Defects in Liver and Muscle Glycogen Metabolism in Neonatal and Adult New Zealand Obese Mice

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Impaired glycogen synthesis is present in subjects at risk for developing non–insulin-dependent diabetes mellitus (NIDDM), suggesting that it is a primary defect in NIDDM. To examine whether defects in glycogen metabolism are present at birth in an animal model of NIDDM, glycogen synthase (GS), glycogen phosphorylase (GP), and total glycogen content were measured in liver and quadriceps muscle of 1-day- and 20-week-old insulin-resistant New Zealand Obese (NZO) mice and control (NZC) mice. In livers of both neonatal and adult NZO mice, active GS was reduced by 54% and 36%, respectively, as compared with that in NZC mice ($P < .03$). Total liver GS activity was the same in neonates, but was 65% higher in adult NZO as compared with NZC mice ($P < .02$). Liver glycogen was 28% lower at birth in NZO mice ($P < .03$), but was 49% higher at 20 weeks of age. Active and total GP were the same in NZO and NZC animals, despite hyperinsulinemia in 20-week-old NZO mice. In muscle, active GS was reduced by 41% in both 1-day- and 20-week-old NZO mice ($P < .02$). Total GS was also lower in NZC mice at 1 day of age ($P < .01$), but not at 20 weeks. No differences were detected in GP activity or in total glycogen content in muscle. Therefore, reduced GS activity is an early defect present at birth in the insulin-resistant NZO mouse in both liver and muscle. However, it is not the sole determinant of the amount of glycogen deposited in tissues. Elevated liver glycogen in adult NZO mice may contribute to hepatic glucose overproduction.

REDUCED GLYCOGEN SYNTHESIS in muscle is an early and major abnormality in non–insulin-dependent diabetes mellitus (NIDDM).[1,2] This finding has resulted in glycogen synthase (GS), the rate-limiting enzyme for glycogen synthesis, being recently investigated as a gene with a potential role in NIDDM (ie, a "diabetogene").[3-5] To date, this search has proved largely fruitless and may indicate that the diabetogene responsible for reduced glycogen synthesis lies elsewhere, for example, in the protein phosphatases that dephosphorylate and activate GS or in the protein kinases that phosphorylate and inactivate GS. Some of these protein phosphatases and kinases are known to be defective in skeletal muscle in NIDDM.[6-9] Alternatively, the initial defect could lie in the action of the protein primer, glycogenin, which initiates glycogen synthesis. It is also possible that since glycogen itself inhibits GS,[10] reduced levels of this enzyme may be a result of higher glycogen levels. If this elevated glycogen is a consequence of reduced glycogen phosphorylase (GP) activity, this may be where the diabetogene lies. Increased liver glycogen levels have been found in NIDDM subjects[11,12] and in some animal models of diabetes, eg, streptozotocin-treated rats,[13] obese fa/fa Zucker rats,[14] and db/db mice,[15] although there are studies contradicting these findings.[16-18] Compared with muscle, relatively little is known of glycogen metabolism in the liver in NIDDM. Liver glycogen may be of crucial importance in regulating basal blood glucose concentration if autoregulation between gluconeogenesis and glycogen breakdown controls the rate of hepatic glucose production.[19-21]

The aim of this study was to investigate whether defects in glycogen metabolism in the liver and skeletal muscle are present at birth and in adult life in an animal model of NIDDM, the New Zealand Obese (NZO) mouse. The enzymes that control the rate of glycogen synthesis (GS) and the rate of glycogen breakdown (GP) plus the total tissue glycogen content were measured.

