

Comparison of plasma ketone bodies, glucose and insulin levels in normal and STZ-diabetic rats (Table 1)

In a 24 h fasted state, the plasma glucose and total ketone body (AcAc + 3-OHB) concentrations of STZ-diabetic rats were significantly higher than those of normal rats. (300 ± 29 mg/dl vs. 96 ± 10 mg/dl, 1242 ± 40 μ M vs. 818 ± 46 μ M, $p < 0.05$). The insulin concentration of STZ-diabetic rats was significantly lower than that of normal rats (8 ± 3 μ U/ml vs. 16 ± 4 μ U/ml, $p < 0.05$).

Ketone body uptake by perfused hindquarter of 24 h fasted normal and STZ-diabetic rats (Fig. 1)

Figure 1 shows the changes in the ketone body concentrations of the perfusate in normal and STZ-diabetic rats under the ketone body-supplemented perfusate condition. The basal values of TKB, AcAc and 3-OHB were 1954 ± 47 , 969 ± 26 , 982 ± 44 μ mol/l in normal rats, and

1964 ± 36 , 974 ± 38 , 984 ± 62 μ mol/l in STZ-diabetic rats, respectively. No significant difference was observed between normal and STZ-diabetic rats in these values.

TKB and AcAc concentrations in the perfusate were gradually decreased during the perfusion in both normal and STZ-diabetic rats and a significant decrease was observed 50 min after the perfusion. On the other hand, there was no significant change in the 3-OHB concentration in either normal or STZ-diabetic rats. Also, no significant change was observed in the ketone body levels during the control experiment (manipulation without muscle perfusion). The cumulative TKB and AcAc uptake by perfused hindquarter in the normal rats were significantly greater than those in the STZ-diabetic rats ($p < 0.05$) (Table 2). The AcAc uptake by perfused hindquarter was significantly greater than the 3-OHB uptake in both normal and STZ-diabetic rats ($p < 0.05$) (Table 2). The ketone body concentrations of perfusate in both normal and STZ-diabetic rats under the ketone body non-supplemented condition were undetectable.

Glucose uptake by perfused hindquarter of 24 h fasted normal and STZ-diabetic rats (Figs. 2, 3)

Figure 2 shows the time course of glucose concentrations of perfusate in both ketone body-supplemented and non-supplemented conditions. The glucose concentration in the perfusate began to elicit a significant decrease ($p < 0.05$) at a sampling time of 40 min and 60 min in the non-supplemented experiment and supplemented experiment, respectively. The glucose concentration in the non-supplemented experiment was significantly lower than that in the supplemented experiment after 50 min of perfusion. The cumulative glucose uptake by perfused hindquarter in the non-supplemented experiment was significantly greater than that in the supplemented

Table 1. Plasma ketone body, glucose and insulin levels of normal and STZ-diabetic rats

	N	Plasma ketone body (μ M)			Plasma glucose (mg/dl)	Plasma insulin (μ U/ml)
		AcAc	3-OHB	Total		
Normal rats	14	240 ± 18	574 ± 49	818 ± 46	96 ± 10	16 ± 4
STZ-diabetic rats	14	$326 \pm 31^*$	$932 \pm 74^*$	$1242 \pm 40^*$	$300 \pm 29^{**}$	$8 \pm 3^*$

Values are mean $\pm$ SEM.

*; $p < 0.05$ and **; $p < 0.01$, Normal vs. STZ-diabetic rats.

Fig. 1. Changes in ketone body concentrations of perfusate during *in situ* hindlimb-muscle perfusion in normal and STZ-diabetic rats. After 25 min of pre-perfusion, aliquots of perfusate were collected to determine ketone body levels every 10 min. $\circ$ — $\circ$ —; Normal rats (N=7), $\triangle$ — $\triangle$ —; STZ-diabetic rats (N=7), $\square$ — $\square$ —; control experiment (N=5). Results are expressed as the mean $\pm$ SEM. *, $p < 0.05$ normal vs. STZ-diabetic rats, $\bullet$ $p < 0.05$ vs. Basal values (0 min).

experiment ($p < 0.05$) (Table 2). Similar results were obtained with STZ-diabetic rats (Fig. 3, Table 2). Namely, the glucose concentration in the perfusate began to show a significant decrease ($p < 0.05$) at a sampling time of 100 min and 70 min in the non-supplemented experiment and supplemented experiment, respectively (Fig. 3). The glucose concentration in the non-supplemented experiment was significantly lower than that in the ketone body-supplemented experiment after 70 min of perfusion. The cumulative glucose uptake by perfused hindquarter in the non-supplemented experiment was significantly greater than that in the supplemented experiment ($p < 0.05$) (Table 2). The lactate level of perfusate during the experiment was similar to those found in previous perfusion studies [11–13].

