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ORIGINAL

Age-related changes in the diurnal variation of ketogenesis in patients with type 2 diabetes and relevance to hypoglycemic medications

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Abstract. To assess the significance of ketogenesis in the management of diabetes mellitus, we analyzed the factors associated with the diurnal variation of the plasma ketone body levels. The subjects consisted of 220 patients with type 2 diabetes, aged 60 ± 15 years, without advanced complications. They ate a standardized, low-fat meal at 8:00, 12:00, and 18:00. The plasma levels of 3-hydroxybutyrate (3HB) and free fatty acid (FFA) were increased before breakfast and before dinner. The plasma glucose concentration was almost the same at any blood sampling time point among age quartiles. However, the 3HB levels were significantly decreased with age, which was most obvious before dinner. The FFA levels also decreased with age, but the decline was mild. A multiple regression analysis with stepwise selection revealed that age was an independent, negative contributor and that the pre-breakfast FFA concentration was an independent, positive contributor to the pre-breakfast 3HB levels. Regarding the pre-dinner 3HB levels, in addition to age and the pre-dinner FFA concentration, the uses of sulfonylurea and dipeptidyl peptidase-4 inhibitors were independent negative contributors. The metabolism of ketone bodies is an alternative energy source for the brain under conditions of starvation. While excessive ketogenesis leads to critical ketoacidosis, inadequate ketone body production could be associated with a propensity to develop neurohypoglycemia in elderly patients treated with insulin secretagogues. Because age-related changes in ketogenesis were the most significant before dinner, attention should be paid not only to fasting but also to the pre-dinner levels of 3HB.

Key words: Ketosis, 3-hydroxybutyrate, Free fatty acid, Diurnal variation, Aging

TRADITIONALLY, increased ketogenesis in diabetic patients has been a sign of profound metabolic disturbances due to deficient insulin action. The key regulatory steps in ketogenesis include lipolysis of triglycerides in the adipose tissue by hormone-sensitive lipases and transport into the mitochondria *via* carnitine palmitoyltransferase 1. Both steps are hormonally regulated by insulin and glucagon. In liver mitochondria, fatty acid-derived acyl-CoA is metabolized to acetyl-CoA through β-oxidation. Acetyl-CoA may enter the Krebs cycle, or it may be converted to one of three forms of ketone bodies, namely, acetoacetate, 3-hydroxybutyrate (3HB), and acetone. The ketogenic fate of acetyl-CoA is determined by mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2, which is transcriptionally regulated by the forkhead box transcription factor FOXA2 [1]. Insulin signaling inactivates FOXA2 by phosphorylation and nuclear export, whereas glucagon activates FOXA2 by p300 acetylation [2].

In type 1 diabetes, insulin deficiency, combined with hyperglucagonemia, causes diabetic ketoacidosis, a life-threatening condition. However, ketone bodies and free fatty acid (FFA) are important energy sources in various tissues under normal physiological conditions. The brain is incapable of using FFA as fuel; therefore, ketone bodies, which are water-soluble and cross the blood-brain barrier, are used by the brain when the glucose supply is inadequate [3]. Furthermore, several lines of evidence have shown that ketone bodies are favorable energy sources for the brain under pathological conditions. The ketogenic diet is an established and effective nonpharmacological treatment for epilepsy

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[4-7]. In an animal model of epilepsy, an intraperitoneal injection of acetoacetate was protective against seizures [8]. Ketosis was neuroprotective in ischemic rat brains after reversible middle cerebral artery occlusion [9]. The ketogenic diet was shown to be beneficial in improving the cognitive function of patients with Alzheimer's disease [10] as well as in a mouse model of Alzheimer's disease [11]. Because ischemic brain infarction is a common chronic vascular complication of diabetes and type 2 diabetes is a major risk factor for the development of Alzheimer's disease, ketone bodies may be an important energy source in the brains of elderly patients with type 2 diabetes.