MATERIALS AND METHODS

Chemical reagents were of analytical grade and were purchased from Sigma (St Louis, MO). All enzymes were purchased from Boehringer Mannheim (Munich, Germany). Radiolabeled compounds were obtained from DuPont New England Nuclear Research Products (North Sydney, NSW, Australia). The NZO mouse is a polygenic model of obesity and insulin resistance obtained by inbreeding for coat color, with agouti giving rise to the NZO mouse while tan-colored control (NZC) mice remain lean.[22] Adult NZO and NZC mice were obtained from the Walter and Eliza Hall Institute (Parkville, Victoria, Australia). Neonatal mice (1 to 4 days old; mean, 1 day; n = 21 NZC and n = 25 NZO animals) were bred in our Departmental Animal House. After decapitation, the liver and quadriceps muscle were extracted rapidly (within 1 minute) and placed in liquid nitrogen. Plasma glucose and insulin levels could not be measured in these animals because of their small size. Adult mice ranging from 19.1 to 21.6 weeks of age, with a mean age of 20.1 weeks (n = 9 NZC and n = 10 NZO animals) were fed *ad libitum* a regular laboratory chow with ready access to water. On the morning of study, they were anesthetized with a 60-mg/kg intraperitoneal injection of pentobarbital sodium (Nembutal; Ceva, NSW, Australia). After 15 minutes, a tail-vein blood sample was taken to measure plasma glucose and insulin levels. A laparotomy was performed 30 minutes after induction of anesthesia, and the liver was rapidly frozen in situ with liquid nitrogen–cooled tongs. A sample of white quadricep muscle was taken within 1 minute of the initial incision and placed immediately in liquid nitrogen. Tissues were stored at −70°C until assayed.

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Analytical Procedures

Plasma glucose level was measured with the glucose oxidase method using a Yellow Springs glucose analyzer (Yellow Springs, OH). Plasma insulin was assayed by radioimmunoassay (Pharmacia, Uppsala, Sweden) using a double-antibody technique to separate free and bound insulin.

GS and GP Assays

The enzyme methods used were slight modifications of those used by Chen et al,[23] incorporating some features of the assays described by Golden et al[24] and Nuttall and Gannon.[25] Frozen tissue samples weighing between 10 and 60 mg were homogenized with a Polytron PT 1200 homogenizer (Kinematica, Switzerland) for 20 seconds in 19 vol cold buffer containing (in millimolars) 100 NaF, 10 EDTA, 5 dithiothreitol, and 50 glycylglycine at a final pH of 7.0. This homogenate was used without centrifugation.

GS level was measured in its less phosphorylated "active" form independent of glucose-6-phosphate (G6P) and also as total activity. Enzyme activity was measured by the incorporation of uridine diphosphate glucose (UDPG) into glycogen in the absence of G6P for measurement of active GS and in the presence of a high G6P concentration for total GS. Active GS was determined by adding 25 μL tissue homogenate to 50 μL of a reaction mixture containing (in millimolars in final reaction mixture) 50 imidazole, pH 7.0, 5 EDTA, 0.8 UDPG, and 15 $NaSO_4$, plus 1% glycogen and ^{14}C-labeled UDPG (~12,000 dpm per tube). A concentration of 0.8 mmol/L UDPG was chosen to approximate physiologic conditions and lies midrange within the various UDPG concentrations described in the literature. After 30 minutes of incubation at 30°C, reactions were stopped by delivering 60 μL of the reaction mixture onto an anion-exchange resin column (Dowex 1-X8, 100-200 mesh, chloride form, bed volume 2 mL; Bio-Rad Laboratories, Richmond, CA). The column was washed four times with 0.5 mL water, and radioactivity in the eluate was determined after adding 10 mL scintillation fluid (Ready Value; Beckman, Irvine, CA). We chose to extract labeled glycogen with columns rather than filter paper because of the lower blanks obtained with columns, as well as a number of other methodological advantages outlined by Golden et al.[24] Total GS activity was measured in the presence of 10 mmol/L UDPG (in place of 0.8 mmol/L UDPG) and 10 mmol/L G6P (in place of $NaSO_4$), and for the liver assays Tris buffer (pH 8.8) was used in place of imidazole in the incubation mixture.

