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Troglitazone Regulates Anaplerosis via a Pull/Push Affect on Glutamate Dehydrogenase Mediated Glutamate Deamination in Kidney-derived Epithelial Cells; Implications for the Warburg Effect

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Key Words

Acidosis • Ammonium • Glutaminolysis • Lactate • Pyruvate

Abstract

Mitochondrial Krebs cycle keto acid pool depends upon input from pyruvate and glutamate to maintain homeostasis. We studied the affect of glucose-derived pyruvate removal on compensatory input from glutamine-derived glutamate by accelerated glutamate metabolism via glutamate dehydrogenase (GDH). In glutamine minus glucose media (Gln-Glc), NH_4^+ production increased 41% without an increase in glutamine uptake consistent with accelerated glutamate metabolism via GDH. Alanine production dropped 40% consistent with a shift of glutamate from alanine aminotransferase (ALT) to GDH. Troglitazone (TRO) added to the Gln-Glc media further enhanced glutamate metabolism via GDH at the expense of glutamate metabolism via ALT since alanine production dropped an additional 70%. TRO reduced cell glutamate content 30% while increasing lactate production 5-fold consistent with blocking of cytosolic pyruvate formed from mitochondrial malate from reentering the cycle and maintaining keto acid pool homeostasis. Consequently mitochondrial keto acid pool deficit pulls glutamate via GDH into the cycle. Additionally TRO reduced cytosolic pH which effectively pushes glutamate via GDH, rather than merely shifting glutamate from ALT to GDH. Providing intramitochondrial pyruvate in the form of methyl pyruvate reduced glutamate metabolism via GDH and elevated glutamate metabolism via ALT to control levels restoring acid-base balance. Our findings are consistent with TRO regulation of anaplerosis dependent upon dual pull (cycle keto-acid deficit)/push (cytosolic acidosis) mechanisms.

Introduction

Mitochondria function to generate ATP from the oxidation of pyruvate and also to provide Krebs cycle intermediates as biosynthetic building blocks. Glutaminolysis [1] and specifically glutamate's oxidative deamination via glutamate dehydrogenase [2] supplies Krebs cycle keto acid intermediates to the mitochondrial pool (anaplerosis) in response to intermediates leaving the pool (cataplerosis) [3], Fig. 1. From this perspective,

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mitochondrial function may be quantitatively different in cancer cells vs. normal cells dependent upon substrate preference [4], for example shifting more to the biosynthetic mode and glutamate utilization [5] with aerobic glycolysis providing ATP (the Warburg effect [6]). One approach to mimic this metabolic difference in normal cells is to challenge keto acid pool homeostasis by limiting the nutrient input as has been done for cancer cells. Thus, withdrawing glucose from glioblastoma cells [7] shifts input into the cycle keto acid pool from glucose-derived pyruvate to dependence on glutamate derived from glutaminolysis reflected in enhanced ammoniagenesis as the result of glutamate's oxidative deamination (2 NH_4^+ formed via GDH, Fig. 1); curiously deamidation via glutaminase (1NH_4^+ formed via PAG, Fig. 1) is essentially little changed [7]. The resulting alpha ketoglutarate (αKG) formed by deamination of glutamate can be converted to malate and, if malate exits the mitochondria and is converted to pyruvate in the cytosol, this keto acid can reenter the cycle contributing to pool homeostasis [7]. However, if pyruvate's entrance into the cycle is blocked as may occur in tumor cells [5, 8], then lowered cycle alpha ketoglutarate effectively "pulls" glutamate via GDH supplying net keto acid just as occurs in glucose withdrawal. However, in contrast to glucose withdrawal, pyruvate formed from malate in the cytosol would be unable to reenter the mitochondria, and, as a default pathway, glycolysis and lactate accumulation could occur (the Warburg effect, Fig. 1) while mitochondria would utilize glutamate dedicated largely to biosynthetic functions [2, 9]. Noteworthy, oncogene inhibition of PDH [10], and, or, reduced pyruvate transport into mitochondria may contribute to the Warburg effect with enhanced glycolysis meeting energy demands while glutaminolysis and specifically glutamate oxidation via GDH supports growth.

The ability to shift from pyruvate to glutamate oxidation on demand would therefore offer strategies for activating and possibly interdicting anaplerosis providing an agent was available to effect this shift. Here we propose that the antihyperglycemic thiazolidinedione troglitazone fulfills this criteria by acting to 1) block pyruvate from contributing to the cycle keto acid pool, reducing α-KG and thus "pulling" glutamate via GDH into the cycle, and simultaneously, 2) lower cytosolic pH and thus "pushing" glutamate through GDH into the cycle as α-KG. To demonstrate this model we utilized LLC-PK_1 cells because they exhibit potentially high PDH and GDH fluxes [11], and, in addition, a high ALT flux [11] which provides a telltale for intramitochondrial pyruvate/glutamate availability in support of alanine formation and indirectly the keto acid pool homeostasis (e.g. a high alanine production may reflect a full keto acid pool and a low alanine production a depleted pool). Accordingly, we first confirmed accelerated ammoniagenesis in response to glucose withdrawal with keto acid pool homeostasis defended by glutamate-derived α-KG. We then demonstrated that TRO further activated GDH as reflected by increased NH_4^+ production without increased glutamine utilization and by reduced alanine formation. Finally, to test this model, we delivered intramitochondrial pyruvate as its methyl ester which reverted the ammonium/alanine production back to control consistent with TRO's regulation of anaplerosis by a novel "pull/push" mechanism.

