Ciglitazone, a New Hypoglycaemic Agent.

3. Effect on Glucose Disposal and Gluconeogenesis in Vivo in C57BL/6J-Ob/Ob and – +/? Mice

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Summary. Ciglitazone is orally active in preventing and reversing the hyperglycaemic syndrome in C57BL/6J-ob/ob mice and it is only mildly and transiently hypoglycaemic in lean littermates (C57BL/6J – +/?). Its effect on glucose disposal in vivo was estimated by injecting glucose-6-³H/¹⁴C and following the specific activity of radiolabelled glucose at 15, 30, 45, and 60 min after injection. The rate constants of glucose turnover were calculated to be as follows in decreasing order: treated obese (0.046/min), treated lean (0.032/min), control lean (0.026/min), and control obese (0.022/min). The obese mice showed less futile Cori cycle activity than the lean mice and ciglitazone had negligible effect on glucose recycling. The control obese mice incorporated more radio-

labels in hepatic lipids, glycogen, and proteins than the control lean mice and ciglitazone further enhanced the incorporations. Ciglitazone also increased hepatic accumulations of radiolabels in the glycogen and lipid fractions in the lean littermates. Using lactate-¹⁴C as precursor, gluconeogenesis in vivo was measured in control and treated obese and lean mice. Ciglitazone significantly lowered the rate of conversion of lactate-¹⁴C to glucose-¹⁴C in the obese mice but not in the lean littermates.

Key words: Ciglitazone, C57BL/6J-ob/ob mice and their lean littermates, glucose turnover rate in vivo, gluconeogenesis in vivo, Cori cycle.

Ciglitazone, 5-[4-(1-methylcyclohexylmethoxy)benzyl]-thiazolidine-2,4-dione, is a new orally active hypoglycaemic agent in obese-hyperglycaemic animals, such as C57BL/6J-ob/ob and C57BL/KsJ-db/db mice [1]. However, it lacks consistent hypoglycaemic effect in lean C57BL/6J – +/? littermates, normal rats, or insulinopenic animals, such as diabetic Chinese hamsters or streptozotocin-diabetic rats [1]. In obese mice, ciglitazone demonstrated multiple pharmacological actions. It enhanced the basal rate of glucose metabolism, insulin binding capacity, and insulin responsiveness in the isolated fat pads using glucose oxidation, lipogenesis, and lipolysis as endpoints [2]. Further, ciglitazone also promoted efficient synthesis and storage of insulin, leading to regranulation of pancreatic islet β cells in the obese mice [1]. The present study reports on the effect of ciglitazone on gluconeogenesis in vivo in the obese mice. In addition, its effect on glucose turnover was also studied using glucose-6-³H/¹⁴C as tracer according to Dunn et al. [3]. Companion studies in the lean C57BL/6J – +/? littermates were also carried out in parallel.

Materials and Methods

Chemicals

Ciglitazone was obtained from Takeda Chemical Industries, Osaka, Japan. Enzymes used in the assays were purchased from either Sigma Chemicals, St. Louis, Missouri or Boehringer-Mannheim, Indianapolis, Indiana.

Animals

The C57BL/6J-ob/ob mice and their lean littermates (genotypes +/ob or +/+) were males obtained from Jackson Laboratories, Bar Harbor, Maine; they were shipped after weaning. The animals used in the glucose-6-³H/¹⁴C disposal study were 37 days old when their baseline blood glucose levels were measured. They were put immediately on ground Purina Mouse Chow alone or admixed with 1 mg/g ciglitazone. Based on food intake and body weight, the dose was calculated to be approximately 190 mg·kg⁻¹·day⁻¹ for the obese or 160 mg·kg⁻¹·day⁻¹ for the lean mice. Drug regimen continued for 12–15 days. The animals used in the gluconeogenesis study were 45 days old when their baseline glucose levels were measured. On day 49, they were orally dosed with 150 mg·kg⁻¹·day⁻¹ late in the afternoon for 5 days and blood glucose levels were measured in samples obtained early in the morning. On day 6, dosing was converted to food admixture at 150 mg·kg⁻¹·day⁻¹ until termination on days 17–19 after the drug regimen had begun.

