Ciglitazone, a New Hypoglycemic Agent

II. Effect on Glucose and Lipid Metabolisms and Insulin Binding in the Adipose Tissue of C57BL/6J-ob/ob and − + /? Mice

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SUMMARY

The fat pads isolated from control and ciglitazone-treated C57BL/6J-ob/ob mice and their lean littermates (− + /?) were incubated in vitro with glucose-1-^{14}C/-5-^{3}H in the presence of insulin. The formation of $^{14}CO_2$ and $^{3}H_2O$ and the levels of free fatty acids and glycerol in the medium and the incorporation of ^{14}C and ^{3}H into esterified lipids and free fatty acids in the fat pads were measured. The basal rates of glucose-1-^{14}C/-5-^{3}H metabolism per unit weight were increased in the fat pads of ciglitazone-treated mice, more pronouncedly in the treated lean than in the treated obese. The insulin-dependent effects were enhanced in the treated ob/ob mice. To see the dose-response of insulin, a second experiment was carried out in which portions of the fat pads were incubated in vitro with glucose-1-^{14}C in the presence of 0–40 ng/ml insulin and isolated adipocytes were used to estimate for ^{125}I-insulin binding. The basal rates of $^{14}CO_2$ and ^{14}C-lipids formation per unit weight of fat pads were increased in both treated obese and treated lean groups but insulin-dependent elevation was seen only in the treated obese group. Ciglitazone significantly increased insulin binding capacity at the low-affinity sites in the adipocytes of obese mice but not in those of lean mice. The data showed that ciglitazone increased the basal rate of glucose metabolism, lipogenesis, insulin receptor numbers, and post-receptor responses in the C57BL/6J-ob/ob mice; it increased the basal rate of glucose metabolism and lipogenesis but not insulin sensitivity in the lean mice. DIABETES 32:839–845, September 1983.

Ciglitazone, a new hypoglycemic agent, has been shown to lower blood glucose and plasma lipids in obese-hyperglycemic and insulin-resistant animal models such as the C57BL/6J-ob/ob and C57BL/KsJ-db/db mice.[1] However, it showed no hypoglycemic activity in the normal or insulinopenic animals such as the streptozotocin-diabetic rats and the spontaneously diabetic Chinese hamsters.[1] This paper presents the results of our efforts to unravel the mechanism of its hypoglycemic action in the C57BL/6J-ob/ob mice. Specifically, we measured the basal and insulin-stimulated glucose metabolism and conversion to lipids in the isolated fat pads using substrate doubly labeled with ^{14}C and ^{3}H. The measured endpoints included the formation of $^{14}CO_2$ and $^{3}H_2O$ and the level of free fatty acids and glycerol released into the medium and the radiolabel incorporations into esterified lipids and free fatty acids in the fat pads. To dissect insulin-dependent effects into receptor and post-receptor responses, ^{125}I-insulin binding in the isolated adipocytes was measured. In addition, parallel studies in lean C57BL/6J − + /? mice, in which ciglitazone showed no consistent hypoglycemic activity, were also carried out to elucidate the cause of its specific bioactivity in the hyperinsulinemic and insulin-resistant animal models.

METHODS

Animals. Male C57BL/6J-ob/ob mice and their lean littermates (genotype: + /ob or + / +) were obtained from Jackson Laboratories (Bar Harbor, Maine) at 6 wk of age. The animals were housed individually in metabolic cages and allowed ground Purina Mouse Chow and water ad libitum. Ciglitazone was given as food admixture at doses indicated in individual studies. Ciglitazone was obtained from Takeda Chemical Industries (Osaka, Japan).

Incubation of epididymal fat pad. The animals were exsanguinated through the orbital sinus and decapitated; the epididymal fat pads were removed and trimmed to pieces weighing 20–160 mg and incubated in 2 ml basal medium[2] containing 5.6 mM glucose (plus 0.5 μCi glucose-1-^{14}C alone or together with 2.5 μCi glucose-5-^{3}H) for 1.5 h at 37°C. In some samples, 100 μU/ml insulin and/or 1 μg/ml epinephrine was also added. After $^{14}CO_2$ was collected, the fat pads

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were removed and frozen in glass tubes for lipid extraction later. In experiments where glucose-5-^{3}H was included, aliquots of 0.24 ml incubation medium were used to measure ^3H_2O formation according to Pollard et al.[3] with samples containing no fat pads as blanks. Aliquots of 0.05 ml medium were measured for glycerol fluorometrically on an Autoanalyzer.[1] Free fatty acids were extracted from 0.5-ml aliquots with 3 ml i-propanol:n-heptane:1 N H_2SO_4 (40:10:1), 0.6 ml n-hexane, and 2 ml H_2O by vortexing for 2 min. Aliquots of 0.75 ml upper phase were dried under N_2 and determined for free fatty acids enzymatically.[4]

The frozen fat pads were homogenized in 2 ml i-propanol:n-heptane:1 N H_2SO_4 (40:10:1) with Polytron at a setting of 7 for 10 s. One milliliter H_2O and 1 ml n-hexane were added and vortexed for 2 min. The upper phase was removed, dried, mixed with 10 ml Liquifluor-Toluene, and estimated for radiolabels associated with total lipids in a scintillation counter. When lipids were further fractionated, the upper phase was dried under N_2; the residues were dissolved in 1 ml petroleum ether and 0.3 ml 4% Na_2CO_3 was added. The mixture was vortexed and centrifuged; the upper phase which contained esterified lipids was removed, dried, and counted in 10 ml Liquifluor-Toluene. After the removal of upper phase, 9 drops of 1 N H_2SO_4 was used to acidify the solution and solid NaCl was added to saturation. One milliliter of petroleum ether was added and the mixture vortexed and centrifuged. The upper phase was removed, dried, and estimated for radiolabels associated with free fatty acids.