Materials and Methods

Experiments were performed on LLC-PK_1 cells grown on DMEM containing 5 mM glucose, 2 mM glutamine and 1 mM pyruvate until confluent and then harvested by trypsinization and reseeded as previously described [11] on 35 mm chambers coated with DMEM for pHi, NHE activity or on 12 well dishes for metabolic studies. After confluency the DMEM was switched to one containing 2 mM glutamine plus 5 mM glucose(Gln+Glc) or 2 mM glutamine minus glucose(Gln-Glc) and incubated for 24h at 37 °C. To test for PPAR gamma dependent effects, cells were preincubated for 15 min with 10 μM GW9662 prior to TRO addition, after which the antagonist was included in the media of 24 h studies. Methyl pyruvate was obtained from SIGMA-Aldrich St. Louis MO.

Metabolite Measurements

Net removal of substrates from and net addition of products to the media were measured at 24 h using HPLC (glutamine, glutamate and alanine) enzymatic assay (lactate and glucose), microgasometry (CO_2) and microdiffusion methods (NH_4^+) as previously described [11]. After removal of the media, cells were placed on ice and were washed 3x with ice cold KHH (Krebs Henseleit Hepes) buffer before being scraped into 5% ice cold TCA (trichloroacetic acid). The cell suspensions were quick frozen at -80 °C, thawed and homogenized using a polytron at full speed for 30 seconds and the pellet and supernatant promptly obtained by centrifugation. The supernatant was analyzed the same day for amino acids with the pellet digested in 0.2N NaOH overnight and protein content estimated using the dye-binding assay of Bradford [12].

Cell pH and NHE activity assays

Cells grown on 35mm chambers for 24 hrs in Gln-Glc media were assayed for cytosolic pH using BCECF and ratiometric spectrometry as previously described [11]. Spontaneous pHi measurements were carried out at 37 °C after 10 minutes of loading with 10 μM BCECF in Krebs Henseleit Hepes buffer,