GP activity was also measured as two different activities in an active phosphorylated state and as total activity. Enzyme activity was assayed in the direction of glycogen synthesis using a low or high concentration of substrate (glucose-1 phosphate [G1P]) to measure active or total phosphorylase activity, respectively. Active GP was determined by adding 25 μL tissue homogenate to 50 μL of a reaction mixture containing (in millimolars in final reaction mixture) 33 morpholinoethanesulfonic acid buffer (pH 6.3), 15 G1P, and 0.5 caffeine, plus 0.35% glycogen and ^{14}C-labeled G1P (~20,000 dpm per tube). The remainder of the assay was identical to the GS assay. Total GP activity was measured in the presence of 273 mmol/L G1P (in place of 15 mmol/L), 5 mmol/L adenosine monophosphate (in place of caffeine), and 1.4% glycogen (in place of 0.35%).

Enzymatic activity was measured as the amount of enzyme that incorporated 1 nmol substrate (ie, UDPG or G1P) into glucosyl units per minute at 30°C per gram of tissue.

Tissue glycogen level was measured using a modified method of Keppler and Decker.[26] Briefly, 10 mg tissue was homogenized with a Polytron homogenizer in 200 μL ice-cold perchloric acid (0.6 mol/L). A sample of this homogenate (40 μL) was added to 20 μL $KHCO_3$ (1 mol/L) plus 400 μL glucoamylase/acetate buffer (20 mg

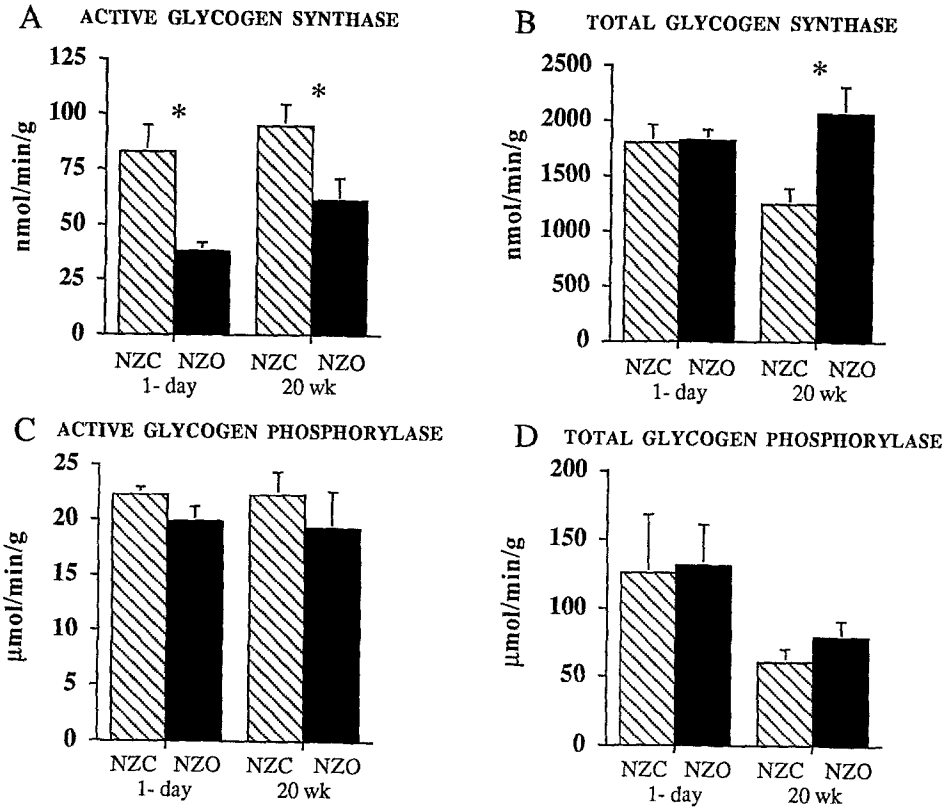

Fig 1. Active and total GS and GP activity in the liver of 1-day-old and 20-week-old NZC and NZO mice. *Significant difference between NZC and NZO animals.