Isolation of and ^{125}I-insulin binding in adipocytes. The adipocytes were isolated from portions of the epididymal fat pads according to published methods[5,6] with slight modifications. Pieces of fat pads were placed into plastic vials containing 1 ml collagenase solution (0.5 mg/ml in Buffer A which was Krebs-Ringer bicarbonate buffer, pH 7.4, with 3.3 mM glucose and 3% BSA). The vials were briefly gassed with a stream of 95% O_2 + 5% CO_2, capped, and incubated in a water bath at 37°C for 1 h with gentle shaking. The mixture was filtered through a nylon mesh screen, which was attached to the end of a cut-off plastic syringe with a rubber band, into a plastic centrifuge tube containing a length of tubing slightly longer than the length of the centrifuge tube. The mixture was centrifuged at 400 × *g* for 1 min. The buffer and nonadipose cells were removed through the plastic tubing using a plastic syringe fitted with an 18G needle. The remaining cells were washed 3 times with Buffer A which was warmed to 37°C. The cell pellets were then resuspended in 10× volume of Buffer A. Cell concentration was determined in a hemocytometer.

The ^{125}I-insulin binding assay was a modified version of published methods.[7,8] Adipocyte suspension (0.4 ml) was added to plastic tubes containing 0.05 ml of ^{125}I-insulin (100 μCi/μg and 2 ng/ml, New England Nuclear, Boston, Massachusetts) and 0.05 ml of unlabeled insulin (25.4 U/mg, pork, Lilly Laboratories, Indianapolis, Indiana). Nonspecific binding was determined by adding an excess of unlabeled insulin (0.05 mg) and was subtracted from total insulin bound to give specific insulin binding. Samples were run in triplicate. The cells were incubated at 24°C for 1 h and 4 ml cold saline were added to the tube to stop the reaction. One milliliter of a 1:1 mixture of silicone fluid and sunflower seed oil was layered on top of the saline. The tubes were centrifuged at 1000 RPM for 4 min at 5°C. The adipocytes which had floated to the top in one or two clumps were removed with a 200-μl MLA pipetter using plastic tips with the tip cut off, placed in a clean tube (tip and cells), and counted.

In the obese mice, adipocytes from individual animals were run for ^{125}I-insulin binding in triplicate samples at each insulin concentration. In the lean mice, adipocytes from two animals were pooled and triplicate samples were run at each insulin concentration. Scatchard plots were constructed for individual runs and binding sites and affinity were estimated from each plot.

Miscellaneous assays. Assays for blood glucose, glycerol, plasma, and pancreatic insulin and glucagon were as described previously.[1] Plasma corticosteroids were measured with Radioimmunoassay Corticosteroid-^{3}H Kit obtained from Radioassay Systems Laboratories, Inc. (Carson, California).

Statistics. All data were reported as means ± SEM (number of samples). The differences were analyzed by Student's *t* test.

RESULTS

Formation of $^{14}CO_2$ and ^3H_2O from glucose-1-^{14}C/-5-^{3}H. The fat pads used were isolated from the control and ciglitazone-treated C57BL/6J-ob/ob mice and their lean − +/? littermates described in a preceding paper.[1] The obese mice had been treated with ciglitazone at a dose of 100 mg/kg/day and the lean mice 150 mg/kg/day for 41–44 days. At termination, the blood glucose levels of control obese were 378 ± 90 mg/dl, treated obese 110 ± 6 mg/dl, control lean 158 ± 10 mg/dl, and treated lean 150 ± 9 mg/dl, respectively. The isolated fat pads were incubated with glucose doubly labeled with ^{14}C and ^{3}H. Basal production of $^{14}CO_2$ appeared to be increased in the isolated fat pads of both ob/ob and +/? mice treated with ciglitazone and the lean group oxidized glucose-1-^{14}C about six times faster than the corresponding obese group (Table 1). The addition of insulin in the incubation medium elevated $^{14}CO_2$ production in treated obese and both control and treated lean mice. Epinephrine showed little or no effect on glucose-1-^{14}C oxidation to $^{14}CO_2$ in the obese group; in the lean littermates, the addition of epinephrine in the incubation medium elicited a 20–25% inhibition in both control and treated groups. The fat pads from the obese mice were also incubated in the presence of both insulin and epinephrine; $^{14}CO_2$ formation under this condition was 142% of basal in the control and 162% in the treated group.

