Metabolic Effects of Rosiglitazone and Pioglitazone on Complex I and Complex II Respiration in Isolated Rat Mitochondria

by
Justin Braveboy-Wagner

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Approved by

Jeffrey MacDonald

Steven Knisley

Lee Graves

ABSTRACT

JUSTIN BRAVEBOY-WAGNER: Metabolic Effects of Rosiglitazone and Pioglitazone on Complex I and Complex II Respiration in Isolated Rat Mitochondria
(Under the direction of Ekhson Holmuhamedov)

Thiazolidinediones (TZDs) are believed to exert their antidiabetic effect through a variety of pathways and mechanisms, some of which relate to the toxic properties of these drugs. Research has proven that TZDs impair cell respiration in vitro and that they have an affect on oxidative stress within the cell. This paper investigates the role of mitochondria in rosiglitazone and pioglitazone action with respect to Complexes I and II of the respiratory chain. Inhibition of Complex I was confirmed via the reduced efficiency of mitochondrial respiration at increasing levels of drug concentration, with malate/glutamate as an energizing substrate, and in relation to Complex I (energized by succinate). Additionally, a decrease in the production of extra-mitochondrial reactive oxygen species (ROS) was detected, particularly on exposure to rosiglitazone, possibly correlating with a lower level of cytotoxicity in comparison to pioglitazone.

Key Words: rosiglitazone; pioglitazone; mitochondria; oxidative stress; drug toxicity

TABLE OF CONTENTS

LIST OF FIGURES

Figures

LIST OF ABBREVIATIONS AND TERMS

Terms

Complex I - Section of the electron transport chain mediated by NADH dehydrogenase ;

Complex II - Section of the electron transport chain mediated by succinate dehydrogenase ;

MMP - (Mitochondrial) Membrane Potential ;

ROS - Reactive Oxygen Species ;

State 2 - Mitochondrial respiration in the absence of Adenosine diphosphate ;

State 3 - Mitochondria respiration in the presence of Adenosine diphosphate ;

TZD - Thiazolidinedione ;

1. Introduction

Thiazolidinediones (also called glitazones) were synthetic compounds introduced in the late 1990s as an adjunctive therapy for diabetes mellitus (type 2) and related diseases. While troglitazone (Rezulin), was withdrawn from the market due to an increased incidence of drug-induced hepatitis in the liver, (Kohlroser, 2000) two other members of this family of drugs, rosiglitazone (Avandia) and pioglitazone (Actos), were approved for the treatment of hyperglycemia (Gillies, 2000) and are believed to be less toxic (Isley, 2003). In vitro studies upheld the differences in cytotoxicity, identifying troglitazone as more toxic than rosiglitazone and pioglitazone (Haskins 2001; Bae, 2003, Yamamoto, 2001).

Metabolic changes due to prolonged TZD treatment, particularly as related to insulin-stimulated glucose disappearance (Maggs, 1998), have been well described in vivo and ex vivo, clinical TZD therapy is built on limited knowledge of metabolic events that occur as a result of TZD action in cells and organelles. A major difficulty in understanding the mechanisms of TZD action is to separate events that are causal for antidiabetic action from other humoral and metabolic phenomenon that arise secondarily to glucose lowering or are unrelated to antidiabetic action. (Furnsinn, 2002) The study of the effects of TZDs on isolated cells or tissues in vitro remains useful as a means to distinguish TZD interaction from causal TZD-induced changes in hormones and metabolites and to provide mechanistic information relevant to clinically observed TZD cytotoxicity.

Mitochondrial dysfunction was observed in cytotoxicity experiments (Haskins, 2001; Shishido, 2003). Mitochondrial dysfunction may play a role in TZD cytotoxicity, but the precise mechanism underlying this action is unclear. Studies have suggested that mitochondrial membrane permeability transition (MPT) has a pathogenic role in mitochondria-mediated cell injury due to chemical agents, characterized by a progressive permeabilization of the inner mitochondrial membrane (Lemasters, 1998). The potent TZD, troglitazone, has induced mitochondrial membrane permeability transition in isolated rat liver mitochondria, while rosiglitazone and pioglitazone have less effect (Masubuchi, 2006).

Thiazolidinediones act by binding to Peroxisome proliferator-activated receptors; many metabolic and anti-inflammatory properties of TZDs are linked to PPAR-γ, a transcription factor that stimulates and represses a number of genes (Delerive, 2001). However, while TZDs have been linked to drug-induced hepatitis in the liver, the healthy liver, like skeletal muscle, does not strongly express PPAR-γ (Auboeuf 1997). Alternative PPAR-independent mechanisms may account for some glitazone responses (Feinstein, 2005). In vitro, TZDs have been observed to reduce fuel oxidation and elevate lactate release in skeletal muscle; an effect independent of PPAR-γ induced gene expression (Brunmair, 2001). A shift to anaerobic respiration indicates a PPAR-γ independent inhibition of cell respiration (Preininger 1999; Dello Russo, 2003), and an inhibitory influence on mitochondrial function.

Mitochondria are major producers of reactive oxygen species (ROS) in response to agents that alter their functions (Perez-Ortiz, 2006). Reactive oxygen species have been implicated in a number of pathologies, such as type II diabetes, atherosclerosis,

ischemia/reperfusion injury (Droge, 2002); the generation of ROS and the release of proteins from the mitochondria have been shown to lead to the activation of different modes of apoptosis (Orrenius, 2003). ROS also regulate the cellular metabolism through the activation of enzymatic cascades and transcription factors.