Although plasma ketone body levels are usually measured in the morning after an overnight fast, there is a marked diurnal fluctuation in the ketone body concentration. Plasma ketone bodies increase around midnight and in the early morning, which is most likely because of the decreased glucose supply and the elevation in the cortisol and growth hormone levels [12, 13]. During the daytime, ketone body levels are largely influenced by the ingestion of carbohydrates, which potently suppress ketogenesis. Additionally, hypoglycemic agents may modulate lipolysis and ketogenesis in diabetic patients. Therefore, in this study, we analyzed the association between age and the diurnal profile of ketogenesis in patients with type 2 diabetes, as well as the possible effects of hypoglycemic medications.

Materials and Methods

The subjects in this study were 220 inpatients with type 2 diabetes, aged 60 ± 15 years, with a body mass index (BMI) of 25.7 ± 5.3 (Table 1). The diagnosis of type 2 diabetes was established based on the American Diabetes Association [14] and Japan Diabetes Society [15] criteria for diabetes, as well as on the absence of pancreatic autoimmune markers, including GAD antibodies and IA-2 antibodies. We excluded patients with renal failure or severe liver disease. They ate a standardized low-fat meal at 8:00, 12:00 and 18:00. The daily carbohydrate intake was 4.3 ± 0.5 g/kg ideal body weight (22 × height^2). They performed only mild exercise, such as walking, during the study period. We measured the pre- and postprandial levels of plasma glucose, 3HB, and FFA (BB, AB, BL, AL, BD, AD, 22:00). The 3HB and FFA values were measured by enzymatic methods using an auto analyzer (Hitachi High-Tech, Tokyo, Japan).

Table 1 Characteristics of the subjects

N (male/female)	220 (132/88)
Age (years)	60 ± 15
BMI (kg/m^2)	25.7 ± 5.3
Glucose (mg/dL)	147 ± 62
HbA1c (%)	8.9 ± 2.0
AST (U/L)	26 ± 18
ALT (U/L)	29 ± 27
γ-GTP (U/L)	50 ± 71
LDL cholesterol (mg/dL)	115 ± 37
HDL cholesterol (mg/dL)	48 ± 14
Triglycerides (mg/dL)	157 ± 154
Albumin (g/dL)	3.9 ± 0.5
eGFR (mL/min/1.73 m^2)	84 ± 31
C-peptide (ng/mL)	2.0 ± 1.0
Carbohydrate intake (g/kg IBW)	4.3 ± 0.5
Pharmacological treatment (n)	
Metformin	73
Sulfonylurea	68
DPP-4 inhibitor	55
α-Glucosidase inhibitor	22
Pioglitazone	8
Prandial insulin	52
Basal insulin	55
GLP-1 receptor agonist	5

Statistics

The data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SAS v.9.3 (SAS Institute, Cary, North Carolina, USA). Pearson's correlations were used to evaluate the factors associated with the pre-breakfast and pre-dinner 3HB levels. One-way analysis of variance (ANOVA) was used for the comparison of quartiles. The unpaired Student's *t*-test was used to test the difference in the means between two groups. Non-parametric data were compared by the Wilcoxon signed-rank test. Multiple regression analysis with stepwise selection was performed using the pre-breakfast or pre-dinner 3HB value as the dependent variable. The serum triglyceride, FFA, 3HB, and C-peptide values were transformed into logarithms to improve the skewed distribution. The results with a $p < 0.05$ were considered statistically significant.

Results

We assessed the diurnal variation of the plasma glucose (Fig. 1a), 3HB (Fig. 1c), and FFA (Fig. 1e) levels in type 2 diabetic patients. The means and SDs of the 3HB and FFA levels were calculated after logarith-

