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Receptor-independent actions of PPAR thiazolidinedione agonists: Is mitochondrial function the key?

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Abstract

Agonists of the peroxisome proliferator activated receptor gamma (PPARγ) are currently used for treatment of type 2 diabetes due to their insulin sensitizing and glucose metabolism stabilizing effects. More recently some of these same agonists were shown to exert anti-inflammatory and anti-proliferative effects as well. Although PPARγ agonists can operate via receptor-mediated events occurring at the genomic level, thereby causing long lasting changes in gene expression patterns, recent studies demonstrate non-genomic as well as genomic actions, and receptor-dependent as well as receptor-independent effects of the thiazolidinedione (TZD) class of PPARγ agonists. In this review we will summarize data describing some of these novel, receptor independent actions of TZDs, review evidence that TZDs directly influence mitochondrial function, and attempt to reconcile how changes in mitochondrial function could contribute to other receptor-independent actions of these drugs.

Keywords: Mitochondria; Glucose; Thiazolidinedione; Fatty acid; Insulin; Stress response; AMPK; Cytokines; Nitric oxide; Metabolism; Mitoneet

1. Introduction

The peroxisome proliferator activated receptor gamma (PPARγ) is a member of the PPAR family, of which three major isoforms have been identified to date (α, β/δ, and γ) with splicing variants now reported for all three subtypes [1–3]. The receptor isoforms show tissue and cell specificity, and upon activation exert related but distinct functions. PPARγ has been the most well characterized, in part due to its therapeutic potential for treatment of diabetes ([4–6] for recent reviews) and related consequences such as metabolic syndrome [7]. PPARγ agonists can be broadly broken down into two major classes, thiazolidinediones (TZDs), and non-TZDs. There are a variety of structurally distinct non-TZD agonists, including synthetic tyrosine based compounds [8], several long chain fatty acids [9], the cyclopentenone prostaglandin 15-deoxy $\Delta^{12,14}$ prostaglandin (PG) J2 (PGJ_2, reviewed in [10]), and certain non steroidal anti-inflammatory drugs including ibuprofen and indomethacin [11] which bind to PPARγ at concentrations higher than those at which they inhibit cyclooxygenase (COX)-2. Although PGJ_2 is

Abbreviations: AICAR, AMPK activator 5-aminoimidazole-4-carboxamide riboside; AMPK, adenosine 5′-monophosphate-activated protein kinase; AP1, activator protein 1; AUC, area under the curve; CBP, CREB-binding protein; CNS, central nervous system; COX, cyclooxygenase; CPT1, carnitine palmityl transferase 1; CREB, cyclic AMP-responsive element binding protein; DMSO, dimethyl sulfoxide; EGR-1, early growth response-1; ERK, extracellular signal regulated kinase; FA, fatty acid; FFA, free fatty acid; GADD45, growth arrest and DNA damage-inducible gene; GLUT, glucose transporter; HL, human leukemia; HSP, heat shock protein; HSR, heat shock response; IFN, interferon; IκB, Inhibitor of nuclear factor kappa B; IKK, IκB kinase; IL, interleukin; JNK, c-Jun NH2-terminal protein kinase; LPS, lipopolysaccharide; MalDC, malonyl-CoA decarboxylase; MAPK, mitogen-activated protein kinases; NAG-1, nonsteroidal anti-inflammatory drug-activated gene-1; NFκB, nuclear factor kappa B; NOS, nitric oxide synthase; PC, pyruvate carrier; NOS2, inducible form of NOS; PDH, pyruvate dehydrogenase; PG, prostaglandin; PGC1α, PPARγ coactivator 1α; PGJ2, prostaglandin J2; Pio, pioglitazone; PK, protein kinase; PPARγ, peroxisome proliferator activated receptor gamma; PPRE, PPAR DNA binding elements; ROS, reactive oxygen species; Rosi, rosiglitazone; siRNA, small interfering RNA; TNF, tumor necrosis factor; Trog, troglitazone; TZD, thiazolidinedione; VCAM-1, Vascular cell adhesion molecule-1; $\Delta\psi_m$, delta psi; mitochondrial membrane potential

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considered a potent, endogenous PPARγ agonist, we will not review its literature here since many of its effects may be due to its ability to directly inhibit the activity of the inhibitor of nuclear factor kappa B (IκB) kinase (IKK) and thereby prevent IκB degradation and nuclear factor kappa B (NFκB) activation [12,13]. The TZD agonists (also referred to as glitazones) include the anti-diabetic drugs troglitazone (Trog), pioglitazone (Pio), and rosiglitazone (Rosi); the latter two currently being used for treatment of type 2 diabetes. Although TZDs were originally approved for treating diabetes, they may have important implications for treating metabolic disease in general, cancer, and various inflammatory and neurodegenerative diseases [14]. A complete understanding of their mechanism of action is therefore key for developing next generation drugs.

2. Examples of receptor-independent actions of TZDs

TZDs have been reported to exert receptor dependent as well as receptor independent effects, as have several PPARγ antagonists [15]. Conclusions that TZDs can exert receptor-independent effects have been based on several criteria, including that: (1) concentrations needed to observe TZD actions were much greater than reported EC_{50} values; (2) the rank order of efficacy for TZDs (Trog > Pio > Rosi) to elicit a response was inverse to their known binding affinities (measured EC_{50} values) for PPARγ; (3) non-TZD agonists showed little or no effect; (4) PPARγ antagonists did not block TZD effects; (5) effects occurred rapidly (within minutes to hours); (6) effects occurred in the absence of PPARγ expression or of PPAR DNA binding elements (PPRE) in gene promoters; (7) effects occurred in the presence of transcriptional or translational inhibitors. Reported receptor independent effects include suppression of inflammatory gene expression; modification of energy and fuel metabolism; suppression of cell proliferation and induction of cytotoxicity; and perturbation of mitochondrial function.