The formation of ^3H_2O from glucose-5-^{3}H was higher in the fat pads isolated from ciglitazone-treated than in those from the untreated lean mice, but no difference was seen in the obese group. The fat pads of the lean mice also produce more ^3H_2O than those of the corresponding obese mice (Table 1). The addition of insulin in the incubation medium increased the formation of ^3H_2O in the lean but not in the obese mice. Similar to its effect on glucose-1-^{14}C oxidation to $^{14}CO_2$, epinephrine decreased glucose-5-^{3}H metabolism to ^3H_2O in the fat pads of the lean group but not in those of the obese mice. Further, the addition of both epinephrine and insulin increased ^3H_2O formation in the "obese" fat pads and the elevation was more pronounced in the fat pads isolated from ciglitazone-treated obese mice.

TABLE 1
Formation of $^{14}CO_2$ and ^3H_2O in fat pads isolated from control and ciglitazone-treated C57BL/6J-ob/ob and − + /? mice and incubated with glucose-1-^{14}C and -5-^{3}H

Measurement	Incubation condition	C57BL/6J-ob/ob Control	C57BL/6J-ob/ob Treated	P values	C57BL/6J − + /? Control	C57BL/6J − + /? Treated	P values
$^{14}CO_2$ (nCi/g)	Basal	3.68 ± 0.45(4)	5.93 ± 0.85(6)	NS	21.5 ± 9.8 (5)	34.9 ± 6.8 (4)	NS
	+100 μU/ml insulin	3.16 ± 1.11(4)	9.04 ± 0.54(6)	<0.001	52.4 ± 8.9 (5)	112.2 ± 10.1(4)	<0.01
	+1 μg/ml epinephrine	3.26 ± 0.91(4)	5.97 ± 0.43(6)	<0.01	11.1 ± 1.9 (5)	24.2 ± 3.3 (4)	<0.01
	+ 1 μg/ml epinephrine 100 μU/ml insulin	5.04 ± 0.36(4)	8.49 ± 0.41(6)	<0.001	—	—	—
^3H_2O (nCi/g)	Basal	23.7 ± 2.7 (4)	21.1 ± 2.1 (6)	NS	36.6 ± 9.4 (5)	74.9 ± 1.6 (4)	<0.05
	+100 μU/ml insulin	22.9 ± 2.0 (4)	25.5 ± 1.7 (6)	NS	77.1 ± 13.9(5)	176 ± 15 (4)	<0.005
	+1 μg/ml epinephrine	25.7 ± 2.8 (4)	24.5 ± 1.3 (6)	NS	30.0 ± 5.5 (5)	65.6 ± 9.7 (4)	<0.05
	+ 1 μg/ml epinephrine 100 μU/ml insulin	29.8 ± 2.3 (4)	36.2 ± 3.0 (6)	NS	—	—	—

Incorporation of glucose-1-^{14}C/-5-^{3}H into esterified lipids. The fat pads isolated from ciglitazone-treated obese and lean mice incorporated more glucose-1-^{14}C/-5-^{3}H into esterified lipids than those from the corresponding control mice (Table 2). The presence of insulin in the medium did not significantly increase the incorporation of both radiolabels in the obese group. Ciglitazone treatment in the obese animal enhanced the insulin-dependent increment in ^{14}C-lipid formation. In the lean group, the insulin-dependent increase was seen only in the conversion of glucose-1-^{14}C into esterified lipids but not in the incorporation of glucose-5-^{3}H into fat. The addition of epinephrine in the medium enhanced lipid synthesis in the fat pads of the obese mice but not in those of the lean mice; ciglitazone treatment in the animal showed no effect on epinephrine response in the isolated fat pads. In the obese group, the ^{14}C/^{3}H ratio in the esterified lipid fractions was lower in the fat pads from drug-treated than in those from control mice; but the addition of insulin or epinephrine, alone or in combination, did not affect the ratio appreciably. On the contrary, the presence of insulin in the medium showed an elevation in the ^{14}C/^{3}H ratio in the esterified lipids extracted from the fat pads of the lean mice and ciglitazone treatment in the animal enhanced the insulin-dependent effect (Table 2).

Incorporations of glucose-1-^{14}C/-5-^{3}H into free fatty acids. The conversion of glucose-1-^{14}C/-5-^{3}H into radiolabeled free fatty acids was much higher in the fat pads of lean than in those of obese mice and ciglitazone treatment in the animals exacerbated the difference (Table 3). The fat pads isolated from the ciglitazone-treated mice also showed higher incorporations of ^{14}C and ^{3}H into the free fatty acids than the corresponding controls. The addition of insulin alone slightly increased the ^{14}C-incorporation, but not ^{3}H-incorporation, into free fatty acids in all four groups of fat pads, whereas epinephrine alone elevated the incorporations of both ^{14}C and ^{3}H. When the fat pads of the obese mice were

TABLE 2
Incorporation into esterified lipids in fat pads isolated from control and ciglitazone-treated C57BL/6J-ob/ob and − + /? mice and incubated with glucose-1-^{14}C and -5-^{3}H

