Characterization of Hepatic Glucose Metabolism Disorder with the Progress of Diabetes in Male Spontaneously Diabetic Torii Rats

Hisayo MORINAGA1)*, Hiromi YAMAMOTO1), Kaoru SAKATA1), Sumiaki FUKUDA1), Makoto ITO1), Tomohiko SASASE1), Katsuhiro MIYAJIMA2), Nobuhisa UEDA1), Takeshi OHTA1) and Mutsuyoshi MATSUSHITA1)

1)Japan Tobacco Inc., Central Pharmaceutical Research Institute, Biological/Pharmacological Research Laboratories, 1–1, Murasaki-cho, Takatsuki, Osaka 569–1125, and 2)Japan Tobacco Inc., Central Pharmaceutical Research Institute, Toxicology Research Laboratories, 23 Naganuki, Hadano, Kanagawa 257–0024, Japan

(Received 13 February 2008/Accepted 16 July 2008)

ABSTRACT: The Spontaneously Diabetic Torii (SDT) rat has recently been established as a new model of non-obese type 2 diabetes. In this study, we examined changes in hepatic glucose metabolism in prediabetic and diabetic SDT rats compared with age-matched control rats. The prediabetic state was confirmed at 16 weeks of age, and the diabetic state was confirmed at 24 and 32 weeks of age. Decreases in glucokinase mRNA levels and activity were observed in the prediabetic state. In this state, glycogen synthase activity and glycogen content were also decreased in the SDT rat. In addition to the above changes, glycogen phosphorylase mRNA and activity were decreased and gluconeogenetic enzyme mRNA levels were significantly increased in the diabetic state. These results indicate there is a great potential that abnormalities in hepatic glucose metabolism play a role in the progression to onset of diabetes. We suggest that the SDT rat is a valuable diabetic model for investigations into mechanisms or causes of progression to diabetes.

KEY WORDS: diabetes, glucose, glycogen, liver, SDT rat.

The Spontaneously Diabetic Torii (SDT) rat is a model of non-obese type 2 diabetes with insulin hyposecretion. Unlike conventional animal models, it is expected to serve as a model of diabetic retinopathy [14, 15, 20, 21]. In addition, onset of diabetes is delayed in the SDT rat until around 20 weeks of age which makes this a highly effective model in which to pursue the mechanism of development of diabetes. To date, the pathogenesis of diabetes in SDT rats has been attributed to malformation and dysfunction of the pancreas. In contrast, Masuyama et al. [15] has reported prediabetic impaired glucose tolerance despite normal insulin secretion, suggesting possible pathogenesis somewhere other than the pancreas.

Due to its complexity, the mechanism of diabetic pathogenesis cannot be sought without accumulating important information through experiments using various animal models, which should be continuously updated. Recently, attention has been focused on high risks of development of cardiovascular diseases in not only diabetic patients but also nondiabetic patients with glucose metabolic disorder. This makes it important to study diabetic pathogenesis to prevent progress to diabetes.

Among the studies available concerning the developmental stage of diabetes, particularly little has been published on hepatic glucose metabolism. This study, therefore, focused on the liver, the major organ involved in glucose metabolism, to investigate changes in hepatic glucose metabolism from the prediabetic to diabetic stages, with the aim of identifying a molecule involved in the onset of diabetes.

Since diabetes is supposed to develop at around 20 weeks of age in the SDT rat, 16-, 24- and 32-week-old SDT rats were used to examine the pre- and post-development stages. Age-matched Sprague-Dawley (SD) rats were used as controls. Under non-fasted conditions, the following mRNA expression levels and enzyme activities were measured: glucokinase, a hepatic glycolytic rate-limiting enzyme, glycogen synthase and glycogen phosphorylase, rate-limiting enzymes involved in glycogen metabolism, which plays an important role in glucose storage and release. Glycogen store was also measured for detailed investigation of glycogen metabolism. We also investigated the changes in gluconeogenetic rate-limiting enzymes, the activities of which have been shown to be elevated in various diabetic animal models. As a result, we succeeded in identifying enzymes implicated in diabetic pathogenesis.

METHODS

Animals: Male SDT rats (CLEA Japan, Tokyo, Japan, 16–32 weeks of age) were used for the study. Age-matched SD rats [Crj:CD (SD); Charles River Japan, Kanagawa, Japan] were used as control animals (the SD rat is the parent strain of the SDT rat) [14]. All the experiments received prior approval from the committee for the humane care and use of animals of Biological/Pharmacological Research Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc., in accordance with the Japanese Law for Humane Treatment and Management of Animals (Law No.105, established October 1, 1973 and revised in 2006). The animals were housed in a controlled room (temperature 23 ± 3°C, humidity 55 ± 15%, 12 hr lighting cycle) and...
allowed free access to diet and water.