2.1. Anti-inflammatory effects

PPAR agonists have now been shown to reduce inflammatory gene activation in a variety of tissues and cells, which has provided the basis for consideration of these drugs for therapeutic use in inflammatory diseases ([14,16–22] for recent reviews). PPARγ TZD agonists have been shown to inhibit different immune functions of macrophages [23]; endothelial cells [24]; and T and B lymphocytes and dendritic cells [25]. TZDs can also exert inhibitory actions within the central nervous system (CNS), directly affecting glial cell [26] and perhaps neuronal cell activation [27]. Observations that some anti-inflammatory treatments replicate the insulin sensitizing effects of TZDs further point to a common mechanism for these drugs [28,29]. However, despite a large number of in vitro and in vivo studies, whether the anti-inflammatory effects of TZDs are due to PPARγ activation remains unclear.

Table 1
Ligand binding affinities for PPARγ

Agonist	EC_{50} (μM)
Rosiglitazone	0.076
Pioglitazone	0.55
Troglitazone	0.78
Ciglitazone	3

EC_{50} values determined using reporter gene chimera genes, for the mouse PPARγ (from ref. [141]).

In vitro, the anti-inflammatory effects of TZDs are often observed at concentrations much higher than their EC_{50} values for binding to or activating PPARγ ([8,141] and see Table 1). For example, Trog reduced cytokine release from activated human monocytes [30] and Trog and Rosi inhibited induction of the inducible form of nitric oxide synthase (NOS2) in activated macrophages [31]; however, inhibitory concentrations needed were 15 to 60-fold (for Trog) and 650-fold (for Rosi) higher than their respective EC_{50} values. High concentrations of TZDs were also required to reduce cytokine release from activated T cells [32]. In endothelial cells, 20–100 μM Trog reduced the expression of proinflammatory adhesion molecules [33] and interferon (IFN)γ dependent induction of several proinflammatory chemokines [34].

In addition to requiring high concentrations, in some studies the rank order of potency to elicit a response (Trog > Pio > Rosi) was inverse of the relative ability to bind to PPARγ (Rosi > Pio > Trog > Ciglitazone), also arguing against a PPARγ dependent mode of action. Thus, in human monocytic THP-1 cells, Trog inhibited phorbol-ester induced tumor necrosis factor (TNF)-α release, while Rosi and Pio were ineffective [35]. Likewise, Trog and ciglitazone inhibited vascular cell adhesion molecule-1 (VCAM1) expression in endothelial cells and monocyte adhesion, but Rosi was found ineffective [36].

Although the above argues for PPARγ independent effects, the ability of a TZD to bind to and activate PPARγ may be influenced by cell properties which limit ligand access to the receptor, or by differential breakdown of individual TZDs, thus requiring higher doses or yielding inverse orders of efficacy. In addition, one mechanism by which PPARγ can inhibit ongoing inflammatory gene expression is via 'trans-repression', in which ligand bound, activated PPARγ sequesters transcriptional cofactors (such as cyclic AMP-responsive element binding protein (CREB)-binding protein (CBP) and p300) that are also needed by other transcription factors including NFκB and activator protein-1 (AP1) [37]. This mechanism requires elevated levels of receptor occupancy and higher agonist

concentrations in order to efficiently sequester the bulk of coactivators [38].

More direct evidence for PPARγ independent effects comes from studies in which PPARγ antagonists were unable to block anti-inflammatory actions. Thus, Pio (10–20 μM) inhibited interleukin (IL)-1β dependent monocyte adhesion to human umbilical vein endothelial cells, while PGF2α, a prostaglandin which can inactivate PPARγ [39] did not reverse this effect [40]. High concentrations (>10 μM) of Rosi and Pio increased lipopolysaccharide (LPS)-stimulated prostanoid (thromboxane A2 and PG E2) levels, however, PPARγ antagonists did not reverse this effect; and similar increases were observed in PPARγ-deficient cells [41]. The expression of nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) is induced by Trog, most likely via direct activation of the transcription factor early growth response-1 (EGR-1) [42], and was not blocked by a PPARγ antagonist. Finally, since inflammatory gene expression can be regulated by mitogen-activated protein kinases (MAPKs) [43], findings that TZDs directly activate ERK1/2 (extracellular signal regulated kinase) ([18,44,45]) provide another means by which TZDs can reduce inflammation in a PPARγ independent manner.

Additional evidence that TZD anti-inflammatory effects may not depend on PPARγ comes from studies using PPARγ null cells. Treatment of macrophages derived from PPARγ null embryonic stem cells with different TZDs inhibited NOS2 and COX2 expression, and reduced TNFα and IL6 release induced by IFNγ. The effects of TZDs observed in the PPARγ knockout cells were approximately the same as in wild type macrophages [46]. Similarly, the LPS/IFNγ induced NOS2 was inhibited by relatively high doses of PPARγ agonists (10 μM) in mouse mesangial cells following transfection with a dominant-negative PPARγ construct, as was NOS2 expression in macrophages prepared from PPARγ conditional knockout mice [47]. In a related study in which PPARγ null macrophages were compared to wild type cells, the anti-inflammatory effects of Rosi (at >10 μM) were concluded to be due to cross-activation of the PPARδ isotype [48]. Thus, although anti-inflammatory effects are observed in the absence of PPARγ, at higher doses activation of other receptors may come into play.

