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Stereoselective effects of 3-hydroxybutyrate on glucose utilization of rat cardiomyocytes

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Abstract

In researches of ketone bodies, D-3-hydroxybutyrate (D-3HB) is usually the major one which has been investigated; in contrast, little attention has been paid to L-3-hydroxybutyrate (L-3HB), because of its presence in trace amounts, its dubious metabolism, and a lack of knowledge about its sources. In the present study we determined the distributions of enantiomers of 3-hydroxybutyrate (3HB) in rat brain, liver, heart, and kidney homogenates, and we found the heart homogenate contained an enriched amount of L-3HB (37.67 μM/mg protein) which generated a significant ratio of 66/34 (D/L). The ratio was altered to be 87/13 in the diabetic rat heart homogenate. We subsequently found this changed ratio of D/L-3HB may contribute to reduce glucose utilization in cardiomyocytes. Glucose utilization by cardiomyocytes with 5 mM of D-3HB was decreased to 61% of the control, but no interference was observed when D-3HB was replaced with L-3HB, suggesting L-3HB is not utilized for the energy fuel as other ketone bodies are. In addition, the reduced glucose utilization caused by D-3HB gradually recovered in a dose-dependent manner with administration of additional L-3HB. The results gave the necessity of taking L-3HB together with D-3HB into account with regard to glucose utilization, and L-3HB may be a helpful substrate for improving inhibited cardiac pyruvate oxidation caused by hyperketonemia.

Keywords: D-3-hydroxybutyrate; L-3-hydroxybutyrate; Glucose utilizaticon; Cardiomyocytes

Introduction

Ketone bodies are by-products in β-oxidation of fatty acids. Acetyl CoA can be generated by ketolysis; thus ketone bodies serve as the alternate energy substrates for organs including the brain, which was considered to use glucose as its sole energy source before 1967 (Paterson et al., 1967). Increased circulatory plasma ketone bodies levels are usually observed when the concentrations of free fatty acids rise and when the relative concentration of insulin decreases, such as starvation or Diabetes Mellitus (DM). Under the circumstances, the body will utilize ketone bodies for the energy requirement to a greater extent. For example, 30–40% of the energy needs are supplied from ketone bodies after a 3-day fast (Laffel, 1999).

D-3-Hydroxybutyrate (D-3HB) is the main ketone body which was under intensive investigation. It is known that D-3HB dehydrogenase can catalyze the oxidation of D-3HB to acetoacetate (Lehninger et al., 1993). The enantiomer of D-3HB, L-3-hydroxybutyrate (L-3HB), is regarded to be nonexistent physiologically. Klee and Sokoloff had identified that only D-isomer of 3HB can function as the substrate for the mitochondrial 3-HB dehydrogenase (Klee and Sokoloff, 1967). Therefore, only 50% of DL-3HB is expected to be utilized when DL-3HB is injected into rats. It was surprising to find that DL-3HB was the more preferred precursor for biosynthesis of lipids than acetoacetate when DL-3HB and acetoacetate were injected in equal amounts (Edmond, 1974). Subsequent studies showed L-3HB as well as D-3HB can be incorporated into hepatic lipids, brain proteins, and amino

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acids during the developmental period of neonatal rats (Swiatek et al., 1981, 1984). In addition, L-3HB was shown to be a more-favorable substrate than other ketone bodies for sterol and fatty acid synthesis in the brain, spinal cord, and kidney (Webber and Edmond, 1977). This superior utilization suggested a physiological role for L-3HB, and Reed and Ozand (Reed and Ozand, 1980) had isolated the enzyme, L-3-hydroxybutyryl CoA dehydrogenase, from the liver which was responsible for L-3HB. Their data also suggested that the liver was the site of L-3HB production. However, Lincoln et al. (Lincoln et al., 1987) showed that L-3HB was not metabolized by a dehydrogenase in the rat liver, but mostly via mitochondrial activation. Scofield et al. (Scofield et al., 1982) also provided evidence that L-3HB was not produced by the liver.

Besides the conflicting results of L-3HB production, distinct biological effects of D- and L-3HB are present. After the depression of excitatory postsynaptic potentials (EPSPs) by 60 min of glucose deprivation, administration of D-3HB restored EPSPs in slices from postnatal day 15 rats. L-3HB, however, was not effective in sustaining synaptic responses (Izumi et al., 1998). Moreover, L-3HB exhibited direct anticonvulsant actions in audiogenic seizure-susceptible mice, but D-3HB was not effective (Rho et al., 2002).

