

# Peroxisome Proliferator-Activated Receptor (PPAR)- $\alpha$ Activation Lowers Muscle Lipids and Improves Insulin Sensitivity in High Fat-Fed Rats

## Comparison With PPAR- $\gamma$ Activation

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Peroxisome proliferator-activated receptor (PPAR)- $\alpha$  agonists lower circulating lipids, but the consequences for muscle lipid metabolism and insulin sensitivity are not clear. We investigated whether PPAR- $\alpha$  activation improves insulin sensitivity in insulin-resistant rats and compared the effects with PPAR- $\gamma$  activation. Three-week high fat-fed male Wistar rats were untreated or treated with the specific PPAR- $\alpha$  agonist WY14643 or the PPAR- $\gamma$  agonist pioglitazone (both  $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ) for the last 2 weeks of high-fat feeding. Like pioglitazone, WY14643 lowered basal plasma levels of glucose, triglycerides ( $-16\%$  vs. untreated), and leptin ( $-52\%$ ), and also muscle triglyceride ( $-34\%$ ) and total long-chain acyl-CoAs (LCACoAs) ( $-41\%$ ) ( $P < 0.05$ ). In contrast to pioglitazone, WY14643 substantially reduced visceral fat weight and total liver triglyceride content ( $P < 0.01$ ) without increasing body weight gain. WY14643 and pioglitazone similarly enhanced whole-body insulin sensitivity (clamp glucose infusion rate increased 35 and 37% and glucose disposal 22 and 15%, respectively, vs. untreated). Both agents enhanced insulin-mediated muscle glucose metabolic index ( $\text{Rg}'$ ) and reduced muscle triglyceride and LCACoA accumulation ( $P < 0.05$ ). Although pioglitazone had more potent effects than WY14643 on muscle insulin sensitization, this was associated with its greater effect to reduce muscle LCACoA accumulation. Overall insulin-mediated muscle  $\text{Rg}'$  was inversely correlated with the content of LCACoAs ( $r = -0.74$ ,  $P = 0.001$ ) and with plasma triglyceride levels ( $r = -0.77$ ,  $P < 0.001$ ). We conclude that even though WY14643 and pioglitazone, representing PPAR- $\alpha$  and PPAR- $\gamma$  activation, respectively, may alter muscle lipid supply by different mechanisms, both significantly improve muscle insulin action in the high fat-fed rat model of insulin resistance, and this effect is proportional to the degree to which they reduce muscle lipid accumulation. *Diabetes* 50:411-417, 2001

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Received for publication 1 June 2000 and accepted in revised form 11 October 2000.

BAT, brown adipose tissue; GIR, glucose infusion rate; LCACoA, long-chain acyl-CoA; NEFA, nonesterified free fatty acid; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor;  $R_d$ , glucose disappearance rate;  $\text{Rg}'$ , glucose metabolic index; TZD, thiazolidinedione.

There is increasing evidence that lipid accumulation in muscle and liver leads to development of insulin resistance (1-3). Reduction of obesity or lowering lipids generally improves insulin sensitivity (4,5). Peroxisome proliferator-activated receptors (PPARs) are members of the superfamily of nuclear transcription factors regulating lipid metabolism. PPAR- $\gamma$  is highly expressed in adipocytes and mediates their differentiation. The insulin-sensitizing thiazolidinediones (TZDs) are PPAR- $\gamma$  agonists, and they improve insulin action in association with lipid-lowering effects. These studies suggest that PPAR- $\gamma$  agonists such as the TZDs improve muscle insulin action by sequestering lipids in adipocytes, a mechanism that ultimately reduces lipid accumulation in muscle (6,7).

In comparison, PPAR- $\alpha$  is widely expressed in liver, muscle, kidney, and intestine (8) and mediates expression of genes promoting fatty acid  $\beta$ -oxidation (7,9,10). Activation of the PPAR- $\alpha$  also lowers circulating lipids. For example, the fibrate class PPAR- $\alpha$  agonists are effective drugs in clinical use to treat hypertriglyceridemia (7,11). Given that lipid oversupply/accumulation leads to insulin resistance, one would expect that lowering lipids with a PPAR- $\alpha$  agonist should also attenuate insulin resistance, as does a PPAR- $\gamma$  agonist in insulin-resistant states caused by lipid oversupply/accumulation (7). However, studies in humans have been inconclusive, with reports showing both improved (12,13) and unimproved insulin sensitivity (14,15). It might be that the PPAR- $\alpha$  agonists, though lowering circulating lipids, have little effect on muscle lipids in those reports in which insulin sensitivity is not improved. Thus far, we are aware of only one study that examined the effects on muscle lipids of a PPAR- $\alpha$  agonist (16). Although this study showed some lowering of muscle triglyceride, no proper assessment of muscle insulin sensitivity was made. In addition, the PPAR- $\alpha$  agonist bezafibrate used in this study (16) may also activate the PPAR- $\gamma$  receptor and PPAR- $\beta$  (10), a third subtype of PPARs with unknown function. A more recent study in diabetic *db/db* mice appears to add more uncertainty by showing that the PPAR- $\alpha$  agonist WY14643 was almost ineffective in overcoming insulin resistance in spite of a marked reduction in circulating triglyceride levels (17). However, this conclusion was based only on the fasting plasma glucose levels and neither muscle insulin sensitivity nor muscle lipid content was examined.