Table 1. Blood glucose, body weight and food intake in control and ciglitazone-treated C57BL/6J-ob/ob and +/? mice before and after treatment

Measurements	C57BL/6J-ob/ob mice			C57BL/6J-+/? mice		
	Control	Treated	<i>p</i>	Control	Treated	<i>p</i>
<i>Glucose disposal study^a</i>						
Fasting blood glucose (mmol/l)						
Baseline	4.3 ± 0.3 (6)	4.8 ± 0.3 (6)	NS	4.3 ± 0.2 (5)	4.4 ± 0.3 (6)	NS
At termination	20.1 ± 1.4 (6)	6.4 ± 0.4 (6)	<0.01	8.4 ± 0.2 (5)	7.2 ± 0.3 (6)	<0.05
Body weight (g)						
Baseline	24.7 ± 0.8 (6)	25.3 ± 1.1 (6)	NS	15.7 ± 0.5 (5)	16.2 ± 0.8 (6)	NS
At termination	37.4 ± 0.8 (6)	37.3 ± 1.7 (6)	NS	22.5 ± 0.9 (5)	23.1 ± 1.1 (6)	NS
Food intake (g/day)	7.56 ± 0.35 (6)	5.96 ± 0.32 (6)	<0.01	5.04 ± 0.07 (5)	4.57 ± 0.6	NS
<i>Gluconeogenesis study^b</i>						
Fasting blood glucose (mmol/l)						
Baseline	18.6 ± 1.0 (6)	15.9 ± 1.2 (7)	NS	9.6 ± 0.4 (7)	8.7 ± 0.4 (7)	NS
At termination	19.8 ± 2.3 (5)	7.9 ± 0.9 (7)	<0.001	8.3 ± 0.5 (7)	7.7 ± 0.6 (7)	NS
Body weight (g)						
Baseline	36.0 ± 1.3 (6)	35.4 ± 0.6 (7)	NS	23.7 ± 0.9 (7)	23.3 ± 0.8 (7)	NS
At termination	45.2 ± 1.2 (5)	43.0 ± 0.9 (7)	NS	26.3 ± 0.81 (7)	25.7 ± 0.6 (7)	NS

Results are expressed as mean ± SEM with number of animals in parentheses; NS = not significant

^a Baseline values were measured on day 37 after birth and the animals were terminated on days 12–15 after treatment had begun

^b Baseline values were measured on day 45 after birth and the animals were terminated on days 17–19 after treatment had begun

Disposal of Glucose-6-³H/¹⁴C

Studies were carried out early in the morning on days 12–15 after treatment had begun. The animals were fasted for 17 h and bled through the orbital sinus; 0.2 ml saline containing 10 µCi glucose-6-³H (30 Ci/mmol) and 3 µCi glucose-6-¹⁴C (5.5 mCi/mmol) were injected SC. The animals were subsequently bled at 15, 30, 45, and 60 min post-injection. From each bleeding, 0.02 ml aliquots were used to measure glucose fluorometrically in a Technicon autoanalyzer (Tarrytown, New York) [4]. From the four blood samples obtained after the injection of labelled glucose, another 0.02 ml aliquot was employed for ³H₂O determination according to Pollard et al. [5]; a third aliquot (0.075 ml) was used to measure glucose-³H and ¹⁴C as described below. At 70 min post-injection, the mice were decapitated and portions of the livers extracted.

The liver portions were quickly frozen and kept at –70°C for 17–20 days. The liver portions were then weighed in the frozen state and homogenized in two volumes of ice-cold 0.9% saline with a Brinkmann tissue homogenizer (Westbury, New York) for 20 s. To measure radiolabelled liver glycogen, 0.075 ml aliquots of the homogenates were spotted on 2.5 × 2.5 cm Whatman 3 mm filter paper discs. After the homogenates dispersed evenly, the discs were dropped into a 66% ethanol bath and, after two subsequent changes of 66% ethanol, the paper discs were dried and counted in 10 ml Liquifluor-Toluene (New England Nuclear, Boston, Massachusetts). To measure liver glycogen content, the homogenates were diluted 1:10 with water; 0.5 ml aliquots of the diluted samples were incubated with 0.45 ml amyloglucosidase (5.6 U/ml) in 0.1 mol/l potassium phosphate buffer (pH 6.0) for 2 h. Glucose content was measured in the samples after deproteinization with HClO₄. A zero-time blank for each homogenate was estimated to correct for the glucose level in the liver extracts. The difference of these estimations represented glucose units present in the glycogen. Rabbit liver glycogen similarly processed served as standards to calculate the level of glycogen present in the mouse liver. To measure radiolabelled liver proteins, 0.075 ml aliquots of the homogenates were spotted on 2.5 × 2.5 cm Whatman 3 mm paper discs, which were then dropped into a 10% trichloroacetic acid bath. After washing twice more in 10% trichloroacetic acid and once in ether, the discs were dried and counted in 10 ml Liquifluor-Toluene. Liver protein content was measured with a BioRad Protein Assay Kit (Richmond, California). To measure radiolabelled liver lipids, 0.1 ml aliquots of

the liver homogenates were diluted with water to 1 ml. Dole's extraction medium (5 ml) (i-propanol:heptane:1N H₂SO₄ = 40:10:1) was added and the mixture vortexed for 5 min. After standing at room temperature for 15 min, 3 ml water and 3 ml hexane were added in succession. After the phases separated, 2.5 ml of upper phase was counted in 10 ml Liquifluor-Toluene.

