Activation of peroxisome proliferator-activated receptors (PPARs) can regulate brain physiology and provide protection in models of neurological disease; however, neither their exact targets nor mechanisms of action in brain are known. In many cells, PPARγ agonists increase glucose uptake and metabolism. Because astrocytes store glucose and provide lactate to neurons on demand, we tested effects of PPARγ agonists on astroglial glucose metabolism. Incubation of cortical astrocytes with the PPARγ thiazolidinedione (TZD) agonist pioglitazone (Pio) significantly increased glucose consumption in a time- and dose-dependent manner, with maximal increase of 36% observed after 4 h in 30 μM Pio. Pio increased 2-deoxy-glucose uptake because of increased flux through the type 1 glucose transporter. However, at this time point Pio did not increase type 1 glucose transporter expression, nor were its effects blocked by transcriptional or translational inhibitors. Pio also increased astrocyte lactate production as soon as 3 h after incubation. These effects were replicated by other TZDs; however, the order of efficacy (troglitazone > pioglitazone > rosiglitazone) suggests that effects were not mediated via PPARγ activation. TZDs increased astrocyte cAMP levels, and their glucose modifying effects were reduced by protein kinase A inhibitors. TZDs inhibited state III respiration in isolated brain mitochondria, whereas in astrocytes they caused mitochondrial membrane hyperpolarization. Pio protected astrocytes against hypoglycemia-induced cell death. Finally, glucose uptake was modified in brain sections prepared from Pio-fed rats. These results demonstrate that TZDs modify astrocyte metabolism and mitochondrial function, which could be beneficial in neurological conditions where glucose availability is reduced.

The peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that can bind to PPAR elements (AGGTCA n AGGTCA) to activate transcription (1–5) normally as heterodimers with the retinoic acid receptor (4–6). Three major subtypes have been identified (α, δ (or β), and γ) with distinct actions on cell physiology and different specificity in their ligand-binding properties (7). PPARγ is widely expressed in the adipose tissue where it controls adipocyte differentiation and lipid metabolism (8). The agonists of PPARγ include fatty acids, non-steroidal anti-inflammatory drugs like ibuprofen (9), the natural ligand 15-deoxy-Δ12,14-prostaglandin J2 (10), thiazolidinediones (TZDs) such as troglitazone (Trog), pioglitazone (Pio), and rosiglitazone (Ros) (11), and high affinity tyrosine-based agonists like GW347845 (7). TZDs were originally designed as antidiabetic drugs because of their insulin-sensitizing effects, and several are in clinical use (12–14).

Recently, it has been shown that PPARγ is involved in the regulation of inflammatory responses (15–18). Thus, PPARγ activation contributes to the secretion of proinflammatory cytokines and neurotoxic substances from activated monocytes (19–21) and microglia (22). Our previous studies showed that PPARγ agonists are able to down-regulate neuronal inducible nitric-oxide synthase expression and to reduce cerebellar granule cell death in vitro (23) and in vivo (24). More recently, we demonstrated that PPARγ agonists reduced the development of clinical symptoms in experimental autoimmune encephalomyelitis, a commonly used animal model for multiple sclerosis (25). These findings have led to the possibility that PPARγ agonists could provide protection in other neurodegenerative diseases, such as Alzheimer’s disease (26), stroke (27), Parkinson’s disease (28), and multiple sclerosis (25, 29–31). PPARγ activation may regulate several functions in brain, in addition to inflammation, including promoting neurite extension (32) and inducing myelin gene expression (33). However, neither the exact targets nor the mechanisms of protection due to PPARγ activation in brain are well known.

Studies carried out in liver, adipose tissue (34–37), and muscle (38–40) showed that in periphery, PPARγ regulates glucose metabolism by increasing glucose uptake through facilitative glucose transporter proteins (GLUTs). Delivery of glucose in the central nervous system is mainly mediated by GLUTs present on the brain-blood barrier, on glial cells, and on neurons (41, 42). However the primary energy source in brain is provided by astrocytes. These cells are able to store glucose in the form of glycogen and provide lactate to neurons on demand (43). Because glucose metabolism could be adversely affected in some neurodegenerative disorders (44–47), we hypothesized that one mechanism of PPARγ agonists protection
during disease could be related to an increase of cerebral metabolism of glucose.

