Original Research

A ketogenic diet impairs energy and glucose homeostasis by the attenuation of hypothalamic leptin signaling and hepatic insulin signaling in a rat model of non-obese type 2 diabetes

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Abstract

Ketogenic diets (KTD) are reported to have beneficial effects on the regulation of energy and glucose homeostasis, but remain controversial. We investigated the effects of KTD and ketones on insulin resistance and secretion in non-obese type 2 diabetic rats and their mechanism. KTD (82% energy as fat), intraperitoneal injection of β-hydroxybutyrate (IHB; 150 mg/kg bw/12 h) with a control diet (COD; 20% energy as fat) or saline injection with COD was given to 90% pancreatectomized (Px) diabetic rats for five weeks. KTD increased epididymal fat pads and serum leptin levels without increasing energy intake, but IHB decreased them. KTD, but not IHB, attenuated hypothalamic signal transducer and activator of transcription 3 and 5′-adenosine monophosphate-activated protein kinase (AMPK) phosphorylation in KTD. Serum glucagon levels were markedly higher in the KTD group than in other groups. During an oral glucose tolerance test, serum glucose levels slowly increased until 80 min in the KTD group and then decreased very slowly. Insulin secretion capacity during a hyperglycemic clamp was significantly lower in the IHB group than in other groups. However, a euglycemic hyperinsulinemic clamp revealed that KTD decreased glucose infusion rates and increased hepatic glucose output in hyperinsulinemic states while IHB had opposite effects to KTD. The increased hepatic glucose output in KTD was associated with increased hepatic phosphoenolpyruvate carboxykinase expression through attenuated tyrosine phosphorylation of IRS2 and phosphorylation of Akt^{Ser473}. Hepatic $AMPK^{Thr172}$ phosphorylation was reduced in KTD. In conclusion, KTD impairs energy and glucose homeostasis by exacerbating insulin resistance and attenuating hypothalamic leptin signaling in non-obese type 2 diabetic rats. These changes are not associated with increased serum ketone levels.

Keywords: β-hydroxybutyrate, insulin secretion, insulin resistance, leptin, insulin signaling, AMPK

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Introduction

A ketogenic diet (KTD) is most commonly used as a weight loss diet due to decreased appetite in obese subjects and it has been reported to reverse insulin resistance and perhaps type 2 diabetes.[1,2] Some studies have supported the assertion that low carbohydrate diets result in a reduction in body fat with improved glycemic control.[3,4] On the contrary, some studies have revealed the opposite results.[5,6] The value or lack of value for using KTD for managing type 2 diabetes remains highly contentious. The putative antidiabetic functions of KTD may be incidental to weight loss. If the antidiabetic effects of KTD are a consequence of weight loss, it would have little or no benefit for non-obese diabetics. In addition, it is unclear what components of KTD regulate energy and glucose metabolism. The important changes in KTD are the increase of serum ketone levels, but the effects of increased circulating ketones as a result of a KTD are little studied in type 2 diabetes. In addition to increasing serum ketone levels, a KTD changes circulating leptin, free fatty acids and glucagon, which are known to lead to insulin resistance and perhaps diabetes.[7] Therefore, KTD would seem to be contraindicated in type 2 diabetes. Furthermore, the action mechanism of KTD in energy and glucose metabolism remains unclear in type 2 diabetes.

The brain, especially the hypothalamus, is a major site for integration of central and peripheral signals that regulate energy homeostasis.[8] Leptin is known to relay the status

of fat stores and proper leptin signaling in the hypothalamus is critical to normal regulation of food intake and body weight.[8] Hypothalamic leptin receptor signaling is mediated by Janus kinase 2 (JAK2) → signal transducer and activator of transcription (STAT3). Hypothalamic leptin resistance, possibly due to defective nutritional regulation of leptin receptor expression and/or reduced STAT3 signaling in the hypothalamus, contributes to the development of obesity associated with high-fat feeding.[8] In addition, hypothalamic 5′-adenosine monophosphate-activated protein kinase (AMPK) plays an important role in regulating food intake and energy balance.[9,10] Activation of AMPK in the hypothalamus is sufficient to increase food intake and body weight by enhancing mRNA expression of orexigenic neuropeptides, neuropeptide Y and agouti-related peptide; suppression of hypothalamic AMPK activity is sufficient to decrease the expression of orexigenic neuropeptides.[9,10]

Type 2 diabetes is typically associated with obesity and overweight in Western countries. However, a substantial minority of Western type 2 diabetic patients are normal weight, as are the majority of Asian type 2 diabetics.[11] Recent studies have revealed that when lean people in Asia develop type 2 diabetes, the diabetic progression is faster in non-obese people than in obese people.[11,12] Insulin resistance easily develops as a result of obesity, but is not sufficient to progress to type 2 diabetes since insulin secretion can compensate for it with hyperinsulinemia to maintain normoglycemia.[13,14] However, lean Asians often develop diabetes easily due to having less insulin secretion capacity to compensate for insulin resistance.[13,14] These results suggest that the etiology and characteristics of obese and non-obese type 2 diabetes are different, although the mechanism remains obscure.

The effects of KTD on obesity and associated type 2 diabetes has been the subject of considerable study with conflicting results, but it has not been studied in non-obese type 2 diabetes that is most common in Asian populations. Therefore, the primary goals of this study were (1) to determine if a KTD and/or ketone injection (as β-hydroxybutyrate [IHB]) are beneficial for energy and glucose metabolism in a model of non-obese type 2 diabetes and (2) to determine what mechanism, if any, KTD and/or ketone have in the management of type 2 diabetes. This study was designed to compare the effects of KTD, a normal diet, and a normal diet with high circulating ketone concentrations in non-obese type 2 diabetic rats, rather than the more commonly used obese murine models. This study was performed in an established type 2 diabetic animal model with non-obese and mild symptoms, the 90% pancreatectomized (Px) rat.[15–17]

Materials and methods

Animals and diets

Male Sprague Dawley rats weighing 198 ± 21 g were housed individually in stainless-steel cages in a controlled environment (23°C and a 12 h light and dark cycle) with unrestricted access to assigned diets and water. All surgical and experimental procedures conformed with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and they were approved by the Institutional Animal Care and Use Committee of Hoseo University, Korea. Rats had a 90% pancreatectomy by the Hosokawa technique[18] or sham-operation (Sham) after being anesthetized with ketamin and xylazine (100 mg and 10 mg/kg body weight [bw], respectively). After one week of surgery recovery, the Px rats showed the characteristics of mild type 2 diabetes mellitus with insulin deficiency and normal body weight while Sham rats showed no symptoms of diabetes.

The compositions of the KTD and control diets (COD) are shown in Table 1. Since our preliminary study showed that rats given a KTD diet consumed 1.8-fold less than those provided a COD diet, KTD contained 1.8-fold more by weight of protein, minerals and vitamins than COD. The COD and KTD diets were based on a modified AIN-93 diet.[19] KTD contained 0% of energy from carbohydrates, 18% from protein and 82% from fat, while COD contained 62%, 18% and 20% energy as carbohydrates, protein and fat, respectively. Since the two diets had different energy densities, and rats in KTD consumed less amounts of foods, vitamin, mineral and cellulose were increased in the KTD to compensate for its energy density.