Thus, we believe that the consequences of PPAR- $\alpha$  stimulation on muscle insulin sensitivity and lipid metabolism should be further investigated, and apparent contradictions between effects of PPAR- $\alpha$  and PPAR- $\gamma$  agonists on insulin sensitivity resolved. The aim of our study was to compare the consequences of PPAR- $\alpha$  activation on muscle lipid accumulation and insulin action in insulin-resistant high fat-fed rats using its specific agonist WY14643 and to compare the responses with specific PPAR- $\gamma$  activation using pioglitazone. Regarding muscle lipid content, recent evidence suggests that levels of the long-chain acyl-CoAs (LCACoAs) may be more important mediators of muscle insulin resistance than triglyceride accumulation (18), and we have therefore also compared effects of the PPAR- $\alpha$  and PPAR- $\gamma$  agonists on these lipid metabolites in muscle.

## RESEARCH DESIGN AND METHODS

**Animals.** All experimental procedures were approved by the Animal Experimentation Ethics Committee (Garvan Institute) and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation. Male Wistar rats supplied from the Animal Resources Centre (Perth, Western Australia, Australia) were conditioned at  $22 \pm 0.5^\circ\text{C}$  with a 12/12-h day/night cycle (lights on 6:00 A.M.) for 1 week in communal cages during which time they were fed ad libitum a standard diet containing 69% carbohydrate, 21% protein, 5% fat, fibers, vitamin, and minerals.

After the acclimatization period, rats (~300 g) were fed a high-fat diet isocalorically (350 kJ/day given at 4:00 P.M.). The nutrient composition of the fat diet expressed as a percentage of energy was as follows: 59% fat, 21% protein, and 20% carbohydrate with quantities of fibers, vitamin, and minerals equal to those in the standard diet. Starting from the second week of the high-fat feeding, rats were administered WY14643 or pioglitazone (Eli Lilly, Indianapolis, IN) as an additive in the high-fat diet (each at  $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ) for 2 weeks. Body weight was recorded daily. No appetite-averting effect was observed for either of the compounds at the dose used.

**Experimental protocol.** Rats were divided into the following three groups: high fat-fed controls, high fat-fed treated with WY14643, and high fat-fed treated with pioglitazone. Subgroups were studied in both basal and insulin-stimulated states. A week prior to study, the left carotid artery and right jugular vein were cannulated under ketamine/xylazine (90 mg/10 mg per kg i.p.) anesthesia. The cannulae were exteriorized in the back of the neck. Rats were handled daily to minimize stress. On the study day, after a 5-h fast, the cannulae were connected to infusion apparatus (via the carotid line) and a blood-sampling syringe (via the jugular line) between 9:00 and 10:00 A.M. The sampling line was filled with sodium citrate (20.6 mmol/l) to prevent blood from clotting. During the experiment, rats were allowed free access to water. After a 50- to 60-min period of settling, two basal blood samples (0.4 ml each) were collected at -30 and 0 min in tubes containing EDTA-K (5  $\mu\text{l}$ ) to act as an anticoagulant. After a rapid centrifugation, erythrocytes were suspended in 0.25 ml sterile normal saline (0.9% NaCl) and returned to the rat. Blood and plasma glucose concentrations were measured immediately and an aliquot of plasma was frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for subsequent measurement of triglycerides, nonesterified free fatty acids (NEFAs), glycerol, insulin, and leptin concentrations.

For the basal subgroups, the rats were usually allowed to settle for ~2 h before being killed by an injection of overdose pentobarbital (~180 mg/kg). Muscles (including red and white quadriceps) were freeze-clamped with aluminum tongs precooled in liquid nitrogen. Visceral (epididymal and retroperitoneal) fat, liver, heart, and intrascapular brown adipose tissue (BAT) were weighed and frozen in liquid nitrogen. The collected tissues were kept at  $-80^\circ\text{C}$  until assay.

**Hyperinsulinemic-euglycemic clamp.** Insulin was infused at a rate of  $0.25 \text{ U} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  while euglycemia was maintained by infusing 30% glucose. During the clamp, 0.05 ml blood was taken every 10 min to measure blood glucose concentration for adjustment of the glucose infusion rate (GIR). After blood glucose levels reached steady state ( $4.5 \pm 0.1 \text{ mmol/l}$  blood glucose), a bolus of 2-deoxy-D-[2,6- $^3\text{H}$ ]glucose ( $150 \times 10^6 \text{ dpm}$ ) and D-[U- $^{14}\text{C}$ ]glucose ( $100 \times 10^6 \text{ dpm}$ ) in 0.1 ml normal saline was quickly injected via the jugular vein. Blood samples (0.2 ml) were taken at 2, 5, 10, 15, 20, 30, and 45 min after the injection to determine the tracer disappearance curve. After 45 min, the rat was killed and tissues collected as described above. Glucose disappearance rate ( $R_d$ ) and hepatic glucose output rate were calculated from the disappearance of [ $^{14}\text{C}$ ]glucose. The area under the tracer disappearance curve of 2-deoxy-D-[2,6- $^3\text{H}$ ]glucose

together with the counts of phosphorylated [ $^3\text{H}$ ]glucose from individual tissues was used to calculate insulin-stimulated glucose metabolic index ( $\text{Rg}^\text{t}$ ), an estimate of tissue glucose uptake (19). Insulin-mediated glycogen synthesis rate during the clamp was assessed by measuring [ $^{14}\text{C}$ ]glucose incorporation into glycogen (20). [ $^{14}\text{C}$ ]glucose incorporation into lipids was determined by counting [ $^{14}\text{C}$ ] in the extracted triglycerides (21). Plasma levels of lipids, insulin, and leptin during the clamp were obtained from averaged values of blood samples taken before the tracer injection and at the end of experiment.

