Pyruvate Dehydrogenase Kinase 4

Regulation by Thiazolidinediones and Implication in Glyceroneogenesis in Adipose Tissue

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OBJECTIVE—Pyruvate dehydrogenase complex (PDC) serves as the metabolic switch between glucose and fatty acid utilization. PDC activity is inhibited by PDC kinase (PDK). PDC shares the same substrate, i.e., pyruvate, as glyceroneogenesis, a pathway controlling fatty acid release from white adipose tissue (WAT). Thiazolidinediones activate glyceroneogenesis. We studied the regulation by rosiglitazone of PDK2 and PDK4 isoforms and tested the hypothesis that glyceroneogenesis could be controlled by PDK.

RESEARCH DESIGN AND METHODS—Rosiglitazone was administered to Zucker *fa/fa* rats, and then PDK4 and PDK2 mRNAs were examined in subcutaneous, periepididymal, and retroperitoneal WAT, liver, and muscle by real-time RT-PCR. Cultured WAT explants from humans and rats and 3T3-F442A adipocytes were rosiglitazone-treated before analyses of PDK2 and PDK4 mRNA and protein. Small interfering RNA (siRNA) was transfected by electroporation. Glyceroneogenesis was determined using $[1\text{-}^{14}C]$pyruvate incorporation into lipids.

RESULTS—Rosiglitazone increased PDK4 mRNA in all WAT depots but not in liver and muscle. PDK2 transcript was not affected. This isoform selectivity was also found in ex vivo–treated explants. In 3T3-F442A adipocytes, *Pdk4* expression was strongly and selectively induced by rosiglitazone in a direct and transcriptional manner, with a concentration required for half-maximal effect at 1 nmol/l. The use of dichloroacetic acid or leelamine, two PDK inhibitors, or a specific PDK4 siRNA demonstrated that PDK4 participated in glyceroneogenesis, therefore altering nonesterified fatty acid release in both basal and rosiglitazone-activated conditions.

CONCLUSIONS—These data show that PDK4 upregulation in adipocytes participates in the hypolipidemic effect of thiazolidinediones through modulation of glyceroneogenesis. *Diabetes* **57:2272–2279, 2008**

Insulin resistance is associated with alterations in the balance between glucose and fatty acid oxidative pathways. This leads to chronic hyperglycemia because of an excessive hepatic glucose production (gluconeogenesis) (1) associated with a decrease in insulin-induced glucose disposal within peripheral tissues, such as skeletal muscle (2). Furthermore, insulin resistance is associated with an excessive plasma concentration of nonesterified fatty acids (NEFAs), which is partly due to a reduction of the antilipolytic action of insulin on white adipose tissue (WAT) in postprandial situation and a decrease in fatty acid reesterification during lipolysis at fast (3,4). Numerous lines of evidence support the notion that this increase in plasma NEFA plays a pivotal role in the early onset of insulin resistance (5–7). The mitochondrial pyruvate dehydrogenase complex (PDC) catalyzes the irreversible decarboxylation of pyruvate to acetyl-CoA and CO_2. This complex regulates the balance between oxidation of glucose and lipids, depending on nutritional status, and thus plays the role of metabolic switch for fuel selection (8). PDC activity is tightly controlled in the short term by a continuous phosphorylation-dephosphorylation cycle (9,10). Phosphorylation of the E1 subunit of PDC is catalyzed by the PDC kinases (PDKs), which inactivate PDC, while PDC phosphatases (PDPs) activate PDC through dephosphorylation. Thus, the relative activities of PDK and PDP regulate the proportion of PDC in the active dephosphorylated form in mitochondria. Four isoforms of PDK (PDK1–4) and two isoforms of PDP (PDP1 and -2) have been described in mammals and are expressed in varying amounts in a tissue-specific manner (11,12). To prevent hyperglycemia in insulin-resistant states, inhibitors of PDK have been developed to activate PDC, thereby decreasing gluconeogenesis in liver and increasing glucose oxidative capacities in skeletal muscle (13,14). However, the role of PDC and its regulation by PDK-to-PDP ratio in other insulin-sensitive tissues, like adipose tissue, has not been extensively studied.

In WAT, lipolytic and reesterification pathways are active and both participate in the control of NEFA release (15). Reesterification into triglycerides of an important part of NEFA arising from lipolysis requires the synthesis of glycerol-3-phosphate (G3P), which mainly arises from noncarbohydrate substrates like lactate or pyruvate through a pathway named glyceroneogenesis (16,17). The key enzyme of this metabolic pathway is the cytosolic isoform of PEPCK-C (18). Pyruvate can be either carboxylated to oxaloacetate by pyruvate carboxylase and used for glyceroneogenesis or decarboxylated to acetyl-CoA by

PDC for the tricarboxylic acid cycle. Hence, we hypothesized that pyruvate flux through glyceroneogenesis was negatively linked to PDC activity. As a consequence, the PDK-to-PDP ratio would participate in the fatty acid reesterification pathway in adipocytes.

We have previously shown that adipocyte PEPCK-C and the whole glyceroneogenic pathway are acute targets for peroxisome proliferator–activated receptor (PPAR)γ agonists, such as thiazolidinediones, in rodents and humans (19–21). *Pck1*, which encodes PEPCK-C, is the earliest and strongest rosiglitazone-activated gene in adipocytes, suggesting an essential role of PEPCK-C in the response of WAT to thiazolidinediones. Such an increase in PEPCK-C precedes activation of glyceroneogenesis, which participates in the sequestration of lipids in adipose tissue observed with these compounds and thus explains, at least in part, the hypolipidemic properties of this class of drugs (22). Because the inactivation of PDC by phosphorylation could favor glyceroneogenesis, we have studied the regulation by thiazolidinediones of the expression of PDK2 and PDK4, the two isoforms of PDK expressed in adipose tissue (11).

Here, we make the original demonstration of a rapid tissue- and isoform-selective upregulation by rosiglitazone of the expression of *Pdk4* in adipocytes. We also demonstrate the implication of PDK4 in the control of glyceroneogenesis, suggesting that this upregulation participates in the thiazolidinedione-induced decrease in NEFA release from WAT.