As TZDs have been reported to affect mitochondria in hepatoma cells (Masubuchi, 2006), and Jurkat cells (Kanunfre, 2004), among others, TZDs have been shown to generate ROS as a result of action on mitochondrial function (Narayanan, 2003). Troglitazone produced intra-mitochondrial oxidant stress leading to mitochondrial permeabilization, leading to organelle injury and cell death (Lim, 2007). With reactive oxygen species implicated in the cytotoxic effects of TZDs, further investigation into the production of the superoxide anion (O_2^-) (a short lived byproduct of oxidative phosphorylation) and hydrogen peroxide (H2O2), both species known to mediate cytotoxicity, could relate to PPAR-γ independent glitazone action.

The present study aimed to better define the mechanisms underlying TZD effects on mitochondrial dysfunction/stress independent of PPAR-ligand pathways and how they relate to Complex I and Complex II activity and mitochondrial function. Previous research indicated that Rosiglitazone and Pioglitazone reduced State 3 respiration in mitochondria primarily by impairing Complex I activity. These drugs could be called Complex I-dependant regarding State 3 respiration (Brunmair 2004). One of the consequences of inhibition of Complexes I, II and III is an increase in mitochondrial ROS generation. Although the exact mechanism of how TZDs trigger cell injury is not fully understood, several lines of evidence point to a pivotal role played by reactive oxygen species (ROS) and oxidant stress (Jung 2007). Research into oxidative stress has

traditionally been assessed in whole cells rather than mitochondria; it is unknown whether changes in ROS production are the source or consequence of cell injury provoked by TZDs (Masubushi 2006). The present study accessed the mitochondrial damage done and changes caused by dose dependant drug exposure, with the aim of understanding the nature of mitochondrial dysfunction under these conditions.

2. Materials and Methods

2.1. *Drugs and Chemicals*

The following need purchase/procurement information: Rosiglitazone, Pioglitazone, TMRM, Amplex Red, Thymadine, ADP, JM2 cells

2.2. *Animals*

Female Sprague–Dawley rats were obtained; the animals were housed in an air-conditioned room (25˚C) under a 12 hour light-dark cycle. All animal experiments were performed under the criteria for humane care as outlined by the National Academy of Sciences in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

2.3. *Isolation of rat liver mitochondria*

Rat liver mitochondria were isolated from fasted mature female Sprague–Dawley rats (180–250 g) as previously described (Pon, 2007). The rats were euthanized by decapitation and the livers removed. The livers were homogenized in an icecold isolation medium consisting of 220-mM mannitol, 70-mM sucrose, 2-mM HEPES, 0.5-mM EGTA, 0.1% BSA (fatty acid free) as a pH of 7.4 using a prechilled homogenizer. The homogenate was then centrifuged at 1000 x g for 10 minutes at 4ºC in a SA-600 rotor. The supernatant obtained was then transferred into a fresh prechilled tube and centrifuged a second time with the same parameters.

The supernatant obtained was then centrifuged at 10,000 x g for 10 minutes at 4ºC. The resulting pellet, enriched with mitochondria, is retained while the supernatant is removed. The pellet is then re-suspended in buffer and gently stirred before being transferred into a chilled 15-ml tube for storage. Mitochondrial protein concentration was determined using a biuret procedure with bovine serum albumin as the standard (Gornall, 1949). The condition of intact mitochondria was then tested by measuring oxygen consumption in the presence of succinate and ADP and determining respiratory control ratio (RCR).

2.4. *Oxygen Consumption*

Oxygen consumption was measured in air-saturated isolation buffer (0.2-M sucrose, 0.02-mM EGTA, 20-mM Tris-Hepes, 1-mM KH2PO4, pH 7.4) at room temperature with a Clark-type oxygen electrode. Mitochondria (1 g protein per liter) were pre-incubated for 3 minutes before the addition of concentrations of rosiglitazone or pioglitazone. Control experiments involving similar TZD-empty volumes of DMSO did not affect rates of oxygen consumption.

For stimulating mitochondria respiration the isolation buffer contained either subtrates for Complex II (5-mM succinate, 5-µM rotenone) or Complex I (5-mM malate, 5-mM glutamate). After 3 minutes, mitochondrial respiration was accelerated by the addition of 250-µM ADP allowing ATP synthesis. The rates of oxygen consumption were measured quantitatively in both State 2 and State 3 (with and without the presence of ADP, respectively) respiration. The Respiratory Control Ratio was then recorded as a

measure of mitochondrial efficiency in ATP synthesis. Measurements were done in triplicate.

2.5. *Detection of Superoxide in Isolated Rat Mitochondria by DHE-derived Fluorescence*

Suspensions of mitochondria (0.1 mg/ml) are preincubated in buffer without modifiers. Plate well suspensions are prepared with either 5-mM succinate, 5-µM rotenone or 5-mM malate, 5-mM glutamate, in addition to 1-µM DHE, with or without ADP (250-µM). Rosiglitazone or Pioglitazone concentrations of 5-µM, 25-µM and 50-µM were also added. Mitochondria were exposed to these conditions at room temperature in 96-well plates and immediately added to a multi-well plate reader BMG (FlouStar, Durham, NC, USA) set at excitation 485 nm and emission 590 nm. The kinetics of O_2^- generation was then recorded in the absence or presence of modifiers for 9 minutes. The level of O_2^- in each experiment was measured in relative fluorescence units (RFU) per minute and the rate of Superoxide anion generation was expressed as RFU.min. The inhibitor, Antimycin A (10µg/ml), was used as a control. (Pon, 2007)

2.6. *Detection of H2O2 in isolated rat mitochondria by Amplex Red-derived fluorescence*

Mitochondrial H2O2 release was measured with Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) horseradish peroxidase method (Pon, 2007). Horseradish peroxidase (HRP, 0.1 units/ml) catalyses the H2O2-dependant oxidation of non-fluorescent Amplex red (50-µM) to fluorescent resorufin red; as HRP is too large a protein to cross membranes , this assay detects only H2O2 that has been released from the mitochondria (it does not measure H2O2 inside mitochondria). 100-µl of a solution

containing HRP and Amplex Red, with or without modifiers in the given concentrations is added to individual wells in a 96-well plate. Reactions are initiated by adding the buffer to the mitochondrial mixture (0.1 mg/ml) with the addition of 1-mM MgSO4. Fluorescence was measured at excitation 560 nm and emission 590 nm at 37ºC at 8 second intervals for 9 minutes. A buffer without mitochondria served as a negative control. A standard curve was created by using known amounts of H2O2 to the assay medium in the absence of mitochondria. Results are calculated as RFUs in the mitochondrial samples minus RFUs in the empty control (background RFUs) and are expressed at pmol H2O2/mg protein/minute.