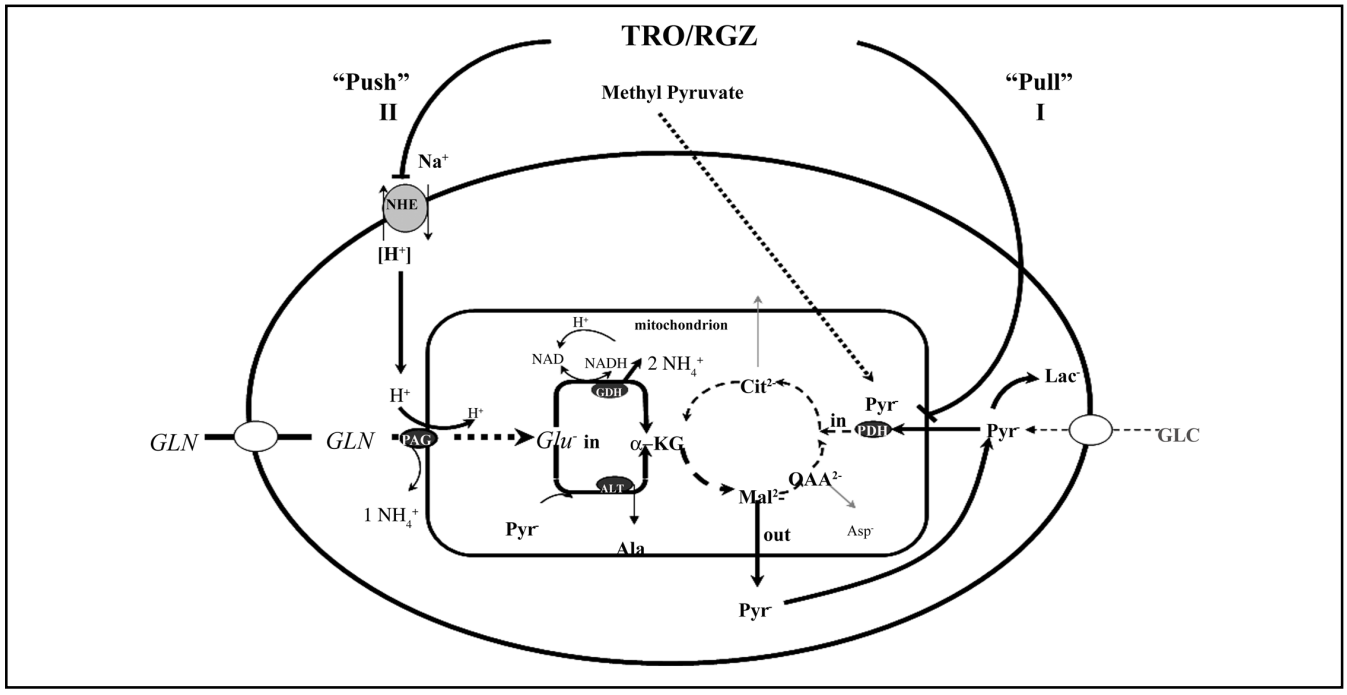

Fig. 1. Model for troglitazone(TRO)/rosiglitazone(RGZ) affect on mitochondrial anaplerosis in LLC-PK$_1$ cells through a "pull/push" mechanism. Mitochondrial keto acid pool depicted as supplied (in) by pyruvate (Pyr$^-$) and glutamate (Glu$^-$) with keto acids leaving the mitochondrial pool (OUT) specifically as malate (Mal), but also as oxaloacetate (OAA) and citrate (Cit). Glucose withdrawal removes pyruvate's contribution with lowered alpha ketoglutarate (αKG) pulling Glu$^-$ through glutamate dehydrogenase (GDH) into the cycle and forming Mal which can leave the mitochondria converting to Pyr$^-$ in the cytosol and reentering the cycle. Glu$^-$ deamination via GDH generates the amino nitrogen ($2NH_4^+$) as NH_4^+ increasing the NH_4^+/produced per glutamine utilized ratio towards two; Glu$^-$ transamination via ALT generates alanine (Ala) and consumes keto acid Pyr-. Proposed glitazone block of Pyr$^-$ entry into cycle and inhibition of acid extrusion by NHE exerts a dual pull (decreased (αKG)/push(increased proton gradient) affect to increase glutamate deamination via GDH and reciprocal fall in glutamate transamination via alanine aminotransferase (ALT). Providing intramitochondrial Pyr$^-$ in the form of methyl pyruvate by-passes glitazone block of mitochondrial Pyr-entry, fills the cycle keto acid pool and eliminates pull effect on GDH; Glu- and $1NH_4^+$ (amide nitrogen) formed by continued glutaminolysis (phosphate activated glutaminase, PAG) defaults into ALT and restored Ala production.

pH=7.40. NHE activity was assayed after NH_4Cl(20 mM NH_4Cl replacing molar equivalent of NaCl) acid loading for 4 min; a standard H^+ stimulus (pHi˜6.75) for NHE-mediated acid extrusion was obtained by 1 min exposure to minus sodium (choline chloride substituted for sodium chloride) KHH buffer followed by recovery measurements in KHH (pH=7.40) media over a 4min time course. NHE activity was obtained from the slope of the rapid recovery over the first 30secs and expressed in ΔpHi/Δtime; all activity measurements were obtained without TRO or rosiglitazone in the recovery media.

Metabolic Pathways

Net utilization and production of metabolites were obtained from the change in media concentration after subtracting the media blank incubated in the absence of cells and expressed as per mg protein. Glutamine metabolized via glutaminase was estimated from the rate of glutamine disappearance from the media after 24 h since LLC-PK$_1$ cells do not express glutamine synthetase and 90% of ammonium produced could be accounted for by the amide and amino nitrogens of glutamine [11]. Alanine production was taken as an estimate of net glutamate metabolized via alanine aminotransferase. Because glutamine utilization was unchanged in the absence of glucose, or, in the presence of TRO [11], or, both combined, increased ammonium production derives from the amino nitrogen and accelerated GDH (Fig. 1). We previously demonstrated using ^{15}N-labelled glutamine in the amide and amino positions [11], that TRO increased ammonium production from glutamine's amino nitrogen. Thus increased ammonium production without increased glutamine utilization is taken as increased net glutamate metabolized via GDH both with TRO and in comparing Gln+Glc vs. Gln-Glc media responses. As a consequence, an increased ratio of ammonium produced to glutamine disappearing reflects accelerated net glutamate deamination while a decreased ratio indicates reduced net glutamate deaminated. The small lactate formation in the Gln-Glc media is taken as reflecting pyruvate formed from malate in the cytosol and entering the glycolytic pathway rather than reentering the mitochondria (Fig. 1); TRO's effect on lactate production in Gln-Glc media is taken to reflect pyruvate blocked from reentering the mitochondrial keto acid pool, instead redirected into cytosolic glycolytic conversion to lactate (Fig. 1).

