Diabetes-Related Changes in Chromatin Structure of Brain, Liver, and Intestinal Epithelium

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To determine whether diabetes alters chromatin structure in vivo, fluorometric analysis of alkali-induced DNA unwinding was carried out in various tissues of streptozocin-induced diabetic rats and genetically obese diabetic (*db/db*) mice. When zero-order kinetics were used to analyze the data, the percentage of double-stranded DNA (%dsDNA) unwinding in brain, liver, and intestinal epithelium of diabetic rats maintained for 4 wk was significantly reduced compared with vehicle-injected control rats (%dsDNA 0.37 ± 0.05 vs. 0.73 ± 0.02 for brain, 0.59 ± 0.1 vs. 0.84 ± 0.02 for liver, and 0.58 ± 0.07 vs. 0.90 ± 0.13 for intestinal epithelium). Insulin treatment of diabetic rats normalized the rate of DNA unwinding in liver (0.82 ± 0.09 %dsDNA/min) and intestinal epithelium (1.05 ± 0.09 %dsDNA/min), but the increase in the unwinding rate of brain DNA (0.51 ± 0.06 %dsDNA/min) did not achieve control values. Similarly, alkali-induced DNA unwinding was significantly slower in brain and liver of *db/db* mice compared with homozygote controls. When first-order kinetics were used to analyze the data, fractional rate constants of DNA unwinding in brain and liver of diabetic rats or mice were significantly smaller than observed in nondiabetic control animals. The fractional rate constant of DNA unwinding in intestinal epithelium was not altered with diabetes. We conclude that chronic uncontrolled hyperglycemia can alter chromatin structure in vivo. *Diabetes* 39:348–53, 1990

There are significant diabetes-related changes in chromatin function. Some of these changes are secondary to diabetes-related alterations in hormonal milieu, whereas other changes can be the direct effect of hyperglycemia (1). In addition, elevated ambient glucose concentrations can directly modify DNA structure (2,3). The biological significance of such glucose-induced changes in DNA has been demonstrated in *Escherichia coli* filamentous bacteriophage (3). High glucose concentrations have been shown to induce DNA damage, hamper replication, and accelerate death in cultured human endothelial cells (4,5). The effect of chronic uncontrolled hyperglycemia on chromatin structure in vivo has not been clarified. In one study, DNA of lymphocytes from diabetic subjects was found to have increased digestibility in alkaline solutions, suggesting increased DNA strand breaks in diabetes (6). However, we have previously shown that alkali-induced digestibility of DNA from predominantly polymorphonuclear cells is reduced in diabetic subjects (7). Differences in cell types studied may account for the discrepancy in the reported findings. An increased alkali-induced DNA unwinding rate has been correlated with increased DNA strand breaks (8,9), whereas a decreased DNA unwinding rate may be due to increased cross-linking of DNA strands. These changes may be tissue specific and dependent on the replicative capacity of tissue. Thus, tissues with high turnover rate, e.g., intestinal epithelium, may be less likely to show changes induced by hyperglycemia. To determine the tissue specificity of diabetes-related changes in the stability of chromatin in alkaline solutions, we studied brain, liver, and intestinal epithelial chromatin of streptozocin-induced diabetic rats and genetically obese diabetic (*db/db*) mice.

RESEARCH DESIGN AND METHODS

Male Fischer 344 rats 3 mo of age were obtained from Harlan (Indianapolis, IN). A 1.3% solution of streptozocin (Upjohn, Kalamazoo, MI) in 0.05 M cold citrate buffer (pH 4.5) was injected at a dose of 45 mg/kg body wt i.p. Control rats (n = 10) were concurrently injected with citrate buffer. Rats

Glucose 1 mM = 18 mg/dl

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were maintained on regular rat chow and water ad libitum. Animals manifesting glucosuria, polydipsia, and polyphagia were considered diabetic (n = 13). The diabetic state was confirmed on the day of death by plasma glucose determination. Urinary excretion of ketones measured with Keto-Diastix (Ames, Miles, Elkhart, IN) was either trace or negative.