2.7. *Assay of thymidine incorporation by liquid scintillation*

JM2 Heptaoma cells were plated at a density of 7.5 x 10^4 cells onto a collagen coated 60-mm culture dish in 2-ml of DMEM (high glucose) supplemented with 1-mM pyruvate, 4-mM glutamine, 0.04-mM Phenol Red and 10% FBS (fetal bovine serum). Cell adhesion was allowed to occur over a period of 2.5 hours incubation with no change in media. Media was then replaced by feed media including thymidine and drugs at concentrations (1-mM, 5-mM, 25-mM, 50-mM) and allowed to culture for 20 hours.

Following incubation with thymidine and the above experimental conditions, cell cultures were harvested, washed with 5% TCA, then with water 5 times sequentially, and then dissolved in 1.5-ml of 0.33 $_N$ NaOH. The dissolved cells were transferred to test tubes on ice and 0.5-ml of a solution of 40% trichloroacetic acid and 1.2 $_N$ HCL was added to each tube. The tubes were centrifuged for 10 minutes at 2000 rpm. The resulting pellets were dissolved in of 0.33 $_N$ NaOH. 0.3-ml aliquots were added to scintillation

vials along with 0.1 ml of trichloroacetic acid-HCL and 5-ml of a Aquasol (New England Nuclear, Boston MA), a xylene-based scintillation fluid. The vials were assayed for radioactivity by a liquid scintillation spectrometer. (Michalopoulos, 1984)

2.8. *Measurement of membrane depolarization during mitochondrial permeability transition*

The electrical transmembrane potential of mitochondria was monitored cytofluorometrically using TMRM in a method previously described (Blattner, 2001). Mitochondria suspended in a solution without Mg2+ but with a respiratory substrate, the presence of CaCl2 leads to Ca2+ uptake into mitochondria through the Ca2+ uniporter, and then to opening of permeability transition pores and membrane depolarization. Mitochondria (0.5-mg/ml) were incubated with TMRM (1-µM), a membrane potential indicating fluorophore, and 100-µM CaCl2. TMRM fluorescence was measured using a multiwell plate reader at excitation 540 nm, emission 590 nm; reduced fluorescence corresponded to a positive shift in voltage potential. Tests were performed according to experimental conditions utilizing a variety of modifiers and respiratory substrates.

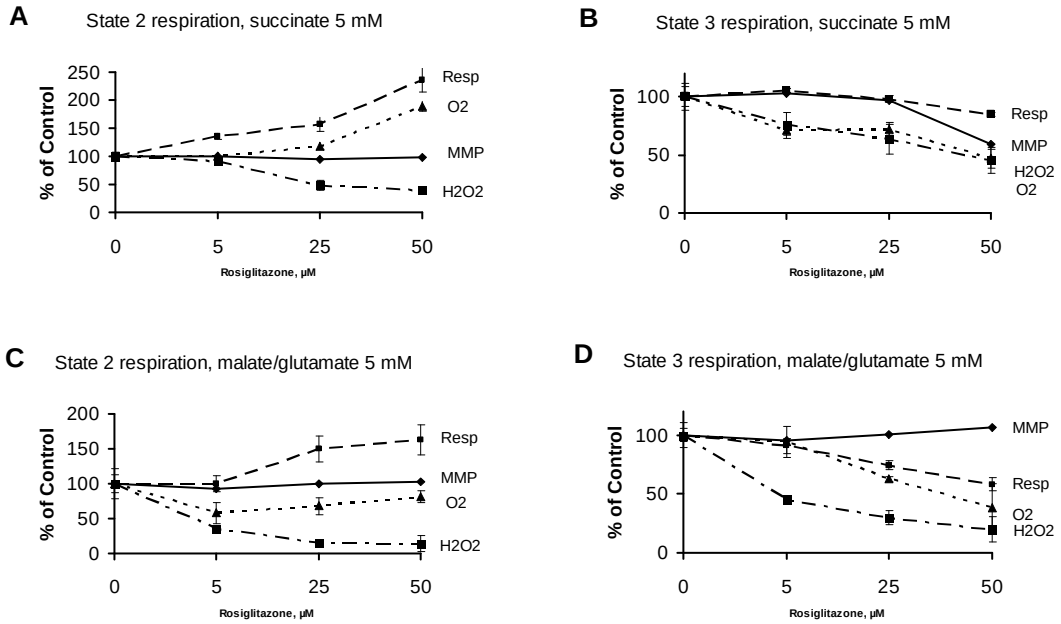

Fig. 1. Effects of rosiglitazone exposure on isolated rat mitochondria given various concentrations of drug exposure. Graphs correlate data, weighted by deviation of that data from a baseline control. Mitochondria were incubated in a reaction medium containing either air-saturated isolation buffer: 200-mM sucrose, 0.02-mM EGTA, 20-mM Tris-Hepes, 1-mM KH_2PO_4, pH 7.4 (during oxygen consumption measurement) or 220-mM mannitol, 70-mM sucrose, 2-mM HEPES, 0.5-mM EGTA, 0.1% BSA; pH of 7.4 (for ROS measurement). Mitochondria were energized with either 5-mM succinate and 5-µM rotenone or 5-mM malate plus 5-mM glutamate, without rotenone. All results represent mean ± S.D. and are expressed as a percentage of untreated individual controls for comparative purposes. **(A)** Effects of rosiglitazone at various concentrations on oxygen consumption (respiration), superoxide production, H_2O_2 generation, and membrane potential (MMP) in State 2, Complex II. **(B)** Effects of rosiglitazone at State 3, in Complex II, due to the presence of 250-µM ADP allowing ATP synthesis. **(C)** Effects of rosiglitazone in State 2, Complex I. **(D)** Effects of rosiglitazone at State 3, Complex I, due to the presence of 250-µM ADP.