**Metabolite measurements.** Plasma glucose was determined using a glucose analyzer (YSI 2300; Yellow Springs Instruments, Yellow Springs, OH). Plasma NEFA was determined spectrophotometrically using an acyl-CoA oxidase-based colorimetric kit (NEFA-C; Wako Pure Chemical Industries, Osaka, Japan). Both plasma triglycerides and glycerol were measured using enzymatic colorimetric methods (Triglyceride INT, procedure 336 and GPO Trinder; Sigma, St. Louis, MO). Plasma insulin and leptin were determined by radioimmunoassay using commercial kits (Linco, St. Louis, MO). Tissue triglycerides were extracted and measured by a Peridochrom Triglyceride GPO-PAP kit (Boehringer Mannheim, Germany) as previously described (22). LCACoAs were extracted from muscle and measured by high-performance liquid chromatography using a symmetry C18-reversed phase column (5  $\mu\text{m}$ ,  $3.9 \times 150 \text{ mm}$ , Waters Corporation) as described previously (20,22,23). The sum of four major species consisting of palmitoyl (16:0), stearoyl (18:0), oleoyl (18:1), and linoleoyl (18:2) is presented to reflect LCACoAs.

**Statistical analyses.** All results are presented as means  $\pm$  SE. A one-way analysis of variance followed by post-hoc (Fisher projected least squares difference [PLSD]) tests was used to assess the statistical significance between groups. All data were processed in Excel 5.0 and statistical analyses were performed using Macintosh Statview SE + Graphic program (Abacus Concepts-Brain Power).

## RESULTS

**Effects on lipid and glucose metabolism in the basal state.** Table 1 shows body weight gain, tissue weights, and basal plasma parameters in the basal state after 2 weeks of drug administration. WY14643 did not significantly alter body weight gain but reduced visceral fat by 32% and enlarged the liver by 58% compared with the high fat-fed controls, whereas pioglitazone increased body weight gain by 16%. Both drugs lowered plasma levels of glucose and leptin (-52 and -57%), respectively. Pioglitazone reduced plasma levels of triglycerides (-30%) and insulin (-67%) to a significantly greater extent than WY14643 (-16 and -39%, respectively).

The effects of WY14643 and pioglitazone on tissue triglyceride content are shown in Fig. 1. Both agonists substantially lowered content of muscle triglyceride (-34 and -28%, respectively, Fig. 1A). WY14643 had an additional action to lower liver triglycerides by -54% when expressed as micromoles per gram of liver weight (Fig. 1B). When adjusted for the increased total liver weight, the total triglyceride content in the organ was still 26% lower in WY14643-treated rats than that of high fat-fed control rats ( $231 \pm 20$  vs.  $314 \pm 17 \mu\text{mol/liver}$ ,  $P < 0.01$ ). In comparison, pioglitazone had no effect on total triglyceride content in the liver ( $308 \pm 33 \mu\text{mol/liver}$ ,  $P > 0.05$  vs. high fat-fed controls).

Consistent with the changes in muscle triglyceride content, total LCACoAs were substantially lowered in muscle of rats treated with WY14643 and pioglitazone (-41 and -42%, respectively) compared with high fat-fed control rats (Fig. 2A). In WY14643-treated rats, most of the measured major species (stearoyl 18:0, oleoyl 18:1, and linoleoyl 18:2) were significantly reduced, whereas in pioglitazone-treated rats, linoleoyl-CoA was the major species reduced (-69%,  $15.2 \pm 2.3$  vs.  $4.7 \pm 0.7 \text{ nmol/g}$  high fat-fed controls,  $P < 0.001$ ).

**Effects on lipid and glucose metabolism during the hyperinsulinemic-euglycemic clamp.** As illustrated in Table 2, both WY14643 and pioglitazone improved GIR to a similar degree (35 and 37%) with markedly increased  $R_d$

TABLE 1

Body weight gain, tissue weights, and the whole-body glucose and lipid metabolism in the basal state

|                               | High fat-fed control | WY14643 (PPAR- $\alpha$ ) | Pioglitazone (PPAR- $\gamma$ ) |
|-------------------------------|----------------------|---------------------------|--------------------------------|
| Body weight gain (g)          | 56.2 $\pm$ 2.5       | 53.7 $\pm$ 4.4            | 65.1 $\pm$ 2.8*†               |
| Visceral fat weight (g)       | 11.6 $\pm$ 0.5       | 7.9 $\pm$ 0.2‡            | 10.1 $\pm$ 0.5§                |
| Liver weight (g)              | 14.4 $\pm$ 0.3       | 22.7 $\pm$ 0.4‡           | 13.0 $\pm$ 0.5§                |
| Plasma glucose (mmol/l)       | 8.9 $\pm$ 0.2        | 8.4 $\pm$ 0.2*            | 8.1 $\pm$ 0.2*                 |
| Plasma insulin (mU/l)         | 50.6 $\pm$ 5.1       | 30.8 $\pm$ 2.7            | 16.7 $\pm$ 2.2  §              |
| Plasma triglycerides (mmol/l) | 0.79 $\pm$ 0.04      | 0.66 $\pm$ 0.04*          | 0.55 $\pm$ 0.04  †             |
| Plasma NEFA (mmol/l)          | 0.52 $\pm$ 0.04      | 0.45 $\pm$ 0.02           | 0.49 $\pm$ 0.05                |
| Plasma glycerol (mmol/l)      | 0.19 $\pm$ 0.02      | 0.14 $\pm$ 0.01*          | 0.17 $\pm$ 0.02                |
| Plasma leptin ( $\mu$ g/l)    | 6.0 $\pm$ 0.8        | 2.9 $\pm$ 0.2             | 2.6 $\pm$ 0.3                  |

