Diabetes in the Toronto-KK Hybrid Mouse Abnormalities in Liver and Pancreatic Islets of Langerhans

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Summary. The activities of six hepatic enzymes were assayed in six-month old Toronto-KK and C57BL/6J mice. Activities of hepatic gluconeogenic enzymes (phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, fructose-1,6-diphosphatase) were significantly elevated in Toronto-KK mice while activities of glycolytic enzymes (glucokinase, hexokinase, pyruvate kinase) remained normal. Isolated islets of Langerhans obtained from Toronto-KK mice demonstrated significantly elevated levels of insulin secretion and of insulin and RNA biosynthesis in response to glucose stimulation. Islets of Toronto-KK mice were markedly hyperplastic and showed

pronounced B-cell degranulation after stimulation with glucose. The data suggest that hyperglycemia appears to result, in part, from excessive gluconeogenesis. Hyperinsulinemia appears to result from excessive insulin synthesis and release from the pancreatic islets in response to glucose stimulation.

Key words: Toronto-KK mouse, diabetes in animals, gluconeogenesis, insulin synthesis and secretion, islets of Langerhans, obesity, gluconeogenic enzymes, glycolytic enzymes, B-cell degranulation, islet RNA synthesis.

In an attempt to establish a colony of KK mice displaying greater homogeneity in the expression of diabetic characteristics, a hybrid between the KK mouse and the C57BL/6J mouse has been inbred and designated as the Toronto-KK mouse. The metabolic disorders associated with the diabetic syndrome of this animal are similar to those of the parent KK strain and have been previously described by Nakamura [20, 21], Dulin [7, 8] and others [3, 16, 19]. Particularly striking abnormalities associated with diabetes in this animal include hyperglycemia and hyperinsulinemia. At present, the etiological factors responsible for these conditions remain unclear. This paper summarizes our efforts to evaluate the roles of hepatic glycolytic and gluconeogenic enzyme activities and of insulin biosynthetic and secretory components from isolated pancreatic islets of Langerhans on these respective metabolic defects.

Materials and Methods

Animals

Toronto-KK mice were obtained from crosses between the Japanese KK mouse and the C57BL/6J mouse (The Jackson Laboratory) [2]. The hybrid progeny resulting from this crossing were maintained in the Upjohn colony [7] and were continuously inbred. Toronto-KK mice were selected on the basis of their fulfilling the following criteria: body weights equal to or greater than 35 g, blood glucose concentration equal to or greater than 200 mg% and plasma immunoreactive insulin levels equal to or greater than 1000 μ U/ml. Male animals six months of age were selected because

the diabetic characteristics were more uniform in male than in female mice, and the diabetic syndrome appears to be climatic at this stage of development. C57BL/6J mice of the same age and sex served as controls. All animals were provided with food (Purina Mouse Breeder Chow) and water *ad libitum*.

Liver Enzyme Assays

Liver supernatant and microsomal fractions were prepared as described by Chang *et al.* [4]. The following enzymes were assayed by methods cited in the references: glucokinase (glukase) — hexokinase (hex-kase) [23], pyruvate kinase (pyr-kase) [24], phosphoenolpyruvate carboxykinase (PEP-CK) [26], glucose-6-phosphatase (glu-6-Pase) [22] and fructose-1,6-diphosphatase (fruct-1,6-dPase) [27]. Inorganic phosphate concentrations were estimated by the method of Nordlie *et al.* [22].

Insulin Content from Plasma, Pancreas and Isolated Islets of Langerhans

Blood samples were collected from the orbital venous sinus using heparinized capillary tubes and 0.1 ml plasma was used for the insulin assay. Pancreatic insulin content was determined by processing individual pancreata according to the method of Gerritsen *et al.* [10] with subsequent insulin assay. Insulin content from isolated islets of Langerhans was determined following sonication of the islets in 0.1 M Tris-Cl buffer (pH 7.4) containing 1% bovine serum albumin and assaying the sonicate at various dilutions. All insulin concentrations were estimated according to the radioimmunoassay procedure of Zaharko and Beck [30].