**Biochemical analysis:** Blood samples were collected from the tail veins of the rats. The blood was centrifuged at 10,000 × g for 5 min. The supernatant was recovered as a serum sample. The concentrations of serum glucose (hexokinase method), triglyceride (enzyme method), and total cholesterol (enzyme method) were determined using commercial kits (Roche Diagnostics, Tokyo, Japan) and an automatic analyzer (7170S; Hitachi, Tokyo, Japan). Serum insulin concentrations were measured with an ultra-sensitive rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Kanagawa, Japan).

**Quantification of mRNA with real-time quantitative PCR:** Total RNA was extracted from the liver using a GenEluteMammalian Total RNA kit (Sigma, St. Louis, MO, U.S.A.). M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) was used to synthesize cDNA. Quantification of mRNA expression was performed by real-time PCR using TaqMan® 1000Rxn Gold/Buffer A Pack (Applied Biosystems, Foster city, CA, U.S.A.). Target primers and probes (glucokinase, glycogen synthase, glycogen phosphorylase, G-6-Pase, FBPase, PEPCK) were designed using the Primer Express Software (Applied Biosystems, Table 1); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified, and the results were used for normalization of these values. The cycle parameters were 50°C × 5 sec, 95°C × 10 min, and then 40 cycles of 95°C × 15 sec and 60°C × 60 sec.

**Glycogen kinase activity:** Livers were isolated and homogenized using a Polytron homogenizer in 3 volumes of ice-cold 20 mM KH₂PO₄-KOH (pH 7.0) containing 100 mM KC1, 1 mM MgCl₂, 1 mM EDTA, 5% (v/v) glycerol, 1 mg/ml BSA and 1 mM DTT. The homogenates were centrifuged at 160 × g for 30 min. The supernatants were ultra-centrifuged for 60 min at 100,000 × g. The supernatants were recovered, and the enzymatic activity was assayed as the enzymatic activity measured at 0.5 mM glucose from the activity measured at 50 mM glucose. Glycogen synthase activity was corrected by subtracting the activity measured based on the glucose concentration of the remaining homogenates. The remaining homogenates were centrifuged at 10,000 × g for 30 min. The supernatants were recovered, and glycogen phosphorylase activity was measured.

**Glycogen synthase activity:** Glycogen synthase (GS) activity was measured as the incorporation of UDP [1-¹⁴C]glucose into glycogen. Fifty µg of Liver homogenates were incubated for 60 min at 37°C with 10 mg/ml bovine liver glycogen, 5 mM UDP-glucose and 3.31 µM UDP-¹⁴C-glucose (0.1 µCi) buffered in 50 mM Tris-HCl (pH7.8), 5 mM EDTA, 50 mM NaF and 50 µM DTT containing 10 mM or 0.1 mM glucose-6-phosphate (G-6-P). Radioactivity was measured following addition of 2 N NaOH to stop the reaction.

The glycogen synthase activity was corrected by subtracting the activity measured at 0.1 mM G-6-P from the activity measured at 10 mM G-6-P.

**Glycogen phosphorylase activity:** The supernatants were incubated at 25°C for 75 min with 4 mg/ml glycogen, 6 U/ml glucose-6-phosphatase dehydrogenase, 0.8 U/ml phosphoglucomutase, 700 µM β-NADP, 15 mM MgCl₂, 4 µM glucose-1, 6-bisphosphate and 7.5 mM glucose buffered in 6 mM KH₂PO₄, 2.4 mM Na₂HPO₄, 50 mM BES (pH 6.8) and 1 mM EDTA. Absorbance at 340 nm was then measured as the enzymatic activity.

**Glycogen content:** Livers were isolated and homogenized using a Polytron homogenizer in 2 volumes of deionized water. The liver homogenates were incubated at 40°C for 2 hr with 5 U/ml amyloglucosidase in 0.2 M acetate buffer. The reaction mixture was centrifuged, and the supernatants were neutralized with 0.25 N NaOH. The glycogen content was determined based on the glucose concentration of the supernatants. The protein concentrations were determined by using the Bio-Rad protein assay reagent.

**Pathological analysis:** Slices of the livers of the rats were collected for histopathological examination and were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with Periodic Acid Schiff’s (PAS) reagent for light microscopic evaluation.

**Statistical analysis:** All results are expressed as the mean ± S.E. Statistical analysis of differences among mean values were performed using F- and t-tests (Student’s t-test or Aspin-Welch t-test).