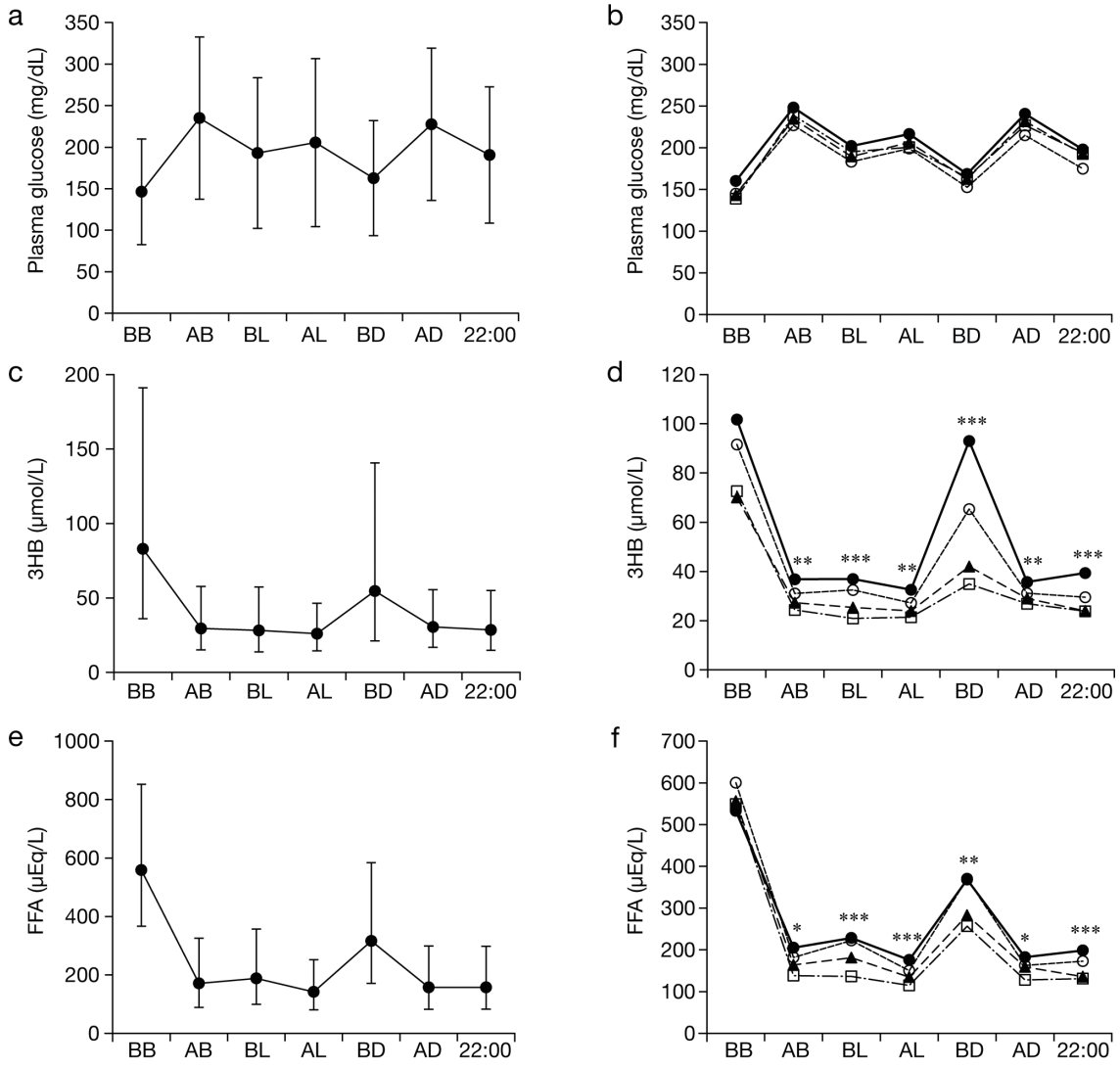

Fig. 1 Diurnal variation of the plasma glucose (a), 3HB (c), and FFA (e) levels. Mean and SD. Means and SDs of the 3HB and FFA levels were calculated after log transformation of the data. Quartile analysis of the plasma glucose (b), 3HB (d), and FFA (f) levels based on age. ●, 1st quartile; ○, 2nd quartile; ▲, 3rd quartile; □, 4th quartile. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ according to ANOVA.