RESEARCH DESIGN AND METHODS

Dulbecco modified Eagle's medium (DMEM) was from Life Technologies (Cercy-Pontoise, France). Rosiglitazone was from Alexis Biochemicals (Coger, Paris). Leelamine was from Cayman Chemicals (Interchim, Montluçon, France). Small interfering RNA (siRNA) was from Invitrogen (Carlsbad, CA). Fetal bovine serum, essentially fatty acid–free BSA, 5,6-dichloro-1B-D-ribofuranosyl benzimidazole (DRB), dichloroacetate (DCA), and all other products were purchased from Sigma (L'isle d'Abeau Chesnes, France).

Male Zucker *fa/fa* rats were purchased from Charles River Laboratories (L'arbresle, France), and male Sprague-Dawley rats were purchased from Janvier Laboratories (Bagneux, France) at 6 weeks of age. They were allowed to acclimate 2 weeks before initiation of treatment and were kept on a 12-h light/dark cycle at constant room temperature. Conventional laboratory diet and tap water were provided ad libitum. In vivo experiments with Zucker rats were carried out as previously described (21). Briefly, rats were given a dose of 5 mg · kg^{-1} · day^{-1} rosiglitazone (maleate) or vehicle (0.5% methylcellulose) via oral gavage in the morning. Four rats were used per treatment group. After 4 days of treatment, animals were fasted during 4 h before killing. Plasma samples were obtained via the jugular vein, and aliquots were stored at −20°C for further glucose (Accu-Check; Roche, Meylan, France), insulin (Ultrasensitive rat insulin ELISA; EuroBio, Courtaboeuf, France), triglycerides (Vitros chemistry system; Johnson & Johnson), and NEFA (Fatty Acids half micro test; Roche) determinations. Samples of subcutaneous, periepididymal, and retroperitoneal WAT, liver, and soleus muscle were dissected and rapidly frozen in liquid nitrogen and stored at −80°C for further RNA analysis. The protocol for the animal studies was conducted according to the French Guidelines for the Care and Use of Experimental Animals.

Culture of human adipose tissue explants. Explants of subcutaneous WAT were obtained from eight women undergoing elective surgery. They were aged 45.7 ± 4.4 (mean ± SE) and had BMI of 30.29 ± 1.42 kg/m^2. None of the subjects suffered from known metabolic or malignant diseases or were taking medications known to alter adipocyte metabolism. The study was performed according to the Declaration of Helsinki. All the patients gave informed written consent.

WAT specimen from the subcutaneous region was obtained within 15 min after the onset of surgery. WAT (300 mg) was cut in small fragments of ~20 mg and cultured in DMEM containing 12.5 mmol/l glucose, 200 IU/ml penicillin, and 50 mg/l streptomycin at 37°C in 10% CO$_2$ atmosphere. After 1 h of preincubation, rosiglitazone was added for 5 h. Explants were then frozen in liquid nitrogen before RNA extraction.

Isolation of adipocytes. Adipocytes from Sprague-Dawley rat periepididymal fat pads were isolated in DMEM containing 5 mmol/l glucose, 0.5 mmol/l pyruvate, and 2% BSA, as described by Weisberg et al. (23). The pellet containing stromavascular fraction was discarded, whereas the floating cells were collected as adipocyte-enriched fraction.

Cell culture and treatment. 3T3-F442A adipocytes were cultured at 37°C in a humidified atmosphere of 10% CO$_2$ in DMEM containing 25 mmol/l glucose, 10% newborn calf serum, 200 IU/ml penicillin, 50 mg/l streptomycin, 8 mg/l biotin, and 4 mg/l pantothenate. At confluence, the medium was changed to DMEM supplemented with 10% FCS and 20 nmol/l insulin to promote adipogenesis. This medium was changed every 2–3 days for 6 days. Twenty-four hours before RNA extraction, cells were placed in serum-free and hormone-free medium.

RNA interference. RNA interference with siRNA was performed by electroporation according to the manufacturer (Cell line Nucleofector kit L; Amaxa). Briefly, 3T3-F442A adipocytes on day 8 of differentiation were detached from culture dishes with trypsin-EDTA (Invitrogen). Tubes containing about 2 million cells were centrifuged for 10 min at 127g. Pelleted cells were then resuspended in 100 μl Nucleofector solution. Control (5′-GGAGGUGGUU GACUUUCAUTT; Invitrogen) or murine PDK4-specific (5′-GGAUUUGGUG GAGUUCCAUTT, Invitrogen) siRNAs were delivered into adipocytes (250 nmol/l) by electroporation. Transfection was stopped by adding DMEM containing 10% FCS. Adipocytes were then reseeded into six-well plates. Twenty-four hours after electroporation, medium was changed to FCS-free DMEM containing rosiglitazone or not. Nineteen hours later, cells were used for RNA analysis or metabolic studies.

RNA analysis. Total RNA was extracted from the cell lines and from rat WAT by the method of Chomczynski and Sacchi (24), whereas RNeasy total RNA kit from Qiagen (Courtaboeuf, France) was used for human WAT explants. Sample quality was controlled by 260:280 nm absorption ratio determination (between 1.8 and 2.0).

For real-time RT-PCR analyses, 1.25 μg total RNA was first reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Courtaboeuf, France). Reverse transcribed RNA were amplified on a thermal cycler (ABI prism 7900 HT; Applied Biosystems) using the SYBR green fluorescence method and specific oligonucleotides. Results were analyzed with the SDS 2.1 real-time detection system software. Quantification of RNA was carried out by comparison of the number of cycles required to reach reference and target threshold values (δ-δ Ct method).

Protein analysis. Mitochondrial fraction was prepared from 3T3-F442A adipocytes, and mitochondrial pellet was resuspended in PBS 1× buffer, containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS to extract mitochondrial proteins. Protein concentrations were determined using the Bradford method using BSA as the standard. Fractions (50 μg) were subjected to SDS-PAGE using a 10% resolving gel with a 6% stacking gel. Resolved proteins were transferred electrophoretically to nitrocellulose membranes, blocked for 1 h at 22°C with Tris-buffered saline (TBS) supplemented with 5% (w/v) nonfat powdered milk and incubated overnight at 4°C with polyclonal antisera raised against mouse PDK4, PDK2 from Santa Cruz Biotechnology (Tebu-bio, France), or PDC-E2 (provided by Dr. J.G. Lindsay, University of Glasgow, U.K.). Nitrocellulose membranes were then washed with 1% Tween in TBS (three times for 5 min) and incubated with horseradish peroxidase–linked secondary antibody anti-rabbit IgG (1:5,000 dilution, in 1% [w/v] BSA in TBS with 0.05% Tween) for 1 h at 22°C. Bound antibody was visualized using ECL according to the manufacturer's instructions. The blots were exposed to Hyperfilm, and the signals were quantified by scanning densitometry.