To test this hypothesis we examined the effects of TZDs on glucose uptake and metabolism in primary enriched cultures of rat cortical astrocytes. Incubation with these drugs increased glucose utilization (assessed as loss of glucose from the incubation media), 2-deoxy-glucose uptake, and lactate production. These effects did not appear to be mediated by activation of PPARγ or to changes in gene expression but instead because of a rapid effect on other cellular signaling systems possibly related to changes in transcriptional activity. Our results demonstrate that TZDs can increase astroglial glucose metabolism, suggesting that this class of drugs may be therapeutically useful in conditions in which brain glucose levels or availability are limited.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents (DMEM and antibiotics) and LPS (Salmonella typhimurium) were from Sigma. Fetal calf serum (FCS) was from Invitrogen. Pio was from Takeda Pharmaceuticals North America (Lincolnshire, IL). Rosi was from GlaxoSmithKline. Trog was from Parke-Davis. GW347845 was a gift of Tim Willson (GlaxoSmithKline). Enhanced chemiluminescence reagents and antibodies conjugated to horseradish peroxidase were from Vector Laboratories (Burlingame, CA). Horseradish peroxidase-conjugated rabbit secondary antibodies were from Vector Laboratories. Reduced and carboxymethylated bovine serum albumin was from Sigma. Immunoprecipitation reagents were from Invitrogen. Pio was from Takeda Pharmaceuticals North America (Lincolnshire, IL). Rosi was from GlaxoSmithKline. Trog was from Parke-Davis. GW347845 was a gift of Tim Willson (GlaxoSmithKline). Polyclonal antibodies directed against GLUT-1 (AB-1340, which corresponds to bases 978–1305, respectively, and which yield a single melting point, and by electrophoresis through 2% agarose gels containing ethidium bromide of the PCR products.

Western Blot Analysis—Astrocytes were grown until confluent and then incubated in DMEM containing 5 mM glucose and 30 μM Pio or the equivalent amount of Me2SO for 6 or 24 h. Cells were washed twice with ice-cold PBS, harvested by trituration, and subcellular fractions were prepared from astrocytes on ice. Subcellular fractionation was carried out as described (51, 52). Briefly, cells were resuspended in lysis buffer (25 mM sucrose, 5 mM Na2HPO4, 2 mM EGTA, 1/100 protease inhibitor mixture (P8320; Sigma), and 20 mM HEPES, pH 7.4) and then homogenized using a Dounce homogenizer. The homogenate was centrifuged at 760 × g for 5 min to remove unbroken cells and nuclei; the supernatant was collected and centrifuged at 31,000 × g for 60 min to pellet the crude plasma membrane and collect the supernatant as cytoplasmic fraction. Protein content was determined by Bradford’s method using bovine serum albumin as standard. Ten μg of protein of each sample were mixed 1:3 with 3× gel sample buffer (150 μM Tris-HCl, pH 6.8, 7.5% SDS, 45% glycerol, 7.5% bromphenol blue, 15% β-mercaptoethanol), denatured at 70 °C for 5 min, and separated through SDS-10% acrylamide gels. Denaturation at 70 °C versus boiling reduces aggregation of GLUT-1 in gels. Apparent molecular weights were estimated by comparison to Benchmark™ prestained protein ladder standards (Invitrogen). After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic transfer. The membranes were blocked with 10% (w/v) low-fat milk in TBST (10 mM Tris, 150 mM NaCl, 0.1% (w/v) Tween 20, pH 7.6) for 1 h and incubated in the presence of antibody (anti-GLUT-1 at 1:5000 dilution, anti-GLUT-3 at 1:1000 dilution) overnight with gentle shaking at 4 °C (53). The primary antibody was removed, and the membranes were washed four times in TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody diluted 1:40,000 for GLUT-1 and 1:8,000 for GLUT-3. After four washes in TBST, bands were visualized by incubation in enhanced chemiluminescence reagents for 1 min and exposure to x-ray film for 1 min (for membranes) or 5–10 min (for cytosolic). Band intensities were determined using ImageJ software (National Institutes of Health) from autoradiographs obtained from at least two different exposure times, and background intensities (determined from an equal-sized area of the film immediately below the band of interest) were subtracted.

mRNA Analysis—Total cytoplasmic RNA was prepared from cells using TRIzol reagent (Invitrogen), aliquots were converted to cDNA using random hexamer primers, and mRNA levels were estimated by real time, quantitative RT-PCR (50). The primers used for GLUT-1 detection were 939F (5′-TGA AAG AGG AGG GTC GCC AGA TAA-3′), corresponding to bases 939–960, and 1301R (5′ AGA TGG CGA CGA TGC TCA GAT AGG-3′), complementary to bases 1278–1301 of the rat GLUT-1 cDNA sequence, which yield a 363-bp product. The primers used for GLUT-3 were 978F (5′-GGG CCG GCC GGC GAG AAG AAG-3′) and 1305R (5′-AGA AAA CCA GGA AGG CAG CAG AGA-3′), which correspond to bases 978–988 and 1281–1305, respectively, and which yield a 328-bp product. The primers used for glyceraldehyde-3-phosphate dehydrogenase detection were 796F (5′ GCC ACG TAT GAT GAC AAC ACT GAA-3′) and 1059R (5′ TCT AGG GGT CTT GGA GAA-3′), which yield a 264-bp product. The primers used for PPARγ were 642F (5′-GGG TTG CTG TGG GGA TGT CTG-3′) and 985R (5′-CGA CAC CTT GGA TGA AAA ATG-3′) from the rat PPARγ sequence NM_013241.1, and they yield a 354-bp product. Cycling conditions were 35 cycles of denaturation at 94 °C for 10 s, annealing at 61 °C for 5 s, and extension at 72 °C for 20 s, followed by 2 min at 72 °C, in the presence of SYBR Green (1:10,000 dilution of stock solution from Molecular Probes, Eugene, OR) carried out in a 20-μL reaction in a Corbett Rotor-Gene (Corbett Research, Sydney, Australia). Relative mRNA concentrations were calculated from the relative take-off point of the PCR reactions using the manufacturer’s software included in the unit. Corrected mRNA concentrations were confirmed by dissociation analysis, which yielded a single melting point, and by electrophoresis through 2% agarose gels containing ethidium bromide of the PCR products.