Experimental design and metabolic analysis

Px rats were weight matched and divided into three groups of 20 each and provided assigned diet and injection for five weeks: (1) saline injection and KTD (Px-KTD); (2) intraperitoneal injection of saline and COD (Px-COD); and (3) intraperitoneal injection of IHB and COD (Px-IHB). Sham rats were used as a normal control (Sham-COD) and they had the same treatment as the COD group.

To confirm that the effects of KTD were due to the increased serum ketone levels, ketone as IHB (Sigma, St Louis, MO, USA) was provided to Px animals fed COD. Therefore, rats in the Px-IHB group were given IHB by intraperitoneal injections (150 mg/kg bw of each) twice a day at the beginning of the dark and light cycles. Rats in the

Table 1 Nutrient composition of experimental diets (g/kg diet)

	Control diet (COD)	Ketogenic diet (KTD)
Corn starch	550	–
Sucrose	80	–
Casein	185	275
Soybean oil	5	8
Hydrogenated corn oil*	85	567
Cellulose	40	63
AIN-76 mineral mixture	35	55
AIN-76 vitamin mixture	10	16
Choline bitartarate	2	3
DL-methionine	3	5
Cholesterol	5	8
Metabolic energy (kcal/g)	4.07	6.28

*Fatty acid content: 14:0 (4.5%), 16:0 (19.2%), 16:1 (4.8%), 18:0 (6.4%), 18:1 (29.9%), 18:2 (n-6) (5.3%), 18:3 (n-3) (0.9%), 20:0 (0.8%), 20:1 (6.9%), 22:0 (0.4%) and 22:1 (6%). Trans fat content: 14.4%

Px-KTD, Px-COD and Sham-COD groups had saline injections by the same procedure as IHB. The calories from ketone in the IHB group were negligible. Overnight-fasted serum glucose levels, food and water intake, and body weight were measured every Tuesday before starting the light cycle. All rats were given an oral glucose tolerance test (OGTT) on the third week following an overnight fast by orally administering 2 g/kg of glucose. Serum glucose and insulin were measured in tail vein blood at 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 120 min after glucose loading, and the average areas under the curve for glucose and insulin were calculated. Serum glucose levels were analyzed with a Glucose Analyzer II (Beckman, Palo Alto, CA, USA) and serum insulin, glucagon and leptin levels were measured using a commercial RIA kit (Linco Research, St Charles, MO, USA). Overnight-fasted serum free fatty acid levels were measured using a NEFA C kit (Wako Chemicals, Osaka, Japan). After the injection of IHB at the light cycle, blood was collected at 1, 6 and 12 h and serum IHB levels were measured with IHB kit (BioVision Inc, Mountain View, CA, USA).

Glucose-stimulated insulin secretion

Half of the rats from each group were randomly selected for a hyperglycemic clamp and the remainder used for a euglycemic hyperinsulinemic clamp. After three weeks of treatment, catheters were surgically implanted into the right carotid artery and left jugular vein of rats anesthetized with intraperitoneal injections of ketamine and xylazine (100 mg and 10 mg/kg bw, respectively). After 5–6 days of implantation, a hyperglycemic clamp was performed in free-moving and overnight-fasted rats to determine insulin secretion capacity as described in previous studies.[20] During the clamp, glucose was infused to maintain serum glucose levels of 5.5 mmol/L above baseline and serum insulin levels were measured at designated times. After the clamp, rats were freely provided diets and water for two days and the following day they were deprived of food for 16 h. Five rats from each dietary group were then anesthetized with a mixture of ketamine and xylazine and 100 nmol/L insulin was injected through the inferior vena cava of the rats for 10 min. Immediately after the 10 min, they were killed and the tissues were collected, frozen with liquid nitrogen and stored at -70°C until the immunoblotting analyses and other experiments were performed.

Insulin resistance

Similar to the hyperglycemic clamp, rats were catheterized in the right carotid artery and left jugular vein on the third week. After recovery from the surgery, a euglycemic hyperinsulinemic clamp was performed on fasted conscious rats to determine insulin resistance as previously described.[17,21] [3-^{3}H] glucose (NEN Life Science, Boston, MA, USA) was continuously infused during a four-hour period at the rate of 0.05 μCi/min. Basal hepatic glucose output was measured in blood collected at 100 and 120 min after initiation of the [3-^{3}H] glucose infusion. Then a primed continuous infusion of a recombinant human insulin (Humulin; Eli Lilly, Indianapolis, IN, USA) was initiated at a rate of 20 pmol $\times$ kg^{-1} $\times$ min^{-1} to raise plasma insulin concentration to approximately 1100 pmol/L. Glucose (25%) was infused at variable rates as needed to clamp glucose levels at about 6 mmol/L. While blood glucose levels were steady between 200 and 240 min, the rate of glucose production was determined by measuring the blood levels of [3-^{3}H] glucose and ^3H_2O every 10 min. Clamped hepatic glucose output was calculated by subtracting glucose infusion rates from the rates of glucose appearance. Whole-body glucose disposal was expressed in terms of mg of glucose per kg of body weight per minute required to maintain euglycemia during hyperinsulinemia.[17,21] At the end of the clamp, the rats were anesthetized with sodium pentobarbital (35 mg/kg bw) (Nembutal, Abbott Laboratories, North Chicago, IL, USA) and were killed by decapitation. Tissues were rapidly dissected, weighed and frozen in liquid nitrogen, and stored at -70°C until further measurements of [3-^{3}H] glucose and ^3H_2O.

In order to determine the glycogen content in the liver, its lysates were centrifuged at 3000 rpm for 10 min and the supernatants deproteinized with 1.5 N perchloric acid. The glycogen content was calculated from glucose concentrations derived from glycogen hydrolyzed by α-amyloglucosidase in an acid buffer.[21] Triglyceride was extracted with a chloroform–methanol (2:1, vol/vol) from the liver and re-suspended in pure chroloform.[21,22] Triacyglycerol concentration was determined using a Trinder kit (Young Dong Pharm., Seoul, Korea).