Previously we had used fluorescence derivatization and HPLC analysis to prove that L-3HB does exist in rat serum under physiological conditions (Tsai et al., 2003). Although the formation source remained unclear, L-3HB is present in a trace amount compared to that of D-3HB in a ratio of 1 to 27. With the consideration in L-3HB properties, including its trace amount in rat serum, superior utilization, and distinct effects from that of D-3HB, we hypothesize that L-3HB may be an intracellular intermediate, not a circulating metabolite. Therefore in the present work, we measured the ratio of D/L-3HB in the rat brain, liver, heart, and kidney homogenates with a readily available analytical technique to explore the potential functional roles of L-3HB. An enriched amount of L-3HB in the rat heart is found and the coexistence of L-3HB is proposed as being necessary for normalization of the decreased glucose utilization caused by D-3HB.

Materials and methods

Animals

Animal experiments were approved by the Animal Care and Use Committee of Taipei Medical University, Taiwan. Male Sprague–Dawley rats (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) weighting 250–300 g were kept in an environmentally controlled room of temperature (25 °C) and under a 12 h light / 12 h dark cycle (lights on at 7:00 a.m.) with food and tap water ad libitum and food at least 1 week prior to use. Diabetes in these rats was induced by administering intraperitoneal injections of streptozotocin (60 mg/kg). The rats after 30 days of the induction of diabetes were used.

Enantiomeric analysis of 3HB in brain, liver, heart, and kidney homogenates

Brains, livers, hearts (including normal and diabetic ones), and kidneys obtained from anesthetized rats were rapidly washed and homogenized in ice-cold PBS buffer. Homogenates were centrifuged at 700 g for 15 min, and the supernatant was kept at −20 °C until used. We analyzed D- and L-3HB in the homogenates with the method which was described in our previous publication (Tsai et al., 2003). Briefly, 10 μL of 1.0 mM propionic acid as an internal standard (I.S.) was added to 50 μL of the homogenate, and was then brought to 200 μL with ethanol for deproteinization. The solution was mixed and centrifuged at 700 g for 5 min. For derivatization, 100 μL of the supernatant was added to 100 μL of 5 mM 4-nitro-7-piperazino-2,1,3-benzoxadiazole (Tokyo Kasei Kogyo, Tokyo, Japan), and then 50 μL each of 280 mM triphenylphosphine and 2,2′-dipyridyl disulfide (Tokyo Kasei Kogyo) was added and mixed. The resultant solution was injected into a column-switching HPLC system. A TSKgel ODS-80Ts column (Tosoh Co., Tokyo, Japan) was used for isolation and quantification of total 3HB in the homogenates; the mobile phase was methanol/H_2O (33/67, v/v) at a flow rate of 0.7 mL/min. Isolated (D+L)-3HB was eluted at about 26 min. Two OD-RH columns (Daicel Co., Osaka, Japan) connected in tandem were used for the enantiomeric separation of D- and L-3HB eluted with acetonitrile/H_2O (40/60, v/v); the flow rate was isocratic at 0.3 mL/min. D- and L-3HB were eluted at about 39 min and 42 min, respectively. Fluorescence detection was performed at 547 nm with a 491-nm excitation wavelength. D- and L-3HB were quantitatively determined from the peak areas on chromatograms.

Aliquots (10 μL) of homogenates were used for protein determination using the BioRad protein assay kit with BSA as a standard. The total 3HB concentration was expressed as micromolar per milligram of protein.

Cardiomyocyte isolation and culture

Primary neonatal cardiomyocytes were isolated according to the publication of Hu et al. (Hu et al., 2004). In brief, the ventricles of neonatal Wistar rats (1 to 2 days old) were minced with scissors into pieces. Ventricle pieces were digested at 37 °C with the digestion buffer containing 137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 10 mM glucose, and 1.25 mg/mL pancreatin. At 20 min intervals, supernatant containing dispersed cells was transferred to sterile tubes containing fetal calf serum, and additional digestion buffer was added to tissue residue. After the digestion was complete, cells suspensions were collected and centrifuged. Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) and were plated to a culture dish. After incubation at 37 °C for 1 h, non-adherent cells as cardiomyocytes were collected. Cardiomyocytes were cultured in DMEM containing 10% fetal bovine serum and 0.1 mmol/L bromodeoxyuridine. The cultured medium was renewed every 48 h, and cells were used for experiments within 3∼7 days.