Rats were fed a high-fat diet for 3 weeks with 2 weeks of drug treatment (3 mg  $\cdot$  kg<sup>-1</sup>  $\cdot$  day<sup>-1</sup>). Data from the basal and clamp subgroups were pooled together for body, visceral, and liver weights ( $n = 14$ –18/group). A combined weight of epididymal and retroperitoneal fat pads was used to represent visceral fat. Plasma parameters ( $n = 6$ –9/group) were the averaged values from two separate blood samples taken at the basal state ( $-30$  and  $0$  min) before the clamp. \* $P < 0.05$ , || $P < 0.01$ , ‡ $P < 0.001$  vs. high fat-fed controls; † $P < 0.05$ , § $P < 0.01$  vs. WY14643.

(22 and 15%, respectively, vs. high fat-fed controls). Compared with the high fat-fed control group values, plasma triglyceride levels were lower in rats treated with WY14643 ( $-21\%$ ) and even lower after pioglitazone treatment ( $-44\%$ ). Plasma leptin levels were also lower in WY14643-treated rats ( $-51\%$ ).

Figure 3 shows insulin-stimulated glucose metabolic index ( $R_g'$ ) in individual tissues. WY14643 enhanced  $R_g'$  in red (47%) and white (63%) muscles as well as in white adipose tissue (90%) ( $P < 0.05$  vs. high fat-fed control values). Compared with WY14643, the improvement of  $R_g'$  induced by pioglitazone was greater ( $P < 0.01$  vs. WY14643) in both the red and white muscles with increases of 125 and 169% above the high fat-fed control values, respectively. Pioglitazone also improved  $R_g'$  in white adipose tissue (110%). Neither agonist had any significant effect on  $R_g'$  in the heart or BAT.

Consistent with the increases in insulin-mediated  $R_g'$  in muscle and fat tissue, pioglitazone enhanced [<sup>14</sup>C]glucose

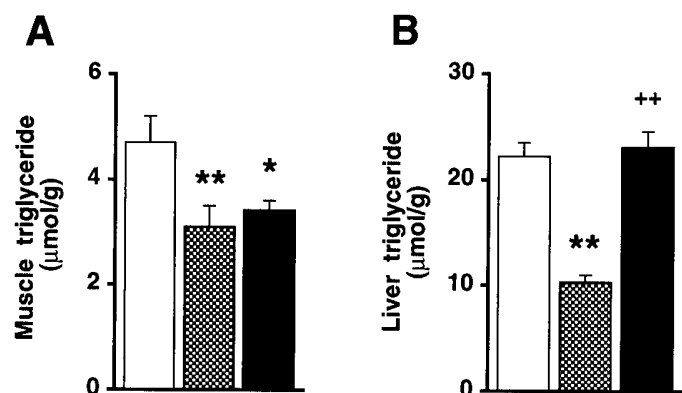


FIG. 1. Effects of WY14643 and pioglitazone on muscle (A) and liver (B) triglyceride content in the basal state. Red quadriceps muscle and the liver were taken for the measurements in the basal state after 3 weeks of the high-fat feeding with 2 weeks of drug treatment ( $n = 6$ –9/group). The tissues were freeze-clamped using tongs precooled with liquid N<sub>2</sub> immediately after the rats were killed with an overdose of pentobarbital. □, High fat-fed controls; ▨, WY14643-treated rats; ■, pioglitazone-treated rats. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the high fat-fed controls; ++ $P < 0.01$  vs. WY14643-treated group.