Comparison of 3-oxoacid CoA transferase and phosphofructokinase activity in hindlimb muscles in normal and STZ-diabetic rats (Table 3)

3-Oxoacid CoA transferase activity in hindlimb muscles perfused in the ketone body supplemented condition was significantly higher than that of hindlimb muscles perfused in the non-supplemented condition ($p < 0.05$) in both normal and diabetic rats. In addition, the 3-oxoacid CoA transferase activity of STZ-diabetic rats was significantly lower than that of normal rats in both ketone body supplemented and non-supplemented conditions ($p < 0.05$).

Table 2. Summary of data obtained from the rat hindlimb-muscle perfusion

Ketone body	Normal rats		STZ-diabetic rats	
	Non-supplemented (N=7)	Supplemented (N=7)	Non-supplemented (N=7)	Supplemented (N=7)
Fasting body wt (g)	225 $\pm$ 20	238 $\pm$ 16	174 $\pm$ 19	170 $\pm$ 21
Hindlimb muscle weight (g)	24.3 $\pm$ 2.1	25.0 $\pm$ 2.6	16.1 $\pm$ 3.2 $\dagger$	15.9 $\pm$ 2.7 $\dagger$
TKB uptake (μ mol/100 min/g muscle)	—	1.78 $\pm$ 0.29	—	1.01 $\pm$ 0.11 $\dagger$
AcAc uptake (μ mol/100 min/g muscle)	—	1.66 $\pm$ 0.16	—	0.95 $\pm$ 0.05 $\dagger$
3-OHB uptake (μ mol/100 min/g muscle)	—	0.12 $\pm$ 0.04	—	0.06 $\pm$ 0.02
Glucose uptake (μ mol/100 min/g muscle)	3.56 $\pm$ 0.26*	2.34 $\pm$ 0.36	1.80 $\pm$ 0.16* $\dagger$	1.42 $\pm$ 0.29 $\dagger$

Values are mean $\pm$ SEM.

$\dagger$ $p < 0.05$ normal vs. STZ-diabetic rats.

* $p < 0.05$ ketone body-supplemented experiment vs. non-supplemented experiment.

Fig. 2. Changes in glucose concentration of perfusate during *in situ* hindlimb-muscle perfusion in normal rats. --□--□--; ketone body (+) (N=7), -□-□-; ketone body (-) (N=7), --△--△--; control experiment (N=5). Results are expressed as the mean±SEM. *; $p<0.05$ ketone body (+) vs. ketone body (-) ■ $p<0.05$ vs. Basal values (0 min).

Fig. 3. Changes in glucose concentration of perfusate during *in situ* hindlimb-muscle perfusion in STZ-diabetic rats. Mean±SEM, N=7. Results are expressed as the mean±SEM. --□--□--; ketone body (+) (N=7), -□-□-; ketone body (-) (N=7), --△--△--; control experiment (N=5) *; $p<0.05$ ketone body (+) vs. ketone body (-) ■ $p<0.05$ vs. Basal values (0 min).

The PFK activity of hindlimb muscles perfused in the ketone body-supplemented condition was significantly lower than that in the non-supplemented condition ($p<0.05$) in both normal and diabetic rats. Furthermore, the PFK activity of the STZ-diabetic rats was significantly lower than that of the normal rats in both ketone body-supplemented and non-supplemented conditions ($p<0.05$).

Discussion

By using an *in situ* rat hindquarter perfusion, we evaluated the resting muscle's ketone body utilization. AcAc uptake was significantly higher than 3-OHB uptake in both the normal and STZ diabetic rats in the perfusate condition supplemented with 1 mM of both AcAc and 3-OHB. In order to utilize 3-OHB in the muscle, 3-hydroxybutyrate dehydrogenase is absolutely necessary. However, this enzyme activity is scarce in the muscle [11] which is the reason why 3-OHB uptake was negligible in this experiment.