Effects of D-, L-, and (D+L)-3HB on glucose utilization by cardiomyocytes

On the day of the study, cells were washed twice with PBS, and each well was loaded with 200 μL of cultured medium supplemented with D-, L-, or (D+L)-3HB. The concentrations of D- and L-3HB were as indicated in the legends of Figs. 2 and 3. After incubation for 4 h at 37 °C, the medium was transferred to Eppendorf tubes, and cells were lysed with 0.1% of Triton X-100 for protein determination. Glucose contents in the cultured medium (the blank), medium in the presence of cells (the control), and medium containing D- or L-3HB in the presence of cells (the samples) were measured using a Sigma glucose assay kit. Glucose utilization was calculated from the difference of the glucose content in the blank minus that in the control or that in the samples. Resultant values were divided by the protein content, and the results are presented as percentages with the control set to 100%. The addition of D-or L-3HB was confirmed to have no influence on glucose measurements, since there was no difference in measurements between cultured medium only and medium containing D- or L-3HB.

Statistical analysis

All data are expressed as the mean ± SEM. The statistical significance of the difference between groups was analyzed by Student's *t*-test for unpaired data. Differences were considered significant when $p < 0.05$ was obtained.

Results

Distributions of D- and L-3HB in normal rat brain, liver, heart, and kidney

Since L-3HB is preferred substrate for lipid synthesis in the brain, kidney, and liver (Webber and Edmond, 1977), we chose these organs as targets for enantiomeric analysis of 3HB. In addition, the heart was also chosen because ketone bodies competed with glucose (Chen et al., 1984), fatty acid (Forsey et al., 1987) or palmitate (Vanoverschelde et al., 1993) as the metabolic fuel for the heart. Table 1 depicts the distributions of D- and L-3HB in these organs. Total 3HB in the brain homogenate could not be determined since the trace amount was below the detection limit of the HPLC method used. Although D-3HB may be an energy substrate for the brain, this result is reasonable because in a normal state, ketone bodies are present in small amounts in the blood (Laffel, 1999). Thus the absence of 3HB is expected, as the brain could not use fatty acids for energy; β-oxidation had not taken place. On the other hand, certain amounts of 3HB were found in the liver, heart, and kidney homogenates. The total 3HB quantities which the various organs contained were in the order of liver ≥ heart > kidney. The liver containing the most-abundant 3HB is in agreement with the finding that the liver is the major site responsible for 3HB formation (VanItallie and Nufert, 2003). When considering the enantiomeric 3HB contents, however, the heart homogenate contained a quite-significant ratio (D/L=66/34) as compared to that of the liver (D/L=98/2) and kidney (D/L=87/13) homogenates. While a moderate ratio of D/L-3HB was found in the kidney homogenate, its amount was quite small; similar to that in the liver. We thus focused our attention on the heart, and D- and L-3HB distribution in the diabetic heart was measured since accumulation of ketone bodies was commonly found in DM.

Table 1
Distributions of D- and L-3HB in various homogenates

	Total 3HB (μM/mg protein)	L-3HB (μM/mg protein)	D-3HB (μM/mg protein)
Brain	ND	ND	ND
Liver	132.65±8.61	2.79 (2.10%)	129.86 (97.90%)
Heart	109.41±8.79	37.67 (34.43%)	71.74 (65.57%)
Kidney	35.39±3.44	4.75 (13.43%)	30.64 (86.57%)

Data are the mean ± SEM ($n=5$). The percentage of D- or L-3HB is expressed in parentheses. ND, not determined.

Chromatograms in Fig. 1 show the enantiomeric analysis of 3HB in normal and diabetic rat heart homogenates. In the diabetic heart homogenate, total 3HB was approximately 8.5-fold greater than that in normal heart homogenate; D- and L-3HB were increased to 788.06 and 128.71 μM/mg protein, respectively (Table 2). The increase in L-3HB (3.42-fold) in the diabetic heart homogenate was lower compared to that of D-3HB (10.98-fold), and this caused a decrease in the ratio of L-3HB from 34.43% in normal to 14.04% in DM rat hearts. The result indicated that it was D-3HB accumulated in DM. As deficient glucose utilization and hyperketonemia are hallmarks in DM, we were thus interesting in exploring the relationship between glucose utilization and altered ratio of D/L-3HB.