Other means by which TZDs can reduce inflammatory activation exist, and most likely additional pathways will be uncovered. The ability of TZDs to induce a stress response is a potent means to induce anti-inflammatory (as well as anti-proliferative) effects, and this pathway will be discussed in detail later on. A more novel means of reducing NFκB activation was recently reported [49], in which ligand binding to the PPARγ activates MAPKs, induces phosphorylation of PPARγ, and facilitates a direct interaction of the phosphorylated PPARγ with the NFκB p65 subunit thereby blocking NFκB transcriptional activity.

2.2. Effects on glucose and lipid metabolism

Several TZDs (Pio and Rosi) are now being used as oral antidiabetic agents ([4–6] for recent reviews). TZDs enhance or partially mimic certain actions of insulin, causing a slowly generated anti-hyperglycaemic effect in type 2 (noninsulin dependent) diabetic patients. This is often accompanied by a reduction in circulating concentrations of insulin, triglycerides and nonesterified fatty acids. The glucose-lowering effect of TZDs is attributed to increased peripheral glucose disposal and decreased hepatic glucose output. This has been generally assumed to be due to the ability of TZDs to bind to and activate PPARγ which is known to increase transcription of certain insulin-sensitive genes [50,51]. However, although TZDs show a positive correlation between their binding affinity for PPARγ and their antihyperglycaemic activity, their interactions with PPARs alone may not provide a full explanation for the spectrum of their biological effects. For example, the predominant expression of PPARγ in adipose tissue compared to liver or muscle suggests that this may be the major site of action of TZDs and that free fatty acids (FFAs) may mediate effects on other tissues. However, in aP2/DTA mice, whose white and brown fat is virtually eliminated by fat-specific expression of diphtheria toxin A chain, Trog alleviated hyperglycemia, and decreased elevated insulin levels without affecting PPARγ mRNA levels in liver, suggesting independence from both adipose tissue and PPARγ [52].

In several studies, the rapid occurrence of agonist-induced metabolic effects, and the lack of correlation between metabolic effects and the affinity for PPARγ suggest a PPARγ independent mechanism. Trog rapidly enhanced glycogen synthase-1 activity in myocytes, and inhibited gluconeogenesis in hepatocarcinoma cells [53]. Similarly, Trog and Rosi rapidly (within 2 h) inhibited lipoprotein lipase activity in adipocyte cell lines and rat adipocytes in culture. Despite the decrease in enzyme activity, TZDs increased lipase mRNA levels in undifferentiated adipocytes but had no effect on mRNA expression or synthesis of lipoprotein lipase in differentiated cells [54]. In hepatocytes isolated from starved rats, Trog rapidly inhibited fatty acid (FA) oxidation and esterification as well as gluconeogenesis [55]. In primary cultured rat cardiomyocytes, acute treatment (30 min) with Trog inhibited phorbol ester-mediated increase in membrane associated protein kinase (PK)C activity without affecting the basal distribution of PKC activity [56].

Effects of TZDs on cholesterol levels also points to receptor independent actions [57]. Trog, ciglitazone and englitazone all inhibited cholesterol biosynthesis in Chinese hamster ovary cells, HepG2 hepatocarcinoma cells, 3T3-L1 adipocytes and rat myoblastic L6 cells. Inhibition was observed as soon as 2 h and reversed within 1 h after drug was withdrawn. Neither actinomycin-D nor cycloheximide affected inhibition of cholesterol synthesis by

Trog, suggesting that this inhibition does not require de novo mRNA or protein synthesis. Finally, the rank order of potency for inhibition of cholesterol synthesis was: ciglitazone > englitazone > Trog > Pio > Rosi, which is inconsistent with their affinities for PPARγ: Rosi > Pio > Trog > ciglitazone > englitazone.

Measurements of fuel utilization in isolated muscle strips [58] further indicated receptor independent effects of TZDs. Thus, Trog inhibited CO_2 production from extracellular as well as intracellular substrate stores under basal conditions. Exposure to Trog for 30 min increased the rate of insulin stimulated lactate release, while after 60 min the lactate release was accompanied by significant reductions in mitochondrial fuel oxidation and glycogen storage. Inhibition of fuel conversion to CO_2, and increased anaerobic glycolysis and glycogen depletion in muscle strips from severely insulin resistant Zucker rats indicated that these actions of Trog are independent of insulin. Failure of actinomycin-D and cycloheximide to block Trog inhibitory action on fuel oxidation and glycogen synthesis showed that these effects were independent of gene expression. All six PPARγ TZD agonists tested shifted fuel utilization from aerobic to anaerobic pathways, with Trog triggering the most robust response, and the high affinity PPARγ agonist Rosi showing the weakest response. In these studies, non-TZD PPARγ agonists did not modulate fuel metabolism as observed with TZDs [59].

2.3. Effects on tumor cell survival

The initial observation that PPARγ agonists mediated adipocyte differentiation led to investigations of potential antiproliferative effects of PPARγ ligands. In the past few years there has been a substantial accumulation of experimental data supporting a key role of PPARγ ligands in inhibiting proliferation in several types of cancer cells (see [60–62] for recent reviews). However, as for other PPARγ agonist effects, the role of PPARγ activation remains controversial. Among TZDs, the anti-tumorigenic activity of Trog has been best established. Trog inhibits proliferation of a variety of human cancer cells including colorectal, prostate, leukemia, and glioblastoma. Analysis of possible mechanism of actions in most cases points to receptor independent effects.