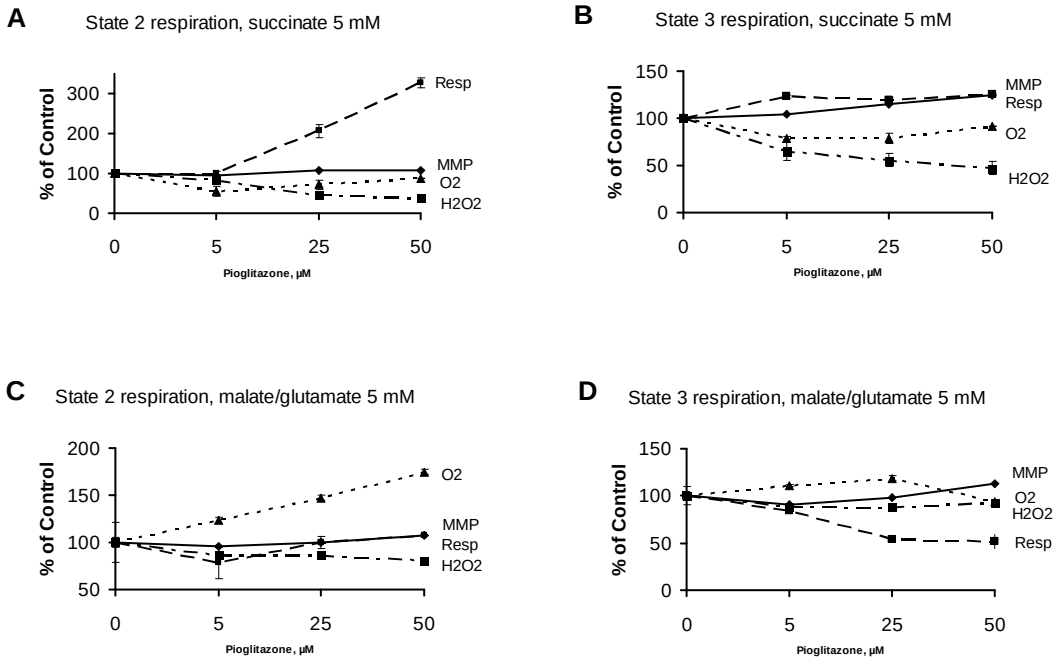

Fig. 2. Effects of pioglitazone exposure on isolated rat mitochondria given various concentrations of drug exposure. Graphs correlate data, weighted by deviation of that data from a baseline control. Mitochondria were incubated in a reaction medium containing either air-saturated isolation buffer: 0.2-M sucrose, 0.02-mM EGTA, 20-mM Tris-Hepes, 1-mM KH2PO4, pH 7.4 (during oxygen consumption measurement) or 220-mM mannitol, 70-mM sucrose, 2-mM HEPES, 0.5-mM EGTA, 0.1% BSA; pH of 7.4 (for ROS measurement). Mitochondria were energized with either 5-mM succinate, 5-µM rotenone or 5-mM malate, 5-mM glutamate. All results represent mean ± S.D. and are expressed as a percentage of untreated individual controls for comparative purposes. **(A)** Effects of pioglitazone at various concentrations on oxygen consumption (respiration), superoxide production, H2O2 generation, and membrane potential (MMP) in State 2, Complex II. **(B)** Effects of pioglitazone at State 3, in Complex II, due to the presence of 250-µM ADP allowing ATP synthesis. **(C)** Effects of pioglitazone in State 2, Complex I. **(D)** Effects of pioglitazone at State 3, Complex I, due to the presence of 250-µM ADP.

3. Results

3.1. *Measurement of changes in relative oxygen consumption in isolated mitochondria as a result of drug exposure*

Incubation of energized mitochondria limited to Complex II based respiration in the presence of rosiglitazone demonstrated an increate in oxygen consumption with respect to increasing drug concentration in State 2, contrasted with a smaller decrease in State 3. Limited to Complex I activity, mitochondria showed a similar increase in State 2 oxygen consumption, and a decrease in State 3. The inhibition of State 3 respiration was more pronounced in mitochondria reliant on malate/glutamate rather than succinate supported respiration. Reactions on exposure to pioglitazone demonstrated an increase in State 2, Complex II respiration, but less change in State 3. Complex I respiration was inhibited in State 3, but not significantly in State 2.

3.2. *Effect of rosiglitazone and pioglitazone on superoxide production*

Investigating the mechanisms underlying the effects of glitazones on cell viability, the oxidative stress response of mitochondria to treatment with rosiglitazone and pioglitazone were tested on isolated mitochondria. The mitochondrial electron chain is a major source of endogenous ROS (reactive oxygen species). Measurement of State 2, Complex II activity in mitochondria in the presence of rosiglitazone demonstrated an increase in mitochondrial O_2^-, and a decrease in relative O_2^- production in State 3. Under Complex I conditions, rosiglitazone affected decreases in O_2^- production in both State 2

and State 3. In the latter case, increases in drug concentration had a similarly increasing inhibitory effect. Pioglitazone inhibited O_2^- production in Complex II, while having an opposite effect on Complex I activity. State 2 Complex I generation of O_2^- increased dramatically with respect to pioglitazone concentration.