Metabolic studies. Isolated adipocytes were submitted to incorporation assay immediately after their collection. [1-^{14}C]pyruvate incorporation was performed with 400 μl adipocyte suspension in 2 ml Krebs buffer containing 2% fatty acid–free BSA, 0.5 mmol/l pyruvate, and [1-^{14}C]pyruvate at isotopic dilution of 25. When indicated, PDK inhibitors were added in the incorporation medium. Untransfected or siRNA-transfected 3T3-F442A adipocytes were treated or not for 18 h with rosiglitazone. Three hours before [1-^{14}C]pyruvate incorporation, medium was replaced with glucose-free, serum-deprived DMEM containing 0.3% free fatty acid BSA. Pyruvate incorporation was carried out with 20 μmol/l [1-^{14}C]pyruvate in Krebs buffer containing 0.3% BSA and leelamine, when indicated. After a 2-h incubation, cells were rinsed in PBS and scraped in 10 mmol/l Tris-Cl, pH 7.4, containing 0.25 mol/l sucrose, 0.1 mmol/l EDTA, 0.1 mmol/l dithiothreitol, and 0.1% Triton and frozen in liquid nitrogen before lipid extraction. Lipids were extracted according to the simplified method of Bligh and Dyer (25), and the subsequent [1-^{14}C]pyruvate incorporation was estimated by counting the radioactivity associated with the chloroformic fraction. NEFA concentration was monitored in the incubation medium. Under each conditions, cells were submitted to a test of toxicity, using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method, which did not show any drug-induced toxicity.

TABLE 1
Effect of rosiglitazone treatment on metabolic parameters in male Zucker *fa/fa* rats

	Control	Rosiglitazone
Body wt (g)	323 ± 14	324 ± 10
Glucose (mg/dl)	120 ± 21	153 ± 35
Insulin (ng/dl)	8.8 ± 0.6	2.7 ± 0.4*
NEFA (μmol/l)	180 ± 70	60 ± 30†
Triglycerides (mg/dl)	145 ± 21.9	87.9 ± 14.5†

Data are means ± SE ($n = 4$ for each group). Eight-week-old male Zucker *fa/fa* rats were or were not treated with rosiglitazone (5 mg · kg^{-1} · day^{-1}) for 4 days. Blood samples were collected for analyses. *$P < 0.01$, †$P < 0.05$.

In vitro functional PDK assay. To test the efficiency of leelamine, a functional PDK assay was performed using a commercially available PDC purified from pig heart and containing intrinsic PDK activity, as extensively described by Jackson et al. (26), with 800 μg/ml purified PDC.

Image quantification and data analysis. Quantitative results of Western blotting were obtained by densitometry in ImageJ software. The nonparametric Mann-Whitney U test for pairwise comparisons was applied due to the small number of experiments. Analyses were performed using the StatView 4.01 (Abacus Concepts, Berkeley, CA) statistical package. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Rosiglitazone induces *Pdk4* expression in adipose tissue and in adipocytes. We showed previously that treating male Zucker *fa/fa* rats with rosiglitazone for 4 days significantly lowered both plasma insulin and serum lipid levels (21). This normalization in lipidemia was linked to a large induction in WAT glyceroneogenesis, thus decreasing NEFA release. Under these conditions, the expression of one of the key genes involved in glyceroneogenesis, PEPCK-C, was stimulated two- to threefold in subcutaneous, periepididymal, and retroperitoneal WAT. A similar rosiglitazone-induced normalization in lipidemia was obtained in the present study (Table 1). We used real-time RT-PCR to analyze the effect that rosiglitazone would have on the expression of *Pdk2*, *Pdk4*, *Pdp1*, and *Pdp2* in the same three WAT depots. PDP1 and PDP2 mRNAs remained unaffected by rosiglitazone treatment (data not shown). In contrast, rosiglitazone induced a large 3- to 4.5-fold increase in PDK4 mRNA in all depots but neither in liver nor in soleus muscle, demonstrating a tissue-specific response of this gene under this acute treatment (Fig. 1*A*). In contrast, *Pdk2* expression remained insensitive to rosiglitazone whatever tissue, demonstrating a selective action of the drug on PDK isoforms (Fig. 1*B*).

To determine whether rosiglitazone acts on PDK4 gene expression through a direct effect on WAT, we treated ex vivo rats and humans WAT explants for 5 h with rosiglitazone and monitored PDK4 mRNA. Both species responded to rosiglitazone by a large three- to fourfold increase in *Pdk4* expression, whereas mRNA concentrations of PDK2 and pyruvate carboxylase (PC) were unaffected (Fig. 2*A* and *B*). WAT is a heterogeneous tissue containing not only adipocytes but also many other cell types, including preadipocytes, macrophages, fibroblasts, and endothelial cells. To study the mechanism of rosiglitazone action on *Pdk4* gene expression in a homogeneous source of adipocytes, we used the 3T3-F442A adipocyte cell line. These cells were previously used to study *Pdk1* regulation and glyceroneogenesis by PPARγ activators (27). A 2-h treatment of differentiated 3T3-F442A adipocytes with rosiglitazone
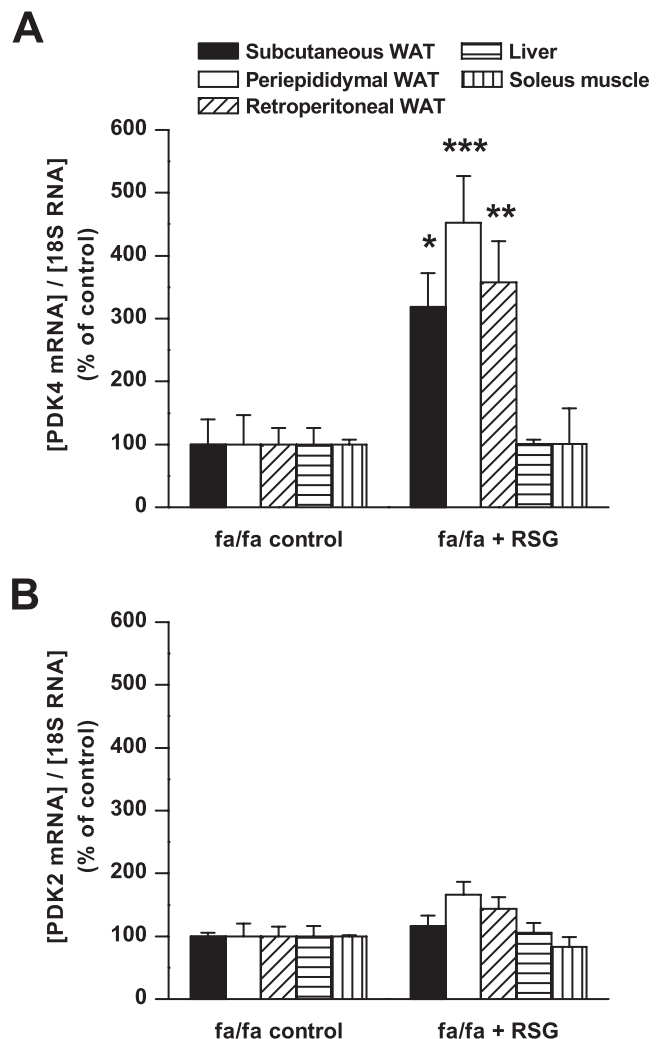