Gluconeogenesis

Mice were fasted for 20 h after being treated at a dose of 150 mg·kg^{–1}·day^{–1} ciglitazone for 17–19 days. Early in the morning, they were bled and immediately 0.2 ml ¹⁴C(U)-lactate (150 mCi/mmol and 10 µCi/ml) was injected SC. The animals were subsequently bled at 5, 10, and 15 min post-injection. From each bleeding, 0.02 ml aliquots were used to measure blood glucose and lactate levels [4]. From the three blood samples obtained after the injection of ¹⁴C-labelled lactate, an additional 0.1 ml aliquot was mixed with 0.2 ml H₂O and heated at 100°C for 1.5 min. After cooling in ice, 0.5 ml chloroform was added and the tubes vortexed. From the upper phase, two 0.075 ml aliquots were removed after centrifugation and mixed with 0.012 ml 0.1 mol/l acetic acid. To one aliquot, 0.15 ml water and 0.265 ml 0.05 mol/l sodium acetate buffer (pH 5.1), which had been gassed with 95% O₂–5% CO₂ for 15 min, were added. To the second aliquot, 0.15 ml glucose oxidase (Sigma No. G6500, Type V, 1400 U/ml) and 0.265 ml 0.05 mol/l sodium acetate buffer (pH 5.1), gassed with 95% O₂–5% CO₂, were added. The tubes were gassed with 95% O₂–5% CO₂ and capped. After incubation at 37°C for 1.5 h, 0.5 ml H₂O was added and the content was introduced into a 0.8 × 6 cm column of QAE-Sephadex A25 (Pharmacia, Piscataway, New Jersey), equilibrated in 0.1 mol/l ammonium formate, pH 7.4. The column was washed four times with 1 ml H₂O. The eluent was collected into vials containing 1.4 g moist Dowex AG50Wx4 (100–200 mesh) (BioRad Laboratories, Richmond, California) in 0.05 mol/l HCl. The vials were shaken for 5 min on an oscillating shaker. The supernatant (4 ml) was withdrawn into a vial containing 15 ml scintillation fluid (Packard, Downers Grove, Illinois). The radioactivity was measured in a scintillation counter and quenching corrected. Radioactivity associated with glucose was represented by the difference between samples in the absence and presence of glucose oxidase. The radioactivity in samples incubated in the absence of glucose oxidase represented the labelled

Fig. 1A and B. Food intake and blood glucose level of (A) C57BL/6J-ob/ob mice and (B) C57BL/6J-+/? mice at pretreatment and post-treatment periods with ciglitazone which was dosed orally at $150 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ late in the afternoons. Bleedings were carried out in the mornings. C57BL/6J-ob/ob mice; control (●—●) and treated (○—○). C57BL/6J-+/? mice; control (▲—▲) and treated (▽—▽). Arrows indicate the initiations of the drug regimen and @ indicates significantly different from controls at <0.05 , # <0.01 , and * <0.001

non-ionic materials in the blood, such as glucose, glycerol, sorbitol, etc., whereas incubation with glucose oxidase specifically converted glucose into gluconic acid which was retained by the ion-exchange resins.

Calculations

Statistical significance of the differences between the means was calculated using one-way analysis of variance and Student's *t*-test. Half-time of radiolabelled glucose, rate constant of glucose turnover, and fraction of glucose recycled were estimated according to Dunn et al. [3].