**RESULTS**

**Biochemical parameters:** Before embarking on our investigation of the changes in hepatic glucose metabolism in

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Probe sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucokinase</td>
<td>AGCAGGTCCACCAACATCCTAAGCC</td>
<td>TCTTGGGCAAGCACATATGGG</td>
<td>CGACCCCTGTCACCGACTGCG</td>
</tr>
<tr>
<td>Glycogen phosphorylase</td>
<td>GAGCCACCAAAGGAGGGTTGGGGGG</td>
<td>GTCAAGGCGCGTTGAGTCAC</td>
<td>GACGCACTGCTGAAGGAGCTGCTG</td>
</tr>
<tr>
<td>Glycogen synthase</td>
<td>GAGAACTCACACGATGGGGGGG</td>
<td>GTCAAGGCGCGTTGAGTCAC</td>
<td>GACGCACTGCTGAAGGAGCTGCTG</td>
</tr>
<tr>
<td>G-6-Pase</td>
<td>GAGCACTACATGAGGCGGAGAGAG</td>
<td>TCTTGGGCAAGCAGCTTGGGG</td>
<td>CCACCTTCGAGCTCGAGCTCGAG</td>
</tr>
<tr>
<td>FBPase</td>
<td>CATCACATACATAGAGGCGGAGAGGA</td>
<td>CGAGGCGGCGAGGAGGAGTTGGG</td>
<td>GCCACCGCTGGAGAGAGGAGGAG</td>
</tr>
<tr>
<td>PEPCK</td>
<td>CCCAGGAGAAGAGGAGAGGAGTGGG</td>
<td>GGAGGCGGCGAGGAGGAGGAGGAGGAGG</td>
<td>GCCACCGCTGGAGAGGAGGAGG</td>
</tr>
</tbody>
</table>

The genes are as follows: G-6-Pase, glucose-6-phosphatase. FBPase, fructose-1,6-bis phosphatase. PEPCK, phosphoenol pyruvate carboxykinase.
We confirmed that SDT rats at 16 weeks of age are prediabetic and that those at 24 and 32 weeks of age are diabetic [14].

Key enzyme of hepatic glucose metabolism: Glucokinase plays a critical role in metabolism of glucose in the liver because it appears at the first step of glycolysis [9]. The glucokinase mRNA level and activity of the 16-week-old SDT rats fell by 54% and 22%, respectively, compared with those of age-matched SD rats (Fig. 1). The levels of glucokinase mRNA and activity in the 24- and 32-week-old SDT rats fell by almost 100% (Fig. 1). Decreases in glucokinase were observed in the prediabetic state.

Glycogen synthase (GS) and glycogen phosphorylase (GP) are rate-limited key enzymes of glycogen metabolism [7, 11]. We investigated changes in their mRNA levels and activity (Fig. 2). There were no significant changes in the levels of GS mRNA; however, GS activity had already decreased by 32% in the SDT rats at 16 weeks of age. On the other hand, there were no changes in GP mRNA or activity at 16 weeks of age. At 24 and 32 weeks, both enzyme levels were decreased. The glycogen content of 16-week-old SDT rats was decreased by 29%, and these decreases were more pronounced at 24 and 32 weeks of age (Fig. 2E). We observed that glycogen staining by PAS was reduced in the 16-week-old SDT rats (Fig. 2F and G), and these reductions were more pronounced in the 24- and 32-week-old SDT rats (data not shown). No other pathological abnor-

---

**Table 2. Physiological characteristics of the animal models**

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>16</th>
<th>24</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>501±19</td>
<td>565±16</td>
<td>678±15</td>
</tr>
<tr>
<td>SDT</td>
<td>474±9</td>
<td>479±21**</td>
<td>465±11**</td>
</tr>
<tr>
<td><strong>Glucose (mg/dl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>137±3</td>
<td>127±2</td>
<td>133±1</td>
</tr>
<tr>
<td>SDT</td>
<td>160±7*</td>
<td>683±39**</td>
<td>697±63**</td>
</tr>
<tr>
<td><strong>Insulin (ng/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>4.3±0.3</td>
<td>5.7±0.6</td>
<td>4.0±0.3</td>
</tr>
<tr>
<td>SDT</td>
<td>3.8±0.5</td>
<td>0.7±0.4**</td>
<td>0.7±0.1**</td>
</tr>
<tr>
<td><strong>Triglyceride (mg/dl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>205±21</td>
<td>312±30</td>
<td>240±31</td>
</tr>
<tr>
<td>SDT</td>
<td>149±14</td>
<td>338±44</td>
<td>368±41</td>
</tr>
<tr>
<td><strong>Total cholesterol (mg/dl)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>79±6</td>
<td>85±2</td>
<td>93±8</td>
</tr>
<tr>
<td>SDT</td>
<td>79±1</td>
<td>84±5</td>
<td>88±3</td>
</tr>
</tbody>
</table>

Data are means ± S.E. (n=6). The animals were weighed before euthanization, blood was taken from tail vein and serum was separated for analysis of biochemical parameters.