Radiolabels	Incubation condition	C57BL/6J-ob/ob Control	C57BL/6J-ob/ob Treated	P values	C57BL/6J − + /? Control	C57BL/6J − + /? Treated	P values
^{14}C (nCi/g)	Basal	10.5 ± 0.5 (4)	12.8 ± 1.3 (6)	NS	31.3 ± 6.2 (5)	70.7 ± 16.4 (4)	<0.05
	+100 μU/ml insulin	11.1 ± 0.9 (4)	15.7 ± 1.5 (6)	<0.05	46.8 ± 6.8 (5)	117 ± 7 (4)	<0.001
	+1 μg/ml epinephrine	13.5 ± 1.3 (4)	18.9 ± 1.2 (6)	<0.05	27.2 ± 5.1 (5)	69.2 ± 5.7 (4)	<0.001
	+ 1 μg/ml epinephrine 100 μU/ml insulin	16.9 ± 0.6 (4)	26.7 ± 2.1 (6)	<0.005	—	—	—
^{3}H (nCi/g)	Basal	0.873 ± 0.004(4)	1.30 ± 0.11(6)	<0.05	3.81 ± 0.63(5)	9.81 ± 2.44 (4)	<0.05
	+100 μU/ml insulin	0.998 ± 0.093(4)	1.41 ± 0.17(6)	NS	3.57 ± 0.47(5)	7.43 ± 0.50 (4)	<0.001
	+1 μg/ml epinephrine	1.20 ± 0.08 (4)	1.69 ± 0.12(6)	<0.05	4.07 ± 0.82(5)	9.03 ± 0.87 (4)	<0.01
	+ 1 μg/ml epinephrine 100 μU/ml insulin	1.43 ± 0.04 (4)	2.55 ± 0.29(6)	<0.05	—	—	—
^{14}C/^{3}H ratio	Basal	12.0 ± 0.5 (4)	9.78 ± 0.53(6)	<0.01	8.23 ± 0.95(5)	7.42 ± 0.72 (4)	NS
	+100 μU/ml insulin	11.2 ± 0.5 (4)	11.2 ± 0.5 (6)	NS	13.1 ± 0.8 (5)	15.8 ± 0.8 (4)	<0.05
	+1 μg/ml epinephrine	11.2 ± 0.3 (4)	11.3 ± 0.4 (6)	NS	7.05 ± 0.70(5)	7.70 ± 0.17 (4)	NS
	+ 1 μg/ml epinephrine 100 μU/ml insulin	11.8 ± 0.3 (4)	10.7 ± 0.5 (6)	NS	—	—	—

TABLE 3
Incorporation into free fatty acids in fat pads isolated from control and ciglitazone-treated C57BL/6J-ob/ob and − + /? mice and incubated with glucose-1-^{14}C and -5-^{3}H

Radiolabels	Incubation condition	C57BL/6J-ob/ob Control	C57BL/6J-ob/ob Treated	P values	C57BL/6J− + /? Control	C57BL/6J− + /? Treated	P values
^{14}C (nCi/g)	Basal	0.492 ± 0.057(4)	0.644 ± 0.108(6)	NS	1.15 ± 0.27 (5)	2.44 ± 0.40 (4)	<0.05
	+100 μU/ml insulin	0.544 ± 0.151(4)	0.758 ± 0.114(6)	NS	1.37 ± 0.26 (5)	3.92 ± 0.27 (4)	<0.001
	+1 μg/ml epinephrine	0.587 ± 0.072(4)	0.904 ± 0.188(6)	NS	1.40 ± 0.37 (5)	3.53 ± 0.26 (4)	<0.005
	+1 μg/ml epinephrine + 100 μU/ml insulin	0.711 ± 0.065(4)	1.440 ± 0.253(6)	<0.05	—	—	—
^{3}H (nCi/g)	Basal	0.040 ± 0.006(4)	0.077 ± 0.014(6)	NS	0.231 ± 0.033(5)	0.36 ± 0.051(4)	NS
	+100 μU/ml insulin	0.048 ± 0.008(4)	0.070 ± 0.014(6)	NS	0.123 ± 0.014(5)	0.227 ± 0.025(4)	<0.01
	+1 μg/ml epinephrine	0.078 ± 0.013(4)	0.109 ± 0.029(6)	NS	0.454 ± 0.062(5)	0.774 ± 0.083(4)	<0.05
	+1 μg/ml epinephrine + 100 μU/ml insulin	0.084 ± 0.008(4)	0.156 ± 0.025(6)	<0.05	—	—	—
^{14}C/^{3}H ratio	Basal	13.4 ± 2.8 (4)	8.92 ± 1.14 (6)	NS	4.95 ± 0.79 (5)	6.71 ± 0.52 (4)	NS
	+100 μU/ml insulin	10.9 ± 1.6 (4)	11.7 ± 1.1 (6)	NS	10.8 ± 1.1 (5)	18.0 ± 2.5 (4)	<0.05
	+1 μg/ml epinephrine	7.65 ± 0.31 (4)	8.70 ± 0.58 (6)	NS	3.42 ± 0.41 (5)	5.33 ± 0.24 (4)	<0.01
	+1 μg/ml epinephrine + 100 μU/ml insulin	8.53 ± 0.38 (4)	9.28 ± 0.56 (6)	NS	—	—	—

treated with both insulin and epinephrine, the elevation in the conversion of glucose-1-^{14}C/-5-^{3}H into free fatty acids was clearly evident (Table 3).