TZDs Modulate Brain Metabolism

C. Dello Russo and D. L. Feinstein, unpublished observations.
assay kit purchased from Biomedical Technologies Inc. (Stoughton, MA) according to the manufacturer’s instructions. Sample concentrations were calculated using a standard curve constructed by plotting relative binding values (B/Bo = cAMP bound/cAMP total) versus log of concentration (pg/ml).

Isolation and Assay of Brain Mitochondria—Rat brain mitochondria were isolated as described (54). Briefly, brains were homogenized in isolation buffer (10 mM per gram of brain, in a 50 ml centrifuge tube with a tight fitting pestle). The homogenate was centrifuged at 5,000 × g for 10 min, the supernatant was removed, and the pellet was re-extracted with isolation buffer. The two supernatants were combined and centrifuged at 16,700 × g. The upper half from this was discarded, and the lower portion combined 1:1 with 15% Percoll and mixed gently. The resulting material was layered onto a Percoll step gradient (25% on top of 40%) and centrifuged at 30,700 × g. The fraction accumulating at the interface was collected, slowly diluted 1:4 with isolation buffer, and centrifuged at 16,700 × g for 10 min to yield the final mitochondrial pellet.

Mitochondrial respiration was carried out at 30 °C and monitored as described previously (55). Respiratory control ratios measured as the ratio of state III (ADP-supported) to state IV (resting) were consistently above ten. Respiration was initiated by the addition of substrate (pyruvate, 10 mM final concentration). Calculations of respiratory rates, in units of ng atoms oxygen per min per mg, were derived from the slopes of oxygen consumption in the reaction chamber, which were linear for over 10 min.

Measurement of Mitochondrial Membrane Potential—Primary rat astrocytes were grown in 25-cm² Petri dishes at a density of about 200,000 cells/cm² in DMEM containing 1% FCS. When confluent, cells were treated with 10 μM of Pio or Trog or the corresponding amount of vehicle (Me2SO). After 6 h, the medium was removed, and cells were trypsinized and used for measurements of JC-1 aggregate fluorescence as described (56). For each sample, the measurements were made three times, and the S.D. values were within 10% of the means. The ratios of FL2 fluorescence of JC-1 aggregation to FL1 fluorescence (because of monomeric JC-1) were determined and normalized to the values obtained for incubation in vehicle alone. Complete mitochondrial depolarization was obtained by addition of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (5 μM for 5 min) to the cells, and that value was assigned as 0%.

In Vivo Experiments—Female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 250–300 g were housed in groups of four at 22 °C and in a 12-h light/dark cycle with free access to food and water. Pio was added to Purina chow 5002 at 0 or 100 ppm. Food and water were available ad libitum. The experiment was carried out two other times with similar results. Incubation with LPS significantly reduced PPAR γ mRNA levels (p = 0.0011, two-way ANOVA).

Pioglitazone Increases Glucose Utilization—Cortical rat astrocytes were incubated in low glucose (5 mM) containing medium, and glucose consumption, assessed as loss of glucose from the incubation media, was measured after different times up to 24 h in the presence of increasing amounts of Pio (0–30 μM). Control cells used glucose from the media (8.1 ± 1.0% per h), most likely because of maintenance of basal energetic needs. Pio time- and dose-dependently increased the rate of glucose utilization (9.0 ± 0.9 and 12.8 ± 0.5% per h, at 10 and 30 μM, respectively; see Fig. 2), and this effect was significant at 30 μM Pio as soon as 4 h of incubation. After 24 h, Pio-treated cells continued to show an increased amount of glucose utilization (control, 80.0 ± 16.0%; Pio with 30 μM, 103.4 ± 16.0%, which was almost 100% of the available glucose used) (data not shown).