3.3. *Effect of rosiglitazone and pioglitazone on extra-mitochondrial hydrogen peroxide generation and release*

H2O2 is the most stable and abundant of the reactive oxygen species produced by mitochondria, due to it being a byproduct of superoxide scavenging by superoxide dismutase (SOD) enzymes (Esposti, 2002). Decreases in the generation and release of H2O2 in isolated mitochondria were detected on exposure to both rosiglitazone and pioglitazone under all experimental conditions. In the rosiglitazone trials quantifiable levels of H2O2 decreased dramatically, particularly with respect to Complex I activity; greater reductions in H2O2 levels occurred in Complex I at similar concentrations of rosiglitazone than Complex II.

3.4. *Effect of Rosiglitazone and pioglitazone on mitochondrial membrane potential (MMP)*

Generation of ROS by mitochondria depends on the mitochondrial membrane potential and flux in the electron transport chain. Mitochondrial ROS production has been shown to decrease with mitochondrial transmembrane electrical potential (Brand, 2004). Previous research into the effect of rosiglitazone and pioglitazone on isolated mouse liver mitochondria indicated that pioglitazone had a greater effect than rosiglitazone,

decreasing membrane potential by approximately 10% relative to control at 50-µM (Masubuchi, 2006). Fluctuations in MMP within the range of standard deviation occurred in State 2 experiments, while there was a slight trend of increasing potential in State 3, except in the case of Complex II activity.

3.5. *Assay of glitazone cytotoxicity by thymidine incorporation*

A thymidine incorporation by liquid scintillation assay was used to determine toxicity of rosiglitazone and pioglitazone, and to determine if the glitazones were halting the cell cycle. JM2 Heptaoma cells were used as thiazolidinediones have been previously reported to affect mitochondria in hepatoma cells (Masubuchi, 2006). The hepatoxicity of pioglitazone was confirmed, but did not halt the cell cycle over the course of the experiment (see Fig 3.).

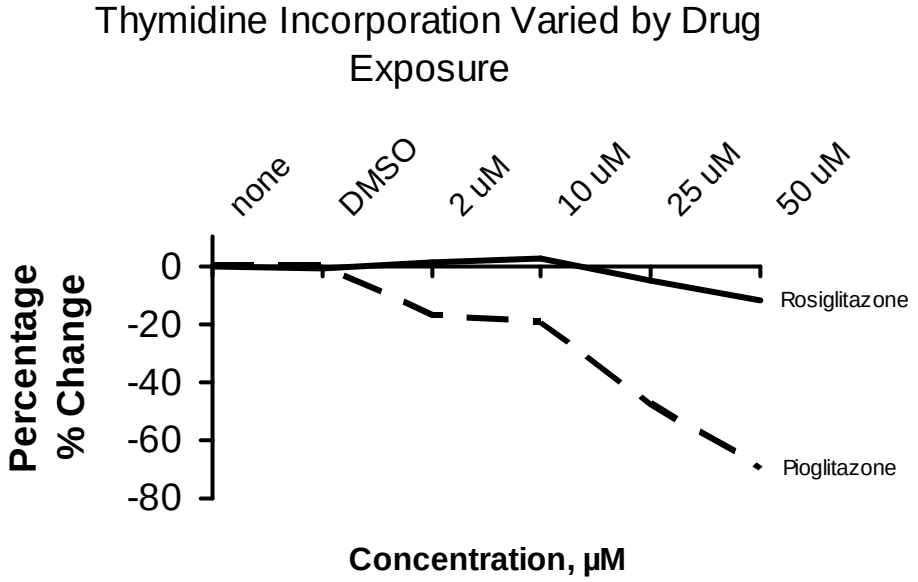

Fig 3. Effects of rosiglitazone and pioglitazone at varied concentrations on cell proliferation. Drug effects were examined via a thymidine incorporation assay, measuring the inhibition of deoxyribonucleic acid (DNA) synthesis following exposure to thiazolidinediones. JM2 Heptaoma cells were plated at a density of 7.5 x 104 cells onto a collagen coated 60-mm culture dish in 2-ml of DMEM (high glucose) supplemented with 1-mM pyruvate, 4-mM glutamine, 0.04-mM Phenol Red and 10% FBS (fetal bovine serum). Incubation offered for 2.5 hours followed by a change in media, and addition of thymidine and drugs at concentrations (1-mM, 5-mM, 25-mM, 50-mM). Cells were then cultured for 20 hours and harvested. Each data point was done in triplicate and expressed as a mean (STDEV of less than 3% was excluded from the graph).