Insulin Secretion

Islets of Langerhans were isolated from the intact pancreas using the method of Lacy and Kostianovsky [17]. Fifty islets were transferred to conical Swinex®-13-Filter units (Millipore Corporation) containing a three micron cellulose filter and connected at both ends with Tygon® tubing. Flow of buffer was maintained constant at 2.0 ml/min by means of a Cole-Parmer Masterflex microperistaltic pump and effluent from the distal portion of the filter unit was recovered using a fraction collector and frozen immediately. A modified Hank's solution (pH 7.4) containing 4.2 mEq/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.0 g/l bovine serum albumin, and 2000 $\mu\text{U/l}$ pancreatic trypsin inhibitor either with or without glucose in one of the following concentrations was used as a "perfusate": 0.9 g/l glucose or 3.75 g/l glucose. The perfusate was maintained at 37°C and gassed continuously with 95% O_2 –5% CO_2 . All islets were equilibrated for fifteen minutes in perfusate containing no glucose and subsequently stimulated with respective glucose test solutions for 90 min.

Insulin Biosynthesis

One hundred islets were isolated from two animals and transferred to a Parafilm® covered watchglass. As a control comparable segments of acinar pancreas were harvested and treated in the same way as the islet tissues. (Hereafter, only treatment of islet tissues is discussed). Isolated islets were incubated in 0.25 ml of medium of the following composition: Krebs-Ringer bicarbonate buffer (pH 7.4) which contained 2 $\mu\text{g/ml}$ 19 amino acid mixture (leucine excluded), 3.75 mg/ml glucose, 1 $\mu\text{g/ml}$ pancreatic trypsin inhibitor, 0.2 mg/ml bovine serum albumin, 0.9 mM KH_2PO_4 , ^{32}P (100 mCi/mmol, New England Nuclear) and 5.7 μM leucine- ^3H (2.2 mCi/mmol, New England Nuclear). Incubation was allowed to proceed at 37°C for four hours under an atmosphere of 95% O_2 –5% CO_2 . At the end of incubation the islets and medium were extracted with 1.0 ml acid alcohol containing 1mM leucine, 0.118 M KH_2PO_4 and 1 mg/ml bovine insulin (Sigma) as carrier according to Davoren [5]. The precipitates thus obtained were fractionated on a Bio Gel P 30 column (0.9 × 60 cm) in 3.0 M acetic acid. The radioactivity of the bovine insulin peak indicated the amount of leucine- ^3H incorporation into murine insulin.

RNA and DNA

The precipitate obtained after islet homogenization was washed 5 × with 1 N HClO_4 containing 1mM leucine and 0.118 M KH_2PO_4 ; 0.91 ml of 0.5 M NaOH was added to the precipitate and the suspension incubated at 37°C for one h. Following incubation, 0.09 ml of 70% HClO_4 was added and the mixture centrifuged. The radioactivity from aliquots of the 0.5 M NaOH hydrolysate were determined and RNA content was estimated according to Fleck *et al.* [9]. The precipitate obtained after alkaline hydrolysis was in-

cubated in 1.0 ml of 1.0 M HClO_4 at 90°C for 20 min. The mixture was centrifuged and the DNA content of the supernatant estimated according to Tsanev *et al.* [25].

Miscellaneous

Tissues prepared for histological demonstration were fixed in Bouin's solution and stained with hematoxylin and eosin [15] and/or with Gormori's aldehyde fuchsin-ponceau [11] stain. Blood glucose was determined according to the method of Hoffman [14] and protein determination was performed according to Lowry *et al.* [18]. Hepatic glycogen was precipitated from 30% KOH extract and measured with the phenol-sulfuric acid method [6]. Spectrophotometric measurements were performed with a Beckman DB spectrophotometer. Radioactivity determinations were made using a Packard Tricarb Scintillation Spectrophotometer and corrected for quenching using the channel-ratio method [13].

Results

General Characteristics

Various characteristics of Toronto-KK and C57BL/6J mice are summarized in Fig. 1. At six months of age, Toronto-KK mice are significantly heavier than controls and are markedly obese as evidenced by the

Fig. 1. Various characteristics of six-month old C57BL/6J and Toronto-KK mice. Numbers indicate the mean of results obtained from studies on N animals

significantly increased weight of their epididymal fat pads. Non-fasting blood glucose levels were significantly higher than controls and plasma immunoreactive insulin was more than thirty times that of controls. Pancreata of Toronto-KK mice contained nearly twice as much insulin per unit tissue weight as those of the controls.