* p<0.05, ** p<0.01 vs. age-matched SD rats.

---

SDT rats, we measured biochemical parameters and body weight to confirm the diabetic state (Table 2). The plasma glucose levels were slightly increased in the 16-week-old SDT rats and markedly increased in the 24- and 32-week-old SDT rats compared with age-matched SD rats. The plasma insulin levels of the SDT rats at 24 and 32 weeks were significantly lower (p<0.01) than those of the age-matched SD rats. Therefore, the marked hyperglycemia of the SDT rats in the non-fasted state was associated with this hypoinsulinemia. Significant decreases in body weight were observed in the 24- and 32-week-old SDT rats. The plasma triglyceride and total cholesterol levels remained unchanged at 16, 24 and 32 weeks of age. Glucosuria appeared in the 24- and 32-week-old SDT rats (data not shown).

---

**Fig. 1. Glucokinase (GK) mRNA level (A) and glucokinase activity (B) in the liver.** Data represent means ± S.E. (n=6).

Open bar, SD rats. Closed bar, SDT rats. (*) p=0.07, * p<0.05, ** p<0.01 vs. age-matched SD rats.
Fig. 2. Changes of key enzymes in the glycogen metabolic pathway. Glycogen synthase (GS) mRNA levels (A) and activity (B), glycogen phosphorylase (GP) mRNA levels (C) and activity (D) and glycogen content (E) in the liver. Histopathological changes in the liver of a 16-week-old SD rat (F) and SDT rat (G) with PAS staining. Data represent means ± S.E. (n=6).

Open bar, SD rats. Closed bar, SDT rats. * p<0.05, ** p<0.01 vs. age-matched SD rats.
GLUCOSE METABOLISM DISORDER IN SDT RATS

malities were determined.

Accordingly, we examined the changes in glycogen content over a fasting and non-fasting cycle [6] (Fig. 3). We then measured the glycogen content in the liver in the fasted and non-fasted states. Although the glycogen content of the liver was almost completely exhausted in the fasted state in the normal SD rat, this change was not observed in the 24- and 32-week-old SDT rats.

We confirmed that the glycogen metabolism pathway of diabetic SDT rats is disordered, and so glycogen could not play a role in glucose supply.

Key enzyme of gluconeogenesis: We measured mRNA levels of G-6-Pase [3, 12, 17], FBPase and PEPCK, which plays an important role as a rate-limiting enzyme of gluconeogenesis (Fig. 4). The mRNA levels of G-6-Pase and FBPase were significantly increased in the 24-week-old SDT rats compared with age-matched SD rats. At 32 weeks of age, the SDT rats showed a tendency for increased mRNAs levels of G-6-Pase, and FBPase compared with the SD rats, but the difference was not significant. The levels of PEPCK mRNA were significantly increased in the 24- and 32-week-old SDT rats. There were no changes in the mRNAs at 16 weeks of age.

These findings showed that key enzymes of gluconeogenesis declined after advance to the diabetic state.

DISCUSSION

The SDT rat is a model in which the pathology gradually progresses to development of diabetes [14]. This study focused on glucose metabolism and glycogen in the prediabetic and diabetic SDT rat liver to investigate the changes associated with development of diabetes.

The diabetic state developed in the SDT rats by 24 weeks of age based on the fact that urine sugar was negative at 16 weeks of age but positive at 24 weeks of age. The changes in physiological parameters included a decrease in body weight, increase in serum glucose and decrease in insulin from 24 weeks of age onward (Table 2), which is consistent with the previously reported characteristics of SDT rats that are diabetic with insulin hyposecretion [14, 21]. Combined with a report by Masuyama et al. [15], which revealed impaired glucose tolerance at 12 weeks of age, the mild increase in serum glucose of the SDT rats at 16 weeks in this study suggests gradual pathological progression occurs in SDT rats.