Five days after streptozocin injection, a group of diabetic rats was treated with NPH insulin (8 U/kg s.c.) twice a day until the end of the 4-wk observation period. Plasma glucose in diabetic rats was 375 ± 35 mg/dl, whereas levels in insulin-treated diabetic rats and control rats were 143 ± 9 and 138 ± 8 mg/dl, respectively. Of the insulin-treated diabetic group, only those with plasma glucose <200 mg/dl were included in the study (n = 13). Animals were killed by exsanguination through the abdominal aorta under pentobarbital sodium anesthesia. Brain (cerebrum) and liver were removed, immediately frozen on dry ice, and kept at −70°C until the day of the experiment. Intestinal epithelium was scraped off the entire length of the small intestine with a glass slide in ice-cold buffer of 0.32 M sucrose and 3 mM $MgCl_2$. Freshly isolated intestinal epithelium was processed for DNA-unwinding assay. Studies with brain and liver were carried out with frozen tissue. Pilot studies did not show any significant differences in DNA unwinding measured in freshly isolated or frozen tissues (data not shown). The number of tissues analyzed in each experimental group varied from 8 to 13.

In addition, studies on frozen brain and liver of 15 *db/db* mice at 90 days of age were compared with measurements in 14 heterozygote (*db/m*) and 6 homozygote (*m/m*) controls at the same age. The number of tissues analyzed in each group varied from 5 to 15. Intestinal tissue was not available from these mice, because these animals had been used by another investigator as controls for studies on memory but had not received any pharmacological treatment. Animals were originally obtained from Jackson (Bar Harbor, ME). Plasma glucose was measured on the day of death. Mean plasma glucose was 685 ± 25 mg/dl in *db/db* mice, 143 ± 11 mg/dl in *db/m* mice, and 140 ± 12 mg/dl in *m/m* mice.

Fluorometric analysis of the DNA-unwinding technique of Birnboim and Jevcak (8) was adapted for soft-tissue chromatin studies as described by Hartnell et al. (9).

Several solutions were used. Solution A was composed of 0.32 M sucrose and 3 mM $MgCl_2$; solution B, 0.25 M *meso*-inositol, 10 mM sodium phosphate, and 1 mM $MgCl_2$ (pH 7.2); solution C, 9 M urea, 10 mM NaOH, 2.5 mM cyclohexanediaminetetraacetate, and 0.1% sodium dodecyl sulfate; solution D, 0.45 vol solution C in 0.20 N NaOH; solution E, 0.40 vol solution C in 0.20 N NaOH; and solution F, 1 ml glucose and 14 mM mercaptoethanol.

Tissue was diluted 1:20 (wt/vol) with ice-cold solution A buffer and gently hand homogenized with seven strokes in a glass homogenizer specially milled to give 0.25-mm clearance. The homogenate was centrifuged at 800 × g for 10 min, and the pellet was washed once with 5 ml of solution A resuspended in 5 ml of solution B. We distributed 0.2-ml aliquots of this suspension in triplicate into three sets of glass tubes. In one set, unwinding in alkali was prevented (total double-stranded [ds] DNA [T]). In another set, unwinding was maximized by sonication for 5 s (90% output of 200-W

TABLE 1
Alkali-induced DNA unwinding rate (b) in brain, liver, and intestinal epithelium