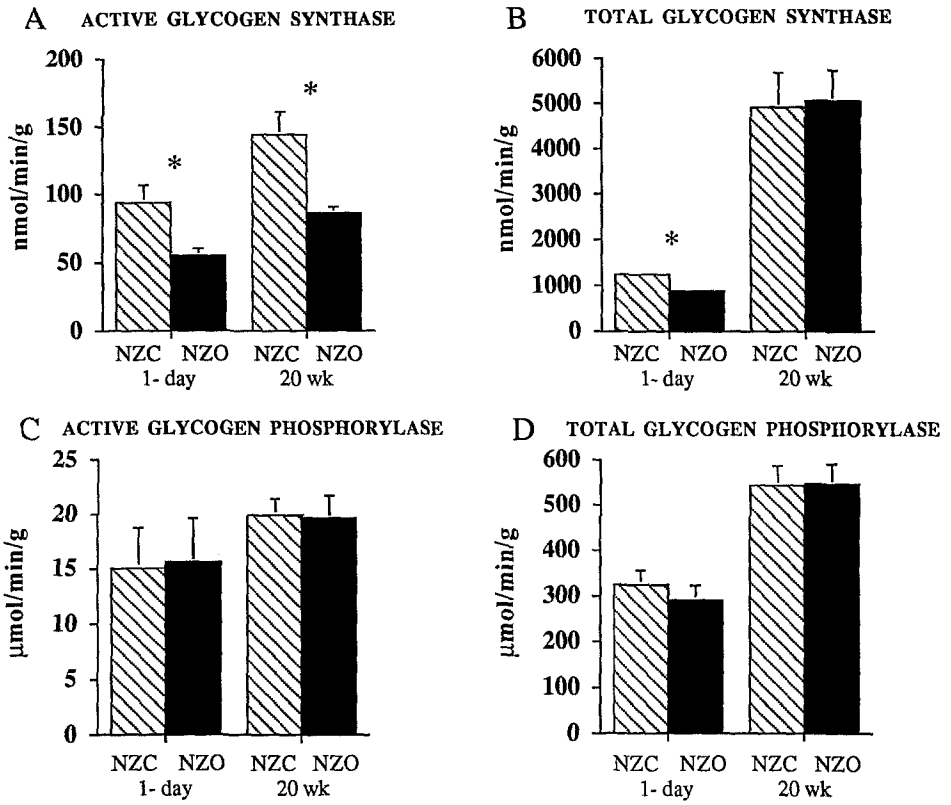

Fig 2. Active and total GS and GP activity in the quadriceps muscle of 1-day-old and 20-week-old NZC and NZO mice. *Significant difference between NZC and NZO animals.

amyloglucosidase in 20 mL acetate buffer, 0.2 mol/L, pH 4.8) and incubated at 40°C for 2 hours. Perchloric acid (0.6 mol/L, 200 μL) was then added, and the sample was centrifuged at 3,000 rpm for 10 minutes. The remaining tissue preparation was also centrifuged. Samples of these supernatants were analyzed for glucose using the method reported by Kunst et al[27] for determination of free glucose and glucose derived from glycogen.

RESULTS

In 20-week-old mice, plasma glucose concentrations were higher in NZO as compared with NZC mice (14.3 ± 0.5 *v* 18.2 ± 1.0 mmol/L, $P < .01$) and plasma insulin levels in NZO mice were twice those of NZC animals (38 ± 6 *v* 74 ± 5 mU/L, $P < .001$). The 20-week-old NZO mice were also twice as heavy as NZC mice (27.8 ± 0.5 *v* 56.9 ± 0.9 g, $P < .001$). However, at 1 day of age there was no difference in body weight between NZC and NZO mice (1.9 ± 0.1 *v* 1.8 ± 0.1 g, respectively).