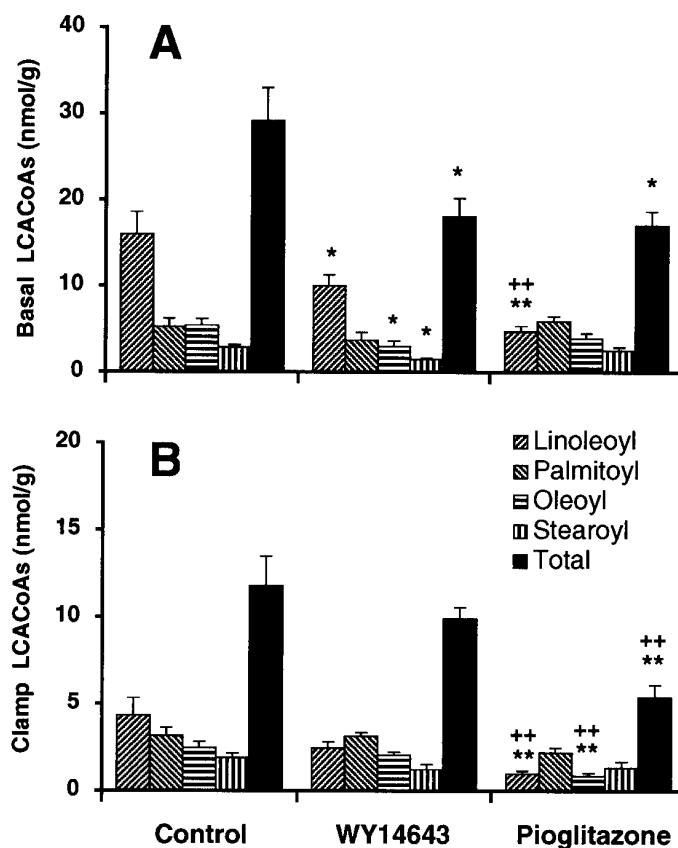


FIG. 2. Muscle LCACoA content in the basal (A) and clamp (B) states. Red quadriceps muscle was taken for the measurements after 3 weeks of the high-fat feeding with 2 weeks of drug treatment ( $n = 6$ –9/group). For the basal values, muscle was taken after 7–9 h of fasting, and for the clamp values the tissue was taken at the end of clamp. The muscle was freeze clamped using tongs precooled with liquid N<sub>2</sub> immediately after the rats were killed with an overdose of pentobarbital. Only major species of LCACoAs are presented: linoleoyl (18:2) (upward bars); palmitoyl (16:0) (downward bars); oleoyl (18:1) (horizontal bars); and stearoyl (18:0) (vertical bars). The sums of the major species are referred to as total LCACoAs (filled bars). \* $P < 0.05$ , \*\* $P < 0.01$  vs. the high fat-fed controls; ++ $P < 0.01$  vs. WY14643-treated group.

TABLE 2  
Whole-body glucose and lipid metabolism during a hyperinsulinemic-euglycemic clamp

|   | High fat-fed control | WY14643 (PPAR- $\alpha$ ) | Pioglitazone (PPAR- $\gamma$ ) |
|---|----------------------|---------------------------|--------------------------------|
| GIR (mg · kg <sup>-1</sup> · min <sup>-1</sup> )            | 15.5 ± 1.5           | 20.9 ± 0.6*               | 21.2 ± 1.2*                    |
| R <sub>d</sub> (mg · kg <sup>-1</sup> · min <sup>-1</sup> ) | 22.1 ± 1.1           | 27.0 ± 0.9*               | 25.4 ± 0.12†                   |
| HGO (mg · kg <sup>-1</sup> · min <sup>-1</sup> )            | 6.3 ± 0.9            | 5.8 ± 0.7                 | 3.6 ± 0.7†                     |
| Plasma glucose (mmol/l)                                     | 7.4 ± 0.2            | 7.6 ± 0.2                 | 7.7 ± 0.2                      |
| Plasma insulin (mU/l)                                       | 86.6 ± 7.6           | 86.8 ± 6.4                | 96.8 ± 7.8                     |
| Plasma triglyceride (mmol/l)                                | 0.52 ± 0.04          | 0.41 ± 0.03†              | 0.29 ± 0.03*‡                  |
| Plasma NEFA (mmol/l)  | 0.21 ± 0.03          | 0.23 ± 0.02               | 0.21 ± 0.04                    |
| Plasma glycerol (mmol/l)                                    | 0.17 ± 0.02          | 0.17 ± 0.02               | 0.10 ± 0.02†‡                  |
| Plasma leptin (μg/l)  | 6.4 ± 0.7            | 3.7 ± 0.4*                | 4.7 ± 0.6                      |

Insulin was infused at a rate of 0.25 U · kg<sup>-1</sup> · h<sup>-1</sup>. The GIR, R<sub>d</sub>, and hepatic glucose output rate were the steady-state values between 60 and 120 min. Plasma parameters were the averaged values from two separate blood samples (75 and 120 min). †P < 0.05, \*P < 0.01 vs. high fat-fed controls; ‡P < 0.05 vs. WY14643 (n = 7–8/group). HGO, hepatic glucose output.

incorporation into glycogen (57%) in muscle and into lipids (250%) in adipose tissue (Table 3). In the WY14643 group, increased [<sup>14</sup>C]glucose incorporation into lipids was found only in fat (106%). Although their values were similar among all groups when expressed as micromoles per gram of weight, the total [<sup>14</sup>C]glucose incorporations into glycogen and lipids in the liver were increased by 80 and 49%, respectively, above the high fat-fed control values in the WY14643 group (Table 3).

Muscle triglyceride contents were similar to respective basal levels in all postclamp groups (data not shown). However, compared with the individual basal values (Fig. 2A), muscle total LCACoAs were decreased by 59, 45, and 68% in the high fat-fed control, WY14643, and pioglitazone groups, respectively, in response to insulin stimulation (Fig. 2B, P < 0.01). Among the clamp subgroups, muscle total LCACoAs in pioglitazone-treated rats were significantly lower than those in high fat-fed controls and the most apparent reduction occurred in linoleoyl- and oleoyl-CoAs. Both circulating triglyceride levels and muscle LCACoA content were inversely correlated with muscle insulin sensitivity (Rg') (Fig. 4).

DISCUSSION

The present study demonstrates for the first time that PPAR- $\alpha$  activation with WY14643 both lowers circulatory and muscle lipids and substantially ameliorates whole-body and muscle insulin resistance in high fat-fed rats. These effects are similar to those produced by the PPAR- $\gamma$  agonist pioglitazone. Like pioglitazone, WY14643 reduces basal plasma insulin levels, showing an insulin-sensitizing action. The magnitude of the insulin-sensitizing action in muscle by both WY14643 and pioglitazone is closely correlated with a reduction of lipid accumulation, particularly muscle LCA-CoA content.