Analysis	Control rats	Diabetic rats	Diabetic rats treated with insulin
Brain			
Zero-order kinetics			
b (%dsDNA/min)	0.73 ± 0.02	0.37 ± 0.05*	0.51 ± 0.06*
r	0.90	0.99	0.99
n	10	11	13
First-order kinetics			
b (%dsDNA/min)	0.99 ± 0.04	0.61 ± 0.11†	0.53 ± 0.09†
r	0.90	0.99	0.98
n	10	11	13
Liver			
Zero-order kinetics			
b (%dsDNA/min)	0.84 ± 0.02	0.59 ± 0.1†	0.82 ± 0.09
r	0.93	0.92	0.96
n	8	13	11
First-order kinetics			
b (%dsDNA/min)	1.77 ± 0.19	1.08 ± 0.14†	1.44 ± 0.14
r	0.99	0.98	0.99
n	8	13	11
Intestinal epithelium			
Zero-order kinetics			
b (%dsDNA/min)	0.90 ± 0.13	0.58 ± 0.07†	1.05 ± 0.09
r	0.97	0.91	0.92
n	10	13	8
First-order kinetics			
b (%dsDNA/min)	2.11 ± 0.29	2.14 ± 0.19	2.42 ± 0.49
r	0.95	0.98	0.98
n	10	13	8

Values are means ± SE. %dsDNA, percentage of double-stranded DNA; r, mean correlation coefficient; n, number of tissues analyzed. *$P < 0.001$, †$P < 0.05$.

TABLE 2
Alkali-induced DNA unwinding rate (*b*) in brain and liver of genetically obese diabetic mice (*db/db*) and heterozygote (*db/m*) and homozygote (*m/m*) controls

Analysis	*m/m*	*db/db*	*db/m*
Brain			
Zero-order kinetics			
b (%dsDNA/min)	0.581 ± 0.02	0.23 ± 0.007*	0.34 ± 0.01*
r	0.99	0.96	0.91
n	5	15	14
First-order kinetics			
b (%dsDNA/min)	1.69 ± 0.10	1.09 ± 0.11†	1.33 ± 0.15
r	0.97	0.98	0.97
n	5	15	14
Liver			
Zero-order kinetics			
b (%dsDNA/min)	0.94 ± 0.07	0.36 ± 0.03*	0.67 ± 0.03†
r	0.92	0.93	0.83
n	6	8	14
First-order kinetics			
b (%dsDNA/min)	1.79 ± 0.09	1.27 ± 0.16†	1.57 ± 0.23
r	0.96	0.94	0.92
n	6	8	14

Values are means ± SE. %dsDNA, percentage of double-stranded DNA; *r*, mean correlation coefficient; *n*, number of tissues analyzed. *$P < 0.001$, †$P < 0.05$.

Bronson 250 Sonifyer II, Danbury, CT; background [B]). In the third set of tubes, unwinding was allowed to occur without manipulations (experimental sample [E]).

We added 0.2 ml of solution C to each tube. The tubes were incubated at 0°C for 10 min to complete cell lysis. We added 0.1 ml of solution D to the E and B tubes, and 0.1 ml of solution E was gently added without mixing. The cell lysates were incubated at 0°C for 20 min to allow the alkali (pH 12.8) to diffuse into the lysate. The E and B tubes were incubated at 15°C for 15, 30, 45, and 60 min. At the end of the desired incubation time in alkali (performed in the dark), the reaction was stopped by neutralizing cell lysates with the addition of 0.4 ml of solution F and chilling the tubes at 0°C as previously described (8). This mixture was then briefly sonicated and diluted with 1.5 ml of freshly prepared dye solution (6.7 g/ml ethidium bromide in 13.3 mM NaOH). Brief sonication was performed for a fraction of a second with the same energy level indicated above. This rendered the lysate more homogeneous.