Pioglitazone Increases 2-Deoxy-glucose Uptake—In control astrocytes, uptake of 2-DG was linear during 90 min of incubation (Fig. 3A). Pre-treatment with Pio (30 μM for 6 h) increased subsequent 2-DG uptake by −33% (from 151 ± 9 to 196 ± 3 fmol per min per mg of protein; p < 0.0001, two-way ANOVA). To characterize which glucose transporter(s) were involved in this increase, a similar experiment was performed in the presence of different amounts of cold β-glucose, and the amount of 2-DG taken up was measured after 45 min (Fig. 3B). The stimulatory effect of Pio was observed primarily at the higher doses of cold β-glucose (0.5–1.0 mM), suggesting an increased glucose flux through GLUT-1, which has a lower affinity for glucose than does GLUT-3 (53).

Effect of Pioglitazone on Glucose Transporter Expression—The effects of Pio on astrocyte glucose utilization measured after 6 h were not significantly reduced by co-incubation with either a transcriptional (5 μg/ml actinomycin D) or translational (10 μg/ml cycloheximide) inhibitor (Fig. 4), suggesting that effects on gene transcription or protein translation were not involved in this process. Consistent with this, we found that Pio treatment (6 h with 30 μM) did not modify the steady state mRNA levels of either GLUT-1, which is mainly expressed in glial and endothelial cells, or GLUT-3, which is mainly expressed in neurons (Fig. 5, A and B). Similar results were obtained after longer incubation periods (up to 24 h) (data not shown) suggesting that increases in GLUT mRNA levels were not responsible for increased glucose uptake.
TZDs Modulate Brain Metabolism

TZD Effects Involve the cAMP/Protein Kinase A Signaling Pathway—To begin to characterize possible mechanisms of action of Pio on astrocyte glucose metabolism, we tested inhibitors of several signaling pathways. The ability of Pio to increase lactate production was not modified by co-incubation with either an ATPase inhibitor (ouabain) or with a phosphatidylinositol 3-kinase inhibitor (wortmannin) (data not shown). However, incubation with a highly selective protein kinase A inhibitor (KT-5720; 100 nM) attenuated the effects of Pio (Fig. 9A). Consistent with this, we found that incubation with Trog significantly increased intracellular cAMP levels after 30 min. Treatment with Pio (10 μM) showed a tendency to increase cAMP levels at this time point, although the values were not significant (p = 0.07, unpaired t test) compared with vehicle (Fig. 9B).

TZDs Influence Mitochondrial Function—Because effects of TZDs on glucose metabolism and lactate production could be secondary consequences of effects on mitochondrial function, we tested whether TZDs directly modified mitochondrial respiration (Fig. 10). In freshly isolated rat brain mitochondria, incubation with Pio or Trog reduced the pyruvate-driven state III respiration, measured by loss of oxygen from the incubation media (Fig. 10A). The consequences of TZD inhibition of mitochondrial respiration was examined in whole astrocytes (Fig. 10B). Mitochondrial membrane potentials (ΔΨ) were measured by using flow cytometry with the fluorescent dye JC-1. Incubation with TZDs significantly increased the ratio of aggregate to monomer JC-1 fluorescence in astrocytes (30% increase by Pio, 43% increase by Trog), indicating a hyperpolarization of mitochondria. The ability of an initial inhibition of respiration to cause subsequent mitochondrial hyperpolarization has been described previously (59, 60) and may involve intramitochondrial hydrolysis of anaerobically derived ATP (see “Discussion”).

Piglittazone Reduces Astrocyte Cell Death—To test whether incubation with TZDs influenced cell viability, we tested the effects of Pio on astrocyte cell death because of glucose deprivation (Fig. 11). Astrocytes maintained for 24 h in either high (25 mM) or low (5 mM) glucose containing media showed some cell death (5 to 10%, assessed by lactate dehydrogenase release), which was not significantly reduced by co-incubation with Pio (30 μM). In contrast, overnight incubation in the absence of glucose increased lactate dehydrogenase release 4-fold over control values (to 18 ± 2%), and this was reduced to control values by Pio (p < 0.001, unpaired t test). These data suggest that the effects of TZDs on astrocytes can provide protection against hypoglycemia-induced injury.

TZD Effects on Glucose Metabolism in Vivo—To establish whether TZDs could influence brain glucose metabolism in animals, we tested the effects of acute and of chronic TZD treatment on brain sections prepared from Pio-fed adult rats (Fig. 12). Incubation of cortical sections prepared from control rats with TZDs significantly increased glucose loss from the media, similar to what we observed in primary astrocyte cultures (data not shown). We then compared glucose utilization in sections prepared from rats that were provided oral Pio (100 ppm) for 1, 3, or 10 days. The glucose utilization in sections from 1 day Pio-fed rats was not significantly different from that of control rats. However, after 3 days of treatment, the glucose utilization was ~50% greater in sections prepared from Pio-fed animals than control animals. However, this potentiating effect of orally provided Pio was transient, because after a longer period (10 days) the sections from Pio-fed animals showed a similar glucose use as did control sections. Although we do not yet know the cellular source of glucose use in these sections, these results demonstrate that oral treatment with Pio can influence brain glucose metabolism.