mic transformation. The plasma glucose levels were lowest before breakfast (146 ± 64 mg/dL). The pre-dinner glucose levels (163 ± 69 mg/dL) were higher than the pre-breakfast glucose levels ($p < 0.0001$), but they were lower than the pre-lunch glucose levels (193 ± 91 mg/dL, $p < 0.0001$). Both 3HB and FFA were increased before breakfast and before dinner. To evaluate the effect of aging, the subjects were divided into four groups (n = 55 each) based on age as follows: 1st quartile < 50.9 years, 2nd quartile < 62 years, 3rd quartile < 71.4 years, and 4th quartile > 71.4 years. There was no difference in the plasma glucose concentration at any blood sampling time point between the age quartiles (Fig. 1b). However, the 3HB levels significantly decreased with age at each time point except before breakfast, which is when the difference did not reach statistical significance ($p = 0.051$ by ANOVA) (Fig. 1d). The age-related decline in the plasma 3HB level was the most obvious before dinner when the levels in the 1st quartile were significantly higher than those in the 3rd and 4th quartiles, and the levels in the 2nd quartile were significantly higher than those in the 4th quartile based on the nonparametric Wilcoxon signed-rank test with Bonferroni correction after ANOVA. The FFA levels, except the fasting level, also decreased with age, although the decline was mild (Fig. 1f). Therefore, when

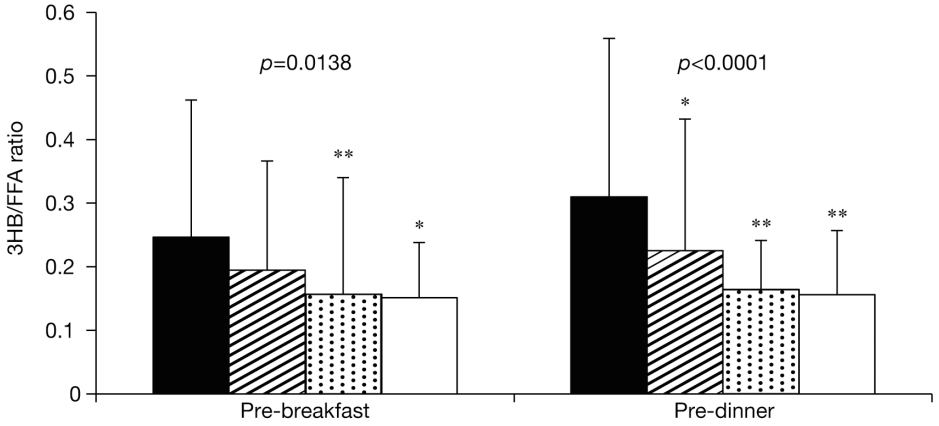

Fig. 2 Age-related reduction in the pre-breakfast and pre-dinner 3HB/FFA ratios.
■, 1st quartile; ▨, 2nd quartile; ⁝⁝, 3rd quartile; and □, 4th quartile. Mean and SD. *P*-values in the figure were obtained by ANOVA. **p* < 0.05, ***p* < 0.01 *vs*. the 1st quartile estimated by the Wilcoxon signed-rank test with Bonferroni correction.

Table 2 Pearson correlation analysis of the factors associated with the pre-breakfast and pre-dinner 3HB levels

	Pre-breakfast		Pre-dinner	
	coefficient	*p*-value	coefficient	*p*-value
Age	-0.1363	0.0435	-0.3657	< 0.0001
Male gender	0.0561	NS	-0.0484	NS
BMI	0.1237	NS	0.1975	0.0033
ALT	0.0397	NS	0.0671	NS
Albumin	0.0278	NS	0.1480	0.0282
eGFR	0.1592	0.0182	0.2669	< 0.0001
log(triglycerides)	-0.1015	NS	0.1305	NS
LDL cholesterol	-0.0417	NS	0.1694	0.0118
log(FFA)	0.6912	< 0.0001	0.7766	< 0.0001
Glucose	0.1745	0.0095	-0.0334	NS
log(C-peptide)	-0.0062	NS	0.0733	NS
Carbohydrate intake	-0.0819	NS	-0.2667	< 0.0001
Metformin	0.0537	NS	0.0127	NS
Sulfonylurea	-0.0862	NS	-0.2328	0.0005
DPP-4 inhibitor	-0.0812	NS	-0.2326	0.0005
α-glucosidase inhibitor	-0.0514	NS	-0.1273	NS
Pioglitazone	-0.0609	NS	-0.1102	NS
Prandial insulin	-0.0438	NS	-0.0864	NS
Basal insulin	-0.0711	NS	-0.1240	NS
GLP-1 receptor agonist	0.0806	NS	0.1672	0.013

the efficiency of ketone body production was shown as the ratio of 3HB to FFA, the ratio declined with age both before breakfast and before dinner (Fig. 2).