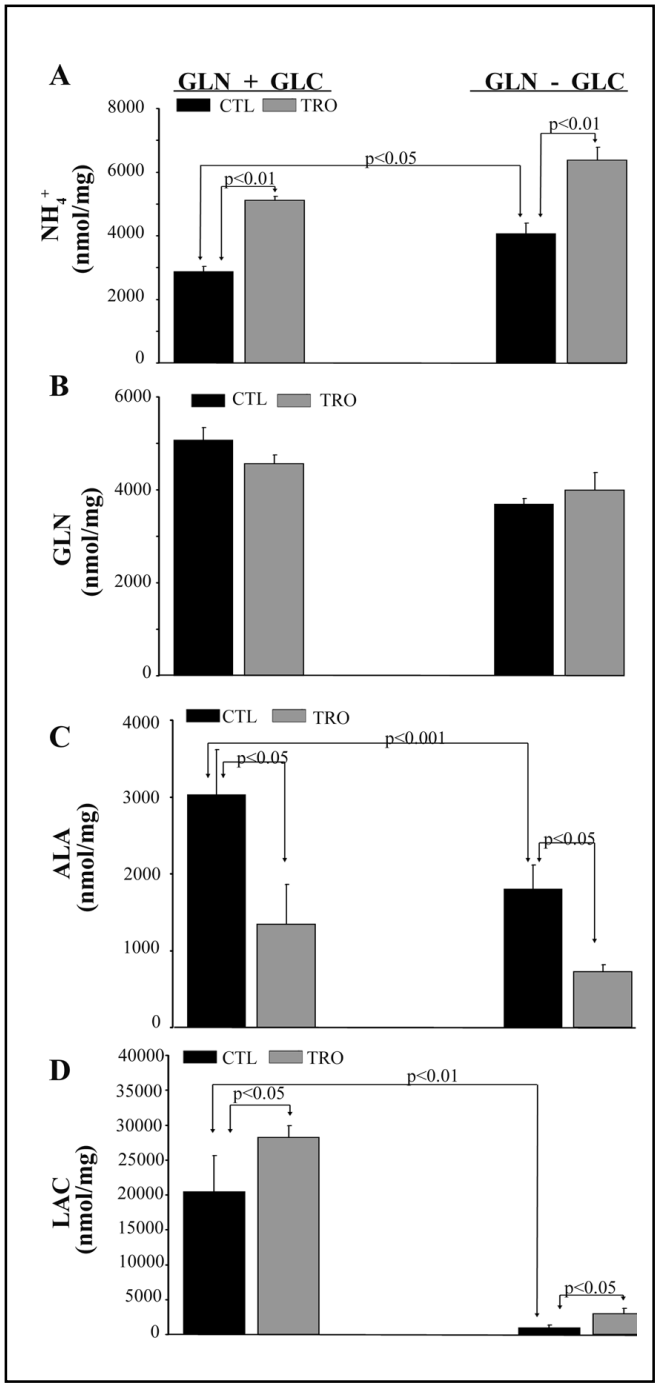

Fig. 2. Glucose withdrawal(Gln-Glc media) accelerates glutamate metabolism via GDH by the "pull" mechanism with alanine production via ALT supported by pyruvate reentry; TRO blocks pyruvate reentry and reduces cycle pool keto acid level enhancing "pull" on GDH. 2A. Ammonium production (nmol/mg protein) by LLC-PK_1 cells incubated for 24hs in either Gln (2mM) + Glc (5mM) media or Gln (2mM) - Glc media in the absence or presence of 25 uM TRO. 2B. Gln utilized in 24hs in nmol/mg protein. 2C. Ala production in 24hs in nmol/mg protein. 2D. Lactate production in 24hs in nmol/mg protein. Results are means ± sem from 5 sets (Gln+Glc vs. Gln-Glc) of experiments with differences judged significant at p<0.05.

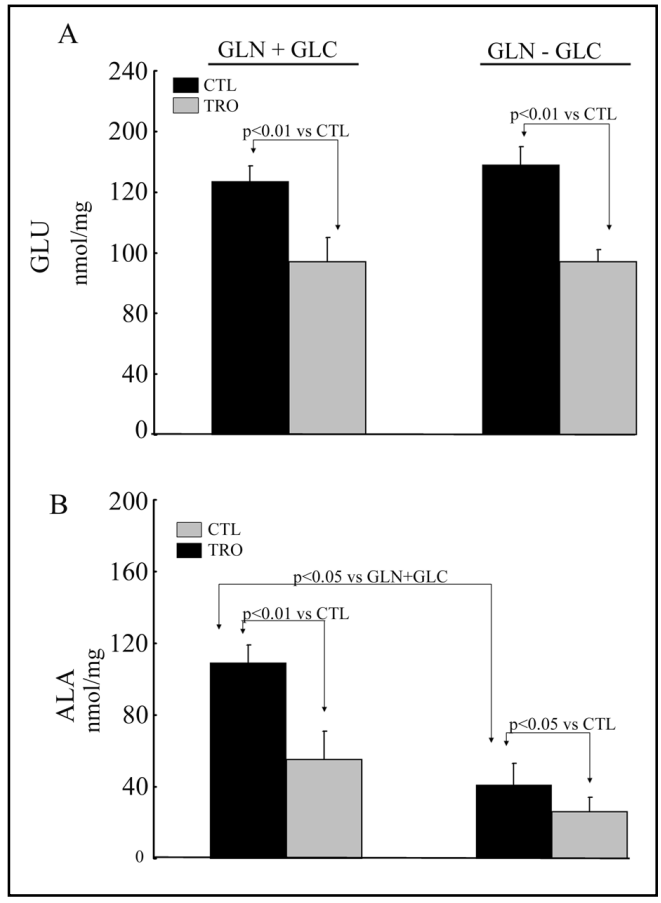

Fig. 3. TRO decreases cell glutamate and alanine content. 3A. Cell glutamate measured at 24hs and expressed in nmol/mg protein from 4-7 sets of experiments. 3B. Cell alanine content measured at 24h and expressed in nmol/mg proteins. Results are means ± sem from 4-6 sets of experiments with differences judged significant at p<0.05.

Statistical Analysis

Comparisons between multiple groups (repeated measurements) were made using ANOVA and a corrected Student's t test (Bonferroni) with the null hypothesis rejected at p<0.05. A one-tailed t table was consulted when an *a priori* hypothesis was tested as depicted in Fig. 1; otherwise a two-tailed table was used.