Analysis of GLUT-1 and GLUT-3 protein levels revealed that incubation with Pio for 6 h did not significantly increase either membrane or cytosolic levels of GLUT-1 (Fig. 6, left panels) although a slight increase (~20% increase in two experiments) in cytosolic levels was observed. However, after 24 h of incubation, we observed a significant increase (greater than 2-fold) in cytosolic levels and a slight increase (roughly 25%) in membrane levels of GLUT-1 in Pio-treated versus control astrocytes (Fig. 6, right panels). These results suggest that increased glucose utilization observed up to 6 h of incubation is not because of an increase in GLUT-1 levels but more likely because of an increase in glucose flux through pre-existing GLUT-1 protein.

Figlittazone Increases Lactate Production—Rat cortical astrocytes were incubated in DMEM containing 5 mM glucose, and lactate levels in the incubation media were measured after different times (Fig. 7). Treatment with Pio significantly increased lactate production (p < 0.0001, two-way ANOVA), suggesting that Pio influences glucose metabolism, as well as its uptake.

TZD Effects Are Not Mediated by PPARγ Activation—Although Pio can bind to and activate PPARγ, it can elicit PPARγ-independent effects, as well. We therefore examined the effects of several different TZDs on astrocyte lactate production (Fig. 8). The order of efficacy for lactate production was found to be Trog > Pio > Rosi, which is the inverse order of their reported efficacies for activating the PPARγ. Furthermore, co-incubation with GW347845, a high affinity, non-TZD PPARγ agonist (7), failed to increase lactate production (Fig. 8A). Indeed, even at a concentration of 10 μM, at which dose Pio shows only a slight increase in glucose utilization, Trog significantly increased glucose utilization (Fig. 8B) whereas GW347845 had no effects. Together, these results suggest that the effects of Pio (and other TZDs) on astrocyte lactate and glucose metabolism could be mediated by a mechanism of action not involving PPARγ activation, in contrast to their more well characterized anti-inflammatory actions (20, 58). In contrast, inflammatory activation of astrocytes by LPS, as assessed by expression of NOS2 and nitrite production (see Fig. 8C), was inhibited by TZDs in approximately the same order of potency as their ability to activate PPARγ (GW347845 > Rosi > Pio > Trog).

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DISCUSSION

Our data demonstrate for the first time that PPAR\textsubscript{\textgamma} agonists of the TZD family can modify glucose metabolism in brain glial cells, despite the fact that the regulation of glucose metabolism in brain is primarily insulin-independent. TZDs increased astrocyte glucose uptake from the media and lactate production and release from the cells. The mechanism underlying this increase in glucose metabolism is not yet clear; however, in contrast to other studies, our results do not support an effect of TZDs, at least at early time points, on glucose transporter expression but instead point to rapid events occurring at the mitochondrial level. Furthermore, our results suggest that the effects of TZDs on astroglial cell physiology are not mediated by PPAR\textsubscript{\textgamma} activation, because the most effective TZD was troglitazone, which of the TZDs tested has the lowest efficacy of PPAR\textsubscript{\textgamma} activation (7), and because the highly selective PPAR\textsubscript{\textgamma} agonist GW347485 was ineffective. Together, our data suggest the existence of a novel rapid and non-genomic action of TZDs upon astroglial cell physiology, which may involve changes in intracellular cAMP. These findings raise the possibility that TZDs could be used therapeutically to increase astroglial energy stores, as well as their ability to provide lactate to neurons when needed.

\textbf{FIG. 3.} \textit{Pio increases 2-deoxy glucose uptake.} A, astrocytes were pre-incubated in DMEM with 5 \textmu M glucose containing 0 or 30 \textmu M Pio. After 6 h, cells were incubated in DMEM containing 0.5 \texttextmu M glucose and 1 \textmuCi/ml 2-DG (24 nm) for indicated times. Data are means ± S.E. of \textit{n} = 3–6 replicates and are expressed as pmol of 2-DG per mg of protein. ***, \textit{p} < 0.001 versus control (two-way ANOVA, Bonferroni multiple post hoc comparison). B, astrocytes were treated as in A, after which 2-DG uptake was measured in the presence of different concentrations of cold \textit{n}-glucose. Data are means ± S.E. and are expressed as nmol of total glucose taken up per mg of protein. *, \textit{p} < 0.05; ***, \textit{p} < 0.001 versus control (two-way ANOVA, Bonferroni multiple post hoc comparison).