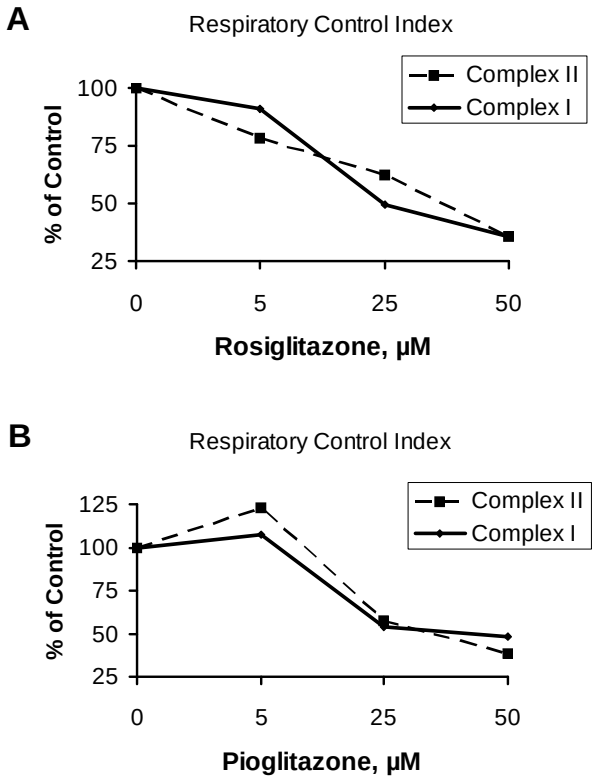

Fig 4. The effect of thiazolidinediones on RCI (respiratory control index) as a function of State-3/State-2 respiration, and sorted by active Complexes (succinate as Complex II substrate; malate/glutamate as Complex I). Mitochondria (1 g protein/liter) were incubated in an air-saturated isolation buffer containing 0.2-M sucrose, 0.02-mM EGTA, 20-mM Tris-Hepes, 1-mM KH2PO4, and in a Clark-type oxygen electrode. **(A)** Displays the results of the rosiglitazone trials; **(B)** displays the results of exposure to Pioglitazone. The results are the representative mean of three experiments.

4. Discussion

In the present study, the incubation of energized mitochondria with rosiglitazone and pioglitazone resulted in impairment of mitochondrial respiration and a decrease in energy conserving capacity as defined by the Respiratory Control Index (State 3/State 2). Both pathways showed a decreased respiratory control index (RCI; state-3/state-2) as a result of drug exposure (Fig 4.). For rosiglitazone, the decreases in RCI were due to both an increase in State 2 respiration and a decrease in State 3. The effect of Pioglitazone was to inhibit oxygen consumption in State 3 with regard to Complex I, and to increase it in State 2 for Complex II.

As in previous research in isolated mitochondria, rosiglitazone and pioglitazone reduced State 3, Complex I respiration (with malate/glutamate as substrates) but did not do so in the presence of succinate and rotenone, along with indications that Complex I dependant oxidation inhibition was taking place (Brunmair 2004). Despite confirming the inhibition of Complex I by rosiglitazone and pioglitazone, a corresponding increase in ROS generation was not detected.

One of the consequences of the inhibition of Complexes I, II and III is an increase in mitochondrial ROS generation (Orrenius, 2007), however rosiglitazone and pioglitazone exposure resulted in a general decrease in ROS (both H2O2 and O_2^-) levels

observed in both State 2 and 3, indicating that a specific inhibition of Complex I or II as would be caused by rotenone or thenoyltrifluoroacetone is not taking place, despite the concentration-dependent decreases in respiratory control. Previous investigations into ROS generation in cells have indicated increased ROS generation in Jurkat T cells (Soller, 2004) but anti-oxidative properties in patients (Garg, 2000). Troglitazone was noted to increase mitochondrial ROS (Lim, 2007).

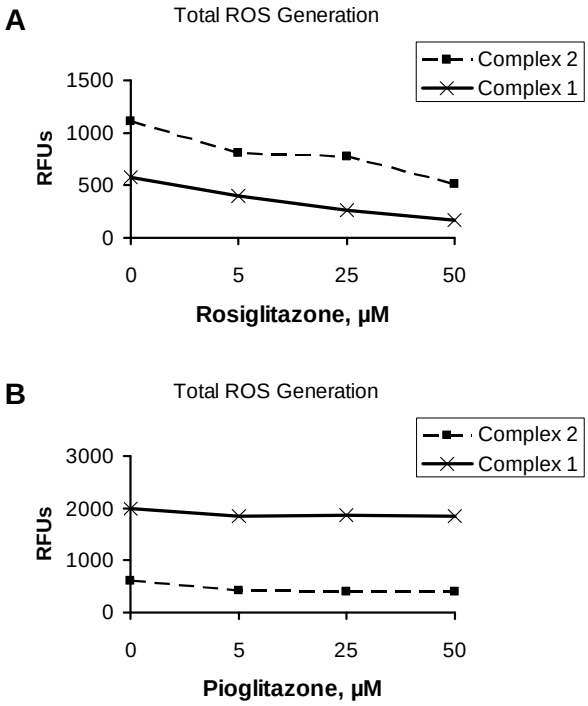

Fig 5. The effect of thiazolidinediones on overall extra-mitochondrial State 3 ROS generation. Net levels of reactive oxygen species are expressed as relative fluorescence units (RFUs). **(A)** Rosiglitazone inhibits the production of ROS in Complex 1 (malate/glutamate) and Complex 2 (succinate/rotenone) respiration. **(B)** The effect of Pioglitazone under Complex 1 and Complex 2 activity.

Drug doses encouraged the release of Superoxide from the mitochondria in State 2, but inhibited it in State 3. This correlates with the increased oxygen consumption observed in State 2 during the Respiration experiments. Oxidative stress is generally

defined as an imbalance that favors the production of ROS over antioxidants; however isolated mitochondria were not shown to produce markedly increased levels of reactive oxygen species that would lead to greater oxidative stress in their environment. Rosiglitazone in particular demonstrates a strong adverse correlation to ROS generation, especially towards Complex 1 activity (Fig 5).

The reduction in Respiratory Control and mitochondrial efficiency (with respect to ATP synthesis) seen are indicative of a contribution to uncoupled respiration without ATP production. Rosiglitazone and Pioglitazone seem to act as uncouplers, both in regards to Complex I and Complex II, but primarily Complex I. This activity has been suggested previously (Brunmair 2004, Lim, 2007); the protonophoretic uncoupling of oxidative phosphorylation is consistent with observed results. However, both rosiglitazone and pioglitazone had little impact on mitochondrial membrane potential, and inhibited extra-mitochondrial ROS generation. As TZD's (primarily troglitazone and c-glitazone) have been linked to oxidative stress and cell death (Soller, 2007), it seems likely that ROS is being generated by an exo-mitochondrial pathway.

