Effect of Tolbutamide and Exogenous Insulin on the Metabolic Responses of Rats (35682)

CAROLYN D. BERDANIER, BELA SZEPESI, SUZANNE DIACHENKO, AND PHYLIS MOSER
(Introduced by D. L. Trout)

Carbohydrate Nutrition Laboratory, Human Nutrition Research Division, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705

Recent reports have indicated that insulin may serve in the synthesis and/or activation of hexokinase (1–3). Studies of the "overshoot" phenomenon (4) showed that when fasted rats were fed a 65% glucose diet an increase in the level of immunoreactive insulin (IRI) preceded an increase in the activities of the hepatic enzymes glucose 6-phosphate dehydrogenase (G6PD) and malic enzyme (ME) and an increase in liver lipid. Subsequent studies revealed that the increased IRI was not continuous. In these laboratories, it had been observed that rats of the BHE[1] strain fed a diet containing purified carbohydrate had higher levels of hepatic pentose shunt dehydrogenases as well as greater amounts of carcass and liver lipid than Wistar animals fed the same diet (5–8). Further, the BHE animal appears to be characterized by hyperinsulinemia early in life (50 days of age) which seems to disappear by 100 days of age (9). Subsequent studies of the metabolic patterns of the BHE and Wistar animals suggested that this early hyperinsulinemia could lead to long-term changes in metabolism (10).

The present study was conducted in an attempt to determine if an artificially induced early hyperinsulinemia would lead to alterations in metabolic patterns. From the age of weaning, male rats of the Wistar strain received either a fixed dose of exogenous insulin administered daily or a fixed percentage of tolbutamide in the diet. The animals were killed at 100 days of age and the effect of these treatments on the activities of glucose 6-phosphatase (G6Pase), G6PD, ME, 6-phosphogluconate dehydrogenase (6PGD), citrate cleavage enzyme (CE), L-α-glycerolphosphate dehydrogenase (L-α-GolPD), and isocitrate dehydrogenate (ICD), and on the lipid content of the blood, liver, and carcass was studied.

Materials and Methods. Three groups of 12 male Wistar weanling rats were used for these studies. One group received daily (9:00 a.m.) subcutaneous injections of 40 μ units of insulin.[2] This dose level was selected because only slight augmentation of the normal circulating IRI level was desired. It was administered in the morning so as to compensate for the expected normal diurnal decline in IRI. Earlier studies have shown that peak IRI levels occur between 8:00 and 12:00 p.m. (4). A second group of animals were given tolbutamide[3] mixed into their diet at a concentration of 0.5 g/kg diet. The third group served as the control group. Food intakes and body weight gains were determined weekly.

Rats were housed individually in wire mesh cages with raised floors in a temperature–humidity controlled room. The animals had access to food and water *ad libitum*. A diet[4] containing 6% corn oil, 45% carbohy-

[1] The BHE strain is a strain resulting from a cross between the Pennsylvania State College strain and the Osborne-Mendel strain (also called the Yale strain).

[2] Iletin, Eli Lilly Co., Ind.

[3] Orinase, Upjohn, Kalamazoo, Mich. This dose level is similiar to that given the human for the control of mild hyperglycemia. That this dose was effective was seen at autopsy; histological examination of the pancreases of these animals revealed islet tissue depleted of insulin granules.

[4] Composition of this diet in percentages: cascin/lactalbumin (1:1) 40, corn oil 6, fiber 3, dextrin 5, mineral mix 5, vitamin mix 1, and 1:2:5 glucose/sucrose/cornstarch 45.