Results

Removal of glucose from the media (Gln-Glc, Fig. 2A) resulted in increased ammonium production by 41% (p<0.05) without an increase in glutamine removal (Fig. 2B), so that the NH_4^+ produced/glutamine utilized ratio rose from 0.57 to 1.10 consistent with accelerated glutamate metabolism via GDH (Fig. 1). Gln-Glc media

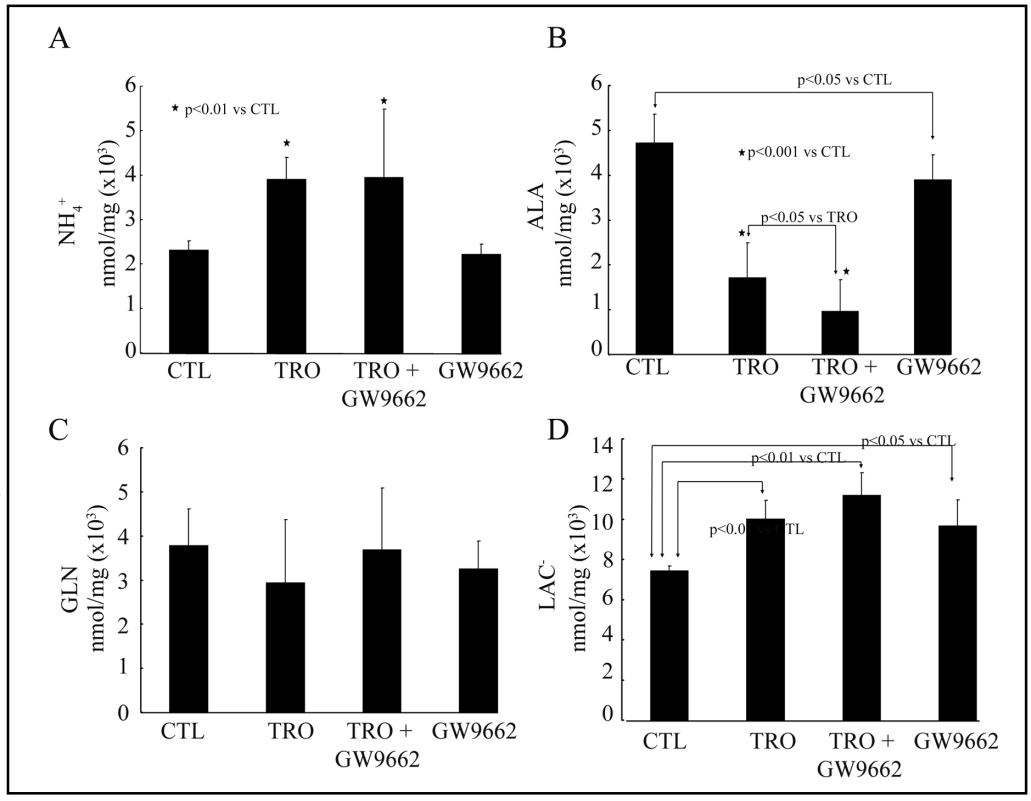

Fig. 4. GW9662, a PPARgamma agonist blocker, does not prevent TRO's affect on ammonium and alanine production. 4A. GW9662,10 uM, does not prevent TRO-induced NH_4^+ production increase; 4B. GW9662 does not prevent TRO-induced alanine production decrease.4C. GW9662 does not affect glutamine utilization. 4D. GW9662 does not prevent TRO-induced increase in lactate production. Results are means ± sem from 6 sets of experiments with differences judged significant at p<0.05.

supported alanine production (Fig. 2C) at 60% (p<0.05) of the Gln+Glc media, consistent with malate leaving the mitochondria and reentering as pyruvate (Fig. 1). On the other hand, lactate production from Gln-Glc was only 5% (p<0.01) of that for Gln+Glc media, Fig. 2D, in line with pyruvate entering the mitochondrial keto acid pool rather than the cytosolic glycolytic pathway and lactate formation (Fig. 1).

Troglitazone, 25 μM, induced a large increase (p<0.01) in ammonium production for both Gln-Glc and Gln+Glc media vs. respective controls (Fig. 2A) (Δ 2295 and 2234 nmol/mg protein) without an increase in glutamine utilization (Fig. 2B); consequently, the NH_4^+ produced/ glutamine utilized ratio increased from 1.1 to 1.6 with Gln-Glc and from 0.57 to 1.12 in Gln+Glc media consistent with an additive increase in glutamate metabolism via GDH in the Gln-Glc media and acceleration of glutamate metabolism via GDH in the Gln+Glc media. Evidence that TRO affects glutamate metabolism via GDH, in part, by shifting glutamate from ALT and alanine production is supported by the large fall (p<0.001) in alanine production, (Fig. 2C) (Δ -1080 and -1694 nmol/mg protein, for both Gln-Glc and Gln+Glc media). Noteworthy, the TRO-induced increase in NH_4^+ production exceeds the fall in alanine production suggestive of additional factor(s) playing a role in acceleration of glutamate metabolism via GDH. The large 3-fold increase in lactate production in the Gln-Glc media, (Fig. 2D) and the 38% increase (p<0.05) in Gln+Glc media suggests that pyruvate generated from malate in the cytosol, or, from glucose in the Gln+Glc media was blocked by TRO from entering the cycle and subsequently entered the glycolytic pathway (Fig. 1).

Cell glutamate concentration, Fig. 3A was similar in the Gln-Glc and Gln+Glc media consistent with reentry of pyruvate maintaining the keto-acid pool. On the other hand, TRO reduced cell glutamate 32 and 36% (both p<0.01) in Gln+Glc and Gln-Glc media respectively consistent with a fall in the cycle keto-acid pool size. TRO also reduced the cellular alanine content by 49 and 37% respectively (Fig. 3B, both p<0.05) an affect that was additive with the lowered cell alanine in the Gln-Glc media consistent with TRO limiting reentry of pyruvate into the mitochondria.