Immunoblot analysis

The livers and hypothalamus collected from the rats with 100 nmol/L insulin stimulation for 10 min were lysed in 20 mmol/L Tris buffer (pH 7.4) containing 2 mmol/L EDTA, 137 mmol/L NaCl, 1% NP40, 10% glycerol and 12 mmol/L α-glycerol phosphate and protease inhibitors. After 30 min on ice, the lysates were centrifuged for 10 min at 12,000 rpm at 4°C. After measuring protein contents in lysates using a Bio-Rad protein assay kit, the liver lysates with equal amounts of protein were immunoprecipitated with IRS2 antibodies prior to being directly resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The precipitates were immunoblotted with tyrosine phosphorylated protein antibody (Py20; UBI, Waltham, MA, USA). For the liver lysates, the antibodies of protein kinase B (PKB or Akt, Cell Signaling Technology, Beverly, MA, USA), phosphorylated PKB^{Ser473} (Cell Signaling Technology), glucokinase, glucose transporter-2 (GLUT2, Santa Cruz Biotechnology, Santa Cruz, CA, USA), AMPK (Cell Signaling Technology) and phosphorylated AMPK^{Thr172}, phosphoenolpyruvate carboxykinase (PEPCK) and β-actin (Santa Cruz Biotechnology) were used for immunoblotting assays as previously described.[23] The hypothalamus lysates were immunoblotted with antibodies of STAT3 (Cell Signaling Technology), phosphorylation STAT3^{Tyr705}, AMPK and phosphorylated AMPK^{Thr172}. The intensity of protein expression was determined using Imagequant TL (Amersham Biosciences, Piscataway, NJ, USA). Five rats from each group were used for the immunoblotting analyses.

Statistical analysis

All results are expressed as a mean ± SD. Statistical analysis was performed using SAS version 9.1. The significance of KTD and ketone injection effects in Px rats was determined by one-way analysis of variance. Multiple comparisons among the Px groups were undertaken by Tukey's tests. The differences between Px and Sham rats were determined by two-sampled *t*-test. Differences with a $P < 0.05$ were considered statistically significant.

Results

Body weight, epididymal fat pads and serum leptin levels

Although non-diabetic rats in the Sham-COD group consumed fewer calories daily, their body weight and epididymal fat pads were higher than those of diabetic rats in the Px-COD group (Table 2). However, serum leptin levels were significantly higher in Sham rats fed COD than Px rats fed COD (Table 2). Serum free fatty acid levels were significantly lower in the Sham-COD group than in the Px-COD group. There was no significant difference of serum IHB levels between the Sham-COD and Px-COD groups. This suggested that insulin secretion in Px rats was sufficient to prevent ketosis. Among Px rats, body weight was higher in the KTD group than in other groups, but not significant ($P = 0.07$). Epididymal fat pad weights, an indicator of visceral fat, were much higher compared with total body weight in the Px-KTD group than in other groups. The epididymal fat pads decreased in the order of KTD, COD and IHB in the Px rats. Serum leptin levels among the Px rats exhibited the same trends of epididymal fat contents (Table 2). However, Px-KTD and Px-IHB had lower caloric consumption than Px-COD. Serum free fatty acid levels, which influence insulin resistance, were higher in the KTD group than in other groups in Px rats. As expected, serum IHB levels were higher in the Px-KTD and Px-IHB groups than in the Px-COD group during the light cycle (Table 2). In the IHB group, serum IHB levels decreased as time passed after injection but the levels were still much higher in the Px-IHB group than in the Px-COD group.

Phosphorylation of STAT3 and AMPK in the hypothalamus

Since leptin signaling and AMPK phosphorylation in the hypothalamus are known to regulate body weight and visceral fats via changing food intake, the phosphorylation of STAT3 and AMPK was measured in the hypothalamus.[8] The phosphorylation of STAT3 was attenuated in Px rats in comparison to Sham rats, but there was greater phosphorylation of AMPK in Px rats (Figure 1). This was related to higher food intake in Px rats than in Sham rats. The lower body weight in Px rats were associated with urinary glucose excretion and insulin deficiency in Px rats, although Px rats had higher food consumption than Sham rats.

KTD attenuated STAT3 phosphorylation in the hypothalamus despite higher serum leptin levels, which suggested that feeding rats KTD induced leptin resistance (Figure 1). Leptin resistance impairs the regulation of food intake and increases body fat.[24] The rats in the KTD group had greater epididymal fat pad weights, but not higher caloric intakes. This may be simply explained by the greater percentage of energy from fat requiring less energy for processing into fat stores than for carbohydrate which must first undergo fatty acid synthesis. In contrast, STAT3 phosphorylation was not significantly different in the COD and IHB groups (Figure 1). However, the rats in the IHB group exhibited significantly lower visceral fat accumulation with less caloric consumption than did COD rats. Thus, caloric intake and body fat were affected by signaling pathways other than leptin. The phosphorylation of hypothalamic AMPK was associated with caloric intake in different groups of Px rats. Unlike STAT3 phosphorylation, AMPK phosphorylation was higher in COD in comparison to KTD and IHB (Figure 1). The potentiation of hypothalamic AMPK phosphorylation in the COD group was associated with higher caloric intake in the COD than the KTD. KTD suppressed AMPK phosphorylation to lower caloric intake than COD. Thus, increased serum ketone levels in

Table 2 Physiological characteristics

	Px-KTD ($n = 20$)	Px-IHB ($n = 20$)	Px-COD ($n = 20$)	Sham-COD ($n = 20$)
Body weight (g)	332 ± 42	295 ± 36	305 ± 38	353 ± 33†
Epidydimal fat pads (g)	4.3 ± 0.7^{a}	2.1 ± 0.4^{c}	2.7 ± 0.4^{b*}	3.8 ± 0.7†
Caloric intakes (kJ/d)	474 ± 57^{b}	478 ± 54^{b}	552 ± 54^{a}	429 ± 51†
Overnight fasted leptin levels (ng/mL)	4.3 ± 0.7^{a}	2.8 ± 0.4^{b}	3.3 ± 0.5^{c*}	4.4 ± 0.7†
Overnight fasted free fatty acid (mmol/L)	0.98 ± 0.13^{a}	0.57 ± 0.09^{b}	0.64 ± 0.11^{b*}	0.51 ± 0.10†
IHB at 1 h after injection (mmol/L)	2.08 ± 0.86^{a}	2.64 ± 0.78^{a}	0.20 ± 0.07^{b***}	0.19 ± 0.07
IHB at 6 h after injection (mmol/L)	2.12 ± 0.79^{a}	1.61 ± 0.63^{b}	0.21 ± 0.08^{c***}	0.18 ± 0.07
IHB at 12 h after injection (mmol/L)	2.07 ± 0.78^{a}	1.08 ± 0.54^{b}	0.19 ± 0.07^{c***}	0.17 ± 0.08
Overnight fasted serum glucose (mmol/L)	7.2 ± 0.8	7.1 ± 0.8	7.4 ± 0.9	4.7 ± 0.6††
Overnight fasted serum insulin (ng/mL)	0.83 ± 0.13	0.84 ± 0.13	0.86 ± 0.11	1.48 ± 0.19†
Overnight fasted serum glucagon (ng/mL)	207.6 ± 35.5^{a}	90.2 ± 11.8^{a}	84.8 ± 10.3^{b*}	92.6 ± 11.0

KTD, Ketogenic diet; COD, control diet; IHB, β-hydroxybutyrate
Values are mean ± SD
^{a,b}Values in the same row with different superscripts were significantly different among the groups of Px rats at $P < 0.05$
*Significantly different among the groups of Px rats at $P < 0.05$ and at $^{***}P < 0.001$
†Significantly different from the control group of Px rats at $P < 0.05$