3-Oxoacid CoA transferase is the enzyme involved in ketone body utilization in its initial step. Our data indicate that 3-oxoacid CoA transferase activity is induced in the presence of 1 mM AcAc and 3-OHB (Table 3). This finding was consistent with the report of Williamson *et al.* [9] stating that 3-oxoacid CoA transferase activity in isolated muscle increased in parallel with the ketone body concentration in the incubation medium.

The ketone body uptake of STZ-diabetic rats was significantly lower than that of normal rats. This result might be explained by the lower 3-oxoacid CoA transferase activity in hindlimb muscles of STZ-diabetic rats (Table 3). Beatty *et al.*

Table 3. 3-Oxoacid CoA-transferase and PFK activity of hindlimb muscles of normal and STZ-diabetic rats

	N	3-Oxoacid CoA-transferase activity ($\mu\text{mol}/\text{min}/\text{g}$ tissue)	PFK activity ($\mu\text{mol}/\text{min}/\text{g}$ tissue)
Normal rats			
ketone body supplemented	7	$0.34 \pm 0.05^{*\dagger}$	$10.0 \pm 0.8^{*\dagger}$
ketone body non-supplemented	7	$0.18 \pm 0.02^*$	$14.1 \pm 2.2^\dagger$
STZ-diabetic rats			
ketone body supplemented	7	$0.15 \pm 0.02^*$	$5.1 \pm 2.1^*$
ketone body non-supplemented	7	0.11 ± 0.01	7.6 ± 0.3

Values are mean±SEM.

† $p<0.05$ Normal vs. STZ-diabetic rats.

* $p<0.05$ Ketone body-supplemented experiment vs. non-supplemented experiment.

reported that insulin promotes ketone body utilization in muscles by activating 3-oxoacid CoA transferase activity [14]. In diabetic ketoacidosis, hyperketonemia might induce 3-oxoacid CoA transferase activity by itself, but severe hypoinsulinemia should depress ketone body utilization by interrupting the induction of 3-oxoacid CoA transferase. Actually our pre-investigation, without insulin, but maintaining the same protocol (ketone body-supplemented condition) showed that the cumulative TKB uptake were significantly lower than those with insulin addition ($10 \mu\text{U/ml}$) in both normal and diabetic rats. (TKB $34 \pm 6\%$ reduction in normal rats, and $42 \pm 8\%$ reduction in STZ diabetic rats. $N=4$ in both cases).

Regarding the glucose uptake, the glucose utilization in the ketone body-supplemented condition was significantly lower than that in the non-supplemented condition. Randle *et al.* hypothesized that ketone bodies spare the oxidation of glucose in muscle by impeding its entry into the cell by inhibiting its phosphorylation to glucose-6-phosphate, and by inhibiting the phosphofructokinase reaction and the oxidative decarboxylation of pyruvate [15]. This hypothesis is supported by our finding that the PFK activity in hindlimb muscles in the ketone body-supplemented experiment was significantly lower than that of in non-supplemented experiment. In addition, Goodman *et al.* indicated that the oxidation of ketone bodies caused an increase in the concentration of intracellular citrate or NADH

which in turn inhibits PFK [16]. This effect of ketone bodies in sparing glucose utilization of muscle seems to be a mechanism in which the energy supply for muscles turns to free fatty acid or ketone bodies from glucose.

We observed that the PFK activity of STZ-diabetic rats was significantly lower than that of normal rats. Nowadays, fructose-2,6-bisphosphate is thought to be a potential activator of PFK, a key enzyme of glycolysis [7]. Also, it is known that the fructose-2,6-bisphosphate level in muscles is regulated by insulin and tolbutamide [18, 19]. Although we did not examine the fructose-2,6-bisphosphate level in muscles in this experiment, the fructose-2,6-bisphosphate level should be low in mild diabetic rats.

In summary, we have clearly demonstrated that AcAc utilization by skeletal muscles decreases in mild diabetic rats and that ketone body utilization causes a reduction of glucose uptake and PKF activity.

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