FIG. 1. Rosiglitazone induces PDK4 gene expression in *fa/fa* rat adipose tissue in vivo. Male Zucker *fa/fa* rats were treated for 4 days with rosiglitazone (5 mg · kg^{-1} · day^{-1}). Rats were killed, and tissues were removed. Total RNA was extracted from subcutaneous, periepididymal, and retroperitoneal WAT, liver, and soleus muscle. PDK4 (*A*) and PDK2 (*B*) mRNA were analyzed by real-time RT-PCR. Values were normalized to 18S rRNA and expressed as percentage of untreated rats. Results are mean ± SE from four rats in each group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

induced PDK4 mRNA 2.5-fold whereas the concentrations of PDK2 and PC mRNAs were unchanged (Fig. 2*C*). We used Western blot experiments with specific antibodies for either PDK2 or PDK4 to monitor the relative expression of both protein isoforms in 3T3-F442A adipocytes at day 6 after confluence. Taking PDC-E2 as a control, results strongly suggest that PDK4 protein amount is about threefold that of PDK2 in adipocytes (Fig. 2*D*).

Rosiglitazone directly increases *Pdk4* transcription and protein abundance in 3T3-F442A adipocytes. Rosiglitazone induction in *Pdk4* expression was acute, with a maximum attained at 2 h in 3T3-F442A adipocytes (Fig. 3*A*). We chose this 2-h treatment to determine the rosiglitazone concentration that produced the half-maximal effect (EC$_{50}$). Incubation of 3T3-F442A adipocytes with rosiglitazone caused a dose-dependent increase in the abundance of PDK4 mRNA (Fig. 3*B*). EC$_{50}$ was at about 1 nmol/l, in agreement with the affinity of rosiglitazone for PPARγ, strongly suggesting that this receptor was involved. The rosiglitazone-induced increase in PDK4 mRNA could be the result of stimulation of *Pdk4* transcription
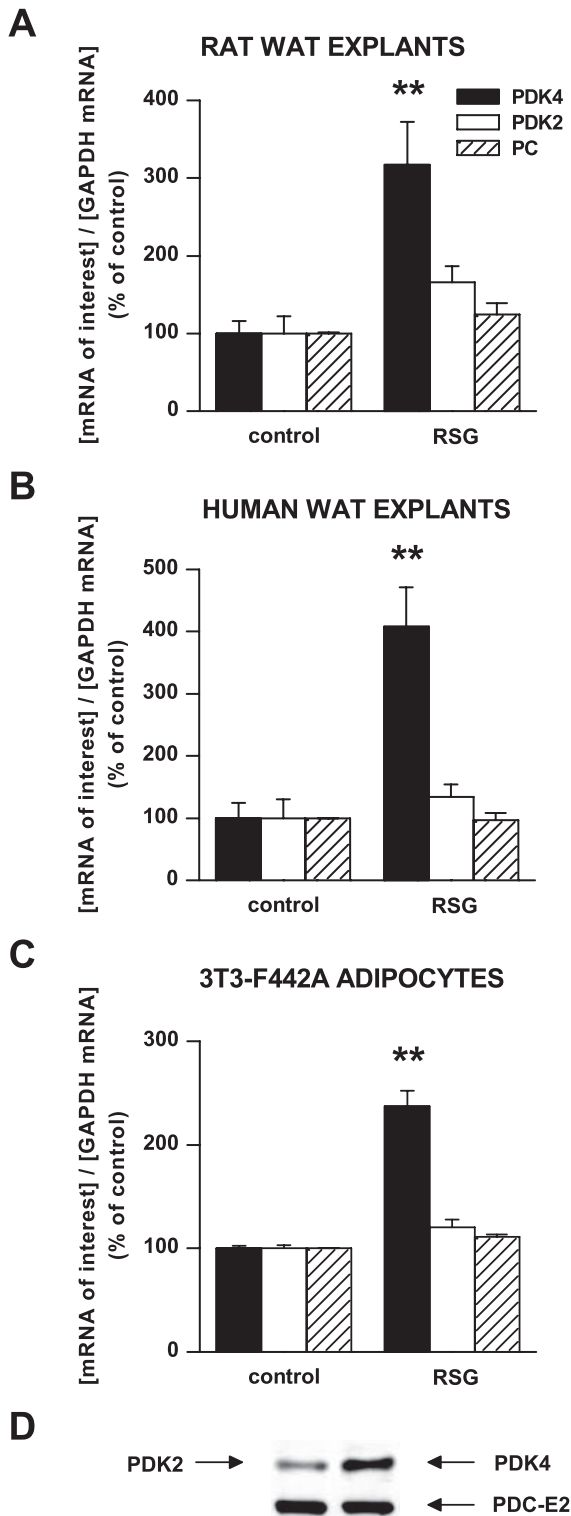
A