Results

Blood Glucose, Body Weight, and Food Intake

In the present study, treatment of C57BL/6J-ob/ob mice with ciglitazone began at two different developmental stages of its obese-hyperglycaemic syndrome. When the drug regimen started at a time when the obese mice were still normoglycaemic, the treated mice remained euglycaemic at $6.4 \pm 0.4 \text{ mmol/l}$, whereas the untreated controls developed hyperglycaemia ($20.1 \pm 1.4 \text{ mmol/l}$) in 2 weeks (Table 1). Ciglitazone lowered food intake in the obese mice but it had no effect on body weight. In the lean mice, ciglitazone showed a very slight hypoglycaemic effect, although it failed to affect either food intake or body weight significantly. When ciglitazone was given to the obese mice after they had already developed hyperglycaemia, blood glucose levels of the treated mice dropped from 15.9 ± 1.2 to

Fig. 2A–D. Blood levels of (A) glucose- ^3H , (B) glucose- ^{14}C , (C) ^3H -labelled non-ionic materials and (D) ^{14}C -labelled non-ionic materials in control and ciglitazone-treated C57BL/6J-ob/ob and C57BL/6J-+/? mice after injection of glucose-6- $^3\text{H}/^{14}\text{C}$ in the fasting state. Ciglitazone was given as food admixture at a dose of $190 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for ob/ob and $160 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for +/? mice for 12–15 days. C57BL/6J-ob/ob mice; control (●—●) and treated (○—○). C57BL/6J-+/? mice; control (▲—▲) and treated (▽—▽)

$7.9 \pm 0.9 \text{ mmol/l}$ in 2½ weeks, whereas blood glucose levels of the untreated controls remained over 16.7 mmol/l (Table 1). Again, no effect on body weight was seen. Figure 1 shows the results of food intake and blood glucose levels in obese and lean mice when ciglitazone was dosed orally by gastric intubation at $150 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 5 days. Although no difference in food intake was seen between treated and control groups in either genotype, significantly lower blood glucose levels were observed in both lean and obese mice as a result of ciglitazone treatment. The dosing was converted to food admixture on day 6 and continued for another 12–14 days. While the fasting blood glucose level in the treated obese mice at the end of treatment remained lower than that of the controls (7.9 ± 0.9 versus $19.8 \pm 2.3 \text{ mmol/l}$, $p < 0.001$), no significant difference was found between treated and control lean mice (7.7 ± 0.6 versus $8.3 \pm 0.5 \text{ mmol/l}$; Table 1).

Glucose-6- $^3\text{H}/^{14}\text{C}$ Disappearance in Vivo

To estimate the glucose turnover rate in vivo, the control and treated obese and lean mice were injected glucose-6- $^3\text{H}/^{14}\text{C}$ after treatment with ciglitazone for 12–15 days. Figure 2 shows the disappearance rate of glucose- ^3H and glucose- ^{14}C in the blood in control and treated obese and lean mice. In both radiolabels, the treated obese mice had a much faster rate of disposal than the control obese mice, whereas the treated lean

Table 2. Blood levels of $^3\text{H}_2\text{O}$, glucose, glucose- ^3H and ^{14}C and $^3\text{H}/^{14}\text{C}$ ratio in glucose in control and ciglitazone-treated C57BL/6J-ob/ob and +/? mice injected with glucose-6- ^{14}C and -6- ^3H