drate, and 40% protein was used to promote good growth. At 100 days of age, half the animals in each group were killed without prior fasting, while the remaining animals were killed after a 24-hr fast. The animals were anesthetized by an intraperitoneal injection of 60 mg/kg of sodium amobarbital, the thoracic cavity opened, and blood drawn by heart puncture. A 0.2-ml aliquot of blood was deproteinized and the glucose concentration determined with glucose oxidase (11). The serum from the remaining blood was collected after centrifugation (30 min, 3000*g*) in a refrigerated centrifuge. Serum IRI (12), cholesterol (13), and triglycerides (14) were determined. The liver was excised, blotted, and weighed. A 1-g sample was taken for enzyme analysis, while the remaining tissue was used for the determination of total lipid (15) and cholesterol (13). Enzyme activities were determined in a 10% liver homogenate prepared with ice-cold 0.14 *M* KCl (pH 7.4), using a Potter Elvehjem homogenizer. Glucose 6-phosphatase activity was determined by measuring the amount of inorganic phosphate liberated in 15 min at 37° (16). The crude homogenate was centrifuged at 0° for 30 min at 20,000*g*. The clear supernatant was used for the determination of soluble liver protein (17), glucose 6-phosphate dehydrogenase (16), malic enzyme (16), isocitrate dehydrogenase (18), 6-phosphogluconate dehydrogenase (16), citrate cleavage enzyme (19), and L-α-glycerolphosphate dehydrogenase (16).

Enzyme activity is expressed as units per 100 grams body weight. One unit of enzyme is defined as that amount of enzyme which can produce one micromole of measured product per minute, or can convert one micromole of substrate per minute under the conditions of the assay. The rationale for expressing enzyme activity per standard body weight has been previously discussed (16).

The ingesta-free carcass was autoclaved (15 lb/in^2 15 min) and ground in a Waring Blendor with crushed ice (carcass:ice, 1:2). Aliquots of this homogenate were taken for total carcass lipid determination (15). Differences between the means of each group for each determination were tested by the Student's *t* test and by an analysis of variance.

Results. The body weight gains and food intakes were unaffected by either the tolbutamide or insulin treatments. All three groups had an average initial body weight of 76 g and an average food intake of 16 g/day. By 100 days of age all three groups consumed an average of 20 g food/day and the average weights were 297, 293, and 292 g for the control, insulin-treated, and tolbutamide-treated rats, respectively.

Similarly, the administration of either tolbutamide or exogenous insulin did not affect either the fasting or nonfasting levels of IRI (Table I) at the 100 days of age. However, a

TABLE I. Serum Immunoreactive Insulin and Blood Glucose Levels of Control, Insulin-Treated, Tolbutamide-Treated Rats.

Group	Treatment	Insulin (μunits/ml serum)		Glucose (mg/100 ml whole blood)	
		Fasting	Nonfasting	Fasting	Nonfasting
1	None	43 ± 5[a]	79 ± 5[b]	84 ± 3	96 ± 3[c]
2	Insulin[d] (40 μunits/day)	37 ± 8	68 ± 6[b]	97 ± 1[e]	99 ± 3
3	Tolbutamide (.5 g/kg diet)	37 ± 7	79 ± 2[b]	92 ± 3	107 ± 8

[a] Standard error of the mean of six rats.

[b] Significant differences between the values from fasting and nonfasting animals in the same group ($p < .01$).

[c] Significant differences between the values from fasting and nonfasting animals in the same group ($p < .05$).

[d] Insulin was not administered the day the animals were killed.

[e] Significant differences between the values from animals in the control and treatment groups ($p < .01$).

TABLE II. Liver and Serum Cholesterol Levels of Control, Insulin-Treated, and Tolbutamide-Treated Rats.

Group	Treatment	Serum cholesterol (mg/100 ml)		Liver cholesterol (mg/g liver)	
		Fasting	Nonfasting	Fasting	Nonfasting
1	None	166 ± 6[a]	189 ± 7	5.68 ± 0.39	4.51 ± 0.50
2	Insulin[b] (40 μunits/day)	155 ± 18	161 ± 14	6.69 ± 0.27	5.27 ± 0.13[c]
3	Tolbutamide (.5 g/kg diet)	140 ± 16	169 ± 19	6.48 ± 0.30	5.22 ± 0.31[c]

[a] Standard error of the mean of six rats.

[b] Insulin was not administered the day the animals were killed.

