



# Effects of Troglitazone and Metformin on Glucose and Lipid Metabolism

## ALTERATIONS OF TWO DISTINCT MOLECULAR PATHWAYS

James M. Lenhard,\* Steven A. Kliewer, Mark A. Paulik, Kelli D. Plunket,  
Jurgen M. Lehmann and James E. Weil

DEPARTMENT OF METABOLIC DISEASES, GLAXOWELLCOME INC., RESEARCH TRIANGLE PARK, NC 27709, U.S.A.

**ABSTRACT.** Troglitazone and metformin are antidiabetic agents that belong to the thiazolidinedione and biguanide classes of drugs, respectively. To evaluate how these drugs influence fuel utilization, we compared their effects on several pathways regulating carbohydrate and lipid metabolism *in vitro*. Both drugs stimulated glucose transport and utilization in C3H10T1/2 cells, a cell line capable of differentiating into adipocytes when treated with thiazolidinediones. However, we observed that these drugs had a number of different *in vitro* effects. Unlike metformin, troglitazone stimulated  $\beta_3$ -adrenergic receptor-mediated lipolysis, lipogenesis, and transcriptional activity of the nuclear receptor peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ). Further, by using a mitochondrial-specific fluorescent dye, we found troglitazone to be more effective than metformin at increasing mitochondrial mass. In contrast to troglitazone, metformin was more effective at increasing mitochondrial fatty acid  $\beta$ -oxidation, peroxisomal fatty acid  $\beta$ -oxidation, and anaerobic respiration (i.e. lactate production). Additionally, metformin stimulated and troglitazone inhibited both aerobic respiration and basal lipolysis. Insulin enhanced the effects of troglitazone, but not those of metformin, on these cells. Taken together, the data show that troglitazone and metformin affect two distinct metabolic pathways: one that is anabolic (i.e. troglitazone) and the other that is catabolic (i.e. metformin). Further, these observations suggest that the metabolic activity of mitochondria may be lower in cells treated with troglitazone than with metformin. *BIOCHEM PHARMACOL* 54;7:801–808, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** troglitazone; metformin; diabetes; metabolism; PPAR $\gamma$ ; insulin

The thiazolidinediones, including troglitazone (CS045), are a new class of oral antidiabetic agents that markedly improve insulin action, hyperlipidemia, and glucose utilization in diabetic patients [1–3]. Recently, the thiazolidinediones were identified as high affinity ligands for PPAR $\gamma$ †, a transcription factor, found in preadipocytes, that is activated by a variety of long chain fatty acids, eicosanoids, and non-steroidal anti-inflammatory drugs [4–7]. Several groups have identified PPAR-binding sites in the regulatory regions of various genes involved in lipid and carbohydrate metabolism [8, 9]. Moreover, treatment of preadipocytes (e.g. C3H10T1/2 cells) with thiazolidinediones [4, 10, 11] or forced expression of PPAR $\gamma$  in fibroblasts enhances adipogenesis [12]. Taken together, these observations indicate that the antidiabetic action of thiazolidinediones may include PPAR $\gamma$ -mediated transcription of genes involved in lipid and carbohydrate metabolism. Although several studies suggest that thiazolidinediones

increase glucose transport and lipogenesis by affecting gene expression, little information is available on the action of thiazolidinediones on aerobic or anaerobic respiration, peroxisomal or mitochondrial fatty acid oxidation, and basal or catecholamine-mediated lipolysis.

Metformin, which belongs to the biguanide class of oral antidiabetic agents, has effects similar to those of troglitazone in diabetic patients [13, 14]. Unlike the sulfonylureas, which stimulate insulin secretion, treatment with metformin is associated with reduced plasma insulin and glucose, indicating that biguanides attenuate insulin resistance. Various biological activities for the biguanides have been reported which may explain in part, their anti-hyperglycemic effect. These include: (1) reduced glucose absorption from the gut, (2) suppressed gluconeogenesis, (3) increased anorexia, (4) enhanced insulin-binding to its receptor, and (5) enhanced glucose transport in fat [15–17] and muscle [18, 19]. Moreover, treatment of type 2 diabetes with metformin is associated with an overall reduction in plasma free fatty acids and body weight. Although this suggests that metformin alters lipid metabolism, its effects on peroxisomal and mitochondrial fatty acid oxidation or basal and adrenergic-mediated lipolysis remain unclear. Similarly, the molecular mechanisms involved in the anti-

\* Corresponding author: J. M. Lenhard, Ph.D., Department of Metabolic Diseases, GlaxoWellcome Inc., 5 Moore Drive, Research Triangle Park, NC 27709. Tel. (919) 483-3022; FAX (919) 483-3731; E-mail: jml29514@glaxowellcome.com

† Abbreviations: DMEM, Dulbecco's modified Eagle's medium high glucose; and PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ .

Received 20 February 1997; accepted 24 April 1997.

hyperglycemic activity of biguanides are far from being certain.

As both thiazolidinediones and biguanides have been reported to affect glucose metabolism in various isolated cell types (e.g. hepatocytes, myoblasts, and adipocytes) and diabetic patients, we wanted to determine if troglitazone and metformin shared similar modes of action. A direct comparison on the metabolic pathways affected by both drugs *in vitro* has not been reported. Likewise, an in-depth analysis of how troglitazone affects fuel metabolism *in vitro* has not been reported. Thus, in the present study we performed a detailed comparison of how troglitazone and metformin affect the multiple pathways regulating metabolism *in vitro*.

## MATERIALS AND METHODS

### Lipogenesis and Glucose Transport

C3H10T1/2 mesenchymal stem cells were grown in DMEM containing 10% fetal bovine serum. The initial concentration of insulin in the medium was 100 pM. Various concentrations of either troglitazone, metformin, and/or insulin were added to the cells 1 day after the cells reached confluence. [<sup>3</sup>H]Glucose (0.5  $\mu\text{Ci}/\text{cm}^2$ ) was added to the cells after 3 days in culture. On day 7, medium was removed, cells were extracted with Econofluor-2 (Packard Inc., Meriden, CT), and radioactivity in the organic phase was determined as a measure of lipogenesis. For measurements of glucose transport activity, [<sup>3</sup>H]2-deoxyglucose (0.5  $\mu\text{Ci}/\text{cm}^2$ ) was added to the cells after 7 days in culture with troglitazone, metformin, and/or 200 nM insulin. The cells were incubated for 30 min at 37° and then were washed three times with DMEM; cellular radioactivity was determined by liquid scintillation counting.