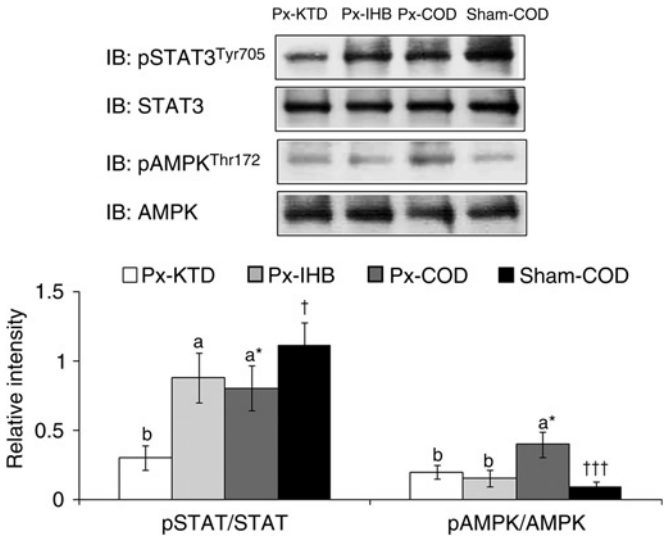

Figure 1 Relative intensity of phosphorylation of STAT3 and AMPK in the hypothalamus. After 10 min of insulin (5 U/kg body weight) stimulation through the inferior vena cava at the end of each experimental period, the hypothalamus collected from the pancreatectomized (Px) diabetic rats administered with ketogenic diet (Px-KTD), β-hydroxybutyrate injection (Px-IHB) or control (Px-COD) or from Sham rats administered control diet (Sham-COD) for five weeks was immediately lysed and phosphorylation and expression levels of the signal transducer and activator of transcription (STAT3) and 5′-adenosine monophosphate-activated protein kinase (AMPK) were determined by immunoblotting with specific antibodies. The bars are mean ± SD. $n = 5$. *Significantly different among the groups of Px rats at $P < 0.05$. [a,b]Values in the bars with different superscripts (a, b) were significantly different among the groups of Px rat at $P < 0.05$. †Significantly different from the control group of Px rats at $P < 0.05$ and at $^{\dagger\dagger\dagger}P < 0.001$

the KTD group might suppress hypothalamic AMPK phosphorylation even though increased body fat induced leptin resistance. KTD increased body fat without elevating caloric intake.

Glucose tolerance by OGTT

The overnight-fasted serum glucose levels of Px rats fed COD were higher than Sham rats fed COD. This increase was accompanied by a decrease in serum insulin levels (Table 2). Serum glucose and insulin levels indicated that Px rats exhibited diabetic symptoms due to the deficient insulin secretion. KTD, IHB and COD did not alter overnight-fasted serum glucose and insulin levels in Px rats (Table 2). However, overnight-fasted serum glucagon levels were not significantly different between the Px-COD and Sham-COD groups (Table 2), but the levels were higher in the Px-KTD group than in the Px-IHB and Px-COD groups and KTD remarkably lowered the ratio of insulin and glucagon in comparison to the IHB and COD groups in Px rats.

In the OGTT, serum glucose levels were approximately 87% higher in Px-COD rats than in normal Sham-COD rats (Figure 2a). Px-COD rats had higher serum glucose levels than Sham-COD rats, but they had similar patterns of serum glucose levels during OGTT. However, KTD exhibited a peculiar shape during OGTT: serum glucose levels were increased with a sigmoid curve at the beginning of OGTT and then decreased slowly after peak serum

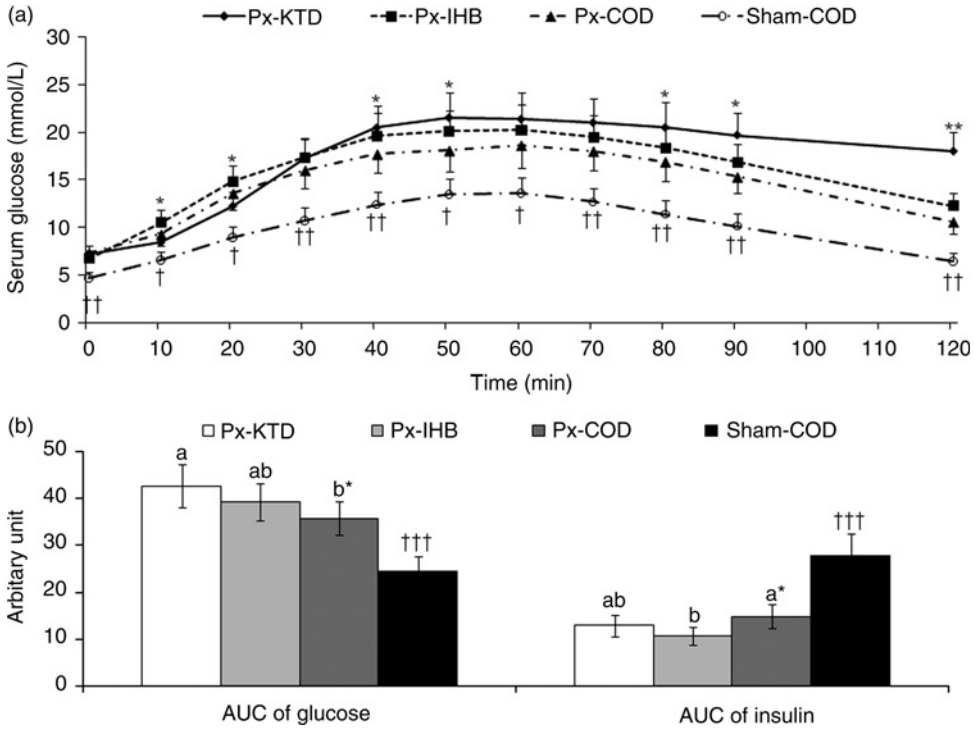

Figure 2 Changes in serum glucose levels and area under the curve of serum glucose and insulin during the oral glucose tolerance test (OGTT). During a four-week treatment period, OGTT was performed in overnight fasted rats by orally administering 2 g/kg of glucose in pancreatectomized (Px) rats administered ketogenic diet (Px-KTD), β-hydroxybutyrate injection (Px-IHB) or control (Px-COD) or in Sham rats administered control diet (Sham-COD). (a) Serum glucose levels at 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 120 min. (b) The average of the area under the curve of glucose and insulin. The dots and bars are mean ± SD. $n = 10$. *Significantly different among the groups of Px rats at $P < 0.05$ and at $^{**}P < 0.01$. [a,b]Values in the bars with different superscripts (a, b) were significantly different among the groups of Px rat at $P < 0.05$. †Significantly different from the control group of Px rats at $P < 0.05$, at $^{\dagger\dagger}P < 0.01$ and at $^{\dagger\dagger\dagger}P < 0.001$

glucose levels were reached (Figure 2a). Areas under the curves of serum glucose and insulin during OGTT are shown in Figure 2b. Px-COD rats exhibited a much larger area under the curve of glucose during OGTT, in comparison with Sham-COD rats ($P < 0.01$). This increase of serum glucose was associated with the smaller area under the curve of insulin in Px-COD rats, compared with Sham-COD rats (Figure 2b). After the oral glucose load, the administration of KTD in Px rats resulted in a significant increase in the area under the curve of serum glucose, compared with COD in Px rats (Figure 2b). However, area under the curve of insulin in KTD did not differ from COD in Px rats whereas that for IHB was lower than COD.