[c] Significant differences between the values from fasting and nonfasting animals of the same group ($p < 0.05$).

residual effect of these treatments on insulin status cannot be ruled out, since insulin-like activity and the various forms of insulin were not specifically determined. The fasting blood glucose level of the insulin-treated group was higher than the glucose level of the other two groups. Fasting affected the glucose level of the control animals, but no effect of fasting was observed in the treated groups.

Serum and liver cholesterol levels are reported in Table II. While no significant treatment effect was observed with respect to serum cholesterol, treatment with either exogenous insulin or tolbutamide elevated liver cholesterol in nonfasted animals. Treatment with either tolbutamide or insulin also served to reduce the level of triglycerides in the serum of nonfasting animals but had little effect on the lipid content of the liver and carcass (Table III). In all groups serum, liver, and carcass lipids tended to fall with fasting.

The effects of tolbutamide and exogenous insulin on the enzymatic responses of the liver before and after a 24-hr fast are presented in Tables IV and V. In general, the effects of both treatments were similar. The nonfasting activity of G6Pase was increased by tolbutamide treatment. The activity of citrate cleavage enzyme was increased by both treatments. Both treated groups had nearly twice the activity of ME in the fasting state as did the control group. As expected, fasting decreased the activity of this enzyme in all three groups indicating decreased lipogenic activity. No significant treatment effects were observed in the activity of L-α-GolPD.

Of the enzymes involved in the generation of NADPH, the differences in the activity of

TABLE III. Serum Triglyceride Level and Lipid Content of the Livers and Carcasses of Control, Insulin-Treated, and Tolbutamide-Treated Rats.

Group	Treatment	Serum triglycerides (mg/100 ml)		Liver fat (mg/100 g body wt)		Carcass fat (g/100 g body wt)	
		Fasting	Nonfasting	Fasting	Nonfasting	Fasting	Nonfasting
1	None	65 ± 39[a]	233 ± 102[b]	127 ± 2	139 ± 4[b]	13 ± 1	17 ± 1[b]
2	Insulin[c] (40 μunits/day)	57 ± 16	111 ± 47	120 ± 4	132 ± 3[b]	15 ± 1	16 ± 1
3	Tolbutamide (.5 g/kg diet)	43 ± 38	143 ± 72	129 ± 3	139 ± 5	15 ± 2	17 ± 2

[a] Standard error of the mean of six rats.

[b] Significant differences between the values from fasting and nonfasting animals of the same group ($p < .05$).

[c] Insulin was not administered the day the animals were killed.

TABLE IV. Activities of Glucose 6-phosphatase, Citrate Cleavage Enzyme, and L-α-glycerol Phosphate Dehydrogenase of Control, Insulin-Treated, and Tolbutamide-Treated Rats.

Group	Treatment	Soluble liver protein (mg/100 g body wt)		Enzyme[a] (units[b]/100 g body wt)					
				G6Pase		CE		L-α-GolPD	
		Fasting	Nonfasting	Fasting	Nonfasting	Fasting	Nonfasting	Fasting	Nonfasting
1	None	311 ± 14[c]	361 ± 13[d]	62.3 ± 1.8	55.4 ± 4.9	0.68 ± 0.04	1.95 ± 0.27	170 ± 6	192 ± 13
2	Insulin[e] (40 μunits/day)	289 ± 16	325 ± 14[d,f]	58.2 ± 5.6	60.6 ± 5.2	1.14 ± 0.16[f]	2.48 ± 0.33	180 ± 8	198 ± 5
3	Tolbutamide (0.5 g/kg diet)	294 ± 3	351 ± 14[d]	61.4 ± 4.5	69.6 ± 3.1[f]	1.16 ± 0.16[f]	2.24 ± 0.20	171 ± 8	185 ± 13

[a] Abbreviations used: G6Pase, glucose 6-phosphatase; CE, citrate cleavage enzyme; L-α-GolPD, L-α-glycerol phosphatase dehydrogenase.

[b] One unit of enzyme activity equals amount of enzyme which can produce one micromole of measured product per minute under the conditions of the assay.