### Lipolysis

Basal lipolysis was determined by incubating the cells in the presence of various concentrations of troglitazone or metformin for 7 days. Accumulation of glycerol in the medium was measured using the Sigma Diagnostic Glycerol-Triglyceride kit (i.e. Trinder reagent 337-B; Sigma, St. Louis, MO).  $\beta_3$ -Adrenoreceptor agonist-stimulated lipolysis was determined by adding fresh DMEM (Gibco, Grand Island, NY) containing 1% BSA to the cells, incubating the cells for 5 hr at 37° in the presence of 100 nM CL316243, and measuring glycerol accumulation in the medium.

### Oxidation of Glucose and Palmitoyl-CoA

The oxidation of glucose and [1-<sup>14</sup>C]palmitoyl-CoA into <sup>14</sup>C-labeled acid-soluble product was performed as described [20, 21]. Mitochondrial and peroxisomal  $\beta$ -oxidations were measured following published methods [22].

### Glucose, Lactate, and pH Determinations

After incubating the cells for 7 days in the presence of drug, lactate and glucose remaining in the medium were measured using a model 2700 Biochemistry Analyzer (YSI Inc., Yellow Springs, OH). The pH was measured using an ABL520 Radiometer (Radiometer American, Inc., Westlake, OH).

### Mitochondrial Staining

C3H10T1/2 cells were incubated for 7 days with various concentrations of metformin or troglitazone in DMEM containing 10% fetal bovine serum. Although the uptake of many mitochondrion-selective dyes is dependent on mitochondrial membrane potential, MitoTracker Green FM is a notable exception enabling the detection of mitochondrial mass [23]. The cells were stained for 30 min at 37° with 0.5  $\mu\text{M}$  MitoTracker Green FM and prepared for fluorescence according to the manufacturer's specifications (Molecular Probes, Inc., Eugene, OR). Mitochondrial mass was determined by measuring cellular fluorescence using a Cytofluor 2350 spectrofluorometer with the filters set at 450 nm excitation and 530 nm emission. The bandwidths were 50 and 25 nm for the excitation and emission filters, respectively. Fluorescence due to accumulation of MitoTracker Green FM was calculated by subtracting cellular autofluorescence from each data point.

### PPAR $\gamma$ Ligand-Binding and Co-Transfection Assays

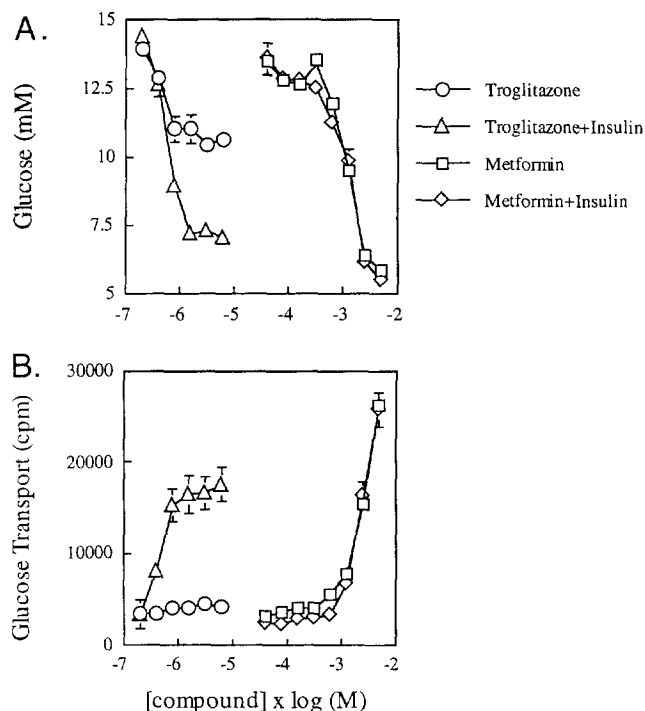
The PPAR $\gamma$  ligand-binding domain (amino acids 174–475) was prepared as described [4]. For competition experiments, recombinant PPAR $\gamma$  was incubated with 40 nM [<sup>3</sup>H]BRL49653 in the presence or absence of troglitazone or metformin for 3 hr at 4°. Bound and free radioactivity were separated by elution through 1-mL Sephadex G-25 columns, and bound radioactivity was measured by liquid scintillation counting. The transient co-transfection assays were performed as described [4]. In this assay, the ligand-binding domain of PPAR $\gamma$  was linked to the DNA-binding domain of the transcription factor GAL4. Expression plasmids for the PPAR $\gamma$ –GAL4 chimera and secretory-placental alkaline phosphatase reporter containing five GAL4-binding sites were co-transfected into CV-1 cells [4].

Each figure presents representative data for experiments performed on three separate occasions. The mean  $\pm$  SD of three data points was determined for each experiment.

## RESULTS

### Troglitazone and Metformin Effects on Glucose Consumption and Transport Activity In Vitro

We have reported previously that the *in vivo* antidiabetic properties of thiazolidinediones with varying potencies correlate ( $r > 0.9$ ) with their ability to stimulate differentiation of C3H10T1/2 cells into adipocytes [24]. Thus,