The release of free fatty acids and glycerol by the fat pads. Ciglitazone treatment in the obese mice did not appreciably affect the release of free fatty acids or glycerol by the isolated fat pads, whereas drug treatment in the lean + /? mice significantly increased the concentrations of glycerol in the medium but not that of free fatty acids (Table 4). The addition of insulin in the medium inhibited lipolysis, whereas epinephrine stimulated lipolysis in the fat pads in all four groups of animals. Ciglitazone treatment in the obese mice enhanced the insulin-dependent inhibition of free fatty acids release but drug treatment did not appreciably affect glycerol release in the obese mice or insulin-dependent inhibition of lipolysis in the lean mice. While, in the control obese mice, the addition of insulin attenuated epinephrine-induced lipolysis, insulin further enhanced the epinephrine-dependent release of both free fatty acid and glycerol in the fat pads of ciglitazone-treated obese mice (Table 4).

^{125}I-insulin binding in adipocytes isolated from control and ciglitazone-treated mice. A second group of C57BL/6J-ob/ob mice was treated with ciglitazone at an approximate dose of 57 mg/kg/day for 3 wk and their lean littermates at 82 mg/kg/day for 5 wk. One of the treated ob/ob mice (#12) showed blood glucose level at pretreatment period of 522 mg/dl and it failed to respond to ciglitazone treatment at 57 mg/kg/day for 10 days. Therefore, on day 11, the dose for mouse #12 was raised to 150 mg/kg/day and, on day 14, it was raised further to 300 mg/kg/day. At termination, it showed blood glucose level of 109 mg/dl, still the highest one in the group but within the normal range. This animal also showed the lowest plasma (32 μU/ml) and pancreatic (1.5 U/g) insulin levels and the highest plasma corticosteroid (150 ng/ml) among the treated obese mice at termination.

TABLE 4
Concentration of free fatty acid and glycerol in the medium where fat pad isolated from control and ciglitazone-treated C57BL/6J-ob/ob and − + /? mice was incubated for 1.5 h at 37°C.

Measurement	Incubation condition	C57BL/6J-ob/ob Control	C57BL/6J-ob/ob Treated	P values	C57BL/6J− + /? Control	C57BL/6J− + /? Treated	P values
Free fatty acids (μmol/g)	Basal	0.449 ± 0.106(4)	0.495 ± 0.091(6)	NS	0.673 ± 0.124(5)	0.744 ± 0.049(4)	NS
	+100 μU/ml insulin	0.288 ± 0.036(4)	0.191 ± 0.058(6)	NS	0.057 ± 0.013(5)	0.274 ± 0.140(4)	NS
	+1 μg/ml epinephrine	0.970 ± 0.354(4)	1.27 ± 0.13 (6)	NS	5.42 ± 1.04 (5)	8.27 ± 1.00 (4)	NS
	+1 μg/ml epinephrine + 100 μU/ml insulin	0.555 ± 0.033(4)	1.48 ± 0.14 (6)	<0.001	—	—	—
Glycerol (μmol/g)	Basal	1.75 ± 0.15 (4)	1.93 ± 0.08 (6)	NS	2.53 ± 0.25 (5)	4.49 ± 0.40 (4)	<0.01
	+100 μU/ml insulin	1.57 ± 0.16 (4)	1.62 ± 0.10 (6)	NS	1.50 ± 0.03 (5)	2.55 ± 0.09 (4)	<0.001
	+1 μg/ml epinephrine	2.64 ± 0.17 (4)	3.15 ± 0.17 (6)	NS	8.17 ± 0.72 (5)	12.8 ± 1.01 (4)	<0.01
	+1 μg/ml epinephrine + 100 μU/ml insulin	2.04 ± 0.10 (4)	3.57 ± 0.13 (6)	<0.001	—	—	—

TABLE 5
Insulin binding sites and affinity in adipocytes isolated from control and ciglitazone-treated C57BL/6J-ob/ob and − +/? mice

		C57BL/6J-ob/ob			C57BL/6J− +/?		
Measurement	Unit	Control	Treated	P values	Control	Treated	P values
High-affinity sites							
Number	sites/cell	3.05 ± 0.85 × 10^3(6)	4.82 ± 0.79 × 10^3(6)	NS	6.07 ± 0.31 × 10^3(3)	5.99 ± 0.93 × 10^3(3)	NS
Affinity	M^{-1}	1.87 ± 0.05 × 10^9(6)	1.72 ± 0.26 × 10^9(6)	NS	2.23 ± 0.13 × 10^9(3)	1.92 ± 0.12 × 10^9(3)	NS
Low-affinity sites							
Number	sites/cell	11.85 ± 3.09 × 10^3(6)	32.10 ± 7.84 × 10^3(6)	<0.05	56.85 ± 6.75 × 10^3(3)	47.92 ± 6.99 × 10^3(3)	NS
Affinity	M^{-1}	0.24 ± 0.06 × 10^9(6)	0.08 ± 0.01 × 10^9(6)	<0.05	0.06 ± 0.01 × 10^9(3)	0.06 ± 0.01 × 10^9(3)	NS