Insulin secretion capacity by a hyperglycemic clamp

To confirm β-cell function, a hyperglycemic clamp was performed. During the hyperglycemic clamp, serum insulin levels peaked between 2 and 5 minutes and then declined to a nadir at 10 min (Figure 3), whereas glucose levels remained elevated and stable. This is known as first-phase insulin secretion, which is known to be associated with insulin resistance. An ascending second phase of plasma insulin was observed at 60–120 min in all rats. Serum insulin levels in first and second phases represent insulin secretion capacity, which is related to β-cell mass. The pattern of insulin secretion was similar in Px and Sham rats but the secretion capacity was changed. Px-COD rats exhibited lower first- and second-phase insulin secretion than Sham-COD rats by 37% and 50%, respectively (Figure 3, Table 3). In Px rats, KTD and IHB decreased first-phase insulin secretion in comparison to COD whereas only IHB lowered second-phase insulin secretion (Figure 3, Table 3). Thus, this suggests that the increase of serum ketone levels attenuated insulin secretion but other components (such as serum free fatty acids) in KTD maintained second-phase insulin at similar levels to COD.

Glucose infusion rates in the hyperglycemic clamp indicate β-cell function and insulin sensitivity in a hyperglycemic state, calculated as the ratio of glucose infusion rates to steady-state serum insulin levels.[16,19] Glucose infusion rates required to maintain serum glucose levels at 5.5 mmol/L above baseline were found to be greater in the Sham-COD group than in the Px-COD group by 2.3-fold. As expected, Sham rats fed COD exhibited greater insulin sensitivity in a hyperglycemic state than Px rats fed COD (Table 3). After administering KTD, IHB or COD to Px rats for five weeks, glucose infusion rates were significantly decreased in an ascending order of IHB, KTD and COD in Px rats (Table 3). Insulin sensitivity in the hyperglycemic state was decreased in the order of COD, IHB and KTD in Px rats during the hyperglycemic clamp. The results of the hyperglycemic clamp revealed that the increment of serum ketone levels attenuated not only insulin secretion but also insulin sensitivity in a hyperglycemic state. Therefore, the increasing serum ketone levels may not be beneficial for lean type 2 diabetics with insulin deficiency.

Insulin sensitivity by a hyperinsulinemic euglycemic clamp

During a hyperinsulinemic euglycemic clamp, whole body glucose utilization was measured to see whether the modulation of glucose tolerance was associated with the changes in insulin resistance in KTD, IHB and COD administered rats. As depicted in Figure 4a, glucose infusion rates and glucose uptake at serum insulin concentrations of about 1100 pmol/L were lower in Px-COD rats than in Sham-COD rats. Glucose infusion rates in hyperinsulinemic clamped states were lower in the KTD group than in other groups in Px rats whereas IHB was slightly, but not significantly, higher than in COD ($P = 0.09$). However, there was no significant difference in glucose uptake among the groups of Px rats.

Hepatic glucose output in basal and hyperinsulinemic clamped states was higher in the Px-COD group than in the Sham-COD group (Figure 4b). Consistent with overnight fasted glucose levels, basal hepatic glucose output

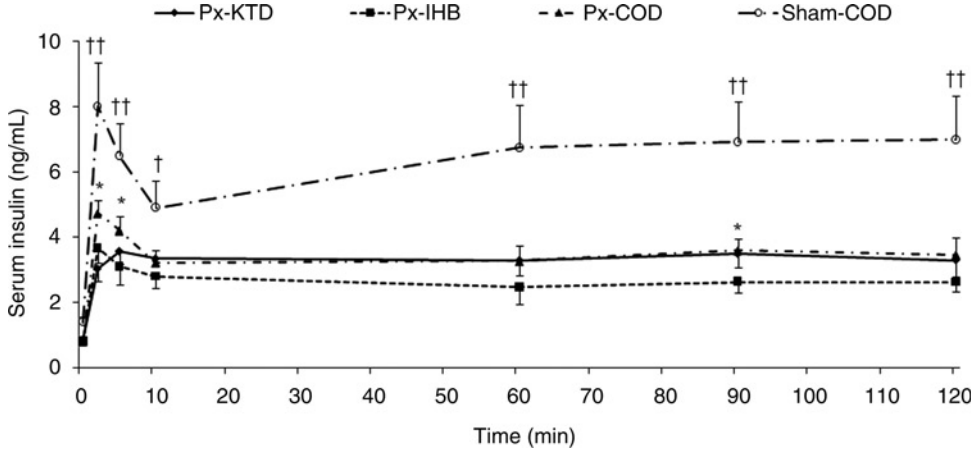

Figure 3 Changes in serum insulin levels during a hyperglycemic clamp. After a five-week treatment with ketogenic diet (Px-KTD), β-hydroxybutyrate injection (Px-IHB) or control (Px-COD) in pancreatectomized (Px) rats or of control diet in Sham rats (Sham-COD), a hyperglycemic clamp was performed in overnight fasted rats to determine insulin secretion patterns and capacity after catheterization of the carotid artery and jugular vein. The changes in serum insulin levels were determined when serum glucose levels were maintained 100 mg/dL above the fasting levels. The dots are mean $\pm$ SD. $n = 10$. *Significantly different among the groups of Px rats at $P < 0.05$. †Significantly different from the control group of Px rats at $P < 0.05$ and at ††$P < 0.01$

Table 3 Insulin secretion capacity during a hyperglycemic clamp

	Px-KTD ($n = 10$)	Px-IHB($n = 10$)	Px-COD($n = 10$)	Sham-COD($n = 10$)
Serum insulin at basal state (ng/mL)	0.84 ± 0.11	0.82 ± 0.12	0.83 ± 0.12	$1.45 \pm 0.18^{\dagger}$
Area under the curve of insulin at first phase (AU)	62.3 ± 7.1^{b}	59.2 ± 8.3^{b}	$75.5 \pm 9.5^{a*}$	$119.4 \pm 17.4^{\dagger\dagger\dagger}$
Area under the curve of insulin at second phase (ng/mL)	408 ± 57^{a}	312 ± 44^{b}	$417 \pm 54^{a*}$	$827 \pm 108^{\dagger\dagger\dagger}$
Glucose infusion rate (mg/kg bw/min)	9.2 ± 1.4^{b}	7.9 ± 1.3^{c}	$11.6 \pm 1.9^{a*}$	$26.5 \pm 3.6^{\dagger\dagger\dagger}$
Insulin sensitivity (μmol glucose/min/ 100 g/μmol insulin/L)	13.5 ± 2.0^{c}	15.2 ± 2.1^{b}	$16.7 \pm 3.1^{a*}$	$19.2 \pm 2.9^{\dagger}$