Blood levels	Time (min)	C57BL/6J-ob/ob			C57BL/6J-+/?		
		Control (n=6)	Treated (n=6)	p	Control (n=5)	Treated (n=6)	p
$^3\text{H}_2\text{O}$ (nCi/ml)	15	14.7 $\pm$ 2.1	27.2 $\pm$ 1.1	<0.001	28.8 $\pm$ 4.3	22.3 $\pm$ 3.6	NS
	30	44.2 $\pm$ 6.6	69.8 $\pm$ 3.4	<0.01	66.3 $\pm$ 5.4	61.3 $\pm$ 3.9	NS
	45	63.0 $\pm$ 8.0	99.9 $\pm$ 4.2	<0.01	93.7 $\pm$ 5.9	90.5 $\pm$ 5.5	NS
	60	78.9 $\pm$ 8.3	116.0 $\pm$ 4.7	<0.01	112.8 $\pm$ 6.3	110.8 $\pm$ 5.1	NS
Glucose (mmol/l)	0	20.1 $\pm$ 1.4	6.4 $\pm$ 0.4	<0.001	8.4 $\pm$ 0.2	7.2 $\pm$ 0.3	<0.05
	15	22.6 $\pm$ 1.8	10.4 $\pm$ 1.0	<0.001	6.6 $\pm$ 0.5	7.3 $\pm$ 0.3	NS
	30	23.2 $\pm$ 1.4	8.3 $\pm$ 1.2	<0.001	6.6 $\pm$ 0.3	6.9 $\pm$ 0.3	NS
	45	23.8 $\pm$ 1.9	9.0 $\pm$ 0.8	<0.001	7.1 $\pm$ 0.4	7.2 $\pm$ 0.3	NS
	60	27.3 $\pm$ 3.1	10.4 $\pm$ 0.9	<0.001	7.1 $\pm$ 0.4	6.8 $\pm$ 0.4	NS
Glucose- ^3H (nCi/ μmol)	15	16.6 $\pm$ 3.0	26.4 $\pm$ 3.5	NS	44.3 $\pm$ 4.0	43.6 $\pm$ 8.3	NS
	30	13.5 $\pm$ 1.2	20.8 $\pm$ 1.9	<0.01	30.7 $\pm$ 3.5	28.1 $\pm$ 4.6	NS
	45	11.3 $\pm$ 1.0	10.1 $\pm$ 1.0	NS	21.0 $\pm$ 2.3	19.2 $\pm$ 2.5	NS
	60	7.1 $\pm$ 0.4	8.1 $\pm$ 1.0	NS	12.9 $\pm$ 1.9	10.9 $\pm$ 1.4	NS
Glucose- ^{14}C (nCi/ μmol)	15	5.50 $\pm$ 0.93	8.46 $\pm$ 0.78	<0.01	15.6 $\pm$ 1.6	14.2 $\pm$ 1.4	NS
	30	4.56 $\pm$ 0.31	7.76 $\pm$ 0.93	<0.01	10.9 $\pm$ 1.1	10.6 $\pm$ 0.9	NS
	45	4.04 $\pm$ 0.12	4.79 $\pm$ 0.54	NS	8.87 $\pm$ 0.45	8.01 $\pm$ 0.49	NS
	60	2.81 $\pm$ 0.20	3.02 $\pm$ 0.45	NS	6.31 $\pm$ 0.32	5.95 $\pm$ 0.38	NS
$\frac{\text{Glucose-}^3\text{H}}{\text{Glucose-}^{14}\text{C}}$	15	3.02 $\pm$ 0.28	3.16 $\pm$ 0.35	NS	2.91 $\pm$ 0.32	2.98 $\pm$ 0.27	NS
	30	2.96 $\pm$ 0.34	2.80 $\pm$ 0.34	NS	2.71 $\pm$ 0.21	2.64 $\pm$ 0.34	NS
	45	2.84 $\pm$ 0.30	2.12 $\pm$ 0.31	NS	2.32 $\pm$ 0.26	2.41 $\pm$ 0.31	NS
	60	2.58 $\pm$ 0.23	2.73 $\pm$ 0.28	NS	2.07 $\pm$ 0.37	1.80 $\pm$ 0.14	NS

Results are expressed as mean $\pm$ SEM

Table 3. Half-time of glucose- ^3H and ^{14}C , rate constant of glucose turnover, and fraction of glucose recycled in control and ciglitazone-treated C57BL/6J-ob/ob and +/? mice in the fasting state

	C57BL/6J-ob/ob		C57BL/6J-+/?	
	Control	Treated	Control	Treated
Half-time of glucose- ^3H (min) $t_{1/2}$	30.9	15.2	27.2	21.8
Half-time of glucose- ^{14}C (min) $t_{1/2}$	37.5	18.9	36.1	29.2
Rate constant of glucose turnover (k) (per min) = $0.693/t_{1/2}$	0.022	0.046	0.026	0.032
Fraction of glucose recycled (f) (%) = $1 - (t_{1/2}/t'_{1/2}) \times 100$	17.6	19.6	24.7	25.3

mice showed a slightly but consistently increased rate than did the control lean mice. The procedure used to estimate the radiolabels associated with glucose also provided data on the radiolabels found in the non-ionic materials among which glucose and H_2O were the major ingredients since most of the metabolites were in ionic forms. As shown in Figure 2C, tritium labels in this fraction remained relatively constant as most of the tritium in glucose was converted into $^3\text{H}_2\text{O}$ which stayed in this fraction with glucose- ^3H . In contrast, ^{14}C -labels in this fraction showed a time-dependent decrease (Fig. 2D) since most of the ^{14}C in glucose was lost as $^{14}\text{CO}_2$. Furthermore, the decrease in ^{14}C -label in the non-ionic fraction was fastest in the treated obese mice

(a $t_{1/2}$ of about 30 min), and slowest in the control obese mice (a $t_{1/2}$ of greater than 60 min). The two lean groups showed a similar rate of disappearance, a $t_{1/2}$ of about 36 min (Fig. 2D).

Table 2 summarizes the blood levels of $^3\text{H}_2\text{O}$ and glucose and the specific activities of glucose- ^3H and glucose- ^{14}C at 15, 30, 45, and 60 min after the injection. As expected, the control obese group showed significantly less $^3\text{H}_2\text{O}$ formation than the other three groups in view of their higher endogenous blood glucose levels. The time courses of $^3\text{H}_2\text{O}$ formation in the treated ob/ob and control and treated lean +/? mice were almost parallel. Blood glucose levels in the control obese group were significantly higher than the other three groups. In the obese mice, increases in blood glucose levels were found at the subsequent four bleedings, whereas the blood glucose level remained relatively constant in the lean mice during repeated bleedings (Table 1).