Ciglitazone treatment in the obese and lean mice significantly reduced the epididymal fat pad weights (7.90 ± 0.23% body weight versus 7.13 ± 0.24% in obese and 3.29 ± 0.20% versus 2.37 ± 0.20% in the lean mice). Adipocytes were isolated from a portion of the epididymal fat pads of these animals and measured for ^{125}I-insulin binding. The remaining parts of the fat pads were cut into pieces and used to measure glucose-1-^{14}C oxidation and incorporation into lipids in response of varying concentrations of insulin (reported below). The experiments were performed on four consecutive days, each experiment containing matched pairs of control and treated mice in obese and lean groups. The adipocytes isolated from the control and ciglitazone-treated lean mice yielded almost superimposable Scatchard plots of insulin binding whereas drug treatment in obese mice induced a shift in the plot which indicated that ciglitazone treatment increased insulin receptor numbers. Based on the plots and multi-site hypothesis,[9] receptor numbers and affinity were calculated for individual Scatchard plots and the mean values are presented in Table 5. In the ob/ob mice, ciglitazone treatment increased the number of low affinity receptors significantly. The affinity in the low affinity binding sites was decreased by the treatment, however. In the lean littermates, ciglitazone treatment failed to elicit any effect on the affinity or number in either type of binding sites. The control obese mice showed half the number of high-affinity binding sites as their lean littermates and the affinity was slightly higher in the lean group, whereas the control obese mice had only about 1/5 the number of low-affinity binding sites as the lean controls but the binding affinity showed significant increase (Table 5).

Insulin-dependent increase in glucose-1-^{14}C oxidation and incorporation into lipids in fat pads. Figure 1A shows insulin dose-response curves of glucose-1-^{14}C oxidation in the fat pads of these animals. The control obese (●——●) showed very mild response to insulin and ciglitazone treatment (○- - -○) significantly increased insulin-responsiveness in glucose oxidation in the isolated fat pads. The lean controls (▲——▲) were highly sensitive to insulin. Ciglitazone treatment in these lean mice did not increase insulin responsiveness in the fat pads as in the early experiment, but slightly increased the basal rate of glucose oxidation. Similarly, glucose-1-^{14}C incorporation into lipids was slightly elevated in the obese control in response to increasing concentrations of insulin in the medium and ciglitazone treatment in the obese mice increased both basal and insulin-dependent synthesis of lipids (Figure 1B). Lipid synthesis in the lean mice fat pads was highly responsive to insulin, and ciglitazone treatment increased the basal lipogenesis but not the insulin-dependent increment of the synthesis of lipids.

Insulin responsiveness in the fat pads versus insulin sensitivity in the adipocytes. Figure 2 shows the insulin-dependent increase in glucose-1-^{14}C oxidation (A) or incorporation into lipids (B) in the fat pads as a function of ^{125}I-

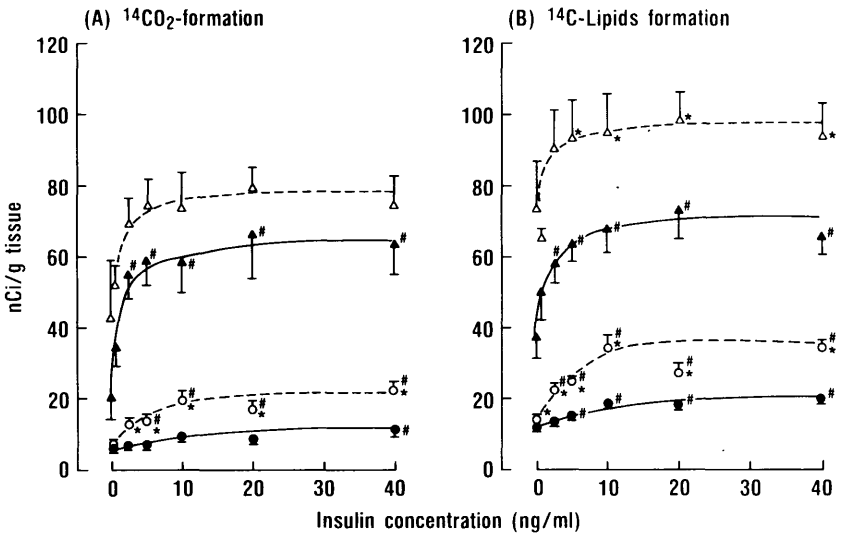

FIGURE 1. Dose response to medium insulin concentration in (A) oxidation of glucose-1-^{14}C to CO_2 and (B) incorporation of glucose-1-^{14}C into lipids by epididymal fat pads isolated from control (●——●) and ciglitazone-treated (○- - -○) C57BL/6J-ob/ob mice and control (▲——▲) and ciglitazone-treated (△- - -△) C57BL/6J− +/? mice. #Significantly different from baseline values, i.e., at no addition of insulin. *Significantly different from control values.