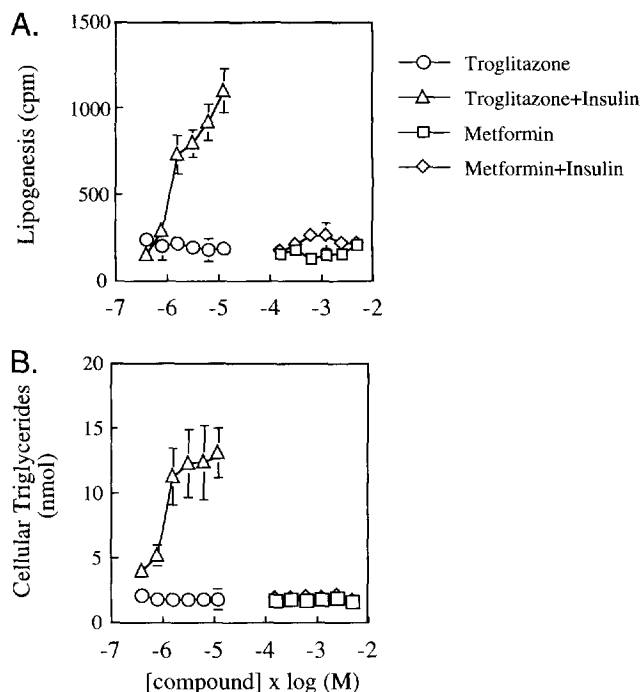


**FIG. 1.** Effects of troglitazone and metformin on glucose consumption and transport. C3H10T1/2 cells ( $10^4$  cells/cm<sup>2</sup>) were cultured in 96-well plates. The cells were incubated with increasing concentrations of troglitazone or metformin in the presence or absence of insulin (200 nM). The amount of glucose remaining in the incubation medium was measured after incubating the cells for 7 days at 37° (A). Glucose transport activity was determined by measuring the incorporation of [<sup>3</sup>H]2-deoxyglucose into the cells (B). Representative data of experiments performed in triplicate are given. The mean  $\pm$  SD from three replicates for each data point is given.

these cells were chosen for this investigation. To compare directly the effects of troglitazone and metformin *in vitro*, we studied their effects on glucose utilization and glucose transport activity in C3H10T1/2 cells grown in the presence or absence of insulin. Exposure of these cells to either compound for 7 days stimulated both glucose utilization (Fig. 1A) and transport (Fig. 1B) in a concentration-dependent manner in the presence of insulin. It was found that insulin enhanced the effects of troglitazone, but not metformin, on glucose utilization and transport. In the presence of  $2 \times 10^{-7}$  M insulin, the  $EC_{50}$  values were  $10^{-6}$  M for troglitazone and  $10^{-3}$  M for metformin, respectively. In the presence of  $10^{-5}$  M troglitazone, the  $EC_{50}$  value for insulin was  $2 \times 10^{-9}$  M. Insulin alone had no effect on these cells.

#### Troglitazone and Metformin Effects on Lipogenesis and Glucose Oxidation

Although troglitazone and metformin stimulated glucose transport and utilization, glucose metabolism involves the coordinated action of several metabolic pathways within the cell (e.g. aerobic respiration, anaerobic respiration, and



**FIG. 2.** Effects of troglitazone and metformin on lipogenesis and accumulation of cellular triglycerides. Cells were incubated with increasing concentrations of troglitazone or metformin in the presence or absence of insulin (200 nM). Incorporation of [<sup>3</sup>H]glucose into lipid (lipogenesis) (A) and total cellular triglycerides (B) were measured after 7 days of incubation with each drug. Representative data of experiments performed in triplicate are given. Plots are the means  $\pm$  SD from three replicates for each data point.

lipogenesis). Thus, we wanted to determine if there was a difference in the fate of glucose consumed by cells treated with troglitazone, metformin, and/or insulin. First, we measured how these agents affected the conversion of [<sup>3</sup>H]glucose into cellular lipids (i.e. lipogenesis). As shown in Fig. 2A, troglitazone enhanced lipogenesis, and this effect was dependent upon the addition of insulin. Metformin had no effect on lipogenesis in the presence or absence of insulin. Consistent with these observations, we found that troglitazone, but not metformin, enhanced insulin-dependent accumulation of total triglycerides within the cell (Fig. 2B).

Little information exists on how thiazolidinediones affect aerobic respiration. Thus, we measured how both anti-hyperglycemic agents affected the conversion of [<sup>14</sup>C]glucose into <sup>14</sup>CO<sub>2</sub> (i.e. aerobic respiration). In contrast to the effects on lipogenesis, troglitazone inhibited and metformin stimulated aerobic respiration in the presence of insulin (Fig. 3). Moreover, we found that troglitazone required insulin in order to inhibit aerobic respiration.

#### Troglitazone and Metformin Effects on Extracellular Lactate (Anaerobic Respiration) and pH

Because biguanides are associated with a well-recognized risk of lactate acidosis [reviewed in Ref. 13] and there are no

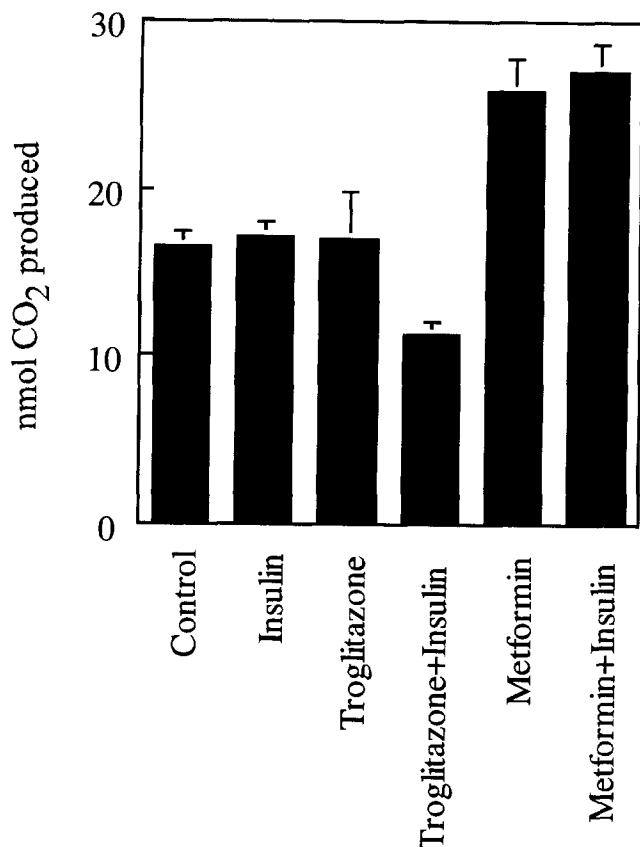


FIG. 3. Effects of troglitazone and metformin on aerobic respiration. C3H10T1/2 cells were incubated with increasing concentrations of troglitazone or metformin in the presence or absence of insulin (200 nM). Incorporation of [<sup>14</sup>C]glucose into CO<sub>2</sub> (aerobic respiration) was measured after 7 days of incubation with each drug. Representative data of experiments performed in triplicate are given. Each data point is the mean  $\pm$  SD from three replicates.