In MCF-7 breast cancer cells, apoptosis induced by Trog was dependent upon GADD45 expression [63]. The authors showed that regulation of GADD45 by Trog occurred at the transcriptional level and was associated with earlier activation by MAPKs; specifically, GADD45 was positively regulated by the c-Jun kinase (JNK) and negatively modulated by ERK 1/2 and p38. In these studies, neither Pio nor Rosi induced GADD45 expression suggesting a receptor-independent action of Trog.

Similarly, the proapoptotic activity of Trog in HCT-116 cells was shown to be dependent on tumor suppressor EGR-1 gene expression [64]. Inhibition of ERK phosphorylation suppressed EGR-1 induction by Trog suggesting that MAPK activation has an important role in the antiproliferative activity of Trog. The authors showed that other PPARγ ligands could not induce EGR-1 and furthermore that ERK activation itself could downregulate PPARγ activity, again suggesting a receptor independent mechanism for Trog toxicity in colon cancer cells. Moreover, ERK activation has been shown to mediate Trog antiproliferative activity in the HCT-15 colon cancer cell line [65], where rapid phosphorylation of ERK (within 15 min) suggested a direct action of Trog, followed by induction of the cyclin-dependent protein kinase inhibitor p21.

Receptor-independent effects of TZDs were also reported in human leukemia (HL) cell lines. In HL-60 cells [66] Trog induced cell arrest and subsequent cell death and was associated with downregulation of c-myc, c-myb and cyclin D2 expression. Since these genes lack a PPRE in their promoter regions, the effect could not be directly PPARγ mediated. Similar findings in a human basophilic leukemia cell line [67] suggested that Trog suppressed cell growth independently of PPARγ via a decrease in cyclin E levels and hyperphosphorylation of retinoblastoma tumor suppressor gene product.

More direct evidence that the cytotoxicity induced by TZDs is independent of PPARγ comes from receptor knockout studies. In PPARγ (−/−) and PPARγ (+/+) mouse embryonic stem cells inhibition of tumor growth by two TZDs, Trog and ciglitazone, was independent of PPARγ [68]. These compounds blocked the G1/S transition by inhibiting translation initiation as a consequence of partial depletion of intracellular calcium stores and resulting activation of PKR, a kinase that phosphorylates the alpha subunit of eukaryotic initiation factor 2, thus rendering it inactive.

Several studies describe the ability of PPARγ ligands to induce glioma cell toxicity, again in a receptor independent manner. In human glioblastoma T98G cells, a novel PPARα/γ dual TZD agonist induced apoptosis, as a consequence of cell cycle arrest [69]. Cell cycle arrest was associated with an increase of p27 levels and the apoptotic events were mediated by downregulation of anti-apoptotic Bcl-2 protein and upregulation of pro-apoptotic Bax protein and caspase-3 activation. In these studies, cytotoxicity was not blocked with either a specific PPARα or PPARγ antagonist.

The mechanisms by which TZDs induce glioma toxicity were investigated in another study [70] using ciglitazone and Rosi to promote cell death in human and rodent glioma cell lines. While ciglitazone (20 μM) was toxic for normal primary astrocytes as well as glioma cells, Rosi at the same dose killed only the glioma cells. The rapidity of mitochondrial damage and the failure to revert cytotoxic effects using the specific PPARγ antagonist GW9662 suggested that receptor activation was not involved. In our own studies using rat C6 glioma cells, mouse GL261 glioblastoma cells, and primary astrocytes we also found that Trog

(and Pio to a lesser extent) induced apoptosis in the transformed cells accompanied by rapid (within hours) decreases in mitochondrial membrane potential ($\Delta\psi_m$) and production of radical oxygen species (ROS), but had little effect in the non-transformed cells (Spagnolo et al., unpublished observations). The basis for differential effects of TZDs on normal versus transformed cells is not yet clear but may be related to the fact that tumor cells are intrinsically under greater oxidative stress (the Warburg effect, and see below).

Interestingly, several studies have shown that in contrast to preventing cytotoxic effects of TZDs, structurally related PPARγ antagonists themselves can induce cell death. Thus, the irreversible PPARγ antagonist GW9662 did not prevent Rosi-induced cell death, and alone induced cell death in different human breast cancer cell lines [71].

2.4. Physiological levels of TZDs

Several studies conclude that TZD effects are not receptor mediated due to the high concentrations (up to 100 μM) required to observe effects. This raises the question if such concentrations could be reached in a therapeutic setting, to activate non-PPARγ pathways. In humans, maximum serum concentrations after a single 30 mg oral dose of Pio were typically about 900 ng/ml (approximately 2.2 μM) [72]; for Trog, maximum serum concentrations reached about 7–8 μM [73]. In animal studies, even higher levels were measured, with serum levels for Pio and its active metabolites peaking between 18 and 145 μM [74]. More importantly, typical AUC values (area under the curve; an index of total drug exposure) reach 20 μM for Pio and about 55 μM for Trog. AUC values give a measure of how much and how long a drug stays in the body, which emphasizes the fact that a long exposure to a low concentration can be as effective as shorter exposure to a higher concentration. Thus, it is likely that sufficiently high serum levels of TZDs could be reached following oral administration at currently approved doses. Furthermore, if a TZD is targeting an intracellular organelle, accumulation could lead to even higher cytosolic levels. This would expected to be both time and concentration dependent, and could help explain why effects in vivo take longer to see.