It is increasingly apparent that mitochondria lie at the centre of the process of cell death: apoptosis and necrosis. Mitochondria may induce mitochondrial permeability transition, and release apoptotic proteins into the cytoplasm, resulting in a biochemical and morphological alteration of cell metabolism. Observations of the effect of rosiglitazone and pioglitazone are consistent with the concept that mild mitochondrial uncoupling contributes to respiratory inhibition and mitochondrial dysfunction, while reducing overall output of reactive oxygen species, particularly in rosiglitazone. These non- PPAR-γ factors likely contribute to the cytotoxic effects attributes to these drugs.

References

1. Bae MA, Rhee H, Song BJ. Troglitazone but not rosiglitazone induces G1 cell cycle arrest and apoptosis in human and rat hepatoma cell lines. Toxicol Lett. 2003 Mar 20;139(1):67-75.

2. Blattner JR, He L, Lemasters JJ. Screening assays for the mitochondrial permeability transition using a fluorescence multiwell plate reader. Anal Biochem. 2001 Aug 15;295(2):220-6.

3. Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, et al. Mitochondrial superoxide: Production, biological effects, and activation of uncoupling proteins. Free Radic Biol Med. 2004 Sep 15;37(6):755-67.

4. Brunmair B, Gras F, Neschen S, Roden M, Wagner L, Waldhausl W, et al. Direct thiazolidinedione action on isolated rat skeletal muscle fuel handling is independent of peroxisome proliferator-activated receptor-gamma-mediated changes in gene expression. Diabetes. 2001 Oct;50(10):2309-15.

5. Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R, et al. Thiazolidinediones, like metformin, inhibit respiratory complex I: A common mechanism contributing to their antidiabetic actions? Diabetes. 2004 Apr;53(4):1052-9.

6. Budd SL, Castilho RF, Nicholls DG. Mitochondrial membrane potential and hydroethidine-monitored superoxide generation in cultured cerebellar granule cells. FEBS Lett. 1997 Sep 22;415(1):21-4.

7. Daiber A, Oelze M, August M, Wendt M, Sydow K, Wieboldt H, et al. Detection of superoxide and peroxynitrite in model systems and mitochondria by the luminol analogue L-012. Free Radic Res. 2004 Mar;38(3):259-69.

8. Degli Esposti M. Measuring mitochondrial reactive oxygen species. Methods. 2002 Apr;26(4):335-40.

9. Degli Esposti M. Measuring mitochondrial reactive oxygen species. Methods. 2002 Apr;26(4):335-40.

10. Delerive P, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors in inflammation control. J Endocrinol. 2001 Jun;169(3):453-9.

11. Dello Russo C, Gavrilyuk V, Weinberg G, Almeida A, Bolanos JP, Palmer J, et al. Peroxisome proliferator-activated receptor gamma thiazolidinedione agonists increase glucose metabolism in astrocytes. J Biol Chem. 2003 Feb 21;278(8):5828-36.

12. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002 Jan;82(1):47-95.

13. Feinstein DL, Spagnolo A, Akar C, Weinberg G, Murphy P, Gavrilyuk V, et al. Receptor-independent actions of PPAR thiazolidinedione agonists: Is mitochondrial function the key? Biochem Pharmacol. 2005 Jul 15;70(2):177-88.

14. Furnsinn C, Waldhausl W. Thiazolidinediones: Metabolic actions in vitro. Diabetologia. 2002 Sep;45(9):1211-23.

15. Gillies PS, Dunn CJ. Pioglitazone. Drugs. 2000 Aug;60(2):333,43; discussion 344-5.

16. GORNALL AG, BARDAWILL CJ, DAVID MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem. 1949 Feb;177(2):751-66.

17. Gottlieb RA, Granville DJ. Analyzing mitochondrial changes during apoptosis. Methods. 2002 Apr;26(4):341-7.

18. Gross MD, Whitty AJ, Foa PP. Respiratory activity of mitochondria isolated from a transplantable islet-cell tumor and from the liver of tumor-bearing hamsters. Cancer Res. 1972 Sep;32(9):1978-82.

19. Haskins JR, Rowse P, Rahbari R, de la Iglesia FA. Thiazolidinedione toxicity to isolated hepatocytes revealed by coherent multiprobe fluorescence microscopy and correlated with multiparameter flow cytometry of peripheral leukocytes. Arch Toxicol. 2001 Sep;75(7):425-38.

20. Holmuhamedov EL, Jahangir A, Oberlin A, Komarov A, Colombini M, Terzic A. Potassium channel openers are uncoupling protonophores: Implication in cardioprotection. FEBS Lett. 2004 Jun 18;568(1-3):167-70.

21. Isley WL. Hepatotoxicity of thiazolidinediones. Expert Opin Drug Saf. 2003 Nov;2(6):581-6.

22. Jung TW, Lee JY, Shim WS, Kang ES, Kim SK, Ahn CW, et al. Rosiglitazone protects human neuroblastoma SH-SY5Y cells against MPP+ induced cytotoxicity via inhibition of mitochondrial dysfunction and ROS production. J Neurol Sci. 2007 Feb 15;253(1-2):53-60.

23. Kanunfre CC, da Silva Freitas JJ, Pompeia C, Goncalves de Almeida DC, Cury-Boaventura MF, Verlengia R, et al. Ciglitizone and 15d PGJ2 induce apoptosis in jurkat and raji cells. Int Immunopharmacol. 2004 Sep;4(9):1171-85.

24. Kohlroser J, Mathai J, Reichheld J, Banner BF, Bonkovsky HL. Hepatotoxicity due to troglitazone: Report of two cases and review of adverse events reported to the united states food and drug administration. Am J Gastroenterol. 2000 Jan;95(1):272-6.