KTD, Ketogenic diet; COD, control diet; IHB, β-hydroxybutyrate; Px, pancreatectomized

Values are mean $\pm$ SD. First-phase insulin secretion was defined as the area under the curve of serum insulin levels from 0 to 5 min, with second phase from 60 to 120 min. Insulin sensitivity at hyperglycemic state was calculated as the ratio of glucose infusion rates to steady-state serum insulin levels

^{a,b,c}Values in the same row with different superscripts were significantly different among the groups of Px rats at $P < 0.05$

*Significantly different among the groups of Px rats at $P < 0.05$

†Significantly different from the control group of Px rats at $P < 0.05$ and at $^{\dagger\dagger\dagger}P < 0.001$

was not significantly different among Px rats administered KTD, IHB and COD. Since glucose uptake was similar among the different groups, a good portion of the difference in glucose infusion rates could be accounted for by the insulin-stimulated reduction in hepatic glucose production, which represents attenuated hepatic insulin sensitivity. Hepatic glucose output in the hyperinsulinemic states was higher in KTD than in COD in Px rats, whereas in IHB it was significantly lower in comparison to COD. This suggested that Px-KTD increased hepatic insulin resistance. In comparison to COD, KTD decreased glycogen storage in the liver of the Px rats and IHB increased the accumulation at the end of the experimental period. However, KTD had higher liver triglyceride contents than the IHB and COD groups in Px rats (Figure 4c). Unlike hepatic insulin signaling, insulin signaling (tyrosine phosphorylation of IRS1 and serine phosphorylation of Akt) in gastrocnemius and quadriceps muscles and adipose tissues was not significantly different (data not shown).

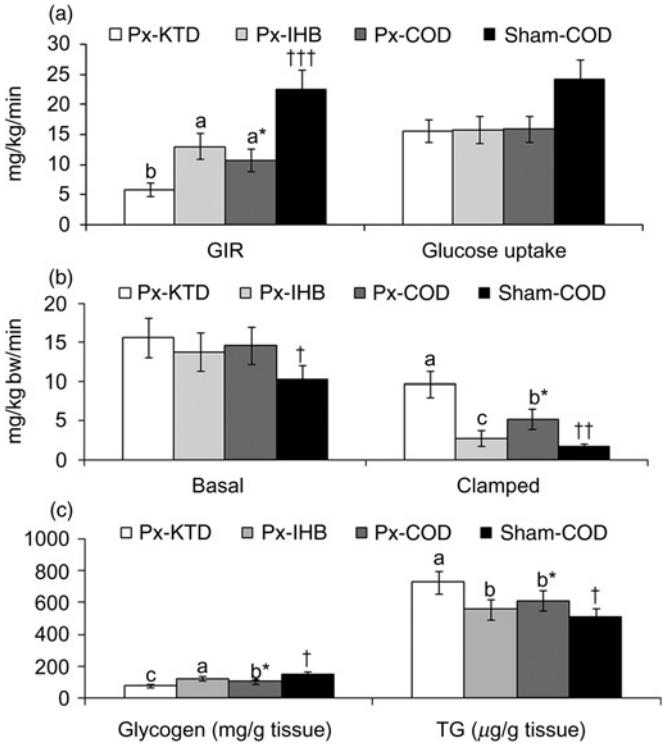

Figure 4 Metabolic parameters under a euglycemic hyperinsulinemic clamp. After a five-week treatment with ketogenic diet (Px-KTD), β-hydroxybutyrate injection (Px-IHB) or control (Px-COD) in pancreatectomized (Px) rats or of control diet in Sham rats (Sham-COD), a euglycemic hyperinsulinemic clamp to maintain euglycemia, when serum insulin levels stayed at 1100 pmol/L was performed in conscious, free moving and overnight fasted rats. (a) Glucose infusion rates (GIR) and whole body glucose uptake. (b) Hepatic glucose output (HGO) at basal and hyperinsulinemic clamp states. (c) Storage of glycogen and triglyceride after a euglycemic hyperinsulinemic clamp. The bars are mean $\pm$ SD. $n = 10$. *Significantly different among the groups of Px rats at $P < 0.05$. ^{a,b,c}Values in the bars with different superscripts were significantly different among the groups of Px rat at $P < 0.05$. †Significantly different from the control group of Px rats at $P < 0.05$, at $^{\dagger\dagger}P < 0.01$ and at $^{\dagger\dagger\dagger}P < 0.001$

Hepatic insulin signaling pathways

Since changes in hepatic glucose homeostasis can be related to the modifications of hepatic insulin signaling, the signaling was examined in order to investigate the molecular pathways. Figure 5 showed the relative intensity of phosphorylation and expression of proteins involved in insulin signaling and glucose sensing in the liver of rats stimulated with insulin. Tyrosine phosphorylation of IRS2 and serine phosphorylation of Akt were attenuated in the Px-COD group in comparison to the Sham-COD group (Figure 5). The expressions of glucokinase and GLUT2 proteins related to glucose sensing in the liver were also lower in Px-COD rats relative to Sham-COD rats (Figure 5). In addition to glucose sensing proteins, the phosphorylation of AMPK, an energy sensing protein, decreased in Px-COD rats compared with Sham-COD rats. As a result of the attenuation of insulin signaling and the decrease of glucose sensing proteins, the expression of PEPCK, a key enzyme of gluconeogenesis, was increased in Px-COD rats (Figure 5). This explained that the increase in hepatic glucose output was associated with the attenuation of hepatic insulin signaling and the decreased expression of glucose and energy sensing proteins in Px rats.

In Px rats, tyrosine phosphorylation of IRS2 was attenuated by KTD compared with COD in Px rats while it was potentiated by IHB. This change was directly delivered into serine phosphorylation of Akt (Figure 5). The expression of glucokinase and GLUT2, glucose sensing proteins, was decreased in the Px-KTD group compared with the Px-COD and Px-IHB groups (Figure 5). These changes in the expression of proteins related to insulin signaling and glucose sensing affected PEPCK expression in the liver. The expression of PEPCK increased in KTD in