3. Effects of TZDs on mitochondrial function

The effects of PPAR agonists on mitochondria have been best characterized with respect to PPARα ligands which can induce mitochondrial gene expression involved in FA metabolism and can regulate mitochondrial biogenesis [75], effects mediated in part by PPARγ cofactor PGC1α (PPARγ coactivator 1α) [76–78]. However, more rapid and direct effects on mitochondria have been described, suggesting that TZDs may bind to target sites in mitochondria to elicit effects. In rat and human hepatocytes, TZDs caused mitochondrial dysfunction, followed by increased permeability, calcium influx, and nuclear condensation, with the order of potency Trog > Pio > Rosi [79]. Trog at doses of 25–100 μM induced a rapid (within minutes) decrease in $\Delta\psi_m$ followed by increases in intracellular calcium levels and caspase-3 activation in human HepG2 hepatocarcinoma cells [80]. In astrocytes, we reported that TZDs (between 10 and 30 μM) caused a rapid increase in glucose consumption and lactate production, associated with an initial decrease in $\Delta\psi_m$ (occurring within 1–2 h) followed by a subsequent hyperpolarization [81]. Similarly, in isolated rat liver, infusion of Trog increased lactate production in less than 10 min [83]. Rapid effects on mitochondria were reported with ciglitazone which increased ROS production [82] in astrocytes, and we observed similar effects using 10 to 20 μM Pio or Trog in mouse astrocytes and astrocytoma cells (Spagnolo et al., unpublished observations).

These findings suggest that TZDs may exert direct and rapid effects on mitochondrial respiration leading to changes in glycolytic metabolism and fuel substrate specificity. Recently, the Furnsinn group characterized the inhibitory effects of TZDs on aerobic metabolism, and showed that TZDs inhibit complex I activity in liver and skeletal muscle tissue as well as in isolated hepatic mitochondria [84]. They also confirmed that metformin inhibits complex I and suggested that alterations in cellular energy state could underlie the insulin-sensitizing effects of some anti-diabetic drugs. An inhibitory effect on complex I by ciglitazone, as well as several PPARα agonists, was previously reported using HL-60 cells [85]. In our studies, we observed inhibition of pyruvate-driven respiration by TZDs in isolated brain [81] and cardiac mitochondria (unpublished data). However, in these studies glutamate-supported respiration was not inhibited by Pio (Fig. 1) (nor by Trog, not shown) suggesting a site of action proximal to complex I. An additional target site for TZDs is consistent with the fact that the degree of inhibition reported to be

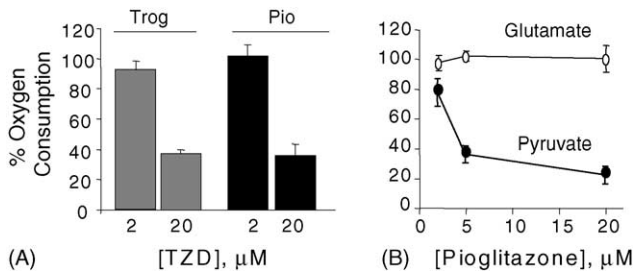

Fig. 1. Substrate specificity of metabolic effects. (A) Freshly isolated rat brain mitochondria were incubated with indicated concentrations of Pio or Trog, or vehicle (DMSO). State 3 respiration was initiated by addition of pyruvate, and oxygen consumption measured over the next 10 min. The data is mean ± S.D. of 3–4 experiments. Adapted from reference [78]. (B) Brain mitochondria were assayed as in A for oxygen consumption (as % no substrate) in the presence of indicated concentrations of Pio, and using either pyruvate or glutamate to initiate state 3 respiration. The data is mean ± S.D. of $n = 3$ experiments.

specific to complex I (18–24%) [84] was substantially less than that found for oxidative metabolism by skeletal muscle as a whole (73% for lipid and 52% for glucose oxidation). This may point to a tissue or cell type specificity with regard to the relative importance of different target sites.

If PPARγ is not the site of metabolic effects, an alternate target may be a newly described protein (''Mitoneet'') which shows relatively high (half maximal binding between 0.1 and 1 μM) affinity for Pio [86]. Mitoneet was identified by labeling mitochondrial lysates with a photoaffinity-labeled Pio, which revealed a single cross linked band of about 17 kDa. Proteomic analysis and subsequent cloning revealed a novel protein containing the sequence Asp-Glu-Glu-Tyr (''NEET''). Binding of radiolabeled Pio to Mitoneet could be dissociated with un-labeled Pio, but not with un-labeled ciglitazone, suggesting specificity in TZD binding. Mitoneet was found associated with several other mitochondrial proteins, including components of the pyruvate dehydrogenase complex, suggesting a means by which TZD binding to Mitoneet could block pyruvate driven respiration.

In initial studies we tested the consequences of reducing Mitoneet levels on astrocyte metabolism (Fig. 2). In control cells, Pio increased lactate production (49% increase) as previously observed [81]. In cells in which Mitoneet levels were reduced by siRNA treatment, lactate production was significantly increased (70%), suggesting that reducing Mitoneet increases anaerobic glycolysis and conversion of pyruvate to lactate. In Mitoneet depleted cells, Pio increased lactate production to approximately the same extent (45%) as observed in control cells (DMSO treated, siRNA infected cells). While this suggests that Pio can influence metabolism despite reduced Mitoneet, it is possible that Pio effects are mediated through interactions with remaining Mitoneet. However, together the data suggests that Mitoneet and Pio share a common target which disrupts mitochondrial respiration, leading to a compensatory increase in anaerobic glycolysis.