reports on how troglitazone may affect anaerobic respiration, we compared the effects of troglitazone and metformin on lactate formation (i.e. anaerobic respiration) and pH in the extracellular medium. When tested at the concentrations that gave maximal stimulation of glucose consumption (see Fig. 1A), metformin was nearly twice as effective as troglitazone (in the presence of insulin) at increasing extracellular lactate (Fig. 4A) and decreasing the extracellular pH (Fig. 4B). Insulin increased the effects of troglitazone, but not metformin, on lactate production and acidification. Taken together, these observations indicate that metformin has a more pronounced effect on lactate acidosis (and anaerobic respiration) than troglitazone plus insulin *in vitro*.

#### Analysis of Lipolysis and Palmitoyl-CoA Oxidation in Cells Treated with Troglitazone or Metformin

Lipid catabolism involves triglyceride hydrolysis to form glycerol and free fatty acids (lipolysis). The fatty acids are catabolized further to form acetyl-SCoA and CO<sub>2</sub> ( $\beta$ -oxidation). As we were interested in determining how

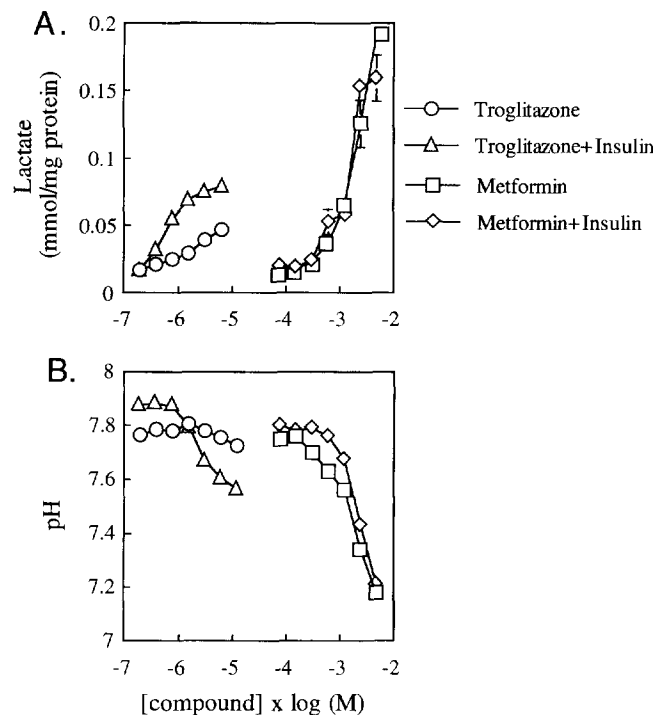
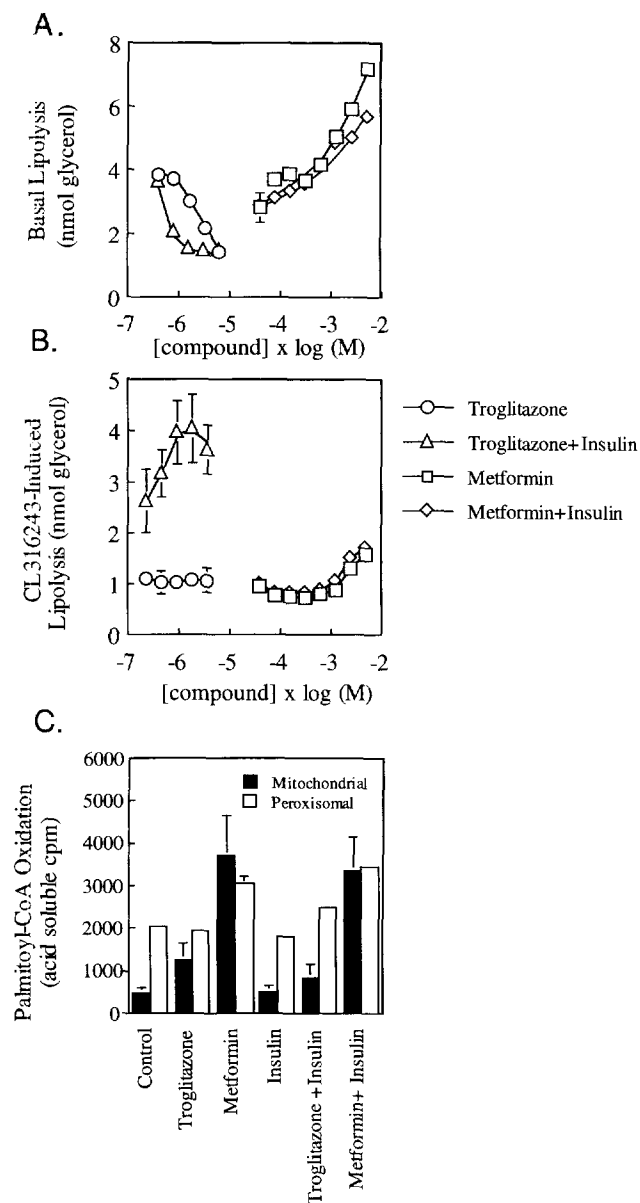


FIG. 4. Effects of troglitazone and metformin on extracellular lactate and pH. The change in anaerobic respiration (lactate production) was measured after treating the cells for 7 days with increasing concentrations of troglitazone or metformin in the presence or absence of insulin (A). Extracellular pH (B) was measured after 7 days to determine the extent of drug-induced acidosis. Representative data of experiments performed in triplicate are given. Plots are the means  $\pm$  SD from three replicates for each data point.

troglitazone and metformin alter lipid catabolism, we measured their effects on lipolysis and  $\beta$ -oxidation *in vitro*.