RAT WAT EXPLANTS

B

HUMAN WAT EXPLANTS

C

3T3-F442A ADIPOCYTES

D

FIG. 2. Rosiglitazone induces *Pdk4* expression in rat and human adipose tissue ex vivo and in 3T3–F442A adipocytes. Rat periepididymal (*A*) and human subcutaneous abdominal WAT (*B*) were cultured in DMEM containing 12.5 mmol/l glucose and treated or not with 1 μmol/l rosiglitazone for 5 h. *C*: 3T3–F442A adipocytes at day 6 of differentiation were cultured in DMEM containing 25 mmol/l glucose and treated or not for 2 h with 1 μmol/l rosiglitazone. Total RNA was extracted, and PDK4, PDK2, and PC mRNA were analyzed by real-time RT-PCR with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. *D*: Protein extracts were obtained from differentiated 3T3–F442A adipocytes and subjected to Western blotting using antibodies against PDK4, PDK2, and the E2 subunit of PDC complex. Data are means ± SE from three to five independent experiments, each performed in triplicate, and are expressed as percent of control. **$P < 0.01$.

and/or of mRNA stability. To address these issues, we used DRB, a transcription inhibitor, and puromycin, a protein synthesis inhibitor. 3T3-F442A adipocytes were first treated with 1 μmol/l rosiglitazone with or without 80 μmol/l DRB for 0.5–2 h before analyzing PDK4 mRNA abundance by real-time RT-PCR. Whatever the time of treatment, DRB abolished the induction of PDK4 mRNA by rosiglitazone (Fig. 3*A*). Under the same conditions of rosiglitazone treatment, puromycin had no effect, demonstrating that de novo protein synthesis was not required (data not shown). These results are strongly in favor of rosiglitazone directly increasing transcription rate of *Pdk4*. Second, we examined the possibility of a rosiglitazone-induced PDK4 mRNA stabilization by incubating 3T3-F442A adipocytes for 2 h with rosiglitazone and then for various times with DRB before monitoring PDK4 mRNA abundance by real-time RT-PCR. In the absence of rosiglitazone, PDK4 mRNA half-life was estimated at ~1.5 h as previously obtained in other cell types (Fig. 3*C*). After a 2-h treatment with 1 μmol/l rosiglitazone, PDK4 mRNA half-life rose to ~2 h, showing a slight rosiglitazone-induced stabilization of PDK4 messenger in adipocytes. mRNA induction was followed by a significant twofold increase in PDK4 protein at 18 h of rosiglitazone treatment (Fig. 3*D*).

Contribution of PDK4 to fatty acid reesterification in basal and rosiglitazone-treated adipocytes. To test the potential involvement of PDC and PDK in the control of pyruvate flux toward glyceroneogenesis, pyruvate incorporation into neutral lipids was monitored in isolated adipocytes in the presence or not of PDK inhibitors. We tested both DCA, one of the few known highly specific synthetic PDK inhibitor, and leelamine, a more recent PDK inhibitor (28,29). Results of MTT test