25. Lemasters JJ, DiGuiseppi J, Nieminen AL, Herman B. Blebbing, free Ca2+ and mitochondrial membrane potential preceding cell death in hepatocytes. Nature. 1987 Jan 1-7;325(6099):78-81.

26. Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, et al. The mitochondrial permeability transition in cell death: A common mechanism in necrosis, apoptosis and autophagy. Biochim Biophys Acta. 1998 Aug 10;1366(1-2):177-96.

27. Lim PL, Liu J, Go ML, Boelsterli UA. The mitochondrial superoxide/thioredoxin-2/Ask1 signaling pathway is critically involved in troglitazone-induced cell injury to human hepatocytes. Toxicol Sci. 2008 Feb;101(2):341-9.

28. Lim PL, Liu J, Go ML, Boelsterli UA. The mitochondrial superoxide/thioredoxin-2/Ask1 signaling pathway is critically involved in troglitazone-induced cell injury to human hepatocytes. Toxicol Sci. 2008 Feb;101(2):341-9.

29. Liza A. Pon, Eric A. Schon. **Mitochondria**. 2nd ed. Academic Press; 2007.

30. Maggs DG, Buchanan TA, Burant CF, Cline G, Gumbiner B, Hsueh WA, et al. Metabolic effects of troglitazone monotherapy in type 2 diabetes mellitus. A randomized, double-blind, placebo-controlled trial. Ann Intern Med. 1998 Feb 1;128(3):176-85.

31. Martinez B, Perez-Castillo A, Santos A. The mitochondrial respiratory complex I is a target for 15-deoxy-delta12,14-prostaglandin J2 action. J Lipid Res. 2005 Apr;46(4):736-43.

32. Masubuchi Y, Kano S, Horie T. Mitochondrial permeability transition as a potential determinant of hepatotoxicity of antidiabetic thiazolidinediones. Toxicology. 2006 May 15;222(3):233-9.

33. Michalopoulos G, Houck KA, Dolan ML, Leutteke NC. Control of hepatocyte replication by two serum factors. Cancer Res. 1984 Oct;44(10):4414-9.

34. Morosetti R, Servidei T, Mirabella M, Rutella S, Mangiola A, Maira G, et al. The PPARgamma ligands PGJ2 and rosiglitazone show a differential ability to inhibit proliferation and to induce apoptosis and differentiation of human glioblastoma cell lines. Int J Oncol. 2004 Aug;25(2):493-502.

35. Morosetti R, Servidei T, Mirabella M, Rutella S, Mangiola A, Maira G, et al. The PPARgamma ligands PGJ2 and rosiglitazone show a differential ability to inhibit proliferation and to induce apoptosis and differentiation of human glioblastoma cell lines. Int J Oncol. 2004 Aug;25(2):493-502.

36. Muller FL, Liu Y, Van Remmen H. Complex III releases superoxide to both sides of the inner mitochondrial membrane. J Biol Chem. 2004 Nov 19;279(47):49064-73.

37. Narayanan PK, Hart T, Elcock F, Zhang C, Hahn L, McFarland D, et al. Troglitazone-induced intracellular oxidative stress in rat hepatoma cells: A flow cytometric assessment. Cytometry A. 2003 Mar;52(1):28-35.

38. Orrenius S, Gogvadze V, Zhivotovsky B. Mitochondrial oxidative stress: Implications for cell death. Annu Rev Pharmacol Toxicol. 2007;47:143-83.

39. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: The calcium-apoptosis link. Nat Rev Mol Cell Biol. 2003 Jul;4(7):552-65.

40. Perez-Ortiz JM, Tranque P, Burgos M, Vaquero CF, Llopis J. Glitazones induce astroglioma cell death by releasing reactive oxygen species from mitochondria: Modulation of cytotoxicity by nitric oxide. Mol Pharmacol. 2007 Aug;72(2):407-17.

41. Perez-Ortiz JM, Tranque P, Burgos M, Vaquero CF, Llopis J. Glitazones induce astroglioma cell death by releasing reactive oxygen species from mitochondria: Modulation of cytotoxicity by nitric oxide. Mol Pharmacol. 2007 Aug;72(2):407-17.

42. Preininger K, Stingl H, Englisch R, Furnsinn C, Graf J, Waldhausl W, et al. Acute troglitazone action in isolated perfused rat liver. Br J Pharmacol. 1999 Jan;126(1):372-8.

43. Reungpatthanaphong P, Dechsupa S, Meesungnoen J, Loetchutinat C, Mankhetkorn S. Rhodamine B as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential in drug-sensitive and -resistant cells. J Biochem Biophys Methods. 2003 Jul 31;57(1):1-16.

44. Scaduto RC,Jr, Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J. 1999 Jan;76(1 Pt 1):469-77.

45. Shishido S, Koga H, Harada M, Kumemura H, Hanada S, Taniguchi E, et al. Hydrogen peroxide overproduction in megamitochondria of troglitazone-treated human hepatocytes. Hepatology. 2003 Jan;37(1):136-47.

46. Shishido S, Koga H, Harada M, Kumemura H, Hanada S, Taniguchi E, et al. Hydrogen peroxide overproduction in megamitochondria of troglitazone-treated human hepatocytes. Hepatology. 2003 Jan;37(1):136-47.

47. Soller M, Drose S, Brandt U, Brune B, von Knethen A. Mechanism of thiazolidinedione-dependent cell death in jurkat T cells. Mol Pharmacol. 2007 Jun;71(6):1535-44.

48. Thurman RG, Scholz R. Interaction of glycolysis and respiration in perfused rat liver. changes in oxygen uptake following the addition of ethanol. Eur J Biochem. 1977 May 2;75(1):13-21.

49. Yamamoto Y, Nakajima M, Yamazaki H, Yokoi T. Cytotoxicity and apoptosis produced by troglitazone in human hepatoma cells. Life Sci. 2001 Dec 14;70(4):471-82.