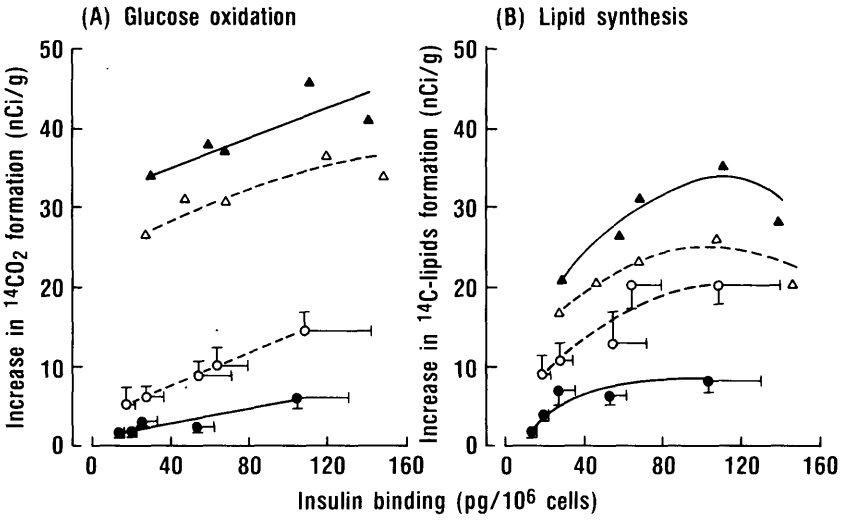

FIGURE 2. Correlation between insulin binding in the adipocytes and insulin-dependent increase in glucose-1-^{14}C oxidation and incorporation into lipids in the fat pads isolated from control (●——●) and ciglitazone (○– – –○) C57BL/6J-ob/ob mice and control (▲——▲) and ciglitazone (△– – –△) C57BL/6J– +/? mice. Bars represent standard error of the means (SEM).

insulin binding in the adipocytes. Each point represents a mean value in the amount of ^{125}I-insulin bound (abscissa) or in the increase above basal level of ^{14}C-incorporation into CO_2 or lipids (ordinate) at a certain concentration of insulin added in vitro, the insulin concentrations being 2.5, 5, 10, 20, and 40 ng/ml. The bars in the graphs represent standard errors of the means (SEM). The lean mice showed very large SEMs in insulin-dependent increments in the $^{14}CO_2$ formation and lipogenesis, i.e., 5–19 nCi/g, whereas the SEMs in the obese group were much smaller, i.e., 0.4–2.8. In Figure 2, only SEMs in the obese group are shown. In the obese group (circles), at a given amount of insulin bound, the drug-treated mice (open) showed higher biologic response to insulin than the controls (solid) in both glucose oxidation and lipid synthesis. Further, the drug-treated group showed steeper slopes than the untreated controls, indicating that ciglitazone-dependent enhancement in insulin responsiveness, as reflected by glucose oxidation and lipogenesis in the fat pads, exceeded the ciglitazone-induced increase in insulin sensitivity, as measured by ^{125}I-insulin binding in isolated adipocytes. Contrariwise, in the lean littermates, the drug-treated group showed a slightly lower biologic response to insulin than the controls at a given amount of insulin bound. Further, the slopes of the curves were similar in drug-treated and control groups in both glucose oxidation and lipid synthesis.

DISCUSSION

Since the hypoglycemic activity of ciglitazone is best illustrated in the obese animals,[1] the adipose tissue must play an important role in its pharmacologic action. Therefore, an extensive study on glucose metabolism in the epididymal fat pads of the C57BL/6J-ob/ob mice and their lean littermates, C57BL/6J– +/?, was carried out in vitro. To obtain as much information as possible, glucose doubly labeled with ^{14}C and ^{3}H at positions 1 and 5, respectively, was used as tracer. The measured endpoints included the formations of ^{14}C- and ^{3}H-labeled free fatty acids and esterified lipids in the tissue and the release of $^{14}CO_2$, $^{3}H_2O$, glycerol, and free fatty acids at basal condition as well as in the presence of insulin and/or epinephrine. When glucose-1-^{14}C/-5-^{3}H was metabolized through the pentose phosphate shunt (PPS), ^{14}C-label was lost as $^{14}CO_2$ in an early step in the shunt and ^{3}H-label as $^{3}H_2O$ via the subsequent metabolism of triose phosphates in glycolysis. When glucose-1-^{14}C/-5-^{3}H went through the glycolytic pathway, ^{14}C-label remained in the end products whereas ^{3}H-label was lost as $^{3}H_2O$ by the action of enolase on 2-phosphoglycerate. Thus, $^{14}CO_2$ formation represented PPS activity, whereas $^{3}H_2O$-formation reflected the combined activities of PPS and glycolysis. The ^{14}C-label in free fatty acids stemmed from acetate formed via glycolysis and pyruvate dehydrogenase, whereas ^{3}H-label in free fatty acids came from $^{3}H_2O$ via reduced pyridine nucleotides consumed in the reductive steps in de novo biosynthesis. The radiolabels in esterified lipids could also be associated with the glycerol moiety in addition to the fatty acids; the ^{14}C-label in glycerol could come from glycolysis only, whereas ^{3}H-label could arise from glycolysis and PPS.