Distinct effects of D-, L-, and (D+L)-3HB on glucose utilization by cardiomyocytes

Primary cultured cardiomyocytes were used for the estimation of the influence on glucose utilization by D- or L-3HB. We loaded D-3HB into the medium to simulate hyperketonemia in a concentration of 5 mM; this concentration was determined according to previous literature (Sultan, 1992; Kashiwaya et al., 1994; King et al., 2001; Hasselbaink et al., 2003). It was found as shown in Fig. 2 that the glucose utilization of cardiomyocytes decreased in the presence of D-3HB, and this decreased glucose utilization caused by D-3HB was dose-dependent. We chose an incubation time of 4 h because prolonged incubation of over 4 h resulted in similar extents of inhibition (data not shown). In a concentration of 5 mM of D-3HB, a maximum inhibition effect of 61% of the control was found, but L-3HB did not interfere with glucose utilization in concentrations of from 0.5 to 5 mM. Additionally, when cells were in medium containing L-3HB and 5 mM of D-3HB, the reduced glucose utilization caused by D-3HB gradually recovered with increasing concentrations of L-3HB (Fig. 3). Although a ratio of 2/1 (D/L) was found in normal rat hearts, in the presence of 5 mM D-3HB, glucose utilization was not

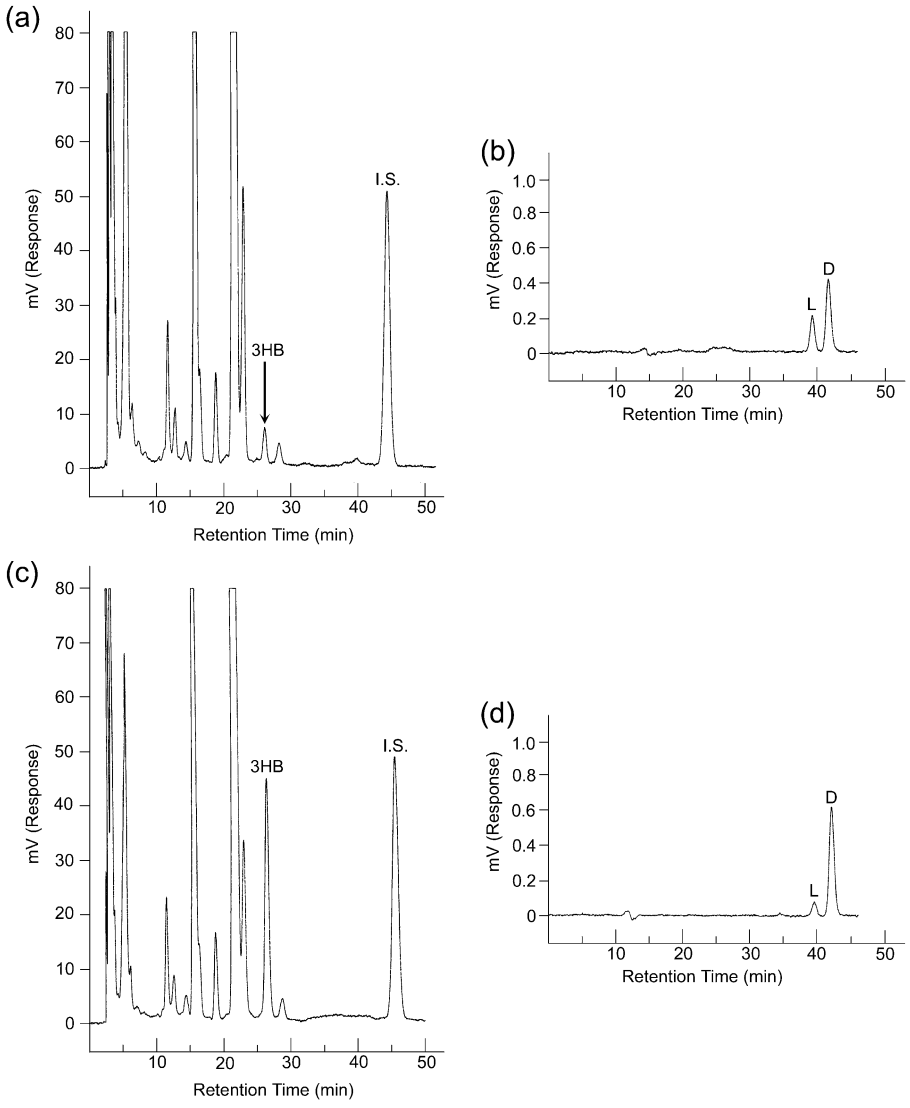

Fig. 1. HPLC chromatograms of enantiomeric analysis of 3HB in normal and diabetic heart homogenates. Total 3HB was first isolated from normal (a) and diabetic (c) heart homogenates, and 3HB was simultaneously separated into D- and L-3HB as shown in (b) and (d) for normal and diabetic heart homogenates, respectively. In diabetic rat heart homogenates, elevated 3HB was found, but the ratio of L-3HB was decreased to 14.04%. I.S., internal standard.