\textbf{FIG. 4.} \textit{Transcriptional and translational inhibitors do not prevent Pio effects.} Cortical rat astrocytes were coincubated with a transcriptional (5 \textmu g/ml actinomycin D) (Act D) or translational (10 \mu g/ml cycloheximide (CHX)) inhibitors in the presence of DMEM containing 5 \textmu M glucose and 0 or 30 \textmu M Pio. Glucose utilization was assessed after 6 h as loss of glucose from the media. The data are means ± S.E. of \textit{n} = 4 samples and are expressed as % loss of starting glucose levels. ***, \textit{p} < 0.001 versus no Pio (one-way ANOVA followed by Newman-Keuls multiple comparison test).

\textbf{FIG. 5.} \textit{Effect of Pio on glucose transporter mRNA expression.} Astrocytes were incubated in DMEM containing 5 \textmu M glucose in the presence of 0 or 30 \textmu M Pio. After 6 h, total cytosolic RNA was prepared and used for RT-PCR analysis of Glut-1, Glut-3, and glyceroldehyde-3-phosphate dehydrogenase (GDH) mRNA levels. A, representative agarose gel electrophoresis showing levels of GLUT-1 and GLUT-3 PCR products from control and Pio-treated samples (results for two different samples from each group, each done in duplicate, are shown). B, results of real-time PCR analysis showing the mean ± S.E. of the calculated cycle number take-off values (\textit{n} = 4 replicates per group).

\textbf{FIG. 6.} \textit{Effect of Pio on GLUT-1 expression.} Astrocytes were incubated 6 (left panels) or 24 h (right panels) in complete media containing 0 or 30 \textmu M Pio. Whole cell lysates were prepared and used to isolate membrane and cytoplasmic fractions, and equal aliquots were used for Western blot analysis of GLUT-1. In each panel, membrane and cytoplasmic samples from different preparation are shown (C1 and P1). The bottom bar graphs show calculated band densities, and the error bars represent analytical error in the densitometric analysis for each sample. Note that film exposure times were 1 min for membrane samples and 5 or 10 min for cytoplasmic samples, and therefore the relative intensities cannot be compared directly.
TZDs Modulate Brain Metabolism

It has been shown that astrocytes are the main source of energy for neuronal activity (43). Therefore, in this study we examined the effect of Pio on glucose metabolism using primary enriched cultures of rat cortical astrocytes. In this model Pio increased glucose utilization as assessed by loss of glucose from the culture media and glucose uptake as measured by influx of radiolabeled 2-DG. Because the delivery of glucose within the brain is mediated primarily by GLUT-1, present in the blood-brain barrier, as well as in glial cells, and to a smaller extent by GLUT-3 presents in neurons (42), we tested whether Pio modified GLUT-1 or GLUT-3 expression. Measurements of 2-deoxyglucose uptake suggested an increased flux through GLUT-1, because significant increases due to Pio occurred at the higher but not the lower glucose concentrations tested (53). In other systems, the regulation of GLUT-1 expression is under both transcriptional and post-transcriptional control (61, 62). However, in our studies the increase in glucose utilization observed at 6 h was not reduced by co-treatment with cycloheximide or actinomycin D, and measurements of GLUT-1 and GLUT-3 mRNA levels did not reveal significant effects due to Pio treatment at either early (6 h) or later (24 h) time points, again suggesting that glucose-modifying effects are mediated by non-genomic mechanisms.

In insulin-sensitive cells, about 50% of the GLUT-1 and most of the GLUT-4 is located in intracellular vesicles, and both are translocated to the plasma membrane in response to insulin (63). Because TZDs can increase GLUT-4 translocation from the intracellular pool to the plasma membrane (51) we assessed whether Pio modified either the cytosolic or membrane content of GLUT-1. However, no significant differences were observed after 6 h of incubation, whereas a slight increase in both cytoplasmic and plasma membrane content of GLUT-1 was observed after 24 h of incubation. We conclude that the effects of Pio on glucose uptake at early time points (within 6 h) are not associated with significant changes in glucose transporter expression. However, at later times, an increase in GLUT-1 content could contribute to or account for the increased rate of metabolism within the cells.

Our data also suggest that the effects of TZDs on astrocyte metabolism are not mediated by PPARγ activation but instead by rapid activation of an intracellular signaling system, possibly leading to modification of mitochondrial function. Measurements of lactate production show that the order of efficacy for increasing production was Trog > Pio > Rosi, inverse of their reported affinities for binding and activating PPARγ. We also found that Trog (at 10 μM) was the only TZD able to increase glucose utilization and moreover that the high affinity PPARγ agonist GW347845 had no effect on glucose metabolism. In contrast, in the same cells the TZDs reduced LPS-dependent nitrite production (presumably because of increased NOS2 expression) with efficacies similar to their ability to activate PPARγ, indicating the presence of functional PPARγ on these cells.