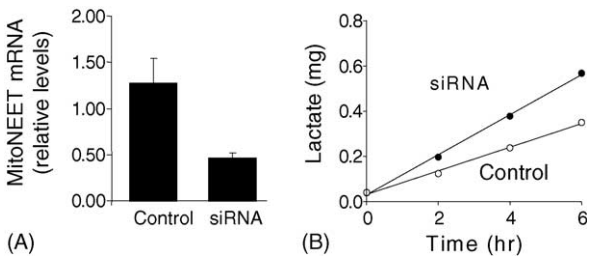

Fig. 2. Mitoneet depletion increases lactate production. Primary astrocytes were infected with adenovirus containing siRNA directed against mouse Mitoneet mRNA. (A) After 3 days, relative Mitoneet mRNA levels were determined by quantitative real time PCR with values normalized to changes in house keeping gene tubulin. The siRNA decreased steady state Mitoneet mRNA levels by approximately 50%. (B) Lactate production was increased approximately 70% in Mitoneet depleted astrocytes compared to control (mock infected) astrocytes. Data is mean ± S.E.M. of $n = 3$ samples.

4. Contribution of mitochondrial effects to other PPARγ independent actions

The above studies suggest that effects of TZDs on mitochondrial function could contribute to reported PPARγ independent actions. Furthermore, changes in mitochondrial function elicit numerous responses, some of which have also been reported in response to TZDs. Mitochondrial dysfunction will cause ATP levels to decrease and ROS to increase, both of which can activate the enzyme adenosine 5′-monophosphate-activated protein kinase (AMPK), which regulates many pathways including inflammation, proliferation, and fuel handling. Increase in ROS production can modulate cellular proliferation, inflammation, and toxicity. Finally, these changes can initiate a stress response leading to de novo expression of anti-inflammatory and cytoprotective genes.

4.1. Induction of a stress response and anti-inflammatory properties

The stress response, better known as the heat shock response (HSR), is a highly conserved cellular response characterized by upregulation of specific proteins (heat shock proteins (HSPs)) and inhibition of intracellular synthetic activity. The HSR is elicited by different stressors aside from temperature, including ROS production [87,88], reduction of ATP [89], and decreased glucose levels [90], and generally helps stressed cells to recover from damage [91]. Among the activities attenuated by HSR are inflammatory responses, which are inhibited by blocking NFκB activation [92]. NFκB activation can be blocked by the HSR either by causing induction of inhibitory IκB proteins [93–95]) or by inactivating IKK, which normally phosphorylates IκB allowing it to be degraded and to release NFκB [90].

Several papers have shown that TZDs can induce a HSR, which may contribute to their anti-inflammatory actions. Although the molecular mechanisms by which TZDs induce a HSR are not yet known, the fact that mitochondrial dysfunction can induce a stress response [103] suggests that TZDs may trigger a HSR due their effects on mitochondrial respiration. In rat insulinoma cells, Trog (100 μM) increased HSP70 expression and reduced the extent of IκBα degradation and JNK phosphorylation induced by IL-1β [96]. Ciglitazone (100 μM) increased HSP32 basal expression and reduced NOS2 expression in astrocytes. However, ciglitazone did not modify HSP32 expression in activated cells, as other PPARγ agonists did [97]. In vivo, Pio (5–40 mg/kg i.p.) increased the rate of gastric ulcer healing and blood flow at the ulcer margin in a dose-dependent manner, and this was accompanied by reduced expression of pro-inflammatory molecules and upregulation of HSP70 [98].

In adult rats, direct injection of Trog into the brain, or provision of oral Pio reduced CNS inflammation due to

LPS/IFNγ [27] or to amyloid beta [99], and was accompanied by increases in IκBα and IκBβ expression [99]. In a mouse model of multiple sclerosis, we showed that oral Pio (5–10 mg/kg day) reduced the incidence and severity of disease, also accompanied by reduction of proinflammatory expression in brain and upregulation of IκB levels [100]. Similarly, oral administration of Pio (20 mg/kg day) increased CNS levels of IκBα and protected animals from neuronal loss induced by 1-methyl-4-phenyl-1,2,3,6, tetrahydropyridine (MTPT) treatment, an animal model of Parkinson's disease [101]. It should be noted that in some studies, TZDs were shown to reduce certain HSPs, such as HSP32 and αB-crystallin [102]. However, in the majority of studies, increased HSP expression was reported.

4.2. Activation of AMPK and effects on glucose handling, inflammation, and cytotoxicity

Recently, activation of AMPK has been implicated in mediating a variety of cellular functions including control of glucose transport, lipid metabolism [104], and regulation of inflammation [105]. AMPK activation occurs under stress conditions which lead to increased AMP levels and higher AMP/ATP ratios. AMPK is activated following phosphorylation of its catalytic alpha subunit by an upstream kinase termed LKB1 [106,107]. Recently, AMPK activation was shown to be mediated by mitochondrially derived reactive oxygen [108] and nitrogen species [109] suggesting a role for NO in AMPK activation, and indicating that AMPK is a redox sensitive enzyme.

AMPK has been shown to mediate glucose transporter (GLUT)-4 translocation during ischemia in heart and skeletal muscle [110–112]. AMPK also has an important role in increasing cardiac FFA oxidation through its inhibition of acetyl-CoA carboxylase and activation of malonyl-CoA dehydrogenase [113], resulting in decreased malonyl-CoA concentrations [104,114]. As malonyl-CoA normally inhibits carnitine palmityl transferase 1 (CPT1), decreased malonyl-CoA levels results in greater CPT1 activity, and increased uptake of FFAs [115].