Pre-incubation with GW9662, 10uM, a potent inhibitor of PPAR gamma agonist binding did not reverse the affects of 25uM TRO (Fig. 4); NH_4^+ production was unaffected by GW9662 alone (Fig. 4A), and in combination with TRO (NH_4^+ production increased 68 and 70% with either TRO and TRO+GW9662 vs. Control,

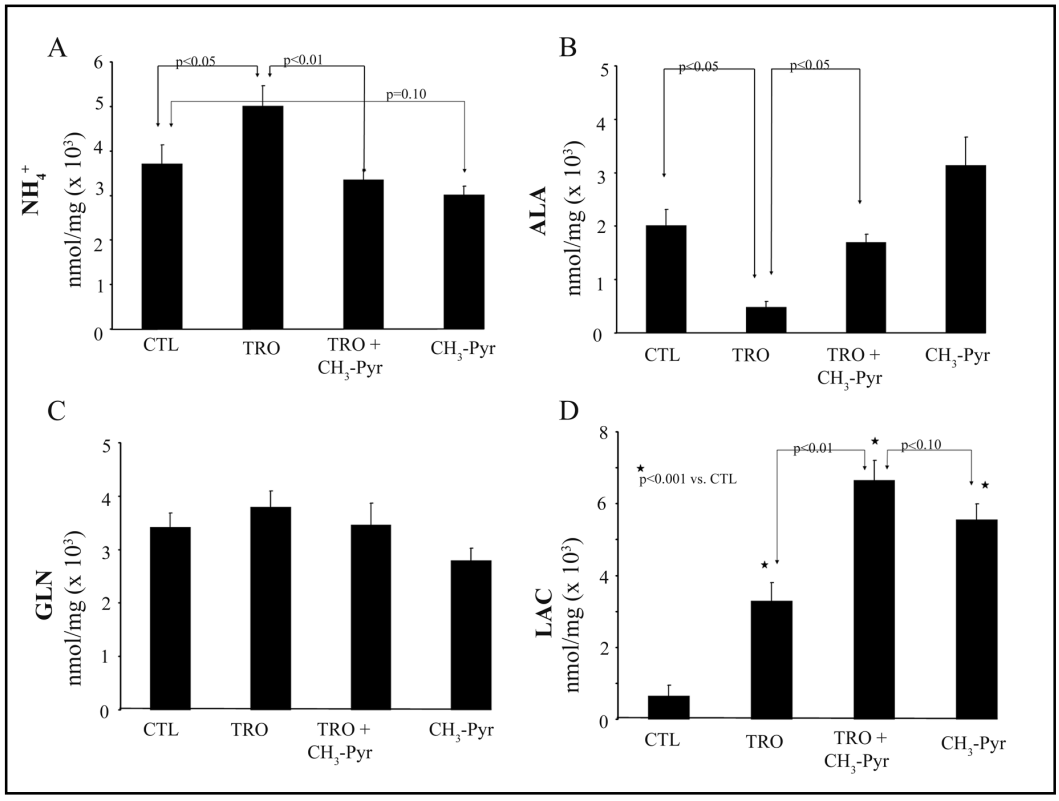

Fig. 5. Methyl pyruvate reverses the affect of TRO on ammonium production via GDH and alanine production via ALT. 3A. NH_4^+ production in 24hs in nmol/mg protein for LLC-PK_1 cells incubated in Gln-Glc media. 3B. Ala production in 24hs in nmol/mg protein. 3C. Gln utilized in 24hs in nmol/mg protein.3D. Lactate production in nmol/mg protein. Results are means ± sem from 5 sets of experiments.

both p<0.01; Fig. 4B). TRO alone induced a 64% reduction (p<0.001) in Ala production which was further exacerbated by GW9662 in combination with TRO (80% reduction, p<0.05 vs. TRO alone). Interestingly, GW9662 alone reduced alanine production (17%, p<0.05 vs. Control) and cellular glutamate content by 12%, p<0.10 (data not shown). Neither TRO alone nor in combination with GW9662 affected Gln utilization (Fig. 4C). The increase in lactate production (Fig. 4D) induced by TRO (35%, p<0.05) was not inhibited by GW9662 but rather increased to 50% in combination (p<0.01 vs. Control) whereas GW9662 alone increased lactate production by 29% (p<0.05). These results are consistent with TRO's affects being via PPAR gamma independent mechanisms.

To test if TRO limits intramitochondrial pyruvate, the highly permeable methyl ester of pyruvate, metabolized in the mitochondria to pyruvate [13], was added to the Gln-Glc media and ammonium production assessed along with alanine production, Fig. 5. Methyl pyruvate, 10 mM, suppressed (p<0.01) the large TRO-induced increase in NH_4^+ production, Fig. 5A, without affecting glutamine utilization, Fig. 5C, so that the NH_4^+/gln ratio falls(1.37±0.19 to 1.01±0.11, p<0.05). Suppressing ammonium production while maintaining glutamine utilization suggests continued glutaminase conversion of glutamine to glutamate which could undergo transamination, specifically alanine formation, instead of deamination. Indeed, alanine production (Fig. 5B) reduced 76% (481±111vs 2,012±303nmol/mg, p<0.001) by TRO is now restored to the control level (1,699±145 vs. 2,012±303nmol/mg). TRO increased (p<0.01) lactate production (Fig. 5D) in the Gln-Glc media as shown above in Fig. 2D consistent with a block in mitochondrial pyruvate uptake while methyl pyruvate increased lactate in the presence of TRO consistent with more pyruvate entering the glycolytic pathway in the presence of TRO (Fig. 1).