demonstrated that neither of these compounds was cytotoxic at the concentrations used (data not shown). Figure 4*A* shows that both inhibitors significantly reduced glyceroneogenic flux in isolated adipocytes from rat WAT. However, the concentrations used to reach a statistical significant reduction differ for the two inhibitors. At 50 μmol/l, leelamine inhibited pyruvate incorporation by 40% ($P < 0.001$), whereas a 10-fold higher DCA concentration was required to produce a significant 60% inhibition ($P < 0.01$) (Fig. 4*A*). Hence, leelamine is more efficient to reduce pyruvate incorporation into triglycerides than DCA. To ensure that leelamine effect was due to PDK inhibition, an in vitro assay was performed, monitoring residual PDC activity after kinase action. At the concentration of 50 μmol/l, leelamine induced an inhibition of 41.00 ± 2.14% in PDK activity ($n = 8$, $P < 0.001$).

To further study the functional involvement of PDK in both basal and rosiglitazone-activated conditions, we treated or not 3T3-F442A adipocytes with 1 μmol/l rosiglitazone for 18 h and then monitored the incorporation of [1-^{14}C]pyruvate into lipids in the presence or not of leelamine for 2 h. The addition of 50 and 100 μmol/l leelamine reduced basal glyceroneogenic flux 25 and 56%, respectively, with a concomitant increase in NEFA release (Fig. 4*B* and *C*). As expected, rosiglitazone induced glyceroneogenesis and reduced NEFA release. The addition of 50 and 100 μmol/l leelamine on rosiglitazone-pretreated cells reduced glyceroneogenic flux 59 and 115%, respectively, with a concomitant increase in NEFA release (Fig. 4*B* and *C*). Under these conditions, glycerol release was not modified (data not shown). Hence, PDK inhibitors did not affect lipolysis. To ascertain the specific implication of
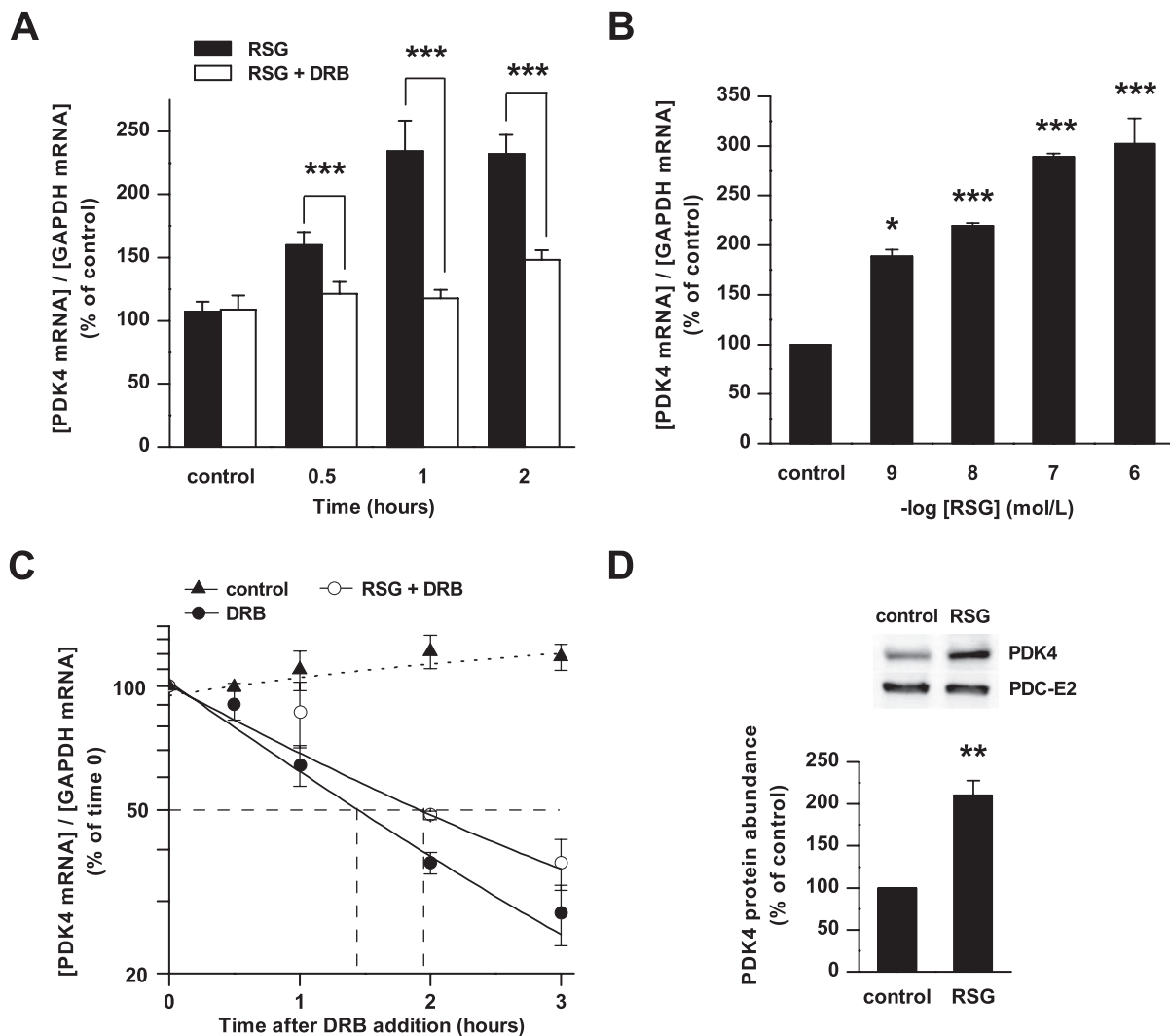
FIG. 3. Mechanism of rosiglitazone stimulation of *Pdk4* expression in 3T3–F442A adipocytes. Quantitative changes in the abundance of PDK4 mRNA in 3T3–F442A adipocytes either cultured for the indicated times with 1 μmol/l rosiglitazone in the absence or in the presence of 80 μmol/l DRB (*A*) or treated for 2 h with the indicated concentrations of rosiglitazone (*B*). In *C*, 3T3–F442A adipocytes were treated or not with 1 μmol/l rosiglitazone for 2 h before DRB addition, and then mRNA for PDK4 was estimated over time. Values were normalized to GAPDH and are expressed as percentage of control. In *D*, 40 μg mitochondrial proteins from adipocytes treated or not with 1 μmol/l rosiglitazone for 18 h were subjected to Western blotting. Densitometry values were normalized to PDC-E2 and expressed as percentage of control. Results are means ± SE of three to five independent experiments, each performed in triplicate. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