normalized to the control level until D- and L-3HB were loaded in a racemic ratio of 1/1. It should be noted that in the experiment we used cardiomyocytes isolated from neonatal rats, and cultured neonatal cardiomyocytes have different physiological properties compared with adult rat hearts. The influence of pH was excluded as we used the sodium salts of D- and L-3HB in the experiment. These data suggest that L-3HB does not participate in the regulation of glucose utilization, but D-3HB does. On the other hand, the metabolism of D-3HB was disturbed in the presence of L-3HB, resulting in normalization of glucose utilization by cardiomyocytes.

Table 2
Determination of total, D-, and L-3HB in normal and diabetic rat hearts

	Normal	DM
Total 3HB (μM/mg protein)	109.41±8.79	916.77±97.84
L-3HB (μM/mg protein)	37.67 (34.43%)	128.71 (14.04%)
D-3HB (μM/mg protein)	71.74 (65.57%)	788.06 (85.96%)

Data are the mean±SEM (n=5). The percentage of D- or L-3HB is expressed in parentheses.

Discussion

In the present work, we showed the distributions of D-and L-3HB in the rat liver, heart, and kidney. We found that the heart contained largest amount of L-3HB among all examined organs. Although the liver is the main site for ketone bodies production, L-3HB only occupied 2.10% of total 3HB, which is identical to that in rat serum (Tsai et al., 2003). Large amount of L-3HB in the heart maybe suggested as the result of the heart possessing active uptake, or L-3HB was produced in the heart. Transport of ketone bodies as well as other monocarboxylates across the plasma membrane is mediated via monocarboxylate transports (Poole and Halestrap, 1993). Using rat heart cells Wang et al. had determined Michaelis constant (K_m) and maximum reaction rate (V_{max}) for transport of either D- or L-

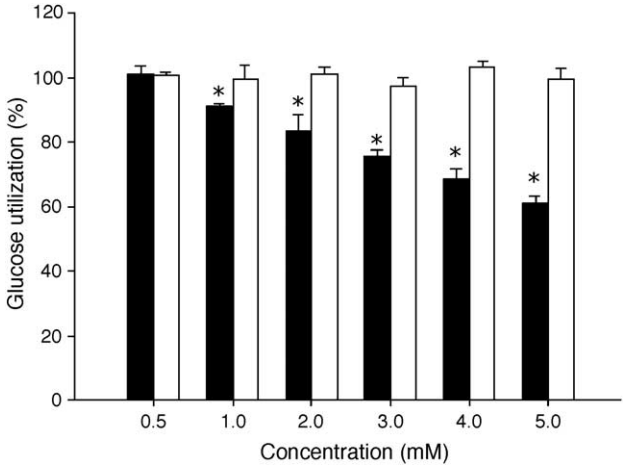

Fig. 2. Glucose utilization of cardiomyocytes in the presence of D- or L-3HB. Cardiomyocytes were independently treated with D- or L-3HB. After an incubation of 4 h, glucose utilization decreased when D-3HB (■) was present, and the decreased glucose utilization caused by D-3HB was dose-dependent. However, L-3HB (□) had no effect on glucose utilization in any of the test concentrations. Each group was from at least 6 independent experiments. * $p<0.05$, the control vs. D- or L-3HB-treated cells.