[c] Standard error of the mean for six rats.

[d] Significant differences between the values from fasting and nonfasting animals of the same group ($p < .05$).

[e] No insulin was administered the day the animals were killed.

[f] Significant differences between the values from animals in the control and treatment groups ($p < .05$).

TABLE V. Liver NADP-linked Dehydogenase Activities of Control, Insulin-Treated, and Tolbutamide-Treated Rats.

		Enzyme[a] (units[b]/100 g body wt)							
		G6PD		6GPD		ME		ICD	
Group	Treatment	Fasting	Nonfasting	Fasting	Nonfasting	Fasting	Nonfasting	Fasting	Nonfasting
1	None	6.1 ± 0.4[c]	7.7 ± 1.1	4.79 ± 0.26	6.22 ± 0.35[d]	2.62 ± 0.68	2.44 ± 0.28	29.3 ± 1.8	38.1 ± 1.3[d]
2	Insulin[e] (40 μunits/day)	12.1 ± 1.9[f]	16.2 ± 2.7[f]	7.12 ± 0.85[f]	8.92 ± 0.66[f]	3.25 ± 0.39	4.23 ± 0.34[f]	30.0 ± 3.8[f]	49.7 ± 6.8[d]
3	Tolbutamide (.5 g/kg diet)	11.5 ± 1.6[f]	12.6 ± 1.4[d,f]	5.36 ± 0.60	8.61 ± 0.80[d,f]	3.36 ± 0.32	4.49 ± 0.32[f]	39.0 ± 3.7[f]	57.6 ± 4.8[d,f]

[a] Abbreviations used: G6PD, glucose 6-phosphate dehydrogenase; 6PGD, 6-phyosophogluconate dehydrogenase; ME, malic enzyme; ICD, isocitrate dehydrogenase.

[b] One unit of enzyme activity equals amount of enzyme which can produce one micromole of measured product per minute under the conditions of the assay.

[c] Standard error of the mean for six rats.

[d] Significant differences between the values from fasting and nonfasting animals of the same group ($p < .05$).

[e] No insulin was administered the day the animals were killed.

[f] Significant differences between the values from animals in the control and treatment groups ($p < .05$).

G6PD due to treatment were most striking. The activity of G6PD both in the fasting and nonfasting state was markedly increased with the administration of either tolbutamide or exogenous insulin. The activity of 6PGD, another enzyme of the pentose phosphate cycle, was also increased. The enhanced activity of these two enzymes suggests that the pentose phosphate cycle activity was increased as a result of the modest insulin increase due to treatment. The nonfasting ME activity was also increased by the administration of either tolbutamide or exogenous insulin indicating an increased activity of the transhydrogenation cycle. Tolbutamide increased the activity of ICD both in the fasting and nonfasting state. The differences between the fasting and nonfasting ICD activities in the insulin-treated group when compared to the control group were not significant.

Discussion. These studies show that changes in the insulin status of weanling rats resulted in observable changes in liver metabolism by 100 days of age. Both the administration of tolbutamide or exogenous insulin resulted in significant increases in G6PD, ME, ICD, and 6PGD activities in the nonfasting state. This is in contrast to the observations of Fabry *et al.* (20) who reported that the administration of 1 unit/kg of insulin did not affect the activity of ME and the pentose shunt enzymes with refeeding after a 3-day fast. However, Fabry's observations were made on adipose tissue rather than on liver. The data from the present study using rats fed *ad libitum* suggest that the increase in enzyme activity with treatment was due to an effect of insulin on the hepatic synthesis of these enzymes.