PDK4 in glyceroneogenesis, a selective PDK4 siRNA was transfected in 3T3-F442A adipocytes. Forty-eight hours after transfection, a specific 60% ($P < 0.001$) decrease in PDK4 mRNA was observed whereas concentrations in PDK2, PDP1, PDP2, PC, and PEPCK-C mRNAs remained unchanged (Fig. 5A). Furthermore, PDK4 siRNA did not induce interferon (IFN)-β mRNA, showing the lack of IFN response (Fig. 5A). Incorporation of [1-^{14}C]pyruvate into lipids was reduced 40% after transfection of adipocytes with PDK4 siRNA ($P < 0.05$) (Fig. 5B). Under these experimental conditions, rosiglitazone induced [1-^{14}C]pyruvate incorporation 3.1-fold in control cells ($P < 0.001$) (Fig. 5B). A 35% decrease in rosiglitazone-induced [1-^{14}C]pyruvate incorporation was observed in the presence of PDK4 siRNA ($P < 0.01$).

DISCUSSION

For a better understanding of the early mechanisms involved in PPARγ effects in WAT, we investigated the regulation of potent primary targets for thiazolidinediones. One important observation of our present report is that the

expression of *Pdk4* is acutely induced by the specific PPARγ agonist rosiglitazone in WAT explants from rats and humans. Induction of PDK4 mRNA occurs in the same time frame as that of PEPCK-C transcript. Furthermore, as we observed previously for PEPCK-C, a 4-day administration of rosiglitazone to Zucker *fa/fa* rats, a model of insulin resistance and dyslipidemia, induced an increase in *Pdk4* expression specifically in WAT from several depots. A longer term in vivo effect of COOH, a PPARγ agonist, on PDK4 mRNA was observed by Laplante et al. (30). Our results are in accordance with these previous results in which, however, the issue of tissue-specific action of the drug was not addressed. In the course of our experiments, we showed that rosiglitazone induction of *Pdk4* expression was tissue-specific because liver and muscle did not respond to such a treatment. Furthermore, in our study, *Pdk2* expression remained unaffected by rosiglitazone treatment, whereas Laplante et al. (30) observed a simultaneous induction of both *Pdk2* and *Pdk4* expression in visceral WAT, probably because of the longer duration of treatment used (3 weeks). In contrast to our results, Way
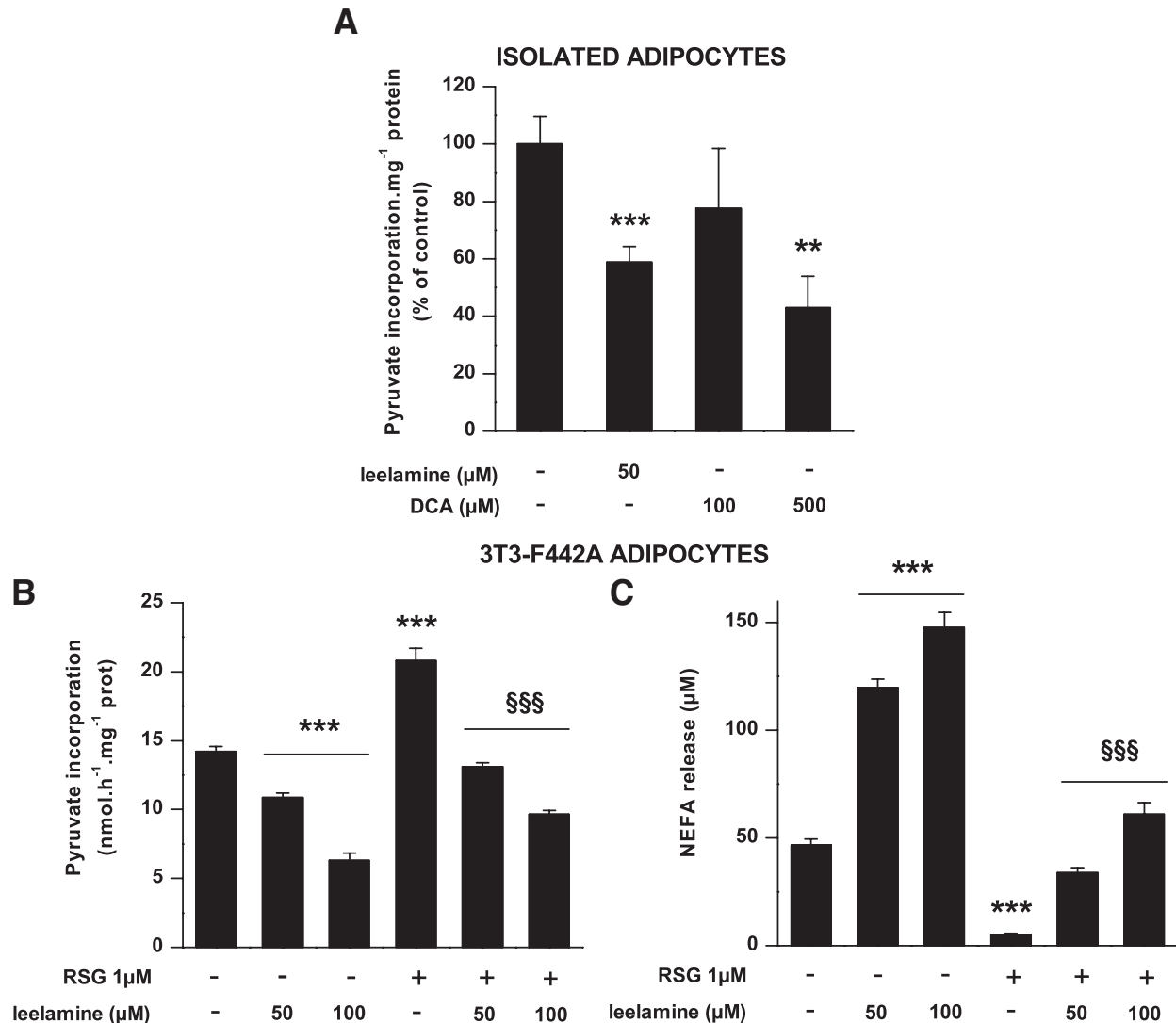
A

ISOLATED ADIPOCYTES

| leelamine (µM) | - | 50 | - | - |
| DCA (µM) | - | - | 100 | 500 |

3T3-F442A ADIPOCYTES

B

| RSG 1µM | - | - | - | + | + | + |
| leelamine (µM) | - | 50 | 100 | - | 50 | 100 |

C

| RSG 1µM | - | - | - | + | + | + |
| leelamine (µM) | - | 50 | 100 | - | 50 | 100 |

FIG. 4. PDK4 inhibition alters basal and rosiglitazone-activated glyceroneogenesis. *A*: Using [1-^{14}C]pyruvate incorporation into neutral lipids, glyceroneogenic flux was monitored in isolated adipocytes from rat periepididymal fat pads for 2 h in the presence or not of PDK inhibitors, leelamine, or DCA at the indicated concentrations. 3T3–F442A adipocytes were treated or not with 1 µmol/l rosiglitazone for 18 h, and then glyceroneogenic flux (*B*) and medium NEFA concentration (*C*) were monitored in the presence or not of leelamine for 2 h. Results are means ± SE of three independent experiments, each performed in triplicate. **$P < 0.01$; ***$P < 0.001$ vs. control; §§§$P < 0.001$ vs. rosiglitazone treatment.

et al. (31) observed that short-term (1-day) treatment with GW1929, a different PPARγ agonist, reduced the concentration of PDK4 transcript in muscles from ZDF Zucker diabetic rats (31). One explanation for this discrepancy could be that ZDF rats are insulin-resistant and diabetic at earlier times than the Zucker *fa/fa* rats we used here. Hence when ZDF rats are 9 weeks old, their insulin-resistant state is correlated with low PDK4 activity in muscle, which could be raised by rosiglitazone. Such an effect is probably indirect as the result of thiazolidinedione-induced rapid decrease in blood NEFA. In 8-week-old *fa/fa* rats, blood NEFA concentrations are near to normal and therefore unable to stimulate PDK4 in muscle (21). Whatever happens in muscle, our data clearly demonstrate that PDK4 is an early-responsive positive target for PPARγ in WAT.

To further study the mechanisms by which rosiglitazone stimulates *Pdk4* expression, we used the 3T3-F442A cell line. Rosiglitazone rapidly stimulated *Pdk4* expression in fully differentiated 3T3-F442A adipocytes as previously found in 7,800 C1 hepatoma cells treated with a PPARα ligand (32). Using transcription and translation inhibitors,

we showed that PDK4 was a transcriptional and direct target for rosiglitazone. Our results are in slight contrast with those from Sears et al. (33) who showed that in 3T3-L1 adipocytes, rosiglitazone induction of PDK4 mRNA is delayed, suggesting that in these cells, PDK4 is an indirect PPARγ target. Furthermore, we found that half-life of the PDK4 transcript was short, i.e., ~2 h, as previously observed for hepatoma cells (32). Interestingly, PC gene expression was not modulated. PC was previously shown as a PPARγ-induced gene during adipocyte differentiation of the 3T3-L1 preadipocytes (34). Our observation that rosiglitazone does not modulate pyruvate carboxylase gene expression in differentiated 3T3-F442A adipocytes is in agreement with a delayed action of PPARγ agonist on this gene during adipocyte differentiation.