Liver Studies

Livers of Toronto-KK mice were greatly enlarged and showed marked fat deposition which in other studies has been identified as "fatty liver" syndrome [1]. Toronto-KK livers weighed more than twice that of control livers, accounted for a significantly greater percentage of total animal body weight and contained significantly less total protein and glycogen per unit tissue weight than the control livers (Fig. 2). The activities of six hepatic "regulatory" enzymes were assayed (Fig. 3). No significant differences were observed in the activities of glycolytic enzymes (glu Kase, hex Kase, pyr Kase) between liver fractions from Toronto-KK and control mice. However, activities of gluconeogenic enzymes (PEP-CK, glu-6-Pase, fruc-1,6, d Pase) were significantly elevated in livers from Toronto-KK mice.

Insulin Secretion

Fig. 4 illustrates the results obtained from typical experiments in which the effects of physiologically "high" and "low" glucose concentrations upon insulin release were studied. The patterns of glucose-induced insulin release were generally similar from islets of both animals. With the absence of glucose in the perfusate, islets from both animals released small and approximately equal amounts of insulin. However, islets from Toronto-KK mice released significantly more insulin than controls when stimulated with either 0.9 g/l or 3.75 g/l glucose solutions. Insulin release appeared to be multiphasic from islets of both animals as was reported by Grodsky [12]; however, the pattern of multiphasic insulin release was most pronounced from Toronto-KK islets. Table 1 summarizes the results of the insulin studies and shows some quantitative variances between Toronto-KK and C57BL/6J islets. Islets of Langerhans from Toronto-KK mice contained more than twice as much insulin per islet than did control islets either before or after perfusion. Toronto-KK islets also released more than four times as much insulin during the perfusion period than the control islets when stimulated with the same concentrations of glucose. Finally, the percentage of insulin released to total islet insulin from Toronto-KK islets was over twice as much as that from islets of controls.

Insulin Biosynthesis

The BioGel P 30 elution profile of leucine-³H labelled acid alcohol soluble proteins may be seen in Fig. 5. Both C57BL/6J and Toronto-KK islets synthesized insulin *in vitro* as evidenced by the peak of radioactivity

Fig. 2. Various characteristics of livers of six-month old C57BL/6J and Toronto-KK mice

Fig. 3. Activities of the hepatic enzymes of six-month old C57BL/6J and Toronto-KK mice. Numbers represent the mean of specific activity of individual enzyme obtained from studies on N animals. Specific activity was defined as nmole substrate turnover per minute per mg total liver protein

coincident with the elution position of the bovine insulin carrier. However, greater incorporation of the label occurred in Toronto-KK islets indicating a greater degree of insulin biosynthesis. Peaks corresponding with proinsulin were also evident in both types of islets, with greater incorporation present in Toronto-KK islets. The relative proportions of proinsulin to insulin synthesized during incubation were approximately equal in both animals. No leucine- ^3H labelled materials were associated with the elution position of the bovine insulin carrier in any of the acinar tissues studied.

Fig. 4. Insulin release profiles by isolated islets of Langerhans in response to continuous stimulation of glucose

RNA and DNA

Islets of Langerhans from Toronto-KK mice contained significantly more DNA and RNA than the control islets (Table 2). In addition, both the total incorporation of $\text{KH}_2\text{PO}_4\text{-}^{32}\text{P}$ into RNA fraction and the specific incorporation of $\text{KH}_2\text{PO}_4\text{-}^{32}\text{P}$ (incorporation into RNA fraction per unit weight of islet DNA) were significantly greater from Toronto-KK islets than from controls.

Histological Examination of Pancreatic Islets

Islets of Langerhans from Toronto-KK mice were markedly hyperplastic (Fig. 6). The ovoid or spherical shape of islets from C57BL/6J mice contrasted sharply with the Toronto-KK islets which appeared frequently as irregularly contoured structures. Large areas of cavitation and occasional blood-filled sinusoids were also observed in Toronto-KK islets. Striking differ-

Fig. 5. BioGel P30 elution profile of acid alcohol extracts of islet and acinar tissues. —●—●— indicates absorbance at 278 nm. —○—○— indicates incorporation of Leucine- ^3H . (ExPr = exclusion proteins; Ins = insulin; PIIns = proinsulin)

ences were seen in the degrees of granulation of Toronto-KK islets during fasted and non-fasted dietary conditions (Fig. 7). Islets from non-fasted Toronto-KK mice showed pronounced depletion of B-granules, whereas non-fasted C57BL/6J islets retained a full component of B-granules.