In contrast to 10 mM methylpyruvate, 10 mM pyruvate did not prevent the TRO-induced increase in NH_4^+ production (5,532±289 vs. 3,364±173nmol/mg, p<0.01 vs. Control Gln-Glc media) nor the decrease in alanine production (2,343±98 vs. 2,261±38nmol/mg). Pyruvate alone reduced NH_4^+ production (2,409± 320 vs. 3,364±173nmol/mg, p<0.05 vs. Control Gln-Glc media) and increased alanine production (8,204 ±1,145 vs. 3,367± 160nmol/mg, p<0.01 vs. Control media). These results are consistent with TRO preventing pyruvate, but not preventing the highly permeant methyl ester, from entering the mitochondria.

The reciprocal relationship between ammonium production and alanine production is consistent with glutamate being "pulled" through GDH in response to a

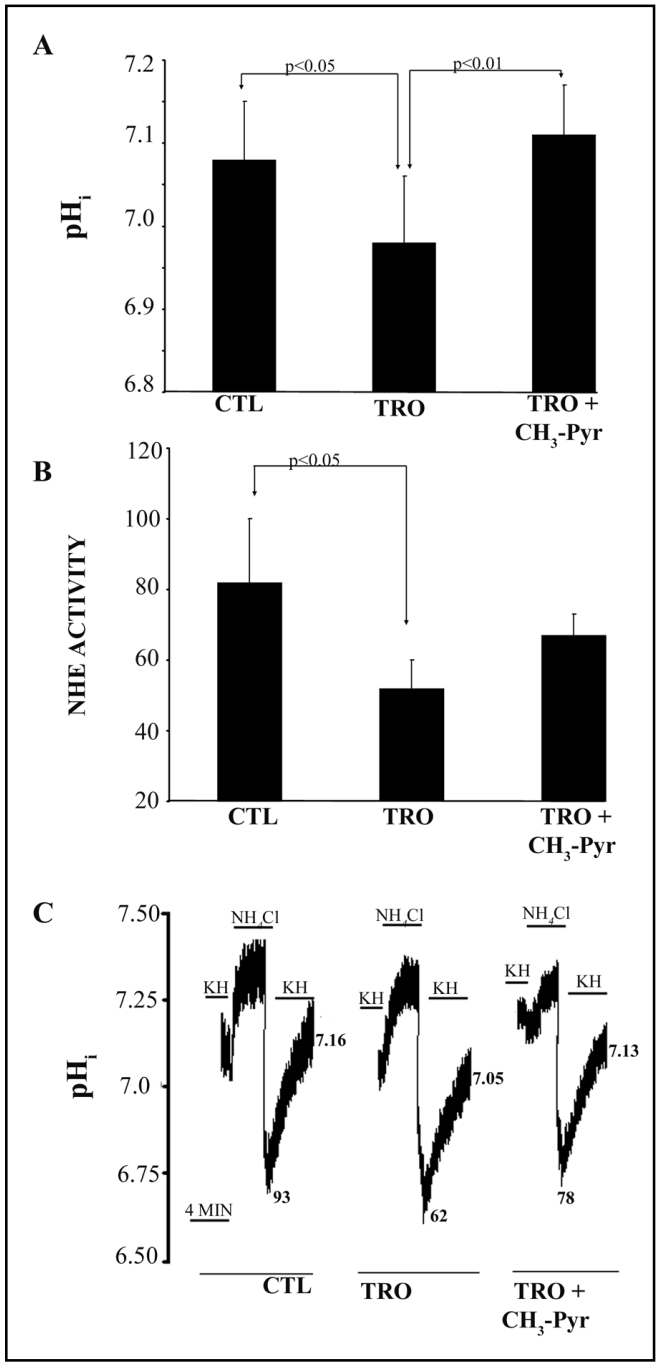

Fig. 6. TRO induces a cytosolic acidosis associated with impaired acid extrusion. 6A. Cytosolic pHi measured as described in Methods after 24 h incubation in Gln-Glc media and TRO (25 μM) and TRO plus methylpyruvate. 6B. NHE mediated acid extrusion following an NH_4Cl administered acid load to cells incubated for 24hs as above. NHE activity expressed as pHi/min in KHH buffer following removal of NH_4Cl; results are means ± sem from 5 sets of experiments.6C. Representative experiment showing 24h TRO exposure reduces acid extrusion activity with TRO absent from the KHH recovery media while TRO plus methyl pyruvate tends to restore NHE activity and recovery pHi.