3HB (Wang et al., 1996). The authors indicated that no significant stereoselectivity was observed with D- or L-3HB. Thus it is unlikely that the heart possesses active uptake of L-3HB. On the other hand, production of L-3HB in cardiomyocytes had been reported during palmitate oxidation (Pinson et al., 1979). The authors described that produced L-3-hydroxybutyryl-CoA would undergo deacylation to be transported outside the cell. However, it is controversial since β-oxidation of fatty acids mainly occurs in the liver, and other investigation failed to identify the formation of L-3HB in the liver (Scofield et al., 1982). The activity of L-3-hydroxybutyryl-CoA deacylase was highest in the liver, but the heart contained <20% of this activity (Reed and Ozand, 1980). In DM when the heart cannot use glucose efficiently, oxidation of fatty acids would be increased to account for ATP production, and our results also showed that the ratio of L-3HB was decreased (Fig. 1). Therefore, if L-3HB is produced in the heart, it is not likely generated via β-oxidation of fatty acids.

In diabetic heart homogenates both D- and L-3HB increased (Table 2). However, these two enantiomers increased to different extents, and the ratio was changed. The increased level of D-3HB is reasonable, due to well-known mechanism that in DM accelerated gluconeogenesis depletes oxaloacetate, diverting acetyl CoA to ketone body production (Lehninger et al., 1993). Decreased activity of D-3HB dehydrogenase also contributes to accumulation of D-3HB, and it results in decreased D-3HB oxidation rate in DM rat (Grinblat et al., 1986).

Concerning on that only limited amount of L-3HB increased in diabetic hearts, we proposed several reasons to account for the result. Production of L-3HB may not be triggered by diabetes, whereas under the circumstance production of D-3HB is stimulated. If L-3HB is increased as well as D-3HB, activities of enzymes responsible for L-3HB may remain as normal, unlike D-3HB dehydrogenase, and therefore L-3HB is utilized to the relative low level. Finally, L-3HB may be produced from the interconversion of accumulated D-3HB. A racemase has been proposed to catalyze the reversible interconversion of L- and D-3-hydroxybutyryl-CoA (Stern et al., 1955). However, we have no exact explanation on the observation so far.

Although glucose is the minor energy fuel for the heart, glucose utilization is associated with cardiac function. Pyruvate oxidation catalyzed by pyruvate dehydrogenase (PDH) is the key step in carbohydrate oxidation (Stanley et al., 1997). In the case of inhibited pyruvate oxidation occurred, such as reperfusion of ischemic myocardium, would lead to a decrease in contractile work (Lewandowski and White, 1995). It had been reported that the products of D-3HB oxidation, NADH and acetyl CoA, could inactivate PDH (Kerbey et al., 1976). Consequently, large amount of D-3HB would decrease glucose utilization, as is the result in Fig. 2. Since L-3HB is a more-favorable substrate than D-3HB, it is surprising that L-3HB had no effect on glucose utilization. This result may suggest that L-3HB is metabolized in a pathway different from that of D-3HB, i.e., no generation of NADH or acetyl CoA during L-3HB metabolism. It had also shown that the oxidation rate of L-3HB was lower than that of D-3HB (Swiatek et al., 1981, 1984). Therefore, unlike other ketone bodies, L-3HB may not be utilized for energy supplements.

Cardiac glucose oxidation is significantly reduced in DM, and diabetic hearts have to utilize fatty acids or ketone bodies to a greater extent. Oxidation of fatty acids or ketone bodies would cause impaired pyruvate oxidation, which results in a vicious circle (Randle et al., 1963, 1966). Fig. 3 showed that the addition of L-3HB could prevent glucose utilization by cardiomyocytes from being influenced by D-3HB. Since monocarboxylate transporters in cardiomyocytes have no significant stereoselectivity with DL-, D- or L-3HB (Wang et al., 1996), uptake of D-3HB may not be inhibited by L-3HB. Our preliminary experiment also showed that uptake of D-3HB was not reduced in the presence of L-3HB (data not shown).