The question as to whether accumulation of muscle lipids is causally related to the development of muscle insulin resistance, and conversely whether lowering cytosolic muscle lipid accumulation leads to improved insulin action, is of importance for our study. One reason for our interest in performing the present study was that, although not directly studied, indirect evidence tended to indicate that PPAR- $\alpha$  agonists might reduce muscle lipid supply without significantly improving insulin action in insulin-resistant states (17). This would appear contradictory to muscle lipid availability as an impor-

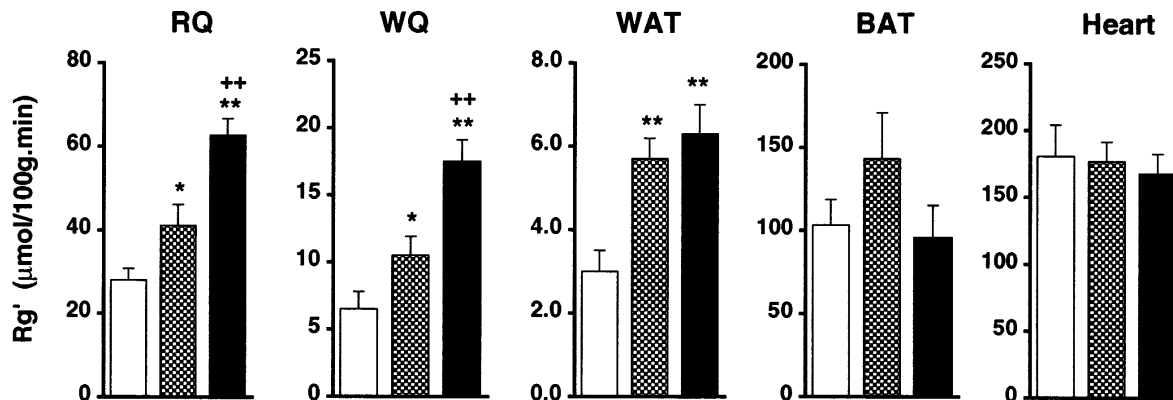


FIG. 3. Glucose metabolic index (Rg') in individual tissues in the clamp state. The hyperinsulinemic-euglycemic clamp was performed at an insulin infusion rate of 0.25 U · kg<sup>-1</sup> · h<sup>-1</sup> for 120 min (n = 7–8/group) and the tissues were freeze-clamped at the end of experiment. □, High fat-fed controls; ▨, WY14643-treated rats; ■, pioglitazone-treated rats. BAT, intrascapular brown adipose tissue; RQ, red quadriceps muscle; WAT, white adipose tissue from the retroperitoneal fat pad; WQ, white quadriceps muscle. \*P < 0.05, \*\*P < 0.01 vs. the high fat-fed controls, ++P < 0.01 vs. WY14643-treated group.

TABLE 3  
 $^{14}\text{C}$ glucose incorporation into glycogen and lipids in tissues in the hyperinsulinemic-euglycemic clamp state

|   | High fat-fed controls | WY14643 (PPAR- $\alpha$ ) | Pioglitazone (PPAR- $\gamma$ ) |
|---|-----------------------|---------------------------|--------------------------------|
| In muscle   |                       |                           |                                |
| $^{14}\text{C}$ glucose into glycogen ( $\mu\text{mol}/100\text{g} \cdot \text{min}$ )        | $5.39 \pm 0.48$       | $5.90 \pm 1.39$           | $8.49 \pm 0.99^*$              |
| $^{14}\text{C}$ glucose into lipids ( $\mu\text{mol}/100\text{g} \cdot \text{min}$ )          | $0.22 \pm 0.05$       | $0.27 \pm 0.03$           | $0.21 \pm 0.02$                |
| In white adipose tissue   |                       |                           |                                |
| $^{14}\text{C}$ glucose into lipids ( $\mu\text{mol}/100\text{g} \cdot \text{min}$ )          | $1.43 \pm 0.39$       | $3.06 \pm 0.56^*$         | $3.47 \pm 0.89^*$              |
| In liver  |                       |                           |                                |
| $^{14}\text{C}$ glucose into glycogen ( $\mu\text{mol}/100\text{g} \cdot \text{min}$ )        | $1.34 \pm 0.12$       | $1.42 \pm 0.24$           | $1.51 \pm 0.15$                |
| $^{14}\text{C}$ glucose into lipids ( $\mu\text{mol}/100\text{g} \cdot \text{min}$ )          | $1.02 \pm 0.20$       | $0.96 \pm 0.07$           | $0.77 \pm 0.17$                |
| Total $^{14}\text{C}$ glucose into glycogen ( $\mu\text{mol}/\text{liver} \cdot \text{min}$ ) | $0.20 \pm 0.02$       | $0.36 \pm 0.06^\ddagger$  | $0.20 \pm 0.01^\ddagger$       |
| Total $^{14}\text{C}$ glucose into lipids ( $\mu\text{mol}/\text{liver} \cdot \text{min}$ )   | $0.15 \pm 0.03$       | $0.22 \pm 0.02^*$         | $0.10 \pm 0.02^\ddagger$       |