To determine the effects of troglitazone and metformin on basal lipolysis, we measured the accumulation of glycerol in the extracellular medium. After treating the cells for 7 days, metformin stimulated and troglitazone inhibited basal lipolysis in a concentration-dependent manner (Fig. 5A). Lipolysis can also be stimulated by activation of  $\beta$ -adrenergic receptors [25], although it is not clear how biguanides or thiazolidinediones may affect this process. To determine if thiazolidinediones or biguanides affect  $\beta$ -adrenergic-mediated lipolysis, we treated the cells with troglitazone or metformin for 7 days and re-treated the cells with fresh medium containing the  $\beta_3$  agonist, CL316243 [25]. CL316243-mediated lipolysis was stimulated only in the cells pretreated with troglitazone and insulin (Fig. 5B).

Since the effects of metformin and troglitazone on mitochondrial and peroxisomal  $\beta$ -oxidation were unknown, we measured the effects of these drugs on the conversion of [<sup>14</sup>C]-palmitoyl-CoA into <sup>14</sup>C-labeled acid-soluble products (e.g. acetyl-SCoA) in the presence and absence of potassium cyanide (an inhibitor of mitochondrial  $\beta$ -oxidation but not peroxisomal  $\beta$ -oxidation). As shown in Fig. 5C, metformin was more effective than



**FIG. 5.** Effects of troglitazone and metformin on basal lipolysis and lipid oxidation. The cells were treated as described in the legend to Fig. 1. The effects of metformin and troglitazone on basal lipolysis were determined by measuring the accumulation of glycerol in the medium after treating the cells for 7 days with either compound (A). The effects of metformin and troglitazone on  $\beta_3$ -mediated lipolysis were determined after treating the cells for 7 days with either compound. Subsequently, the cells were incubated for 5 hr with 100 nM CL316243, and the glycerol in the medium was measured as described in Materials and Methods (B). Panel C shows the effects of each drug treatment on mitochondrial and peroxisomal fatty acid oxidation (i.e. the generation of acid-soluble products derived from [1- $^{14}$ C]palmitoyl-CoA). Representative data of experiments performed in triplicate are given. The mean  $\pm$  SD from three replicates for each data point is shown.

troglitazone at stimulating both peroxisomal and mitochondrial fatty acid oxidation. Additionally, metformin was found to be more effective at stimulating mitochondrial  $\beta$ -oxidation than peroxisomal  $\beta$ -oxidation (Fig. 5C).

### Effects of Troglitazone and Metformin on Mitochondrial Mass

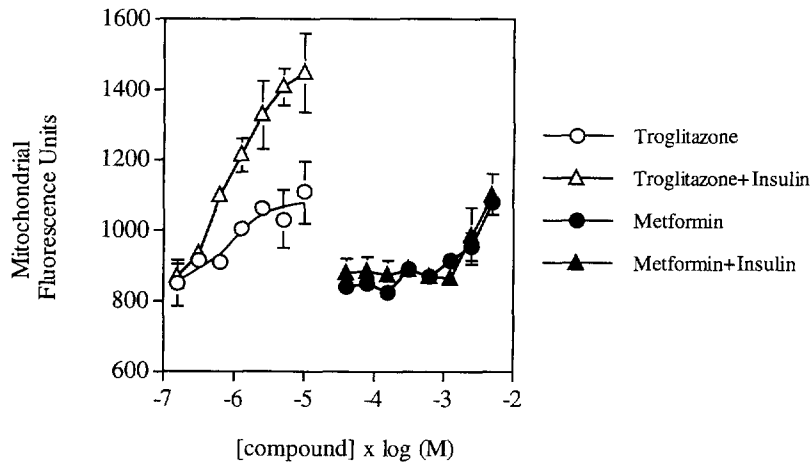
Mitochondria are the primary site for oxidative metabolism within the cell. Thus, we studied the effects of troglitazone and metformin on mitochondrial mass within C3H10T1/2 cells. Cells were pretreated for 7 days with either anti-hyperglycemic agent, stained using MitoTracker Green FM, and mitochondrial accumulation of the dye was determined using a spectrofluorometer. Figure 6 shows that troglitazone was more effective than metformin at increasing mitochondrial mass within these cells. Further, insulin enhanced the effects of troglitazone on mitochondrial mass. These observations were confirmed by fluorescence microscopy (data not shown).

### Troglitazone and Metformin Effects on Binding and Activation of PPAR $\gamma$

While some thiazolidinediones (e.g. BRL49653 and pioglitazone) bind and activate the adipocyte nuclear receptor PPAR $\gamma$  [4], binding of metformin or troglitazone to PPAR $\gamma$  has not been shown. Thus, we tested if troglitazone or metformin could displace binding of the radiolabeled thiazolidinedione [ $^3$ H]BRL49653 to the ligand-binding domain of PPAR $\gamma$ . As shown in Fig. 7A, troglitazone inhibited binding of [ $^3$ H]BRL49653 to recombinant PPAR $\gamma$  ( $K_i = 2 \mu\text{M}$ ), while metformin had no effect. As part of the effort to determine if troglitazone and metformin are PPAR $\gamma$  ligands, these compounds were also tested in an established transient transfection assay for activation of PPAR $\gamma$  [4, 5]. As shown in Fig. 7B, troglitazone activated ( $EC_{50} = 0.4 \mu\text{M}$ ) whereas metformin had no effect on PPAR $\gamma$ .