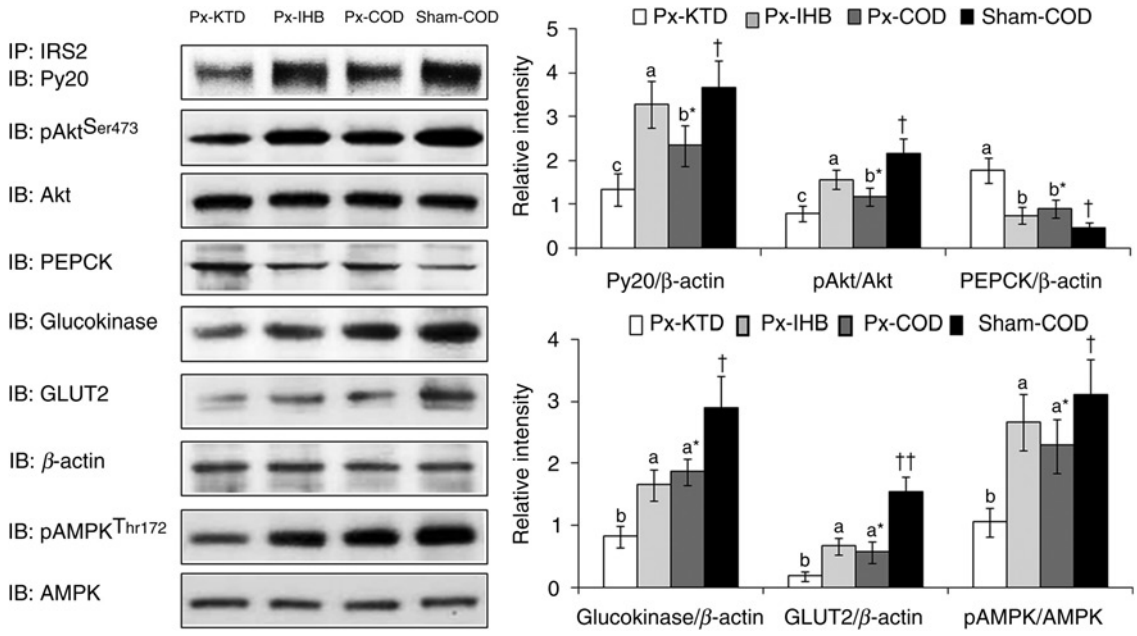

Figure 5 Relative intensity of phosphorylation and expression of proteins involved in insulin signaling and glucose sensing in the liver. After 10 min of insulin (5 U/kg body weight) stimulation through the inferior vena cava at the end of each experimental period, the liver collected from the pancreatectomized (Px) diabetic rats administered with ketogenic diet (Px-KTD), β-hydroxybutyrate injection (Px-IHB) or control (Px-COD) or from Sham rats administered control diet (Sham-COD) for five weeks was immediately lysed with a lysis buffer. After the immunoprecipitation with IRS2 antibody, the tyrosine phospnorylation was detected by immunoblotting with Py 20 antibody. The phosphorylation and expression levels of Akt, glucokinase, GLUT-2, AMPK, and PEPCK, involved in insulin signaling were determined by immunoblotting with specific antibodies. The bars are mean $\pm$ SD. $n = 5$. *Significantly different among the groups of Px rats at $P < 0.05$. [a,b,c]Values in the bars with different superscripts were significantly different among the groups of Px rat at $P < 0.05$. †Significantly different from the control group of Px rats at $P < 0.05$

comparison to COD in Px rats while it slightly decreased in IHB. These changes were consistent with hepatic glucose output in the hyperinsulinemic clamp state. In addition, KTD attenuated hepatic insulin signaling to decrease glucose utilization in the liver, which was accounted for by a decreased glycogen accumulation and increased lipid storage. The phosphorylation of AMPK in the liver was attenuated in the Px-KTD group in comparison to the Px-IHB and Px-COD groups. This change might account for the increase of triglyceride accumulation in the liver.

Discussion

Animal model

The animal model used in this study is specific to non-obese type 2 diabetes,[16,18,20] the most common type of diabetes in Asia and an important subtype in Western countries. Since most type 2 diabetes in Western countries develops as a consequence of obesity-related insulin resistance, this non-obese type 2 diabetes model provides little input into the ongoing debate on the merits of KTD as an intervention for obesity and obesity-related type 2 diabetes. The emphasis of this study was on the effects of KTD and increased ketone alone on type 2 diabetes with an etiology unrelated to diet and body weight, using an established model in which rats are made insulin deficient by a partial pancreatectomy. This animal model exhibits non-obese type 2 diabetic symptoms. Goto-Kakizaki rats are also a good model for non-obese type 2 diabetic model but they have a somewhat more complicated impairment of insulin secretion which appears to be the result of a defect in the release of insulin in response to glucose[25] and not related to insulin secretion capacity. However, the partial pancreatectomized rats do share the trait of low insulin secretory capacity. Thus, these two animal models have differences and it would be useful to conduct another experiment to confirm the results of ketone effects in a spontaneous non-obese type 2 diabetic animal model. It is interesting that the decreased insulin secretion capacity in this model is not compensated for by enhanced insulin sensitivity, but on the contrary it causes the animals to progress rather rapidly to an insulin-resistant state. The effects of ketone and KTD were evaluated by measuring body composition, insulin, and glucose production and metabolism, and the expression and activity of metabolic regulatory molecules.

Effects of 90% pancreatectomy

The rats with partial pancreatectomies and normal diets (Px-COD) displayed typical symptoms of type 2 diabetes resulting from insulin insufficiency and resistance. Diet-induced type 2 diabetes is known to induce leptin resistance in rats and increase caloric intake with concomitant weight gain.[26] However, despite higher energy intakes due to attenuation of leptin signaling and increased hypothalamic AMPK phosphorylation, our Px-COD rats had lower body weights than Sham rats, possibly due to increased urinary glucose excretion and insulin deficiency. Fasting serum glucose was elevated and insulin levels suppressed in the Px-COD rats. Moderate type 2 diabetes was also revealed by the elevated serum glucose during an

OGTT and the impaired insulin secretion capacity and insulin response during the hyperglycemic clamp study. The hyperinsulinemic euglycemic clamp confirmed a peripheral (mostly adipose and muscle tissue) insulin resistance that was unaffected by the different diets and a modest hepatic insulin resistance.[21] The hepatic insulin resistance was associated with attenuated insulin receptor signaling and increased hepatic glucose output, which was related to the increased gluconeogenesis as reflected in the higher expression of PEPCK, which catalyzes the rate-controlling step in gluconeogenesis. This inappropriate increase in gluconeogenesis and glucose release occurred simultaneously with decreased hepatic expressions of two glucose sensing proteins, glucokinase and GLUT2, as well as decreased activation of the energy sensing AMPK. Therefore, it was well established that the pancreatectomy resulted in decreased insulin secretion coupled with insulin resistance in both liver and peripheral tissues.

Effect of KTD on non-obese type 2 diabetes

The present study used hydrogenated corn oil for dietary fat sources since it has less harmful effect on diabetes than lard, even though it contained trans fat.[27] The role of trans fat in the development of type 2 diabetes has not been as widely investigated. The Nurses' Health Study,[28] the largest and most detailed epidemiological study, showed a positive association between trans fat intake and risk of diabetes, with a clear dose–response relation. However, small epidemiological studies or those that did not include repeated measures of diet did not indicate a positive association.[29] Tardy *et al.*[30] showed that it does not seem to impair insulin sensitivity, at least in the muscle of rats. KTD did not reverse, but rather exacerbated, the effects of pancreatectomy-induced diabetes. Thus, hydrogenated corn oil was used as a dietary fat instead of lard.