Detailed examination of enzymes involved in hepatic glucose metabolism demonstrated that the glucokinase mRNA level and activity decreased in the prediabetic state (Fig. 1). Such decreases were not observed in the SDT rats at 8 weeks of age (data not shown). These findings suggest that a decrease in glucokinase, which plays a key role in the first step of hepatic glucose metabolism [1], is partly responsible for the mild increase in serum glucose. A decrease in glycogen synthase activity and moderate decrease in glycogen store also preceded development of diabetes (Fig. 2). The former decrease is explained by down regulation of glycogen synthetase activity in response to decreased glucose availability resulting from a decrease in glucokinase, whereas the latter decrease is thought to be associated with a decrease in the glycogen synthesis rate.

After onset of diabetes, a decrease in the glycogen phosphorylase mRNA level and enzyme activity and a further decrease in glycogen store occurred in addition to the changes in the prediabetic state (Fig. 2). Glycogen phosphorylase is known to exert high activity in response to attenuated insulin signal due to insulin deficiency, and low expression or activity in association with an increase in serum glucose, a decrease in glycogen store, enhancement of gluconeogenesis (due to glycerol and FFA influx resulting from lipolysis) [7] and an increase in glucagon/insulin ratio [18] (ex. hyperglucagonemia in the STZ rat). In SDT rats, GP mRNA and activity are considered to be decreased as a consequence of elevation of serum glucose and reduction of glycogen store.

Thus, caught in a vicious cycle of abnormalities such as a decrease in glucose availability and decline of glycogen synthesis and decomposition pathways, glycogen continues...
involved in the onset of diabetes in the SDT rat. The question then becomes, "Why does glucokinase begin to be suppressed before onset of diabetes?" One possible answer is expression control by insulin [1]. Testing of the glucose tolerance of SDT rats at 16 weeks of age revealed mild impaired glucose tolerance, with suppressed additional insulin secretion [15, 21]. Hence, suppressed expression of glucokinase is attributable to insulin hyposcretion already present in the prediabetic state. For other expression-regulating hormones, including glucagon, glucagon-like peptide-1 (GLP-1), catecholamine and glucocorticoids, it remains undetermined whether or not there is secretion disorder, which presents future challenges. It is uncertain whether the phenomenon affecting only glucokinase far ahead of all other glucose metabolism-involved proteins for which expression is regulated by insulin is characteristic to this model or a common process in diabetic pathogenesis. However, given reports of decreased glucose disposal rate (glucose uptake) in patients with impaired glucose tolerance as in diabetic patients [8, 23], it is possible that decreased glucokinase partly contributes to a decrease in glucose availability.

A possible cause other than hormones is gene abnormality, as noted in other animal models [10, 27]. Particularly, prediabetic pathology has been shown to have many similarities to the pathology of glucokinase deficiency (MODY II) [19, 23, 25], including mild increase in serum glucose, decrease in glycogen, slow progress of pathology and insulin hyposcretion, and this warrants investigation of glucokinase and other gene abnormalities. Glucokinase is suppressed in human NIDDM [4, 5], STZ [13], NOD [22] and ZDF [16] rats and is elevated in db/db and ob/ob mice [26] and GK, OLETF [24] and ZF [2] rats. Although it has not been clarified how this difference is produced, it may be certain that suppressed insulin is always accompanied by suppressed glucokinase. Suppressed glucokinase in human NIDDM [4, 5] is of special interest in associating human diabetic pathology with animal model pathology.

Previous studies in the SDT rat have mainly investigated changes in the pancreas associated with insulin hyposcretion [15] and complications at older ages [20, 21], whereas we have focused on the liver to investigate the progress of diabetic pathology. We have provided evidence for the involvement of hepatic glucose metabolic disorder in the onset and progression of diabetes in the SDT rat. To elucidate the mechanism of the onset of diabetes and to develop drugs to prevent diabetic pathological progression, the SDT rat should offer a highly useful model of spontaneous diabetic onset for such increasingly important studies.

REFERENCES


---

**Fig. 4.** Changes of gluconeogenic key enzyme mRNA levels. Glucose-6-phosphatase (G-6-Pase) (A), fructose-1,6-bisphosphatase (FBPase) (B) and phosphoenolpyruvate carboxykinase (PEPCK) (C) mRNA levels in the liver. Data represent means ± S.E. (n=6). Open bar, SD rats. Closed bar, SDT rats. * p<0.05, ** p<0.01 vs. age-matched SD rats.

---

to be suppressed, thereby affecting glycogen store- and glucose-releasing response to feeding conditions. The liver, with depleted glucose materials, has no choice but to release glucose, resulting in enhancement of gluconeogenesis (Fig. 4).

These findings suggest that hepatic glucose metabolic disorder, beginning with suppressed glucokinase, is greatly