Pearson's correlation analysis showed that the pre-breakfast 3HB levels were correlated with age, eGFR, log (FFA), and plasma glucose levels and that the pre-dinner 3HB levels were correlated with age, BMI, plasma albumin, eGFR, LDL cholesterol, log (FFA), carbohydrate intake per ideal body weight, use of sulfonylureas, use of dipeptidyl peptidase-4 (DPP-4) inhibitors, and use of GLP-1 receptor agonists (Table 2). A multiple regression analysis with stepwise selection revealed that age was an independent negative contributor and that the pre-breakfast FFA concentration was an independent positive contributor to the pre-breakfast 3HB levels (Table 3). Regarding pre-dinner 3HB levels, in addition to age and pre-dinner FFA concentration, uses of sulfonylureas and DPP-4 inhibitors were independent negative contributors (Table 4).

Table 3 Independent contributing factors to the pre-breakfast 3HB levels according to multiple regression analysis and stepwise selection

	Parameter estimate	SE	*p*
Age	-0.1854	0.0475	0.0001
Pre-breakfast FFA level	0.7042	0.0475	< 0.0001

Table 4 Independent contributing factors to the pre-dinner 3HB levels according to multiple regression analysis and stepwise selection

	Parameter estimate	SE	*p*
Age	-0.1844	0.0390	< 0.0001
Pre-dinner FFA level	0.7154	0.0404	< 0.0001
Use of sulfonylureas	-0.1109	0.0435	0.012
Use of DPP-4 inhibitors	-0.0981	0.0403	0.025

Discussion

It is obvious that plasma ketone bodies increase at fasting, and decrease after food intake or glucose load [16, 17]. However, to our best knowledge, the diurnal variation of plasma ketone bodies has not been characterized yet. Most studies so far have focused on ketogenesis in the morning, and the significance of pre-dinner ketogenesis remained to be elucidated. In this study, we showed that both FFA and 3HB, the most abundant circulating ketone bodies, were elevated when the plasma glucose levels were low, namely, before breakfast and before dinner. The diurnal profiles of 3HB and FFA were approximately the mirror image of the plasma glucose profiles.

It is well known that young children frequently develop ketosis in response to glucose shortage. Among non-diabetic children beyond infancy, idiopathic ketotic hypoglycemia is the most common cause of hypoglycemia [18]. The episodes usually become milder and more infrequent by the age of 5 years and rarely occur after the age of 9 years. However, age-associated changes of ketogenesis in adulthood have not been well described, although some animal studies have shown an age-related reduction of ketogenesis [19, 20].

In this study we found that the plasma 3HB levels significantly decreased with age, and the decline was most obvious before dinner. The pre-dinner 3HB levels were comparable to the pre-breakfast levels in younger subjects even though the duration of pre-dinner fasting was much shorter than that of pre-breakfast fasting. In the 3rd and 4th age quartiles, however, the pre-dinner elevation of 3HB levels was nominal. The age-related decline may be, at least in part, attributable to the reduced lipolytic activity in elderly people because in vitro studies have shown that hormonal and pharmacological stimulation of lipolysis diminishes with age [21, 22]. The decline in the plasma 3HB levels was larger than that in the plasma FFA levels, suggesting that ketogenetic activity may also decrease with age, which is most likely due to impaired mitochondrial function. The reason why the age-related difference was marked before dinner but not before breakfast is not clear. One explanation is that younger individuals may have a higher energy demand and, therefore, consume glucose fully in a short time. Another possible explanation is that it may take longer for elderly people to switch from burning glucose to burning fat. The age-related decrease in ketogenetic activity may explain why ketosis-prone atypical diabetes induced by the over-consumption of a sugar-containing beverage is predominantly observed in younger individuals [23], while hyperglycemic hyperosmolar nonketotic syndrome occurs most often in older people.