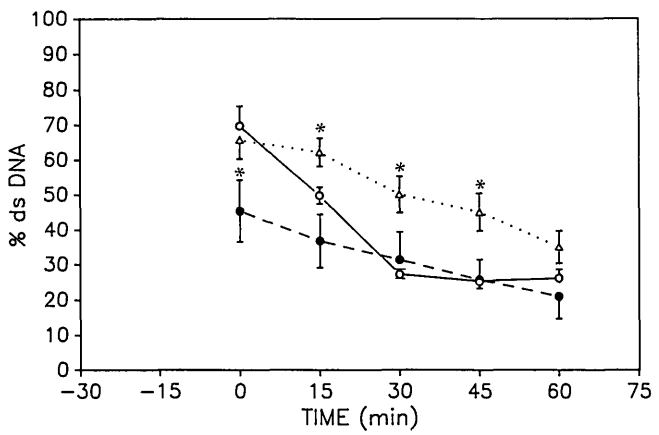

FIG. 1. Diabetes-related changes in percentage of double-stranded DNA (%dsDNA) in rat brain at different incubation times with alkali. ○, Control rats; ●, diabetic rats; △, insulinized diabetic rats. *$P < 0.05$ vs. control rats.

Fluorescence was read in an Aminco-Bowman spectrofluorometer (Silver Spring, MD) at an excitation wavelength of 520 nm and an emission wavelength of 590 nm. The %dsDNA remaining after different times of exposure to alkali was calculated by the equation %dsDNA = (E − B/T − B)100.

Data at each time point were compared. The DNA unwinding rate for each tissue was calculated for the individual experiment from the slope of the linear regression correlating %dsDNA with time of incubation in alkali. The correlation coefficient of the linear regression for each experiment was >0.87. Data were also analyzed as semilog regression. This analysis improved the fit of the data for most of the curves (Tables 1 and 2). The DNA unwinding rate in alkaline solutions was compared in control, diabetic, and insulinized diabetic rats by one-way analysis of variance. All results are means ± SE.

RESULTS

Diabetes-related changes in %dsDNA in the brain at different incubation times with alkali show that the %dsDNA at time 0 in diabetic rats (45.4 ± 8.1 %dsDNA) was significantly reduced compared with that in control rats (69.8 ± 5.6 %dsDNA, $P < 0.05$) or insulinized diabetic rats (65.7 ± 5.4 %dsDNA, $P < 0.05$) (Fig. 1). There were no significant differences between control and diabetic rats at subsequent incubation times with alkali. The %dsDNA in brain of insulinized diabetic rats compared with that in controls was significantly higher at 15, 30, and 45 min of incubation.

The %dsDNA in liver at time 0 was modestly but not significantly lower in diabetic rats (46.9 ± 5.8 %dsDNA) compared with controls (52.9 ± 0.9 %dsDNA) or insulinized diabetic rats (62.8 ± 8.1 %dsDNA) (Fig. 2). There were no significant differences at subsequent incubation times. The %dsDNA in intestinal epithelium at time 0 was significantly

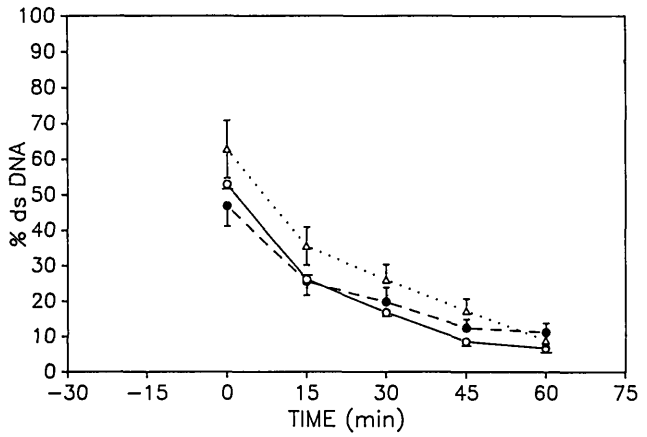

FIG. 2. Diabetes-related changes in percentage of double-stranded DNA (%dsDNA) in rat liver at different incubation times with alkali. ○, Control rats; ●, diabetic rats; △, insulinized diabetic rats.

lower in diabetic rats (37.5 ± 5.1 %dsDNA) compared with control rats (54.6 ± 5.3 %dsDNA, $P < 0.05$) or diabetic rats treated with insulin (73.4 ± 7.9 %dsDNA, $P < 0.01$). In addition, the %dsDNA in intestinal epithelium of diabetic rats compared with controls was significantly lower at 15 and 30 min of incubation (Fig. 3).