Discussion

Since levels of blood glucose reflect the dynamic equilibrium between its utilization and production, one may infer that hyperglycemia in Toronto-KK mice results from a decreased utilization of glucose and/or an overproduction of glucose. Several investigators have observed glucose intolerance and diminished sensitivity by adipose and muscle tissues to insulin in the KK mouse [7, 16]. Such evidence is strongly supportive of the notion that hyperglycemia in the Toronto-KK

peritoneally administered pyruvate- ^{14}C to glucose- ^{14}C of whole blood, suggesting that gluconeogenesis is likewise elevated *in vivo*.

That hepatic gluconeogenesis is elevated in diabetic Toronto-KK mice is surprising from at least several points of view. Hepatic gluconeogenesis is normally more active during periods of starvation in an attempt to accommodate metabolic requirements. Hepatic gluconeogenic enzymes from Toronto-KK mice had increased activity during non-fasting dietary conditions despite the obese physical state of the animal. In addi-

Table 1. Comparison of islet insulin content and insulin release between six-month old Toronto-KK and C57BL/6J mice

Type mouse	Islet insulin non-perf. ^a ($\mu\text{U}/\text{islet}$)	Islet insulin post-perf. ($\mu\text{U}/\text{islet}$)	Total insulin released ($\mu\text{U}/50$ islet/90 min)	% Islet insulin released ^b
C57BL/6J	2318 ± 536^c (4)	1760 ± 578 (4)	6866 ± 1944 (5)	6.77 ± 3.10 (4)
Toronto-KK	4988 ± 969 (4)	4067 ± 1105 (4)	29556 ± 13601 (5)	14.00 ± 2.95 (4)
(P) ^d	< 0.005	< 0.010	< 0.005	< 0.01

^a Values were obtained using islets other than those used in perfusion studies. (Toronto-KK islet insulin content was determined from 25–100 islets per animal. C57BL/6J islet insulin content was determined from 35–50 islets per animal.)

^b Values were derived by the following expression:

$$\frac{\text{Insulin content per islet following 90 min of perfusion}}{\text{Insulin content per nonperfused islet}} \times 100$$

^c Values represent means $\pm$ S.D. Number of mice used are in parenthesis.

^d P is the probability for the difference between C57BL/6J and Toronto-KK mice as determined by T-test.

Table 2. Comparison of islet characteristics and rates of incorporation of KH_2PO_4 - ^{32}P into RNA fraction of isolated islets of Langerhans of C57BL/6J and Toronto-KK mice

Type mouse	Islet DNA·P (ngDNA·P/islet)	Islet RNA·P (ngRNA·P/islet)	Total islet incorp. (dpm/ml/islet)	Specific islet incorp. ^a (pmole/ml/ μg DNA·P)
C57BL/6J	2.4 ± 0.3^b (3)	12.6 ± 3.6 (3)	456 ± 309 (3)	320 ± 181 (3)
Toronto-KK	6.9 ± 0.6 (3)	56.9 ± 37 (3)	2128 ± 1210 (3)	809 ± 232 (3)
P ^c	< 0.001	< 0.001	< 0.050	< 0.050

^a Specific islet incorporation is defined as pmole of KH_2PO_4 - ^{32}P incorporated per ml RNA fraction per μg of islet DNA·P.

^b Values represent means $\pm$ S.D. Number of animals used are in parenthesis.

^c P is the probability for the difference between C57BL/6J and Toronto-KK mice as determined from T-test.

mouse results from a decreased utilization of available glucose. The present studies also implicate overproduction of glucose as an additional etiological component of hyperglycemia in this animal. The elevated levels of gluconeogenic enzymes but normal levels of glucose catabolizing enzymes suggest that hepatic glucose production is accelerated in Toronto-KK mice. In addition, Wyse *et al.* [29] reported that Toronto-KK mice demonstrated an increased ability to convert intra-

tion, insulin has been implicated to have an inductive effect on hepatic "regulatory" glycolytic enzymes and a suppressive effect on "regulatory" gluconeogenic enzymes [28]. Enzymes of livers from Toronto-KK mice, therefore, do not respond to insulin in the manner expected since elevated activities of hepatic "regulatory" gluconeogenic enzymes were coincident with exorbitantly increased levels of plasma immunoreactive insulin.