Several studies (64–66) have now shown that TZDs can
trigger specific effects within a relatively short time range, consistent with the existence of transcription-independent mechanisms of action. Incubation with 15-deoxy-D_{12,14}-prostaglandin J2 (as well as Pio and Trog) induces a rapid and transient activation of the mitogen-activated protein kinase/ extracellular signal-regulated kinase pathway in vascular smooth muscle cells by an activation of phosphatidylinositol 3-kinase (65) suggesting that some actions of TZDs might be mediated via this protein kinase. However, incubation of astrocytes with a phosphatidylinositol 3-kinase inhibitor (wortmannin) had no significant effect on the Pio-dependent increases in glucose uptake or lactate production. Because glucose uptake and the rate of glycolytic metabolism are increased by activation of Na\(^+\)/K\(^+\) ATPase in astrocytes (67) we also tested the effect of a Na\(^+\)/K\(^+\) ATPase inhibitor (ouabain). However, under

Fig. 9. Pio affects glucose metabolism via the cAMP/protein kinase A pathway. A, the stimulatory effect of Pio (30 \(\mu\)M) on lactate was reversed by a selective protein kinase A inhibitor (KT-5720) used at 100 \(\mu\)M (Ki = 56 \(\mu\)M). Data were expressed as mg/100 ml of lactate and were analyzed by one-way ANOVA, followed by Newman-Keuls multiple comparison test. **, \(p < 0.01\) versus control. B, astrocytes were incubated in low glucose DMEM for 30 min in the presence of 10 \(\mu\)M Pio or Trog, or vehicle alone, after which intracellular cAMP levels were determined. Trog (10 \(\mu\)M) increased cAMP levels (1.86 ± 0.5 versus 0.08 ± 0.02 pg of cAMP per ml of Trog and Me\(_2\)SO, respectively, \(p < 0.05\), unpaired \(t\) test); Pio (10 \(\mu\)M) induced a slight but non-significant increase (to 0.29 ± 0.08, \(p = 0.07\), unpaired \(t\) test).

Fig. 10. TZDs modify mitochondrial function. A, freshly isolated rat brain mitochondria was incubated in the presence of the indicated concentrations of Pio or Trog or the appropriate amount of Me\(_2\)SO vehicle. State III respiration was initiated by addition of pyruvate, and oxygen consumption was measured over the next 10 min. The data are means ± S.E. of three-four experiments. B, astrocytes were incubated for 6 h in 10 \(\mu\)M Pio or Trog, or vehicle only. At that time the mitochondrial membrane potential was determined by fluorescence measurement of JC-1 aggregation state. The data are means ± S.D. of \(n = 3\) experiments. **, \(p < 0.05\); ***, \(p < 0.01\) versus vehicle, unpaired \(t\) test.

Fig. 11. Pio reduces hypoglycemia-induced cell death. Astrocytes were incubated in DMEM containing 1% FCS and either 0, low (5 mM), or high (25 mM) glucose and in the presence or absence of Pio (30 \(\mu\)M). After 24 h, lactate dehydrogenase release and total lactate dehydrogenase levels were determined. Data are means ± S.E. of three-four measurements. ***, \(p < 0.001\), unpaired \(t\) test.

Fig. 12. Pio increases glucose utilization in rat cortical slices. Cortical slices were prepared from adult rats kept on a diet containing Pio for 1, 3, or 10 days and further incubated in vitro. Glucose levels in the incubation media were measured after 4 h. Data are expressed as % of glucose utilization and are means ± S.E. of \(n = 4\) replicates per group. The same experiment was carried out twice. Data are analyzed by unpaired \(t\) test. *, \(p < 0.05\) versus control.
the conditions used, ouabain did not reverse Pio effects.

In contrast, we found that inhibition of protein kinase A with the selective inhibitor KT-5720 (100 nM) significantly reversed the metabolic effects of Pio. Consistent with this, we also found that astrocyte cAMP levels were increased (albeit slightly) after 30 min of incubation with Pio and significantly increased upon incubation with Trog. To our knowledge these results are the first demonstration of an effect of TZDs on intracellular cAMP levels. The greater effect due to incubation with Trog is consistent with our findings that Trog more potently increased glucose utilization and lactate production than did Pio. However, further studies are needed to better characterize the effects of TZDs on the kinetics and magnitude of intracellular cAMP levels. There are numerous pathways linking changes in cAMP to effects on glucose metabolism. For example chronic treatment with cAMP analogues increased glucose utilization by up-regulating GLUT-1 expression in human choriocarcinoma cells (68), although opposite results were observed in primary mouse placental cells (69). In insulin-sensitive tissues, several studies have shown that glucose uptake was enhanced by regulation of GLUT-1 expression through the cAMP/protein kinase A pathway. However, in myoblasts these effects were observed only after 24 h (70) and only at the higher concentrations (within the mM range) of cAMP analogues (71). Similarly, cAMP increased glucose uptake in 3T3-L1 adipocytes by increasing GLUT-1 protein content in the plasma membrane after 8 (72) or 16 h (73). Interestingly, in brain adipocytes norepinephrine enhanced glucose transport across the membrane by a mechanism involving cAMP and increased GLUT-1 affinity for its substrate (63). Taken together, these reports indicate that glucose uptake can be regulated by cAMP via several distinct mechanisms. Finally, and in light of the fact that TZDs directly inhibit mitochondrial respiration, reports demonstrating inhibition of the pyruvate dehydrogenase complex by cAMP-dependent phosphorylation of pyruvate dehydrogenase kinase (74, 75) suggest an additional mechanism to account for suppression of pyruvate-driven respiration.