The turnover rate of glucose in vivo was estimated on the basis of its specific activity in the blood [3]. In the obese groups, the treated mice showed higher specific activity in both glucose- ^3H and glucose- ^{14}C than the controls at 15 and 30 min after injection; the difference was no longer evident at 45 and 60 min (Table 2). There was no apparent difference in the specific activity of either glucose- ^3H or glucose- ^{14}C between the control and treated lean mice at all time points. As expected, the control obese mice showed significantly lower specific activity of radiolabelled glucose than the lean controls since the obese mice had much higher endogenous

Table 4. Radiolabels incorporated into liver proteins, glycogen and lipids in control and ciglitazone-treated C57BL/6J-ob/ob and +/? mice

Liver	C57BL/6J-ob/ob			C57BL/6J-+/?		
	Control (n=6)	Treated (n=6)	p	Control (n=5)	Treated (n=6)	p
Protein						
Content (mg/g)	86.1 ± 2.6	85.4 ± 2.8	NS	92.3 ± 1.8	93.8 ± 1.7	NS
³ H-label (nCi/g)	19.3 ± 1.2	32.6 ± 2.1	<0.001	24.3 ± 1.0	28.3 ± 2.6	NS
¹⁴ C-label (nCi/g)	11.0 ± 0.7	20.2 ± 1.6	<0.001	11.6 ± 0.5	13.8 ± 1.1	NS
³ H/ ¹⁴ C-ratio	1.76 ± 0.06	1.63 ± 0.06	NS	2.10 ± 0.05	2.04 ± 0.07	NS
Glycogen						
Content (mg/g)	11.0 ± 3.3	2.93 ± 1.19	<0.05	0.450 ± 0.134	0.775 ± 0.166	NS
³ H-label (nCi/g)	20.4 ± 1.0	36.2 ± 2.2	<0.001	20.5 ± 0.5	25.7 ± 2.3	NS
¹⁴ C-label (nCi/g)	11.2 ± 0.7	21.0 ± 1.2	<0.001	8.52 ± 0.38	11.2 ± 0.9	<0.05
³ H/ ¹⁴ C-ratio	1.85 ± 0.08	1.72 ± 0.04	NS	2.42 ± 0.08	2.32 ± 0.11	NS
Lipids						
³ H-label (nCi/g)	10.1 ± 0.6	19.7 ± 1.5	<0.001	3.72 ± 0.27 (n=4)	8.78 ± 1.03	<0.005
¹⁴ C-label (nCi/g)	7.01 ± 0.45	14.5 ± 1.2	<0.001	4.69 ± 0.32 (n=4)	6.89 ± 0.55	<0.05
³ H/ ¹⁴ C-ratio	1.46 ± 0.06	1.37 ± 0.03	NS	0.792 ± 0.021 (n=4)	1.27 ± 0.10	<0.005

Results are expressed as mean ± SEM

blood glucose levels. Surprisingly, despite the fact that the treated obese mice had comparable blood glucose levels to the two lean groups, the specific activities of radiolabelled glucose were significantly lower in the treated obese than in the two groups of lean mice, suggesting that ciglitazone-treated obese mice had a faster glucose turnover rate than the lean mice (Table 2). The ratio of glucose-³H/glucose-¹⁴C remained similar in all four groups of mice and it decreased during the course of the experiment (Table 2). Based on these data, the half-times of glucose-³H and glucose-¹⁴C were estimated (Table 3). For both radiolabels the control ob/ob mice showed the longest half-time (30.9 min for glucose-³H and 37.5 min for glucose-¹⁴C), whereas the ciglitazone-treated obese mice showed the shortest half-time (15.2 min for glucose-³H and 18.9 min for glucose-¹⁴C). The half-times of glucose-³H and glucose-¹⁴C were also slightly shorter in the treated than in the control lean mice. The glucose turnover rates were found to decrease in the following order: treated obese > treated lean > control lean > control obese. The obese mice showed a smaller fraction of recycled glucose than the lean mice and ciglitazone had negligible effect on the Cori cycle (Table 3).