The Px-KTD rats had significantly higher visceral fat accumulation (epididymal fat pad), leptin levels and circulating free fatty acids than other Px rats, which would seem to indicate a similar metabolic state to the one that leads to insulin resistance in humans.[26] This may have been due in part to the severely impaired leptin signaling as revealed by the much lower activation of STAT3, a downstream mediator of leptin signaling, despite high circulating leptin concentrations. Type 2 diabetes is known to induce leptin resistance in rats and increase caloric intake with concomitant weight gain,[26] and this was worsened in the diabetic rats fed KTD in this study. This KTD-induced leptin resistance probably caused the increased visceral fat accumulation compared with other Px rats since leptin resistance in aging rats is known to favor visceral fat accumulation and insulin resistance[31] and providing leptin to insulin-deficient mice has been demonstrated to preferentially decrease visceral fat accumulation.[32]

KTD also exacerbated hepatic insulin resistance, but not peripheral insulin resistance, as evidenced by the equal glucose uptake among all Px rats in the euglycemic hyperinsulinemic clamp study, but increased hepatic glucose output in the Px-KTD rats. Hepatic gluconeogenesis was responsible for the higher glucose output in a hyperinsulinemic state as revealed by the very high PEPCK expression during a hyperinulinemic state. The elevated hepatic insulin resistance was further confirmed by low tyrosine phosphorylation of insulin receptor substrate-2 and decreased activation of its downstream mediator, Akt. The signaling of insulin receptor substrate-2, the first substrate of hepatic insulin signaling, is essential for the suppression of gluconeogenesis and apoptosis in immortalized hepatocytes.[33] Activated insulin receptor substrate-2 contributes to potentiating the serine^{473} phosphorylation of Akt, which results in decreased PEPCK expression.[34]

The decreased expressions of the two glucose sensing proteins, GLUT2 and glucokinase, were also significantly lower than any of the other groups. Therefore, there is an abundance of evidence that in non-obese diabetic rats, KTD induces a profound hepatic insulin resistance, causing the liver to react to a postprandial state in a manner more appropriate for a fasted state, with a resultant inappropriate release of glucose from hepatic gluconeogenesis, increased circulating non-esterified fatty acids and further impaired glucose handling. The impairment of hepatic insulin signaling, possibly due to increased circulating free fatty acids, decreased the ability of insulin to suppress hepatic glucose output. This suggests an increase in gluconeogenesis in a hyperinsulinemic state, indicating the induction of hepatic insulin resistance.[35,36]

When mice on a 75% corn syrup diet consumed the same calories as mice on KTD, the former nevertheless rapidly lost weight whereas the latter gained weight with increased body fat and insulin resistance.[37] Although their control diet and KTD were different from those used in this study, the results are consistent with the findings of the present study in which KTD increased whole body and hepatic insulin resistance as determined by the euglycemic hyperinsulinemic clamp study in Px rats. By contrast, Kennedy *et al.*[38] found that KTD promotes weight loss in mice made obese by a standard high-fat diet by activating AMPK in the liver, indicating that KTD decreased insulin resistance. Their results appear inconsistent with ours, possibly due to the different animal models; for instance, the much higher expression of uncoupling protein-1 in brown adipose tissue in their mice fed KTD, suggests that energy wasting may have been responsible for the weight loss, increased heat production and greater oxygen consumption. In addition, it is well known that KTD has different effects on energy and glucose metabolism in different species: KTD may have better protective action against increased body fat in mice than in rats.[5] We cannot be sure why our rats responded differently than the mice in the study by Kennedy *et al.*,[38] but certainly the different species and the impaired insulin secretion capacity of our rats were major factors. It should not be surprising that the rat model for non-obese type 2 diabetes would respond differently to KTD than obese animal models since the etiology of the two diseases are very different. In addition, Kennedy *et al.*[38] gave a very low protein diet to invoke negative nitrogen balance while the present study provided a normal protein diet. A very low protein diet decreased body weight due to loss of lean body mass without decreasing fat mass in comparison to the control group. Thus, the

weight loss might be primarily associated with a low protein content in the diets in the study of Kennedy *et al.*[38]

Effect of ketone alone on non-obese type 2 diabetes

The intraperitoneal injections of IHB at the beginning of the dark and light cycles resulted in a sustained release of the ketone, with circulating concentrations that were similar to the Px-KTD rats during feeding times and procedures. The effects of ketone on metabolism should fully reflect that which occurs in diet-induced ketosis. Our preliminary acute study revealed that serum IHB levels peaked at one hour post-injection and then decreased by 15, 27 and 34% at two, four and six hours, respectively; but serum IHB levels remained elevated until the next injection. The serum IHB levels were maintained throughout the day. Rossi *et al.*[39] also revealed that intraperitoneal injection of IHB was used in goats to evaluate the effects of elevated ketone levels on dietary intake.

Unlike other studies in which young children and rodents on a KTD frequently exhibit poor weight gain and have lower blood glucose levels compared with those on standard diets,[40,41] in the present study the Px-KTD group had the highest visceral fat accumulation among all the groups in contrast to the Px-IHB group which had the lowest. This was probably due to the leptin-associated STAT3 signaling that was impaired by KTD, but this impairment was not due to ketone itself since ketone injection (IHB) did not attenuate leptin signaling in comparison to COD. This indicated that KTD and IHB affect energy metabolism by different mechanisms.

Hepatic insulin receptor expression and signaling (AKT phosphorylation), which were impaired by pancreatectomy, were also partially restored by ketone injection, in contrast to KTD which exacerbated the impairment. This may have been attributable to the partial normalization of circulating non-esterified fatty acids in the Px-IHB rats. These results suggest that ketone itself may provide some protection against the untoward effects of a high-fat diet. However, most of the effects of KTD in this study appeared to be distinct from the effects of ketone itself.

Summary

We found that in animals with type 2 diabetes resulting from surgically induced insulin insufficiency, KTD exacerbated leptin resistance in the hypothalamus leading to increased visceral fat without raising energy intake. Ketone injection decreased visceral fat, and improved hepatic insulin resistance by potentiating insulin signaling. However, KTD decreased the phosphorylation of AMPK in the hypothalamus, impaired glucose tolerance by increasing hepatic insulin resistance and decreased first-phase insulin secretion. Therefore, KTD exacerbated energy and glucose dysregulation in diabetic rats, which was not associated with increased serum ketone levels but possibly related to other factors such as increased serum glucagon and/or free fatty acid levels. Therefore, KTD, but not ketone, exacerbates impaired energy and glucose metabolism in type 2 diabetic rats, suggesting that it may not be an appropriate dietary intervention for non-obese type 2 diabetic patients.

Author contributions: SP designed the experiment, performed statistical analysis and wrote the manuscript. DSK and SK performed animal experiments and biochemical assays. JWD participated in designing the experiment and writing the manuscript.

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