Table 1 summarizes the alkali-induced DNA unwinding rate in brain, liver, and intestinal epithelium of vehicle-injected control rats, streptozocin-induced diabetic rats, and diabetic rats treated with insulin. The DNA unwinding rate was calculated for individual experiments from the slope of the linear regression correlating the %dsDNA with time of incubation in alkali. Although differences between two comparison groups at each time point may not reach statistical significance, differences of the mean DNA unwinding rate calculated from individual experiments may be significant. Compared with controls, alkali-induced DNA digestibility is reduced in brain, liver, and intestinal epithelium of diabetic rats. The DNA unwinding rate in the brain of the diabetic rats (0.37 ± 0.05 %dsDNA/min) was significantly slower than that seen in control rats (0.73 ± 0.02 %dsDNA/min, $P < 0.001$). Similarly, DNA unwinding rates in liver and intestinal epithelium of diabetic rats (0.59 ± 0.10 and 0.58 ± 0.07 %dsDNA/min, respectively) were significantly slower than rates in control rats (0.84 ± 0.02 and 0.90 ± 0.13 %dsDNA/min, respectively, $P < 0.05$). Insulin treatment with normalization of plasma glucose could normalize the DNA unwinding rate in liver (0.82 ± 0.09 %dsDNA/min) and intestinal epithelium (1.05 ± 0.09 %dsDNA/min) of diabetic rats. In brain, however, the DNA unwinding rate was increased with insulin therapy (0.51 ± 0.06 %dsDNA/min) but did not reach control values.

When first-order kinetics were used to analyze the data, fractional rate constants of DNA unwinding in brain and liver of diabetic rats were significantly smaller than observed in control rats (Table 1). The fractional rate constant of DNA unwinding in intestinal epithelium was not altered with diabetes (Table 1).

A similar pattern of resistance to alkali digestion was seen in *db/db* mice. The %dsDNA in brain of *db/db* mice at time 0 (18.7 ± 0.6 %dsDNA) was significantly lower than seen in *db/m* mice (25.8 ± 0.7 %dsDNA) and *m/m* controls (39.7 ± 2.2 %dsDNA, $P < 0.001$) (Fig. 4). The %dsDNA measurements in *db/db* or *db/m* brain at 15 and 30 min of incubation were significantly lower than measured in *m/m* controls (Fig. 4).

The %dsDNA in liver of *db/db* mice (39.4 ± 3.8 %dsDNA) at time 0 was significantly lower than that in *db/m* mice (51.9 ± 2.8 %dsDNA) and control *m/m* mice (52.1 ± 2.1 %dsDNA) (Fig. 5). There were no significant differences in subsequent incubation times except that at 60 min, %dsDNA in liver of *db/m* mice was modestly higher than in *m/m* controls.

Table 2 shows the mean rate of alkali-induced DNA unwinding in brain and liver of *db/db* mice and in *db/m* and *m/m* controls. The DNA unwinding rate in brain of *db/db* mice (0.23 ± 0.007 %dsDNA/min) was significantly reduced compared with *m/m* controls (0.58 ± 0.02 %dsDNA/min, $P < 0.001$) or *db/m* controls (0.34 ± 0.01 %dsDNA/min, $P < 0.05$). Similarly, the DNA unwinding rate in liver of *db/db* mice (0.36 ± 0.03 %dsDNA/min) was significantly lower than in *m/m* mice (0.94 ± 0.07 %dsDNA/min, $P < 0.001$). Unexpectedly, DNA unwinding rates in both brain and liver of *db/m* mice were significantly reduced compared with *m/m* control mice. The DNA unwinding rate in liver of *db/m* mice was 0.67 ± 0.03 %dsDNA/min. However, this was not the case when first-order kinetics were used to analyze the data. The fractional rate constant of DNA unwinding in brain and liver of diabetic mice was significantly smaller than in *m/m* controls (Table 2). Values observed in *db/m* controls were not significantly different from those seen in *m/m* controls (Table 2).