In inflammatory scenarios, activation of AMPK was shown to reduce inflammatory gene expression. Treatment of glial cells as well as macrophages with the AMPK activator 5-aminoimidazole-4-carboxamide riboside (AICAR) inhibited LPS-induced cytokine and NOS2 expression [105]. In the same study the authors found that AICAR blocked inflammatory protein expression in CNS of rats injected with LPS to induce a central inflammatory response. In peritoneal macrophages AICAR inhibited LPS-induced increases in TNF-α levels, as well as activation of phosphoinositide 3 kinase and Akt, although it had no effect on MAPK activation [116]. In a third study, NOS2 induction in myocytes, adipocytes, and macrophages was blocked by AMPK activation due to insulin sensitizing drugs, primarily due to down-regulation of NOS2 protein [117]. AICAR also reduced cytokine production (TNFα and IL-6) from adipose tissue, suggesting that its anti-inflammatory effects may contribute to insulin sensitizing effects [118]. Most recently, in endothelial cells, NFκB activation following exposure to palmitate or to TNFα was reduced by AICAR or by a constitutively active form of AMPK [119]. Together, these data suggest that AMPK activation is a viable therapeutic target for decreasing inflammatory activation in response to a variety of stimuli.

AMPK activation has recently been shown to reduce angiotensin-II induced proliferation of rat vascular smooth muscle cells [108]. In those studies, AMPK activation was sensitive to free radical scavengers, suggesting that mitochondrially derived ROS could directly activate AMPK. The relationship of AMPK activation to induction of apoptosis is not yet clear, as some papers report increased apoptosis [120,121] while others indicate protection [122]. However, the finding that AMPK as well as upstream activators of AMPK are targets of the tumor suppressor gene LKB1 [106,107] supports a role for AMPK in tumor cell growth and regulating cell proliferation and tumor cytotoxicity.

That TZDs can activate AMPK has been reported several times. Incubation of muscle cells with Rosi increased the AMP:ATP ratio and activated AMPK [123]. The same group showed that the anti-diabetic drug metformin, which impairs mitochondrial function, also activated AMPK although without effect on the ratio of AMP:ATP. Pio was shown to activate AMPK in adipose and liver tissue [124], thought to be mediated by changes in cellular ATP levels. Activation of AMPK may therefore be dependent upon TZD induced changes in metabolic state and mitochondrial impairment, as well as to the fact that AMPK is a redox sensitive enzyme [109].

4.3. Production of reactive oxygen species and cytotoxicity

Most ROS are generated in the mitochondria as by-products of oxidative phosphorylation. ROS at high levels have toxic effects but at low levels have physiological functions such as activation of signal transduction pathways [125], transcription factors [126,127] and mitochondrial enzyme activities [128]. Although mitochondria are the major source of cellular ROS, they can also be produced by membrane-bound enzymes such as NADPH oxidases, which can affect cell proliferation and apoptosis [129–131].

Tumor cells are metabolically more active than normal cells and under an intrinsic oxidative stress [132], and can generate increased amounts of ROS due to malfunction of the mitochondrial respiratory chain [133]. Excessive production of free radicals can damage different cellular components including DNA, protein, and membranes. In particular, since much of the ROS is generated in mitochondria, a major consequence is damage of mitochondrial membrane with release of cytochrome-c and activation of apoptotic cascades [134–136]. The intrinsic oxidative

stress of cancer cells makes them highly sensitive to any further increases in ROS production, so any drug able to induce ROS generation or inhibit the activity of antioxidant enzymes can be useful in the attempt to develop new therapeutic strategies for cancer treatment.

In this regard some of the cytotoxic effects induced by PPARγ ligands have been clearly associated with their ability to promote ROS generation. In N1S1 rat hepatoma cells, Trog at concentrations at least of 5 μM induced superoxide production with simultaneous decrease of mitochondrial $\Delta\psi_m$ [137]. That this effect was not PPARγ dependent was indicated by the rapidity (within 1 h) of the effect, and that Rosi did not cause any toxicity. Likewise, in HepG2 hepatocarcinoma cells, Trog-induced toxicity was reduced by cyclosporin A, an inhibitor of mitochondrial permeability transition pore [138]. Although in this study the mechanism involved in mitochondrial impairment was not investigated, the rapid loss of $\Delta\psi_m$ suggests that toxic effects were not mediated by PPARγ activation.

Finally, in astrocytes and preadipocytes, MAPK activation by PPARγ agonists required ROS production since the ROS scavenger *N*-acetyl cysteine (NAC) blocked activation [139]. Similarly, in mouse myoblast cells, different PPARγ ligands promoted ERK1/2 activation and production of ROS [140], but a PPARγ antagonist did not prevent ERK activation, nor did overexpression of wild-type or mutant PPARγ influence ERK activation. Since MAPK activation (particularly ERK1/2) is involved in anti-proliferative effects of TZDs [63–65], these observations provide a link between TZD-induced ROS and inhibition of proliferation.