We show unambiguously here that PDK4 participates in adipocyte glyceroneogenesis because leelamine, a PDK inhibitor, and a specific PDK4 siRNA strongly reduces basal and rosiglitazone-induced pyruvate incorporation into neutral lipids, with a coordinated stimulation of NEFA release. These results are illustrated in Fig. 6 in which pyruvate crossroad is shown as the central switch for the
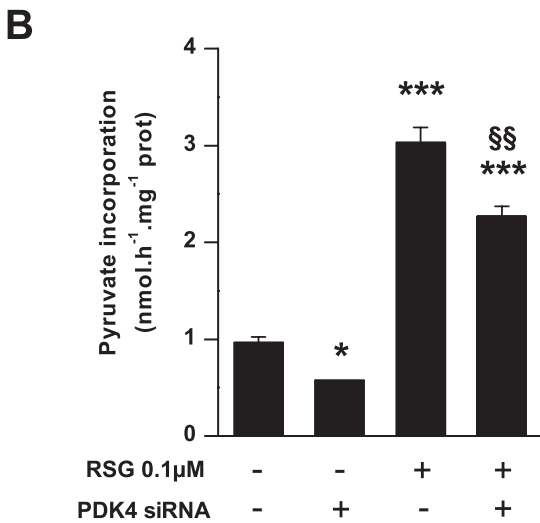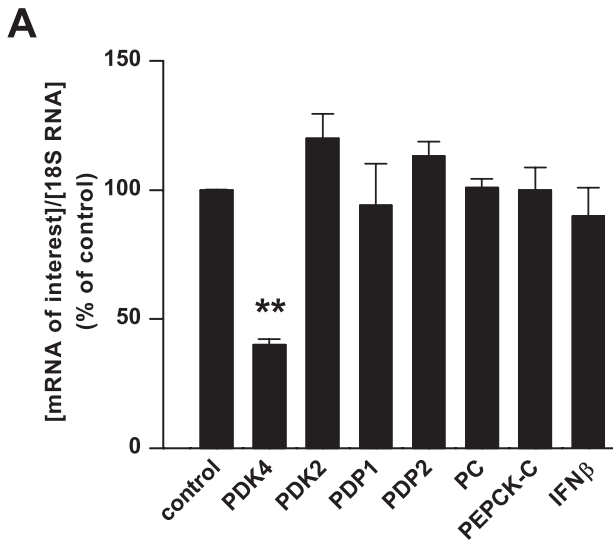
A

B

FIG. 5. Influence of PDK4 siRNA on gene expression and glyceroneogenesis in 3T3–F442A adipocytes. 3T3–F442A adipocytes were transfected with a control or a PDK4-specific siRNA. Forty-eight hours after transfection, cells were used for mRNA studies (A) or [1-^{14}C]pyruvate incorporation experiments (B). A: Total RNA was extracted, and concentration of PDK4, PDK2, PDP1, PDP2, pyruvate carboxylase, PEPCK-C, and IFN-β mRNA was analyzed by real-time RT-PCR with normalization to 18S rRNA. Control or PDK4 siRNA-transfected adipocytes were treated or not with 0.1 μmol/l rosiglitazone for 18 h, and glyceroneogenic flux was monitored. Results are means ± SE of three independent experiments, each performed in triplicate. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. control; §§$P < 0.01$ vs. rosiglitazone treatment.
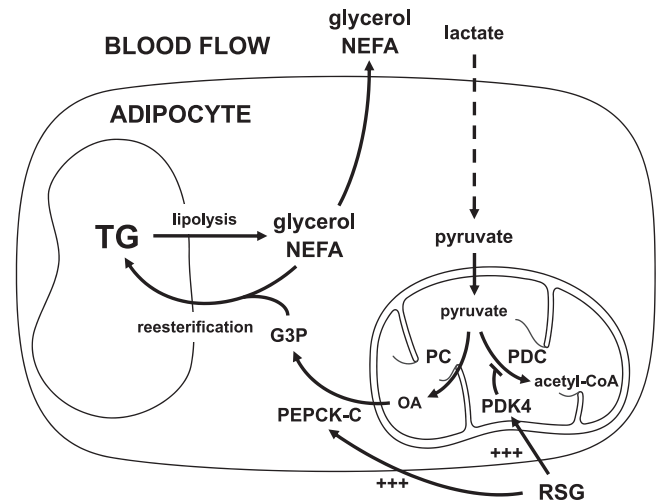

FIG. 6. Pyruvate routing for fatty acid reesterification: influence of thiazolidinediones. Pyruvate is either decarboxylated by PDC into acetyl-CoA or carboxylated to oxaloacetate (OA) by PC in mitochondria. Oxaloacetate is the precursor of G3P, and PEPCK-C is involved in this process, allowing for reesterification into triglyceride (TG) of NEFA arising from lipolysis. This could lead to decreased NEFA release in the blood flow, without affecting glycerol release. PDC is mainly regulated by a phosphorylation/dephosphorylation cycle. PDK4 inactivates PDC, thereby regulating pyruvate availability for glyceroneogenesis in adipocytes. Rosiglitazone induces both PDK4 and PEPCK-C, thereby reducing pyruvate flux toward acetyl-CoA and favoring G3P production.

glucose oxidation is not a predominant pathway during fasting and in the absence of insulin.

Elevated concentration of plasma NEFA is now recognized as a key factor in the onset of insulin resistance and type 2 diabetes (35). Our previous results indicate that WAT glyceroneogenesis is important to lipid homeostasis and that a disregulation in this pathway has profound pathophysiological effects (19–21,36). This would result in dramatic changes in net NEFA output, hence in the concentration of plasma NEFA, which in turn would mediate metabolic alterations, for instance by affecting insulin sensitivity of peripheral tissues. Here, we provide clear evidence that PDK4 is a new player in the glyceroneogenic process and is a thiazolidinedione target selectively in WAT. Therefore, by means of PEPCK-C and of PDK4, glyceroneogenesis is a target for thiazolidinediones, the antidiabetic action of which seems clearly to be linked to their hypolipidemic effect (22,37).

modulation of G3P production. We postulate that the circulating substrate for the whole process is lactate, which is abundant in the blood under the physiological situation during which lipolysis is activated, i.e., fasting. Rosiglitazone induction of both PDK4 and PEPCK-C results, respectively, in reduction of pyruvate flux toward acetyl-CoA because PDC activity is strongly inhibited by PDK4 and in increased G3P synthesis via PEPCK-C. Such a combined action of thiazolidinediones allows increased reesterification during lipolysis leading to decreased NEFA release from adipocytes. The reduced PDC activity under rosiglitazone treatment raises the intriguing possibility that thiazolidinediones may reduce glucose oxidation to CO_2 in adipocytes, in contrast with what occurs in muscle and at the whole-body level. Although this issue requires further development, we can emphasize here that

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