DISCUSSION

Experiments validating the assay in our study have been published (9). This study is the application of this assay to the diabetes-related changes in chromatin structure. In streptozocin-induced diabetic rats, the digestibility of DNA in alkali was clearly reduced. This finding is in agreement with our previous observations in human leukocytes (7). Except in intestinal epithelium, diabetes-related changes were

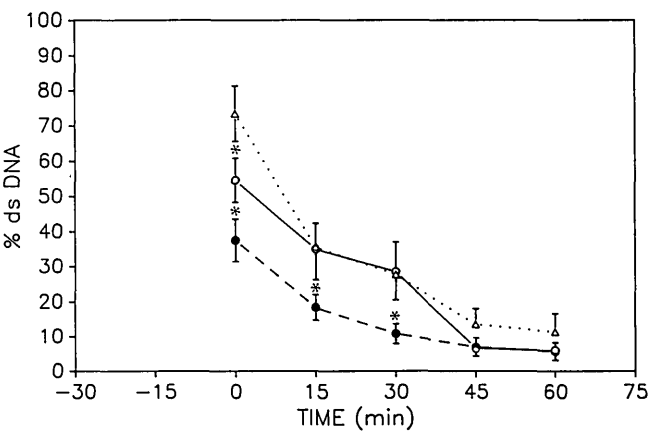

FIG. 3. Diabetes-related changes in percentage of double-stranded DNA (%dsDNA) in rat intestinal epithelium at different incubation times with alkali. ○, Control rats; ●, diabetic rats; △, insulinized diabetic rats. *$P < 0.05$ vs. control rats.

demonstrable when either zero-order or first-order kinetics were used to analyze the data. Insulinization with near normalization of plasma glucose could prevent diabetes-associated changes in hepatic and intestinal chromatin structure. In brain, however, insulinization reduced the diabetes-related change in DNA digestibility but did not correct it. This may suggest that brain nuclei are more susceptible than liver nuclei to glucose-induced changes. Insulinization probably did not achieve a perfect normalization of plasma glucose levels in these rats, although single plasma glucose determinations on the day of the experiment showed that insulinized diabetic rats had plasma glucose concentrations comparable to those of nondiabetic controls. We have recently found that the kinetics of chromatin digestion with micrococcal nuclease are altered in diabetic rat brain but not in liver (A.D.M., unpublished observations).

Precise biochemical mechanisms underlying this in vitro chemical reaction, i.e., alkali-induced DNA unwinding, are not known. This assay is capable of detecting single-stranded (ss) DNA lesions in leukocytes (8) and soft tissues (9). Endothelial cells but not fibroblasts incubated in the presence of high concentrations of glucose have increased ssDNA breaks, as suggested by enhanced alkali-induced digestibility (5). However, this assay is by no means a specific test of ssDNA lesions, and it can detect dsDNA breaks and alkaline-labile sites (10). Also, glucose-induced DNA-protein cross-links may alter alkali-induced DNA digestibility. In our pilot studies, duration of diabetes <7 days was not associated with changes in hepatic chromatin digestibility, suggesting that chronic exposure to hyperglycemia is necessary to see these changes in chromatin structure. An alternative interpretation is that although the DNA unwinding rate is a measure of alkaline-labile sites, %dsDNA at time 0 (i.e., 30 min after preincubation at 0°C) reflects in situ DNA breaks. Thus, in diabetic rats, the increased unwinding may occur at the beginning of exposure to alkali as a result of in situ DNA lesions, whereas the subsequent slow rate of unwinding is perhaps secondary to reduced alkali-labile sites in diabetes. However, this assay cannot distinguish between