## DISCUSSION

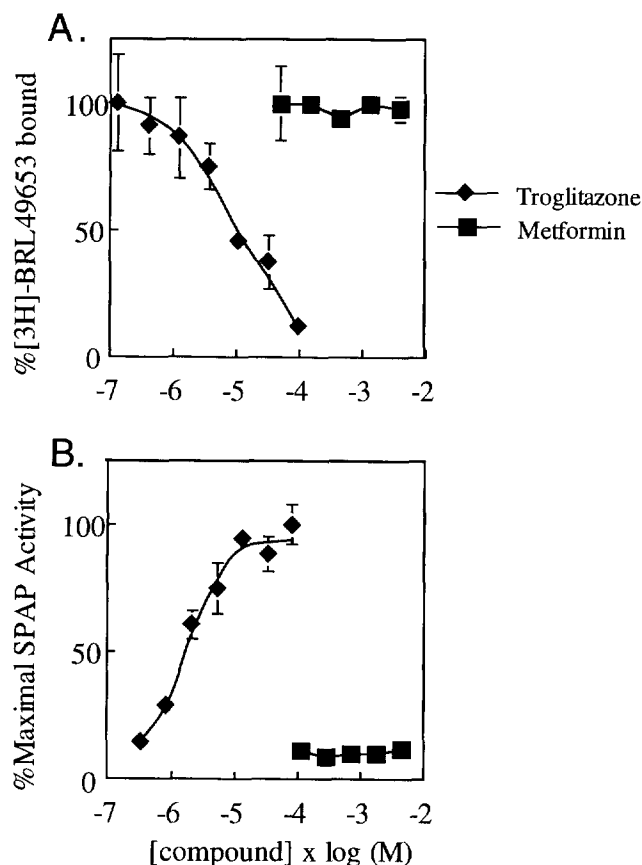
As diabetes covers a spectrum of clinical and metabolic abnormalities linked to multiple cellular defects, it is likely that various pharmacological approaches will be required for the treatment of this disease. It is therefore necessary to understand the underlying mechanisms by which different therapies (e.g. thiazolidinediones or biguanides) improve glycemic control. While troglitazone and metformin are known to enhance glucose disposal *in vivo* and *in vitro*, it was unclear whether similar molecular mechanisms mediated these effects. In this report, we compared the effects of metformin and troglitazone on glucose and lipid metabolism in C3H10T1/2 preadipocytes and evaluated their capacity to bind to the nuclear receptor PPAR $\gamma$ . The results, which are summarized in Table 1, demonstrated clearly that thiazolidinediones and biguanides differ in their modes of action. In sum, metformin promoted catabolism (i.e. it stimulated aerobic respiration, basal lipolysis, and fatty acid oxidation), whereas troglitazone promoted anabolism in these cells (i.e. it increased lipogenesis and mitochondrial mass while inhibiting aerobic respiration and basal lipolysis in the presence of insulin). The effects of



**FIG. 6.** Effects of troglitazone and metformin on mitochondrial mass. Changes in mitochondrial mass were measured after treating cells for 7 days with troglitazone or metformin in the presence or absence of insulin. Cells were stained with Mito-Tracker Green FM, and cellular fluorescence was measured as described in Materials and Methods. Representative data of experiments performed in triplicate are given. Plots are the means  $\pm$  SD from three replicates for each data point.

troglitazone, but not metformin, may be attributed to activation of PPAR $\gamma$ -mediated gene transcription and adipogenesis.

While others have shown that insulin increases the



**FIG. 7.** Effects of troglitazone and metformin on binding and activation of PPAR $\gamma$ . Competition-binding experiments were performed using a histidine-tagged PPAR $\gamma$  ligand-binding domain and 40 nM [ $^3$ H]BRL49653 in the presence of vehicle or increasing concentrations of troglitazone or metformin (A). CV-1 cells were co-transfected with an expression plasmid containing PPAR $\gamma$ -GAL4 and a reporter plasmid containing secretory placental alkaline phosphatase (SPAP) (B). Representative data of experiments performed in triplicate are given. The mean  $\pm$  SD from three replicates for each data point is given.

effects of metformin in adipocytes, hepatocytes, and myoblasts *in vitro* [4–9], our results indicate that metformin did not require insulin to stimulate glucose and lipid metabolism in C3H10T1/2 preadipocytes. These observations suggest that two pathways mediate the effects of metformin: an insulin-regulated pathway present in fat, liver, and muscle, and an insulin-independent pathway present in stem cells. Consistent with this hypothesis, treatment of type II diabetic patients with metformin improves glycemic control both in the basal and insulin-stimulated states [26]. In contrast to metformin, troglitazone required insulin as a cofactor for maximal stimulation of glucose and lipid metabolism in C3H10T1/2 cells. In streptozocin-treated mice, an insulin-deficient diabetic animal model, troglitazone is ineffective at lowering glucose [27]. These data support the postulate that the antidiabetic effect of troglitazone is attributed to enhancement of insulin action. Similarly, the antidiabetic effect of insulin may be attributed to enhancement of thiazolidinedione (i.e. PPAR $\gamma$ ) action.

Treatment of type II diabetic patients with metformin results in decreased plasma triglycerides, free fatty acids, and body weight [28, 29]. Our *in vitro* data indicate the effect of metformin on serum lipids and weight loss may involve increased basal lipolysis and fatty acid  $\beta$ -oxidation. The unique observation that metformin stimulated both mitochondrial and peroxisomal fatty acid  $\beta$ -oxidation indicates that biguanides do not selectively affect either pathway of fatty acid catabolism. This is in contrast to other hypolipidemic drugs (e.g. fibrates) which appear to selectively increase peroxisomal  $\beta$ -oxidation. How might troglitazone treatment lower serum lipids *in vivo* [2, 3, 27]? One possibility is that troglitazone increases lipid accumulation in preadipocytes by increasing activation of gene transcription by PPAR $\gamma$  and adipogenesis. An alternate explanation is that increased cellular responsiveness to  $\beta$ -agonists (i.e. catecholamines) may clear the body of excess fats via increased lipolysis, mitochondrial  $\beta$ -oxidation, and heat production [25, 30]. The data suggesting that troglitazone increases CL316243-induced lipolysis and mitochondrial biogenesis *in vitro* support the latter hypothesis. This raises

TABLE 1. Summary of the *in vitro* effects of troglitazone and metformin

Assay	Troglitazone	Metformin
Glucose utilization	+	+
Glucose transport	+	+
Insulin requirement	+	○
Lipogenesis	+	○
Total cellular triglycerides	+	○
Aerobic respiration	-	+
Lactate (anaerobic respiration)	+	++
Acidosis	+	++
Basal lipolysis	-	+
$\beta_3$ -Adrenergic-mediated lipolysis	+	○
Peroxisomal $\beta$ -oxidation	○	+
Mitochondrial $\beta$ -oxidation	○	+
Mitochondrial mass	++	+
PPAR $\gamma$ binding	+	○
PPAR $\gamma$ activation	+	○

Stimulation of a given activity is represented by (+), inhibition is represented by (-), and no significant effect is represented by (○). If both agents stimulated an activity, the agent with greater efficacy was assigned a (++). These data are summarized from at least three independent experiments.

the intriguing possibility that PPAR $\gamma$  may regulate  $\beta_3$ -adrenoreceptor expression and mitochondrial abundance *in vivo*.