Incorporation of Glucose-6-³H/¹⁴C into Hepatic Proteins, Glycogen, and Lipids

Table 4 contains data on radiolabels recovered in proteins (trichloroacetic acid-precipitable), glycogen (ethanol-precipitable), and lipid fractions in the livers of these mice at 70 min after the injection of glucose-6-³H/¹⁴C. The ciglitazone-treated obese mice retained significantly greater amounts of both ¹⁴C and ³H in hepatic protein, glycogen, and lipid fractions than the other three groups. The treated obese mice also showed less liver glycogen content than the control obese mice. No differences in liver protein content or ³H/¹⁴C ratios in

hepatic protein, glycogen, or lipid fractions were found between the control and treated obese groups. In the lean +/? mice, ciglitazone increased ¹⁴C and ³H incorporation into hepatic glycogen and lipids and the ³H/¹⁴C ratio in the lipids. No significant difference in the protein fraction was seen between the two lean groups (Table 4).

Gluconeogenesis in vivo

The rate of gluconeogenesis in vivo using lactate-¹⁴C as the precursor was measured in control and ciglitazone-treated C57BL/6J-ob/ob mice and their lean littermates after a treatment at 150 mg·kg⁻¹·day⁻¹ for 12–15 days. Figure 3 shows the blood levels of glucose during the course of the experiment. Due to the stress of repeated bleedings within a short interval of 15 min, blood glucose levels increased in all four groups of mice, particularly pronounced in the obese groups. At all four timepoints, the drug-treated obese mice showed statistically significantly lower blood glucose than the control obese, whereas no significant difference was seen in the lean group. Further, the initial blood glucose levels of ciglitazone-treated obese mice were similar to those of the control and treated lean mice, but stress-induced increase in blood glucose was much more pronounced in the treated obese than the two groups of lean mice. The blood lactate levels also increased during the course of the experiment and the increase was more striking in the control groups than in the treated groups (Fig. 3). The lean groups also showed higher blood lactate levels than the corresponding obese groups.

The blood samples obtained at 5, 10, and 15 min after the injection of lactate-¹⁴C were estimated for total radioactivity and radiolabel specifically associated with glucose (Fig. 4). The ciglitazone-treated obese mice had less total radioactivity in the blood than the control

Fig. 3A and B. Blood levels of (A) glucose and (B) lactate in control and ciglitazone-treated C57BL/6J-ob/ob and C57BL/6J-+/? mice after injection of lactate- ^{14}C in the fasting state. Ciglitazone was orally dosed by gavage feeding for the first 5 days and subsequently given as food admixture at a dose of $150 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for a total of 17–19 days. C57BL/6J-ob/ob mice; control (●—●) and treated (○—○). C57BL/6J-+/? mice; control (▲—▲) and treated (▽—▽)

Fig. 4A–C. Blood levels of (A) total radioactivity (B) glucose- ^{14}C and (C) percentage radioactivity in glucose in control and ciglitazone-treated C57BL/6J-ob/ob and C57BL/6J-+/? mice after injection of lactate- ^{14}C in the fasting state. C57BL/6J-ob/ob mice; control (●—●) and treated (○—○). C57BL/6J-+/? mice; control (▲—▲) and treated (▽—▽)

obese and the difference was statistically significant at 5 and 10 min after injection (Fig. 4). At all three time points the radioactivity associated with glucose was significantly lower in the drug-treated obese than in the control obese (Fig. 4B, upper panel). When percentage radiolabel associated with glucose was calculated, the treated obese mice showed less percentage of ^{14}C in glucose than the controls and the difference was statistically significant in the 15 min sample (Fig. 4C, upper panel). In the lean littermates, total radioactivity in blood was similar in control and treated groups at 5 and 10 min after the injection but the treated lean mice showed significantly less radioactivity in the blood than the controls at 15 min (Fig. 4A, lower panel). The radioactivity associated with blood glucose was not significantly different between control and treated lean mice (Fig. 4B, lower panel). At 5 and 10 min after injection of lactate- ^{14}C , no difference in percentage of ^{14}C in glucose was seen between control and treated lean mice, but at 15 min the treated lean mice showed 31% ^{14}C in glucose and the control only 18% (Fig. 4C, lower panel).

Discussion

The data presented here demonstrate that ciglitazone is orally active in preventing and reversing the hyperglycaemic syndrome in C57BL/6J-ob/ob mice. Although ciglitazone reduced food intake when it was given as food admixture, it did not affect food intake during the first 4 days of oral dosing by gastric intubation. The blood glucose level was still significantly decreased in the treated obese mice. Therefore, the hypoglycaemic activity of ciglitazone was independent of its effect on food intake. During the gluconeogenesis study, the blood glucose level in both the obese and lean mice increased due to the stress caused by repeated bleedings in a short interval. The stress-induced increase in blood glucose was much more pronounced in the obese than in the lean mice. Ciglitazone, however, showed no effect on stress-induced hyperglycaemia in either genotype. The data agreed with our previous observation that ciglitazone had no effect on adrenaline-induced hyperglycaemia [1].