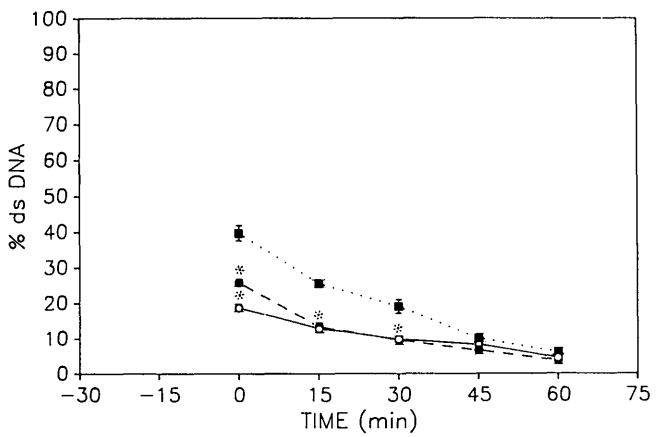

FIG. 4. Percentage of double-stranded DNA (%dsDNA) at different incubation times with alkali measured in brain of genetically obese diabetic mice (○, *db/db*) and heterozygote (●, *db/m*) and homozygote (■, *m/m*) controls. *$P < 0.05$ vs. *m/m* controls.

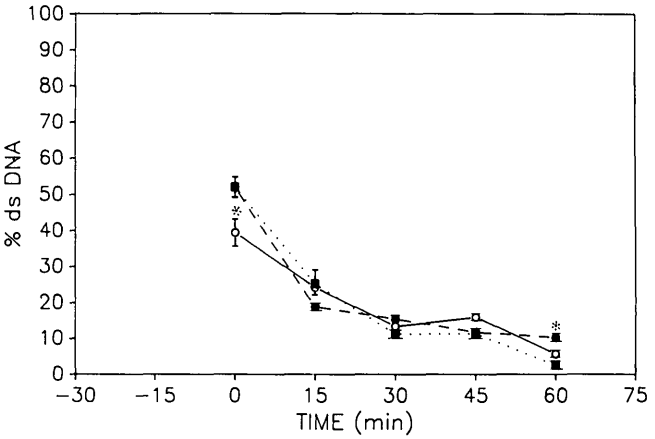

FIG. 5. Percentage of double-stranded DNA (%dsDNA) at different incubation times in alkali measured in liver of genetically obese diabetic mice (○, *db/db*) and heterozygote (●, *db/m*) and homozygote (■, *m/m*) controls. *$P < 0.05$ vs. *m/m* controls.

these possible interpretations. These changes in chromatin structure could be observed in two different animal models of diabetes. Thus, changes seen in streptozocin-induced diabetic rats could essentially be reproduced in *db/db* mice. Unexpectedly, however, the DNA unwinding rate in normoglycemic *db/m* mice was intermediate between that of *db/db* and *m/m* control mice. By use of first-order kinetics to analyze the data, differences between the DNA unwinding rate in *db/db* and *db/m* mice were not any more significant.

The similarity between these observations in diabetic animals and those previously published in aged animals is remarkable (9). Alkali-induced DNA digestibility may be a biochemical marker of aging, and certain tissues in diabetes may undergo changes simulating premature aging (11). Similarity in age- and diabetes-related changes seen in brain and liver and the lack of changes in intestinal epithelium with rapid turnover underscore the importance of cellular replicative capacity as an important determinant of tissue aging (11). Different lines of evidence suggest that intestinal epithelium, unlike postmitotic tissue, may not show senescent changes (12–15).

There is an increase in the euchromatin-heterochromatin ratio in cell nuclei of aortic adventitia within 2 wk of diabetes (16). This observation can be interpreted as indirect evidence that chromatin digestibility should increase in early diabetes. However, similar studies in chronically diabetic animals have not been performed.

Chronic uncontrolled hyperglycemia can alter chromatin structure in vivo, and these changes may be similar to those seen in aging. These alterations may involve some but not all cell types within a specific tissue. This cannot be ascertained by the method used in this study. The biological significance of diabetes-related changes in chromatin structure remains to be determined.

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