5. Conclusions

A general scheme depicting receptor dependent and independent actions, and an attempt to integrate effects of TZDs on mitochondria with reported receptor independent actions is presented in Fig. 3. This scheme proposes that

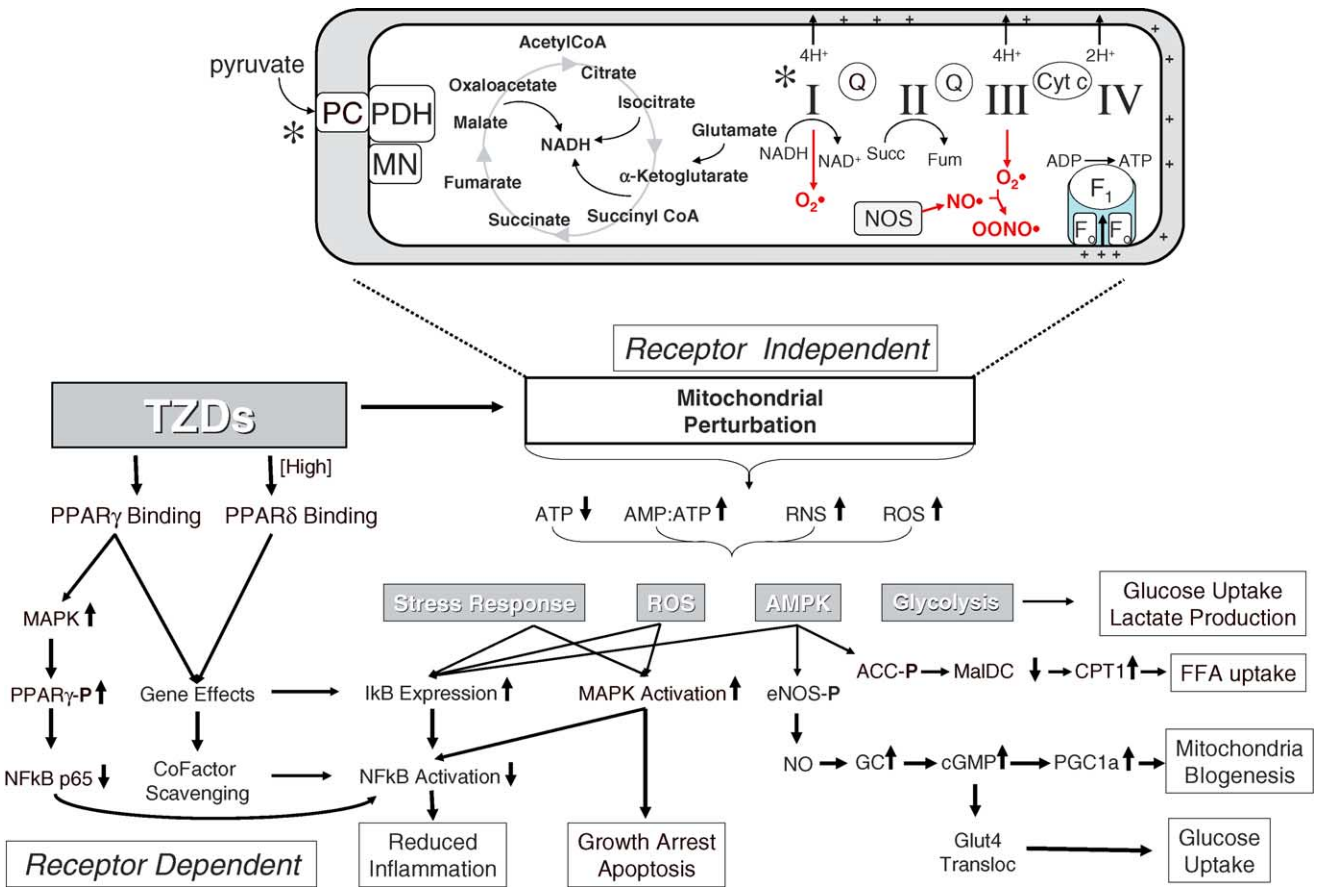

Fig. 3. Schematic illustrating receptor dependent and independent actions of TZDs. TZDs can exert cellular effects via activation of PPARγ leading to changes in gene expression patterns due to binding to PPR elements in promoter regions of target genes. By reducing levels of available transcription co-factors, PPARγ can also 'trans-repress' other ongoing transcriptional activity. Genomic changes will influence all cellular targets discussed (shown in open boxes), including inflammation, growth, apoptosis, glucose, and fatty acid uptake, as well as mitochondrial biogenesis. At high concentrations, TZDs can cross-activate PPARδ, eliciting genomic effects via that receptor. Receptor occupation by TZDs can also inhibit inflammatory gene expression via inhibition of the NFκB p65 subunit by phosphorylated PPARγ. Receptor-independent actions are postulated to depend on direct actions of TZDs with mitochondria. Candidate targets sites include binding to Mitoneet (MN), a component of the PDH complex (PDH), or a component of complex I, all leading to a reduction in overall mitochondrial function. Mitochondrial dysfunction can result in reduced ATP levels (and associated increase in AMP:ATP ratio), increased ROS levels, and increased RNS. In turn, changes in these signaling molecules can activate the same target responses (shown in grey boxes), namely the stress response, AMPK, and glycolysis. Finally, acting alone or in concert, these pathways lead to induction of observed cellular effects as discussed in the text.

direct interactions of TZDs with mitochondria, possibly mediated via interactions with a protein such as Mitoneet, or by direct actions with other respiratory complexes, impairs oxidative respiration and activates multiple downstream pathways. Decreased mitochondrial function rapidly leads to ROS (and possibly reactive nitrogen species) production and ATP depletion, which in turn can activate AMPK and induce a stress response. These responses, as discussed, can contribute to observed effects on inflammation, glucose and fatty acid metabolism, and cellular proliferation and apoptosis. Finally, it is also possible that changes in PPARγ expression or activation state, and thus subsequent changes in PPRE mediated gene expression, occur as a consequence of mitochondrial perturbation and therefore at later times TZDs may begin to exert PPARγ dependent as well as independent effects. Further characterization of these pathways will provide an understanding of the relative importance of receptor independent versus receptor dependent actions of PPAR agonists, and should facilitate development of new agonists which selectively activate one or both pathways.

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