Figure 1 illustrates GS and GP activities in livers of NZC and NZO mice at 1 day and 20 weeks of age. Active liver GS was markedly reduced in NZO mice in both neonates (by 54%, $P < .002$) and adults (by 36%, $P < .03$; Fig 1A). Total liver GS (measured at saturating concentrations of G6P) was the same in NZC and NZO mice just after birth, but by 20 weeks of age total activity was actually 65% higher in obese animals ($P < .02$; Fig 1B). Figures 1C and D show active and total GP activities in livers of NZC and NZO mice, respectively. No differences were detected between the animals at either age.

Figure 2 illustrates GS and GP activities in the quadriceps muscle of NZC and NZO mice at 1 day and 20 weeks of age. As in the liver, active GS in muscle was reduced in NZO mice in both neonates and adult animals (by 41% at both ages, $P < .02$; Fig 2A). Total muscle GS was 30% lower in NZO mice just after birth ($P < .01$), but by 20 weeks of age total activity was the same in NZC and NZO mice (Fig 2B). Figures 2C and D show active and total GP activities in muscle of NZC and NZO mice, respectively. Again, as in the liver, no differences were detected between the animals at either age.

Total glycogen contents in the liver and muscle of 1-day- and 20-week-old mice are shown in Fig 3. In liver, glycogen levels were 28% lower in NZO animals just after birth ($P < .03$), but at 20 weeks the situation had reversed and glycogen levels were 49% higher in NZO mice ($P < .01$). No differences were detected in muscle glycogen content between NZC and NZO mice at either age.

DISCUSSION

This study has shown that defects in GS are present at birth in both liver and quadriceps muscle in the insulin-resistant NZO mouse, which suggests that impaired GS activity occurs early in the development of NIDDM. Therefore, the GS defect may be of primary rather than secondary origin, although it could still be secondary to an *in utero* effect of glucose toxicity. By restricting G6P flux into glycogen, reduced GS could have a dominant role in producing peripheral insulin resistance in NIDDM. However, our results confirm other reports[28,29] showing that