Under basal condition, fat pads isolated from ciglitazone-treated obese mice produced more $^{14}CO_2$ than control obese, indicating that ciglitazone raised the activity in PPS which is the major insulin-dependent pathway of glucose metabolism in the fat pads. The basal rate of lipogenesis, as evidenced by the increased incorporation of glucose-5-^{3}H into esterified lipids, was also elevated in the fat pads of treated obese mice. In the subsequent experiment where insulin dose-dependence was measured, insulin-dependent increase in both glucose oxidation and lipogenesis was substantially augmented in the ciglitazone-treated obese mice fat pads when compared with those of obese controls. Thus, in the obese mice, ciglitazone treatment increased the biologic response to insulin in the adipose tissue. Furthermore, the adipocytes of treated obese mice showed higher numbers of insulin receptors than those of the control obese, indicating that ciglitazone augmented insulin sensitivity in the adipose tissue of the obese mice. When insulin sensitivity was plotted against the biologic response to insulin at varying insulin concentrations, the treated obese showed higher biologic response, in both glucose oxidation and lipogenesis, and a steeper curve than the control obese. The increase in insulin receptor numbers could arise, at least in part, from drug-induced lowering of plasma insulin[1] whereas

the increases in post-receptor responses and in basal rates of glucose metabolism and lipogenesis were not directly related to the decrease in plasma insulin.

In contrast to its effect in the C57BL/6J-ob/ob mice, ciglitazone treatment in the lean littermates failed to affect insulin binding in the adipocytes and did not produce consistent effects on the biologic responses to insulin in the isolated fat pads. In the first experiment, fat pads of ciglitazone-treated lean mice showed slightly greater incremental response to insulin, whereas, in the second experiment, the incremental response to insulin was similar in control and treated lean mice fat pads. The discrepancy may be due to the fact that the drug dosage in the first experiment was almost twice (150 mg/kg/day) as high as in the second experiment (82 mg/kg/day). However, in both experiments, ciglitazone increased the basal rates of glucose oxidation and lipogenesis in the fat pads of these animals. Since ciglitazone showed only mild and transient hypoglycemic activity in the lean mice but striking and consistent effect in the obese mice, it was surprising that it exerted equally, if not more, impressive increase in the basal rates of glucose oxidation and lipogenesis in the fat pads of the lean mice as those in the obese mice. Why ciglitazone exerted such diverse effects in C57BL/6J-ob/ob and − +/? mice remains unknown.

In the present study, the effect of epinephrine in vitro on glucose metabolism, lipogenesis, and lipolysis in the isolated fat pads was also measured. Epinephrine showed no effect on glucose metabolism in the obese mice fat pads but caused a slight depression in glucose oxidation in the adipose tissue of lean mice. Epinephrine-induced lipolysis was evident in both groups and the effect was much more pronounced in the lean mice fat pads. Epinephrine also increased the incorporation of glucose-1-^{14}C/-5-^{3}H into free fatty acids in both obese and lean groups. Ciglitazone, however, showed no effect on these epinephrine-responsive metabolic processes in the isolated fat pads of either lean or obese mice. The data obtained here in vitro agreed with the previous observation that ciglitazone had no effect on epinephrine-induced hyperglycemia and lipolysis in the C57BL/6J-ob/ob and − +/? mice in vivo.[1] In the obese group, the combined effects of insulin and epinephrine on these processes were also followed since, under certain conditions, these hormones exert opposite effects on lipolysis.[10] In the present study, both insulin and epinephrine showed a positive effect on lipogenesis in the control obese mice fat pads and ciglitazone treatment did not change the additive effect of insulin and epinephrine.

The lipolytic effect of epinephrine and anti-lipolytic action of insulin were in full evidence in both control and drug-treated obese mice fat pads when either hormone was present alone. In the control obese group, the opposing effects of these hormones on lipid metabolism cancelled out each other when both were added to the incubation medium. Contrariwise, in ciglitazone-treated obese mice fat pads, the antilipolytic effect of insulin was reversed in the presence of epinephrine. Kono and Barham studied the effects of insulin on lipolysis under various conditions and observed multiple and opposite effects.[10] In addition to its well-known inhibitory effect on lipolysis, insulin also stimulated lipolysis induced by 1 mM dibutyryl cAMP or 0.1 to 1 mM norepinephrine.[10] Therefore, a study on the effect of ciglitazone on cAMP metabolism in response to insulin and epinephrine in the fat pads may provide us with a clue to explain our present observation.

In summary, ciglitazone treatment in the C57BL/6J-ob/ob mice, in which the drug showed striking hypolycemic activity, affected adipocyte metabolism in the following aspects: (1) increased insulin binding sites, (2) increased insulin post-receptor responsiveness, (3) increased basal rates of glucose metabolism and lipogenesis, (4) altered insulin-response to epinephrine-induced lipolysis. Ciglitazone treatment in the C57BL/6J − +/? lean littermates, in which the drug lacked consistent hypoglycemic activity, showed the following changes in the adipocytes: (1) increased basal rates of glucose oxidation and lipogenesis, and (2) increased basal rate of lipolysis.

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