It has been shown that the glutamate released upon neuronal activity is taken up by astrocytes, causes an increase in astroglial glycolysis, and subsequently increases the uptake of glucose from the incubation media and lactate release into the media, thereby coupling in this way neuronal activity with energy metabolism (43). We therefore hypothesized that increased glucose uptake because of Pio might also increase lactate production by astrocytes. We observed that Pio induced a rapid increase in lactate production that was evident as soon as after 2 h of treatment, suggesting that the effect of Pio is to increase glucose metabolism and subsequently cause uptake from extracellular pools. A similar effect has been demonstrated in rat muscle cells in which inhibition of insulin-stimulated mitochondrial fuel oxidation by Trog leads to an increase of anaerobic glycolysis and glucose transport (76).

Our current findings demonstrate that TZDs also directly inhibit the ability of acutely isolated brain mitochondria to catalyze pyruvate-driven state III respiration, consistent with previous findings that TZDs directly inhibit mitochondrial fatty acid metabolism (64, 76). Although inhibitory effects on mitochondrial respiration are difficult to reconcile with subsequent protective actions, it has been hypothesized previously (56, 60) that the increase in anaerobically derived ATP due to mitochondrial impairment by nitric oxide subsequently contributes to sustained mitochondrial membrane potential, and we observe a similar phenomenon after 6 h of incubation with TZDs. Furthermore, mitochondrial hyperpolarization has been shown to protect cells against cytotoxic damage, and in our experiments we find that treatment with Pio reduced astrocyte cell death due to hypoglycemia.

Based upon the above considerations, we propose the following scheme to explain the ability of TZDs to increase astroglial glucose metabolism. A similar scheme has been proposed for the ability of low doses of nitric oxide to inhibit initial oxidative respiration but that eventually lead to higher intracellular ATP levels and protective effects. We suggest that after entry into the cell, TZDs modulate (either directly or possibly by a mechanism involving cAMP) enzyme activities present within the mitochondrial membrane causing inhibition of ongoing pyruvate-driven respiration. Whether this is a selective effect on pyruvate oxidation (i.e. by inhibition of pyruvate dehydrogenase) or is a more general inhibition of mitochondrial substrate utilization is not yet known. Increased cytosolic pyruvate results in greater lactate production, observed as an increase in extracellular lactate levels. In astrocytes, this inhibitory effect on mitochondrial function is compensated by an increase in anaerobic glycolysis allowing for continued ATP production, as well as further pyruvate and lactate synthesis. Eventually, the reduced intracellular glucose levels are replenished by glucose transport through the GLUT-1 because of mass action through the transporter and without significant change in GLUT-1 levels (at least initially).

At later times, mitochondrial respiration recovers, because the actions of TZDs are neither toxic nor irreversible. Accumulated ATP can be translocated via the adenine nucleotide translocase into the mitochondria, where hydrolysis to ADP releases protons that contribute to maintenance and increase of the membrane potential. Because hyperpolarization of the mitochondrial membrane is postulated to be protective, for example by conferring resistance to depolarization and release of pro-apoptotic factors, the net result of TZD treatment, at least in astrocytes, is protective and allows cells to withstand subsequent noxious stimuli.

It has been shown recently, both in vitro and in vivo, that PPARγ activation can exert anti-inflammatory effects (25), regulate myelin gene expression (33), and increase neurite extension (32) in brain cells. For these reasons, PPARγ agonists, including TZDs like Pio and Rosi, are being considered as potential neuroprotective agents in neurodegenerative disorders, including Alzheimer’s disease, Parkinson’s disease, stroke, and multiple sclerosis. It is possible that increases in intracellular glucose could contribute to the anti-inflammatory effects by increasing expression of stress proteins, such as inhibitory IκB proteins, which would reduce glial inflammation. However, because glucose metabolism is perturbed in the early stages of Alzheimer’s disease (77–79) and in multiple sclerosis (80–82), the effects described herein suggest an additional mechanism, i.e. maintenance of brain energy supplies, by which TZD treatment could provide benefit.

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