Several observations indicate that mitochondrial metabolism was greater in metformin-treated cells than in troglitazone-treated cells. First, aerobic respiration, which occurs predominately in mitochondria, was stimulated by metformin and inhibited by troglitazone. Second, metformin was three times more effective than troglitazone at increasing mitochondrial  $\beta$ -oxidation. In contrast, troglitazone was twice as effective as metformin at increasing mitochondrial mass. Thus, when mitochondrial fatty acid  $\beta$ -oxidation is corrected for mitochondrial mass, metformin is six times more effective than troglitazone at increasing mitochondrial  $\beta$ -oxidation. These observations suggest that changes in mitochondrial mass do not correlate with changes in mitochondrial activity (i.e. aerobic respiration) as a result of drug treatment. Although it is unclear if the effects of metformin and troglitazone on mitochondrial mass represent a change in size, shape, or number of mitochondria, it is clear that these drugs have different effects on fuel metabolism within the mitochondria.

As indicated here and in previous work [4, 5, 10, 11, 30], stimulation of adipocyte differentiation with thiazolidinediones is linked to increased insulin sensitivity, glucose transport, and lipid accumulation within the cell. Thus, the novel effects that we report for troglitazone on fatty acid oxidation, lipolysis, respiration, and mitochondrial mass in C3H10T1/2 cells may also be attributed to increased adipogenesis. While troglitazone appears to enhance adipogenesis *in vitro*, published clinical studies indicate that troglitazone does not cause weight gain [2]. However, recent data indicate that thiazolidinediones increase the mass of intrascapular brown adipose tissue in rats [31]. Moreover, *in vitro* thiazolidinediones induce differentiation of C3H10T1/2 cells into brown adipocytes [30]. Indeed, the adipogenic effects of thiazolidinediones in

C3H10T1/2 cells correlate with their anti-hyperglycemic effects in diabetic rodents [24]. This raises the interesting possibility that troglitazone may improve glucose utilization without changing body weight by increasing brown adipose tissue function (i.e. thermogenesis).

It should be noted that the effective concentration of troglitazone observed in this study is similar to its therapeutic concentration in serum [35]. Likewise, the effective concentration of metformin in this study is similar to its therapeutic concentration in the intestine [32], although it is much greater than that found in the serum [13, 14]. Whereas the use of C3H10T1/2 cells provides detailed comparative information on the mode of action of troglitazone and metformin, it should be noted that biguanides and thiazolidinediones may affect other cell types [10, 33–35]. Thus, the differential effects of either drug *in vivo* may be due to different tissue sites of action (e.g. preadipocytes, fat, intestine, and/or muscle) as well as distinct molecular pathways within the same cell.

Although metformin increased free fatty acid oxidation in C3H10T1/2 cells, *in vivo* metformin decreases free fatty acid oxidation [36]. One possibility for this discrepancy is that metformin may have distinct metabolic effects depending on the tissue type. Consistent with this, metformin increases glucose oxidation in muscle [18, 19] while inhibiting glucose oxidation in the splanchnic bed [32]. Additionally, *in vivo* metformin is likely to affect various endocrine systems that regulate whole body homeostasis. These endocrine systems may not be represented accurately in the cell culture conditions used in this study. Nonetheless, the results indicate that metformin and troglitazone act in different ways at the cellular level.

In summary, troglitazone and metformin altered glucose and lipid metabolism within C3H10T1/2 cells through distinct molecular mechanisms. This raises the intriguing possibility that in combination these antidiabetic agents may be more efficacious than either drug alone. Clearly,

future research is needed to more fully understand the molecular mechanisms underlying the antidiabetic activity of these agents and whether these drugs could be more effective if used in combination.