The tissues were freeze clamped at the end of the hyperinsulinemic-euglycemic clamp ( $n = 7-8/\text{group}$ ). Measurements for muscle were from red quadriceps muscle, and the retroperitoneal fat was used to represent white adipose tissue.  $*P < 0.05$ ,  $^\ddagger P < 0.01$  vs. the high fat-fed controls.  $^\ddagger P < 0.05$  vs. WY14643 treatment ( $n = 7-8/\text{group}$ ).

tant causative factor modulating muscle insulin resistance, and also against a reduction of lipid supply to muscle being a principal factor in the insulin-sensitizing action of PPAR- $\gamma$  activators. However, the findings from both PPAR- $\alpha$  and PPAR- $\gamma$  activation in the present study are consistent with a lipid supply hypothesis of muscle insulin resistance. Although PPAR- $\alpha$  and PPAR- $\gamma$  agonists lower lipids by entirely different mechanisms, with PPAR- $\alpha$  mediating lipid oxidation mainly in the liver (7,9) and PPAR- $\gamma$  sequestering lipids in adipose tissue (6), both enhance muscle insulin action accompanied by a lowering of muscle lipid accumulation.

Although resolving an apparent contradiction of the lipid supply hypothesis of modulation of muscle insulin sensitivity does not prove the causality implicit in the hypothesis, our group has recently demonstrated that incubation of isolated soleus muscle with physiological concentrations of various fatty acids can significantly inhibit insulin-mediated glucose uptake and glycogen synthesis and impair insulin-mediated Akt/protein kinase B phosphorylation (24). Similar findings have also recently been reported in cultured murine C2C12 myotubes (25). Furthermore, in the isolated soleus preparation, fatty acid-induced insulin resistance was accompanied by muscle LCACoA accumulation (24). These in vitro studies support a causal link between muscle lipid accumulation and insulin resistance.

The present study provides evidence that improved muscle insulin action in vivo by a PPAR- $\alpha$  or PPAR- $\gamma$  agonist is associated with a reduction of LCACoAs. LCACoAs are the activated state of fatty acids within cells, and their intramuscular levels are closely associated with various insulin-resistant states, such as high-fat feeding (18), chronic glucose infusion (26), triglyceride emulsion/heparin infusion (2), or an acute human growth hormone infusion (23). Recently, we have found an inverse association of muscle LCACoA levels with insulin sensitivity in humans (22). Although precise mechanisms in which increased LCACoAs cause insulin resistance are still unclear, LCACoAs have been reported to inhibit the activity of hexokinase and pyruvate dehydrogenase (27) and glycogen synthesis in the liver (28), and it is possible that similar inhibitory effects of LCACoAs may also occur in muscle. In addition, they are precursors for the synthesis of diacylglycerol, an endogenous activator of protein kinase C (PKC) (29). Lipid-induced activation of some PKC subtypes may impair the insulin signaling pathway (3). Therefore, it is likely that lowered muscle LCACoA content by WY14643 and pioglitazone may improve muscle response to insulin action by alleviating these factors that cause insulin resistance.

In spite of a number of similarities, several apparent differences were found between effects of PPAR- $\alpha$  and PPAR- $\gamma$  activation. At a dosage that produced similarly increased levels of

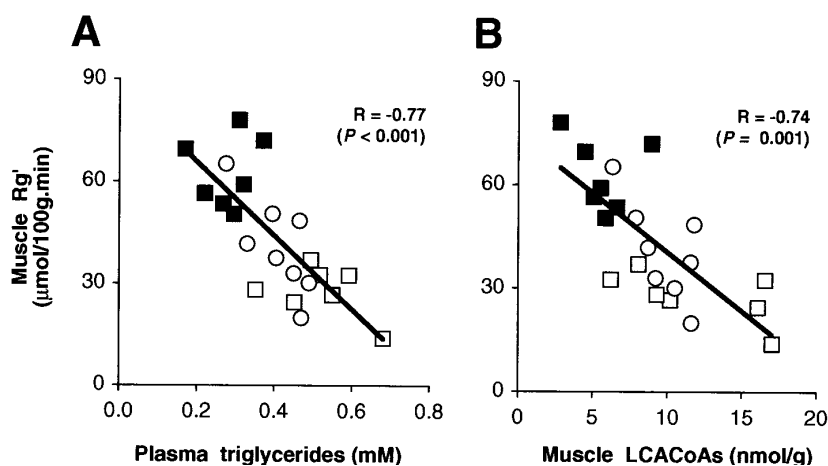


FIG. 4. Correlation of muscle  $Rg'$  with circulating triglyceride levels and local LCACoAs content in the clamp state. Data of plasma triglycerides are the averaged values from two separate blood samples in the last hour of the hyperinsulinemic-euglycemic clamp. The results for LCACoAs and  $Rg'$  were both obtained from red quadriceps muscle at the end of the hyperinsulinemic-euglycemic clamp. Results from the high fat-fed controls ( $\square$ ), WY14643 ( $\circ$ ), or pioglitazone ( $\blacksquare$ ) groups are pooled for the correlation analysis.

whole-body GIR and  $R_{di}$ , pioglitazone had a greater effect than WY14643 on enhancing muscle  $R_g'$  as well as [ $^{14}C$ ]glucose incorporation into glycogen. Although dose-response relations and muscle drug bioavailability might elucidate reasons for this, it was noteworthy that the greater muscle insulin sensitization with pioglitazone was in parallel with a greater lowering of muscle LCACoAs and circulating triglyceride levels during the clamp. Compared with WY14643, pioglitazone reduced muscle linoleoyl CoA (18:2) and oleoyl-CoA (18:1) more effectively. The most abundant LCACoA species—linoleoyl CoA—is the predominant fatty acid component in safflower oil used to make the high-fat diet. In cultured murine C2C12 myotubes (25) and the incubated rat soleus preparation (24), linoleate significantly inhibited insulin-mediated glucose uptake/phosphorylation at physiological concentrations. Therefore, a greater reduction in linoleoyl-CoA levels may be involved in the greater improvement of muscle insulin sensitivity with pioglitazone treatment.