The obese mice showed lower fasting blood lactate levels than the lean mice and ciglitazone treatment did not affect the fasting blood lactate level. However, it slightly attenuated the increase in blood lactate caused by repeated bleedings in both genotypes. In the obese mice, the appearance of subcutaneously injected lactate- ^{14}C in the blood was retarded in the treated group. The difference could be due to a slower rate of absorption in the treated than in the control obese mice. A more likely explanation would be an accelerated disposal rate of lactate- ^{14}C to $^{14}\text{CO}_2$ in the peripheral tissues in the ciglitazone-treated obese mice. Indeed, in the study on glucose turnover with glucose-6- $^3\text{H}/^{14}\text{C}$,

the ciglitazone-treated obese mice showed a rate constant twice as high as that of the control obese mice. The conversion of lactate- ^{14}C to glucose- ^{14}C and the percentage of blood radiolabel in glucose were markedly lower in treated than in control obese mice, suggesting that ciglitazone suppressed gluconeogenesis *in vivo* in the ob/ob mice. The difference, however, could partially arise from an accelerated channelling of lactate- ^{14}C to the oxidative pathway. In the treated, lean littermates, the total blood radiolabel began to decrease at 10 min after injection of lactate- ^{14}C , in contrast to the data observed in the control lean mice and in the two groups of obese mice, but the glucose- ^{14}C level continued to increase with time. Therefore, 31% of blood radiolabel was associated with glucose at 15 min in the treated lean mice, compared to only 18% in the control lean mice. The facilitated disposal of lactate- ^{14}C in the ciglitazone-treated lean mice must have contributed, at least in part, to this difference.

The current study indicates that ciglitazone increased glucose turnover rate pronouncedly in the ob/ob mice and, less strikingly, in the lean littermates. Although ciglitazone greatly enhanced blood $^3\text{H}_2\text{O}$ formation and radiolabel incorporation into hepatic lipids, glycogen, and proteins in the obese mice, the data were difficult to interpret quantitatively on account of the vastly different endogenous glucose levels in the treated and control obese groups. In the lean mice, which showed relatively comparable blood glucose levels, ciglitazone failed to affect blood $^3\text{H}_2\text{O}$ formation but significantly increased the incorporation of radiolabels into hepatic lipids. More importantly, the $^3\text{H}/^{14}\text{C}$ ratio in hepatic lipids was greater in the treated lean mice than in the controls, suggesting considerable recycling of tritium present in H_2O into lipids. Therefore, ciglitazone must have a stimulatory effect on fatty acid synthesis, a process requiring many reductive steps which took up tritium from $^3\text{H}_2\text{O}$. Ciglitazone also increased the conversion of glucose to liver glycogen in the lean mice. As expected, no significant effect on the $^3\text{H}/^{14}\text{C}$ ratio in liver glycogen was found to be exerted by ciglitazone since the synthesis of glycogen from glucose involves few reactions and is minimally affected by the recycling processes. Ciglitazone had no significant effect on hepatic protein synthesis.

The genetically obese mice showed less futile Cori cycle activity than the corresponding lean littermates. This cycle represents the recycling of glucose and metabolic inefficiency. The thrifty gene concept of the diabetes-obesity syndrome [6] and the observations of Coleman [7, 8] that obese mice survived longer than their lean littermates during prolonged fasting were in full accord with our finding that obese mice had lower activity in the Cori cycle.

The present study confirmed our previous observations that ciglitazone is a highly efficacious hypoglycaemic agent in insulin-resistant, obese-hyperglycaemic animals [1, 2]. This agent appears to affect the glucose-fatty acid cycle proposed by Randle et al. to explain the metabolic disturbances in non-insulin dependent diabetes mellitus [9]. The ob/ob mice use non-esterified fatty acid predominantly for energy supply, leading to underutilization and accumulation of glucose. Our data on the remarkably low glucose disappearance rate in the control ob/ob mice attest to this phenomenon. Ciglitazone greatly enhanced glucose utilization in the ob/ob mice, as evidenced by the pronouncedly accelerated glucose disappearance rate. The enhanced insulin responsiveness which follows the correction in the "Randle" cycle [9] may further increase insulin-dependent metabolic activities such as glycogen synthesis and lipogenesis.

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