## References

- DeFronzo RA, Bonadonna RC and Ferrannini E, Pathogenesis of NIDDM. *Diabetes Care* **15**: 317–368, 1992.
- Kumar S, Boulton AJM, Back-Nielsen H, Berthezene F, Muggeo M, Persson B, Spinass GA, Donoghue S, Lettis S and Stewart-Long P, Troglitazone, an insulin action enhancer, improves metabolic control in NIDDM patients. *Diabetologia* **39**: 701–709, 1996.
- Kuzuya T, Iwamoto Y, Kosaka K, Takebe K, Yamanouchi T, Kasuga M, Kajinuma H, Akanuma Y, Yoshida S and Shigeta Y, A pilot clinical trial of a new oral hypoglycemic agent, CS-045, in patients with non-insulin dependent diabetes mellitus. *Diabetes Res Clin Pract* **11**: 147–153, 1991.
- Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM and Kliewer SA, An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ). *J Biol Chem* **270**: 12953–12956, 1995.
- Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC and Lehmann JM, A prostaglandin  $J_2$  metabolite binds peroxisome proliferator-activated receptor  $\gamma$  and promotes adipocyte differentiation. *Cell* **83**: 813–819, 1995.
- Forman B, Tontonoz P, Chen J, Brun R, Spiegelman B and Evans R, 15-Deoxy- $\Delta^{12,14}$ -prostaglandin  $J_2$  is a ligand for the adipocyte determination factor PPAR $\gamma$ . *Cell* **83**: 803–812, 1995.
- Lehmann JM, Lenhard JM, Oliver BB, Ringold GM and Kliewer SA, Peroxisome proliferator-activated receptors  $\alpha$  and  $\gamma$  are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem* **272**: 3406–3410, 1997.
- Castelein H, Gulick T, Declercq PE, Mannaerts GP, Moore DD and Baes MI, The peroxisome proliferator activated receptor regulates malic enzyme gene expression. *J Biol Chem* **269**: 26754–26758, 1994.
- Tontonoz P, Hu E, Devine J, Beale EG and Spiegelman BM, PPAR $\gamma$ 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol Cell Biol* **15**: 351–357, 1995.
- Hiragun A, Sato M and Mitsui H, Preadipocyte differentiation *in vitro*: Identification of a highly active adipogenic agent. *J Cell Physiol* **134**: 124–130, 1988.
- Kletzien RF, Clarke SD and Ulrich RG, Enhancement of adipocyte differentiation by an insulin-sensitizing agent. *Mol Pharmacol* **41**: 393–398, 1992.
- Tontonoz P, Hu E and Spiegelman B, Stimulation of adipogenesis in fibroblasts by PPAR $\gamma$ 2, a lipid-activated transcription factor. *Cell* **79**: 1147–1156, 1994.
- Bailey CJ, Biguanides and NIDDM. *Diabetes Care* **15**: 755–772, 1992.
- Wiernsperger N and Rapin JR, Metformin–insulin interactions: From organ to cell. *Diabetes Metab Rev* **11**: S3–S12, 1995.
- Cigolini M, Bosello O, Zancanaro C, Orlandi PG, Fezzi O and Smith U, Influence of metformin on metabolic effect of insulin in human adipose tissue *in vitro*. *Diabetes Metab* **10**: 311–315, 1984.
- Matthaei S, Hamann A, Klein H, Benecke H, Kreyman G, Flier J and Greten H, Association of metformin's effect to increase insulin-stimulated glucose transport with potentiation of insulin-induced translocation of glucose transporters from intracellular pool to the plasma membrane in rat adipocytes. *Diabetes* **40**: 850–856, 1991.
- Kozka IJ and Holman GD, Metformin blocks downregulation of cell surface GLUT4 caused by chronic insulin treatment of rat adipocytes. *Diabetes* **42**: 1159–1165, 1993.
- Bailey CJ and Puhah JA, Effect of metformin on glucose metabolism in mouse soleus muscle. *Diabetes Metab* **12**: 212–218, 1986.
- Wilcock C and Bailey CJ, Sites of metformin-stimulated glucose metabolism. *Biochem Pharmacol* **39**: 1831–1834, 1990.
- Abdel-aleem S and Frangakis C, Stimulation of fatty acid oxidation by phosphodiesterase III inhibitors in rat myocytes. *J Cardiovasc Pharmacol* **18**: 293–297, 1991.
- Veerkamp JH, Herman TB, van Moerkerk B, Glatz J, Zuurveld J, Jacobs A and Wagenmakers A,  $^{14}\text{C}$  production is no adequate measure of  $^{14}\text{C}$  fatty acid oxidation. *Biochem Med Metab Biol* **35**: 248–259, 1986.
- Lazarow PB, Assay of peroxisomal  $\beta$ -oxidation of fatty acids. *Methods Enzymol* **72**: 315–319, 1981.
- Haugland RP, *Handbook of Fluorescent Probes and Research Chemicals* (Ed. Spence MTZ), 6th Edn, pp. 266–273. Molecular Probes, Inc., Eugene, OR 1996.
- Lenhard JM, Hamacher L, Paulik M, Beck K, Kliewer SA, Lehmann JM, Cobb J, Henke B, Willson T, Parks D and Blanchard S, Analysis of thiazolidinedione, biguanide, and retinoid effects on adipogenesis and the nuclear receptors PPAR $\gamma$  and RXR. *Diabetologia* **39**[Suppl 1]: 234, 1996.
- Giacobine JP,  $\beta_3$ -Adrenoreceptor: An update. *Eur J Endocrinol* **132**: 377–385, 1995.
- Riccio A, Del Prato S, Vigili de Kreutzenberg S and Tiengo A, Glucose and lipid metabolism in non-insulin dependent diabetes. Effect of metformin. *Diabetes Metab* **17**: 180–184, 1991.
- Fujiwara T, Yoshioka S, Yoshioka T, Ushiyama I and Horiuchi H, Characterization of new oral antidiabetic agent CS-045. Studies in KK and *ob/ob* mice and Zucker fatty rats. *Diabetes* **37**: 1549–1558, 1988.
- Fedele D, Tiengo A, Nosadini R, Marchiori E, Briani G, Garotti MC and Muggeo M, Hypolipodemic effects of metformin in hyperprebetalipoproteinaemia. *Diabetes Metab* **2**: 127–133, 1976.
- Hermann LS, Metformin. A review of its pharmacological properties and therapeutic use. *Diabetes Metab* **5**: 233–245, 1979.
- Paulik MA and Lenhard JM, Thiazolidinediones inhibit alkaline phosphatase activity while increasing expression of uncoupling protein, deiodinase and increasing mitochondrial mass in C3H10T1/2 cells. *Cell Tissue Res*, in press.
- Tai TAC, Jennermann C, Brown KK, Oliver BB, MacGinitie MA, Wilkison WO, Brown HR, Lehmann JM, Kliewer SA, Morris DC and Graves RA, Activation of the nuclear receptor peroxisome proliferator-activated receptor  $\gamma$  promotes brown adipocyte differentiation. *J Biol Chem* **271**: 29909–29914, 1996.
- Bailey CJ, Wilcock C and Day C, Effect of metformin on glucose metabolism in the splachnic bed. *Br J Pharmacol* **105**: 1009–1013, 1992.
- Fulgencio J-P, Kohl C, Girard J and Pégrier J-P, Troglitazone inhibits fatty acid oxidation and esterification, and gluconeogenesis in isolated hepatocytes from starved rats. *Diabetes* **45**: 1556–1562, 1996.
- El-Kebbi IM, Roser S and Pollet RJ, Regulation of glucose transport pioglitazone in cultured muscle cells. *Metabolism* **43**: 953–957, 1994.
- Hiroyoshi H, Yoshioka T, Kawasaki T, Nakamura K, Matsunuma N, Yamaguchi Y and Sasahara K, Troglitazone (CS-045), a new antidiabetic drug. *Annu Rep Sankyo Res Lab* **46**: 1–57, 1994.
- Stern J, Pharmacology and mode of action of hypoglycaemic guanidine derivatives. In: *Oral Hypoglycaemic Agents* (Ed. Campbell GD), pp. 193–245. Academic Press, London, 1969.