However, we cannot exclude possible local effects of both PPAR- $\alpha$  and PPAR- $\gamma$  in muscle. There is evidence that PPAR- $\gamma$  receptor levels in muscle may be higher (8,30) than previously thought (31). The possibility of local intramuscular action of TZDs is also suggested in some (32) but not other (31) *in vitro* studies. It remains to be further investigated as to whether muscle PPAR- $\gamma$  also contributes to pioglitazone-induced improvement of insulin sensitivity or whether some TZDs have additional insulin-sensitizing action independent of PPAR- $\gamma$ -mediated responses. In comparison, PPAR- $\alpha$  is abundantly expressed in muscle (8,30). Although it is not clear whether PPAR- $\alpha$  also mediates lipid  $\beta$ -oxidation in muscle, WY14643 can increase the expression and activity of muscle pyruvate dehydrogenase kinase similarly to starvation (33). Activation of this kinase could phosphorylate and thus inactivate the pyruvate dehydrogenase complex. Therefore, it may well be that the local effects mediated by PPAR- $\alpha$  have partially offset, by substrate competition (34), an otherwise much greater improvement of muscle insulin action in response to WY14643-reduced lipid accumulation.

The apparent lesser effectiveness of WY14643 than pioglitazone in enhancing  $R_g'$  in muscle on the basis of similar GIR and  $R_{di}$  suggests that enhanced glucose uptake in tissues other than muscle and fat may also contribute to the glucose disposal. Because PPAR- $\alpha$  is highly expressed in liver (7,9), we assessed a possible hepatic contribution by determining [ $^{14}C$ ]glucose incorporation into glycogen and lipids in the liver. As expected, there were substantial increases in total [ $^{14}C$ ]glucose incorporation into both glycogen (80%) and lipids (49%) in WY14643-treated rats, suggesting that the liver may be another important site of glucose disposal induced by PPAR- $\alpha$  stimulation.

Insulin resistance is closely correlated with an excess accumulation of visceral fat (35), and lowering central fat has been a target to improve insulin action. In animals such as rats, insulin resistance is ameliorated when visceral fat is reduced by surgical removal (36), pharmacological interventions (4), or food restriction (37). WY14643 treatment substantially reduced total liver triglyceride content and visceral fat (estimated as epididymal and retroperitoneal fat stores). This may be also involved in its enhancement of whole-body insulin sensitivity.

The human PPAR- $\alpha$  shares functional characteristics with the rodent PPAR- $\alpha$ , in that the activation of PPAR- $\alpha$  leads to

reduction of circulating lipids (38). However, PPAR- $\alpha$ -induced hepatomegaly—presumably by promoting peroxisome proliferation—is thought to be species-specific for rodents (39). In fact, the fibrate class of PPAR- $\alpha$  agonists was safely used in humans to treat hypertriglyceridemia, and no hepatomegaly was reported (11). In the present study, we used WY14643 because of its high specificity as a PPAR- $\alpha$  agonist with virtually no affinity for PPAR- $\gamma$  and PPAR- $\beta$  (10,17,40). Our results are consistent with recent reports showing that other PPAR- $\alpha$  activators also improve insulin sensitivity and lower lipid availability in insulin-resistant rodent models, such as obese Zucker rats, high fat-fed mice (41), and sucrose-lard-fed rats (17). These data taken together suggest that lowering muscle lipid accumulation might be a key factor for an improvement of insulin sensitivity in humans with PPAR- $\alpha$  activators. Although still controversial, some recent clinical studies have shown improved insulin sensitivity by fibrates (12,13). The controversy over the consequence of PPAR- $\alpha$ -induced lipid lowering in humans may be resolved by using a hyperinsulinemic-euglycemic clamp technique together with muscle lipid measurement.

In summary, using the hyperinsulinemic-euglycemic clamp technique, we have found that the PPAR- $\alpha$  activation with WY14643 ameliorates insulin resistance in the high fat-fed rat along with its lipid-lowering action. The association of reduced lipids, particularly muscle LCACoAs, with the improvement of insulin sensitivity was similar for both PPAR- $\alpha$  and PPAR- $\gamma$  agonists. These results are consistent with reduced muscle lipid accumulation being central to PPAR-mediated improvement in insulin action. Because the finding of PPAR- $\alpha$  activation-reduced visceral fat mass was consistent with another recent report (41), we suggest that compounds with combined PPAR- $\alpha$  and PPAR- $\gamma$  stimulating action may have significant therapeutic potential in insulin-resistant states, with less tendency toward increased adiposity compared with PPAR- $\gamma$  activation alone.

#### ACKNOWLEDGMENTS

This study was supported by Australian National Health and Medical Research Council and a Novo Nordisk Research Grant.

We wish to thank J. Edema, L. Croft, and D. Wilks for their excellent technical assistance and the Biological Testing Facility staff for their support of animal care and maintenance.

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