The current studies also showed an effect of the treatments on liver cholesterol. Since the liver is the major site of cholesterol synthesis, these data suggest that insulin may be of importance in the regulation of cholesterol level in the serum and liver. Penhos *et al.* (21), using the perfused rat liver technique, was unable to show an effect of insulin on the level of liver cholesterol. However, other investigators (22), using partially depancreatectomized (90–95%) rats have shown that insulin-treated rats have greater amounts of liver lipids and liver cholesterol than their untreated controls. Further, rats given sodium acetate-1-^{14}C and insulin intravenously had significantly greater amounts of ^{14}C-labeled cholesterol in their aortic tissue than rats given labeled acetate alone (23). This suggests that insulin enhances cholesterol synthesis in selected tissues; however, it is also possible that insulin affects the rate of degradation without any effect on cholesterol synthesis. It is possible that the form of insulin, *e.g.*, proinsulin or active insulin, is important in the regulation of the synthesis, deposition, and degradation of cholesterol. Detailed studies are needed before final conclusions can be drawn concerning the relationship of insulin and cholesterol metabolism.

Summary. The effect of tolbutamide or exogenous insulin on the levels of certain enzymes, blood glucose, serum IRI, and lipids in the blood, liver, and carcass of Wistar rats was studied. The drugs, administered from weaning to 100 days of age, appeared to increase the activity of G6PD, ME, ICD, CE, and the level of liver cholesterol. Blood glucose, IRI, and the level of lipid in the liver and carcass were unaffected by the treatments. These findings show that slight changes in insulin status early in life may result in markedly different metabolic patterns at maturity.

1. Hansen, R. J., Pilkis, S. J., and Krahl, M. E., Endocrinology **86**, 57 (1970).

2. Il'in, V. S., Vestn. Akad. Med. Nauk SSSR **24**, 3 (1969).

3. Il'in, V. W., Probl. Endokrinol. Gormonoter. **12**, 54 (1966), as translated in Fed. Proc., Fed. Amer. Soc. Exp. Biol. **25**, T1034 (1966).

4. Szepesi, B., and Berdanier, C. D., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 569 (1970).

5. Marshall, M. W., Hildebrand, H. E., Dupont, J. L., and Womack, M., J. Nutr. **69**, 371 (1959).

6. Marshall, M. W., and Hildebrand, H. E., J. Nutr. **79**, 227 (1963).

7. Taylor, D., Conway, E., Schuster, E., and Adams, M., J. Nutr. **95**, 275 (1967).

8. Chang, M. L., Schuster, E. M., Lee, J. A., Snodgrass, C., and Benton, D. A., J. Nutr. **96**, 368 (1968).

9. Berdanier, C. D., Marshall, M. W., and Moser,

P., Life Sci. **10**, 105 (1971).

10. Berdanier, C. D., Szepesi, B., Moser, P., and Diachenko, S., Proc. Soc. Exp. Biol. Med. **137**, 668 (1971).

11. Saifer, A., and Gerstenfeld, S., J. Lab. Clin. Med. **51**, 448 (1958).

12. Hales, C. N., and Randle, P. J., Biochem. J. **88**, 137 (1963).

13. Koval, G. J., J. Lipid Res. **2**, 419 (1961).

14. Van Handel, E., and Zilversmit, D. B., J. Lab. Clin. Med. **50**, 152 (1957).

15. Association of Official Agricultural Chemists: Fat, acid hydrolysis method. *in* "Official Methods of Analysis," p. 176. Washington, D.C. (1960).

16. Freedland, R. A., J. Nutr. **91**, 489 (1967).

17. Mokrasch, L. C., and McGilvery, R. W., J. Biol. Chem. **221**, 909 (1956).

18. Freedland, R. A., Avery, E. H., and Murad, S., Can. J. Biochem. **46**, 141 (1966).

19. Srere, P. A., J. Biol. Chem. **234**, 2544 (1959).

20. Fábry, P., Kleinfeld, R., Tepperman, H. M., and Tepperman, J., Proc. Soc. Exp. Biol. Med. **133**, 577 (1970).

21. Penhos, J. C., Wu, C. H., Lemberg, A., Daunas, J., and Brodoff, B., Metabolism **17**, 246 (1968).

22. Wilson, R. B., Martin, J. M., and Hartroft, W. S., Diabetes **18**, 225 (1969).

23. Stout, R. W., Lancet II, 467 (1969).

Received Jan. 26, 1971. P.S.E.B.M., 1971, Vol. 137.