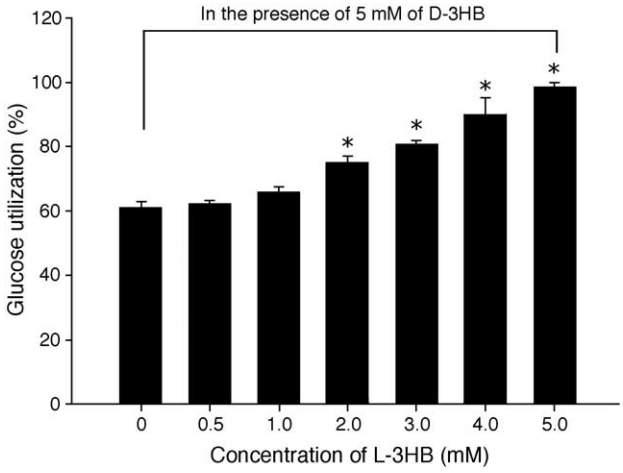

Fig. 3. Glucose utilization of D-3HB-treated cardiomyocytes in the presence of L-3HB. The inhibition of glucose utilization by cardiomyocytes in the presence of 5 mM D-3HB could be overcome in a dose-dependent manner with administration of additional L-3HB. In a ratio of 1/1 (D/L), glucose utilization remained at 98% of the control. Data are from at least 6 independent experiments. * $p<0.05$, significantly different from cells without the presence of L-3HB.

The preventive effect of L-3HB was in a dose-dependent manner; therefore, the simplest explanation maybe that the metabolism of D-3HB might compete with that of L-3HB. Although physiological D-3HB dehydrogenase activity is stereo-specific, Swiatek et al. had proposed some acyl-CoA synthetase enzymes might be capable of activating either isomer of 3HB (Swiatek et al., 1981). The superior utilization of L-3HB might decrease the metabolism of D-3HB. However, due to the lack of clarified physiological routes for the production and utilization of L-3HB, it is uncertain how actually L-3HB effects against D-3HB on glucose utilization. The exact mechanism remained to be investigated.

The term "β-hydroxybutyrate" is also used in many studies on ketone bodies. Yet it is notable that "β-hydroxybutyrate" as well as "3-hydroxybutyrate" are often used to represent D-3HB (Christopher et al., 1995; Jovanovic et al., 1998; Boden and Chen, 1999; Alam et al., 2001) or DL-3HB (Edmond, 1974; Siess, 1985; Poole et al., 1990). Fischer et al. reported that administration of 3 mM of β-hydroxybutyrate to cardiomyocytes could cause a reduction in glucose uptake to 84% of the control within 1 h (Fischer et al., 1997). On the contrary, Tardif et al. showed that incubation of cardiomyocytes with 5 mM of β-hydroxybutyrate for 4 h did not affect glucose uptake (Tardif et al., 2001). Our data that using D-or (D+L)-3HB had different influences on glucose utilization may provide an explanation for the inconsistent results. Assuming that β-hydroxybutyrate refers to D-3HB in Fischer et al.'s work, Fig. 2 showed similar results in which glucose utilization was decreased to the extent of 76% of the control when incubation with 3 mM of D-3HB for 4 h. Assuming that β-hydroxybutyrate refers to DL-3HB in Tardif et al.'s work, Fig. 3 also revealed that DL-3HB had no influence on glucose utilization. While we are uncertain about the true situation, this in part enhances the necessity of a clear indication of which enantiomer of 3HB is used in studies.

Similarly, it is interesting to find a key role of L-3HB when studies of the same subjects used D-3HB or DL-3HB as the substrate. In Zou et al.'s work administration of DL-3HB could protect hearts from ischemia–reperfusion-induced myocardial damage (Zou et al., 2002), but King et al. proved that ketone bodies (4 mM D-3HB plus 1 mM acetoacetate) did not protect hearts during low-flow ischemia (King et al., 2001). Additionally, in rat hearts the rate of glucose uptake increased when 10 mM of (D+L)-3HB was present (Chen et al., 1997), but the glucose flux and net glycolytic flux were reduced in the presence of 4 mM D-3HB plus 1 mM acetoacetate (Kashiwaya et al., 1994). The presence of L-3HB may be critical in investigating cardiac glucose utilization, as L-3HB exists in a significant amount in normal rat hearts.

In conclusion, among various organs we had found an enriched amount of L-3HB in normal rat hearts and D-3HB dramatically increased in diabetic hearts. The 'unbalanced' ratio of D/L-3HB could contribute to reduce glucose utilization in cardiomyocytes, and therefore a physiological role of L-3HB on glucose utilization rather than the energy substrate was proposed.

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