Although uncontrolled up-regulation of ketogenesis results in critical ketoacidosis, ketone bodies are physiological metabolites serving as alternative energy substrates for glucose in a variety of tissues, such as the brain, heart, kidney, and skeletal muscles. 3HB is converted to acetoacetate in mitochondria by β-hydroxybutyrate dehydrogenase and then transformed to acetoacetate-CoA by oxoacid-CoA transferase. Acetyl CoA, generated from acetoacetate-CoA, enters the Krebs cycle and produces ATP. Ketone bodies may have a neuroprotective effect, and they represent an alternative fuel for both the normal and injured brain [24, 25]. Experimental studies using rats

have shown that ketone bodies are protective against hypoglycemia-induced neuronal damage [26, 27]. Therefore, impaired ketogenesis could make the brain vulnerable to hypoglycemia.

In addition to age, hypoglycemic drugs may affect ketone body production. The multiple regression analysis revealed that sulfonylurea use and DPP-4 inhibitor use are negative contributors to the pre-dinner 3HB levels. Sulfonylureas stimulate insulin secretion, and DPP-4 inhibitors augment glucose-induced insulin secretion as well as inhibit glucagon release. Lipolysis is controlled by β-adrenergic stimulation and suppression by insulin, both of which affect the cytoplasmic cyclic AMP levels [28, 29]. However, ketogenesis is subject to bihormonal control through the relative blood concentrations of insulin and glucagon [30]. Sulfonylureas and DPP-4 inhibitors may modulate the plasma 3HB levels through their effects on insulin and glucagon secretion. GLP-1 receptor agonists most likely have similar effects on ketogenesis. However, their role was not determined in this study because only five subjects were treated with GLP-1 receptor agonists. It has been shown that the administration of sulfonylureas resulted in the reduction of fasting plasma ketone body levels [31, 32]. As far as we could search, however, the effect of DPP-4 inhibitors on plasma ketone bodies has not been reported in the literature. On the other hand, the potent antiketogenic effect of insulin has been established in clinical and experimental studies. The insignificant association between insulin use and plasma 3HB levels did not exclude the role of insulin treatment in the regulation of ketogenesis.

Although Pearson's correlation analysis showed a significant inverse correlation between the pre-dinner 3HB levels and carbohydrate intake per ideal body weight, the carbohydrate intake did not remain an independent contributor to the pre-dinner 3HB levels in the multiple regression analysis with stepwise selection. This was most likely because the subjects in this study were inpatients receiving hospital foods containing an adequate amount of carbohydrates. However, it is obvious that strict carbohydrate restriction up-regulates lipolysis and ketogenesis. Similarly, the administration of an SGLT-2 inhibitor, which enhances urinary glucose excretion, resulted in a carbohydrate shortage and increased ketogenesis in rats [33]. Therefore, ketogenesis has a greater physiological and pathological effect on patients with a low-carbohydrate diet and those who are given SGLT-2 inhibitors. The significant association of eGFR with the pre-breakfast and pre-dinner 3HB levels obtained by the Pearson correlation analysis was attributable to an inverse correlation between eGFR and age. eGFR did not remain in the model by the multiple regression analysis with step-wise selection.

In conclusion, the daily profiles of the plasma 3HB and FFA levels in patients with type 2 diabetes mirrored the plasma glucose levels; they showed peaks before breakfast and before dinner. Although both 3HB and FFA levels decreased with age, the decline was more marked in the former. The age-related reduction of 3HB was minimal before breakfast, but the pre-dinner 3HB levels obviously decreased with age. Furthermore, the use of sulfonylurea and DPP-4 inhibitors was negatively associated with pre-dinner 3HB levels. The age-related decline in 3HB levels may result from the reduction in lipolysis and the impairment in mitochondrial function. While increased ketogenesis in younger individuals may be associated with a higher risk of ketoacidosis, inadequate ketogenesis in elderly subjects may be associated with a propensity to develop neurohypoglycemia. The observations in this study indicate that attention should be paid not only to the fasting but also the pre-dinner levels of 3HB when treating diabetic patients with hypoglycemic agents.

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