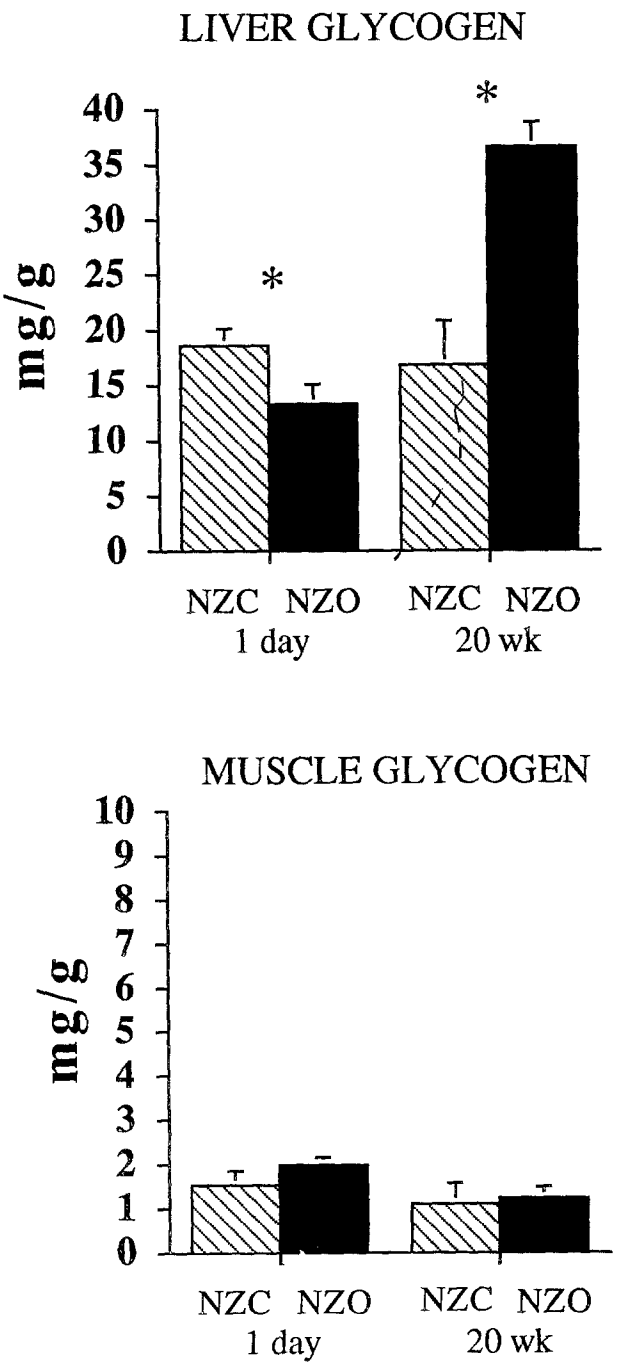

Fig 3. Glycogen concentration in liver and quadriceps muscle of 1-day-old and 20-week-old NZC and NZO mice. *Significant difference between NZC and NZO animals.

reduced GS activity does not always correlate with lower glycogen levels. In the present study, although reduced GS activity was accompanied by reduced glycogen content in the liver at birth, this was not the case in muscle at birth nor in either tissue later in life. In muscle, we could detect no differences in glycogen at 1 day or 20 weeks of age, whereas in the liver, glycogen levels had increased dramatically by 20 weeks of age. Increased glycogen content in livers of adult NZO mice may be caused by increased gluconeogenic enzyme activity and substrate flux through gluconeogenesis known to occur in this animal at this age.[30,31] This increased flux through gluconeogenesis to glycogen would use the indirect pathway of glycogen synthesis (ie, 3C intermediates → G6P → G1P → UDPG → glycogen). This pathway is increased in diabetic rats,[32] and the flux through it is believed to determine the total mass of glycogen synthesized. The increased liver glycogen levels found in adult insulin-resistant mice in the present study have been documented in diabetic rats,[13,14] diabetic mice,[15] and also in livers of NIDDM subjects,[11,12] and may have important physiological significance. There is evidence of intrahepatic autoregulation in nondiabetic subjects whereby an increase in gluconeogenesis is accompanied by a reduction in glycogen breakdown to maintain a stable rate of hepatic glucose production.[19,20] In other words, gluconeogenesis "spares" glycogen. This autoregulatory system may be defective or have a different setpoint in NIDDM subjects,[19,21] in whom gluconeogenesis and glycogen are markedly elevated, resulting in hepatic glucose overproduction. This is in line with the hypothesis of Felber et al[33] that insulin resistance is a consequence of excess substrates (glucose and nonesterified fatty acids) increasing glycogen levels and causing an inhibition of glycogen turnover.

In our study, hyperinsulinemia present in the NZO mouse at 20 weeks of age was unable to reduce GP activity despite the fact that insulin is normally a potent inhibitor of glycogenolysis. Therefore, insulin is ineffective not only in activating GS but also in inactivating GP. This insulin-resistant GP activity, in the presence of elevated glycogen, may contribute to the elevated hepatic glucose production that has been shown to occur in 20-week-old NZO mice.[34]

The changes in total GS activity found in the muscle and liver of the NZO mouse could be due to alterations in either the intrinsic activity or the turnover rate of the protein. The increased total activity found in livers of 20-week-old NZO mice may be due to a response to hyperinsulinemia, since hyperinsulinemia has been shown to increase total GS activity in liver.[35] In muscle of 1-day-old NZO mice, we found the opposite with a decrease in total enzyme activity. A similar decrease was found basally and over a range of insulin concentrations in skeletal muscle of NIDDM subjects as compared with nondiabetic subjects.[36] This may be related to impaired expression of GS mRNA in muscle, which has been reported in NIDDM subjects.[3] The reason that this decrease in total enzyme activity was not found in muscle at 20 weeks of age and the reason for the different responses in muscle and liver are not known.

In conclusion, reduced GS activity is an early defect present at birth in the insulin-resistant NZO mouse in both liver and muscle. Later in life, increased gluconeogenesis, elevated liver glycogen, and insulin-resistant GP activity may combine to cause hepatic glucose overproduction.

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