Our data concerning the isolated pancreatic islets indicate that insulin biosynthesis and secretory mechanism within the B-cells of Toronto-KK mice are not impaired, but are hyperresponsive when stimulated by glucose. Supportive evidence for this notion is based on several experimental observations. Both Toronto-KK and C57BL/6J islets released minimal and essentially equal quantities of insulin in the absence of glucose. Therefore, insulin release from Toronto-KK islets does not proceed spontaneously and requires prior

stimulation by those physiological agents directing normal insulin release. The biphasic patterns of insulin release and elution profiles of leucine- ^3H incorporation into acid alcohol-soluble proteins were qualitatively similar from islets of both animals. However, the extent of insulin release and of leucine- ^3H incorporation following glucose stimulation was much greater from Toronto-KK islets. It is therefore tempting to infer that insulin synthesis and release proceed in a similar manner in islets from both animals, but at an acceler-

Fig. 6a and b. Pancreatic islets of six-month old C57BL/6J and Toronto-KK mice. a) C57BL/6J mouse (blood glucose 105 mg%, plasma immunoreactive insulin 41 $\mu\text{U/ml}$). Hematoxylin and eosin stain 90 $\times$. b) Toronto-KK mouse (blood glucose 294 mg%, plasma immunoreactive insulin 2560 $\mu\text{U/ml}$). Islet demonstrates pronounced hyperplasia. Central cavitation (C) is apparent. Hematoxylin and eosin stain 90 $\times$

Fig. 7a–c. Pancreatic islets of six-month old C57BL/6J and Toronto-KK mice during fasted and non-fasted dietary conditions. a) Small islet from a Toronto-KK mouse fasted for 24 h (blood glucose 85 mg%, plasma immunoreactive insulin 81 $\mu\text{U/ml}$). Islet appears deeply stained with well granulated aldehyde-fuchsin positive B-cells. Aldehyde fuchsin-ponceau stain 600 $\times$. b) Small islet from a Toronto-KK mouse fed *ad libitum* (blood glucose 272 mg%, plasma immunoreactive insulin 2560 $\mu\text{U/ml}$). A small number of beta cells contain aldehyde fuchsin positive granules. Most beta cells are markedly degranulated (arrows). Aldehyde fuchsin-ponceau stain 600 $\times$. c) Normal sized islet from a C57BL/6J mouse fed *ad libitum* (blood glucose 120 mg%, plasma immunoreactive insulin 42 $\mu\text{U/ml}$). Islet is well granulated. Aldehyde fuchsin-ponceau stain 600 $\times$

ated rate in Toronto-KK islets. In addition, the similar incorporation patterns suggest that an abnormal conversion of proinsulin to insulin would not appear to be

a factor in the elevated levels of plasma insulin observed from Toronto-KK mice.

The histologic appearance of pancreatic islets from

Fig. 7

Toronto-KK mice during fasted and non-fasted dietary states was consistent with the above observations. The striking loss of aldehyde fuchsin positive granulation with feeding suggests that exaggerated quantities of insulin are released in response to appropriate nutrient stimulation. Thus hyperinsulinemia observed from non-fasted Toronto-KK mice probably reflects an accelerated rate of insulin synthesis and secretion from an increased volume of B-cells, the absolute rate of secretion being, in part, dependent upon the insulin reserves contained within the islet.

The precise role of the islets of Langerhans in the diabetic syndrome of Toronto-KK mice remains unclear. The data indicate that hyperinsulinism in Toronto-KK mice is accompanied by hyperresponsive mechanisms of RNA and insulin biosynthesis and of insulin release. However, though rates of insulin biosynthetic and secretory activities are elevated, little is known concerning the causal effect of these processes on the observed B-cell hyperplasia. The specific causative factors ultimately responsible for this proliferation presently remain obscure.

In conclusion, our studies reveal that hyperglycemia appears to result, in part, from excessive and inappropriate hepatic gluconeogenesis. Hyperinsulinemia appears to result from excessive insulin biosynthesis and release from the pancreatic islets in response to glucose stimulation. This accelerated rate of insulin biosynthesis and release probably reflects both an increased volume of B cells and concomitant hyperresponsiveness displayed by these component B cells.

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