decreased keto-acid pool size in turn reducing alanine production by limiting pyruvate availability. However, this relationship between the increased NH_4^+ production and decreased alanine production may not simply reflect shifting glutamate from ALT to GDH, rather TRO may exert an additional influence to accelerate glutamate metabolized via GDH by reducing pHi [14]. As shown in Fig. 6A**,** TRO does in fact reduce ($p<0.01$) the cytosolic pH (7.06±0.04 vs. 6.96.±0.05) creating an acidosis that is in the range which is known to accelerate glutamate metabolism via GDH. This reduction in pHi was associated with a 37% reduction ($p<0.05$) in the NHE-mediated acid extrusion rate (Fig. 6B) following an NH_4Cl acid load as depicted in a representative experiment shown in Fig. 6C; TRO-treated cells display a slower rate of recovery and achieve a lower recovery pHi following the acid load than do control cells with both the rate of recovery and recovery pHi tending to be restored with methyl pyruvate. Consequently by reducing NHE mediated acid extrusion TRO establishes a sustained cytosolic acidosis that may "push" glutamate formed from PAG [15] into the GDH pathway down a proton gradient [16].

Rosiglitazone, 25uM, increased NH_4^+ production 86% (2,188± 287 to 4,079± 338nmol/24h/mg,$p<0.01$) and decreased alanine production by 79% (3,794 ±215 to 788±156nmol/24h/mg,$p<0.001$) without affecting glutamine utilization (3,381±713 and 3,452±483nmol/24h/mg respectively) consistent with a shift from ALT to GDH shown above for TRO. Lactate production increased (8,036±391 to 10,143±1020nmol/24h/mg, $p<0.05$) while cellular glutamate decreased (208±23 to 172±17nmol/mg protein, $p<0.05$) and the cytosol was decidedly acidotic (spontaneous pHi 6.87±0.07 vs. 7.20±0.03 RGZ vs. Control, $p<0.05$).

Discussion

Mitochondrial keto acid pool homeostasis requires input from pyruvate and, or, glutamate, or, both pyruvate and glutamate as normally occurs in order to maintain ATP generation and cycle biosynthetic functions, Fig. 1 [2, 3]. With glucose withdrawn from the media, mitochondrial keto-acid pool homeostasis is defended by an increased glutamate deamination and ammonium production via GDH with pyruvate generated from malate in the cytosol reentering the cycle pool. This net gain in the keto acid pool derives from a shift in glutamate-derived from glutamine to deamination via GDH (and net keto-

acid production of alpha-ketoglutarate) at the expense of transamination via ALT (no net gain since pyruvate is consumed cancelling alpha-ketoglutarate) rather than increased glutamine utilization (Fig. 1**,** PAG flux) in LLC-PK_1 cells similar to what was previously shown in glioblastoma cells [**7**]. Thus, acute glucose deprivation in LLC-PK_1 cells results in increased ammonium production (Fig. 2A) without increased glutamine utilization (Fig. 2B) consistent with accelerated GDH flux which in turn apparently defends cycle keto acid homeostasis as suggested by the normal cell glutamate level in the absence of glucose (Fig. 3A).

Troglitazone and rosiglitazone, are antihyperglycemic agents that can act acutely [17], increasing glucose uptake coupled to enhanced glycolysis and lactate production [18]. How this is brought about is not clear but one possibility is limited mitochondrial ATP generation by blocking pyruvate uptake, PDH activity and oxidation [18-20] with compensatory activation of aerobic glycolysis. If so, mitochondrial keto acid homeostasis would necessitate another keto-acid input; here we propose an increased GDH flux and alpha ketoglutarate entry into the cycle. In contrast to the well studied affects of these agents on glucose metabolism, little attention has been paid to glitazones affect on glutamine metabolism, particularly glutamate fluxes via GDH [11], and ALT [11] in relation to anaplerosis.

The effect of TRO to accelerate ammoniagenesis and inhibit alanine production could not be blocked by GW9662 indicating that the underlying mechanism(s) is PPARgamma receptor-independent. Furthermore these affects of TRO are apparent over therapeutic plasma concentrations, 5 μM (data not shown), and a similar response can be elicited by rosiglitazone. Although the simplest explanation for the inverse relationship between GDH/ALT is reduced keto acid pool size and the shift of glutamate from ALT to GDH in compensation for the pool deficit ("pull" mechanism), that may not be the complete picture since the increase in ammoniagenesis may (but not always) exceed the decrease in alanine production suggesting an additional factor. From this perspective (Fig. 1), inhibition of acid extrusion by NHE results in a cytosolic acidosis that specifically activates GDH flux [14, 16] that would constitute an additional 'push" mechanism for glutamate to contribute net keto acid to the mitochondrial keto acid pool. TRO does in fact induce a sustained cytosolic acidosis that can be explained by the combination of a reduced NHE activity, Fig. 6C and increased acid production, Fig. 2D [21]. Noteworthy, both TRO and rosiglitazone have been shown to inhibit NHE activities through direct and indirect mechanisms [22, 23, 24]. These findings are consistent with, and, predictable from the proposed "push" (cytosolic acidosis)/"pull"(reduced keto-acid pool) mechanism. Consequently, glutamates' contribution to anaplerosis via GDH might be regulated under physiological conditions such as exercise or metabolic acidosis and pathologic conditions like tumor growth by the judicious use of TRO or rosiglitazone.

To test whether TRO does in fact limit pyruvate's contribution to the keto acid pool by inhibiting access to the mitochondrial Krebs cycle pool, we used the highly permeant methyl ester of pyruvate to restore intramitochondrial pyruvate availability [13]. If TRO inhibits delivery of pyruvate into the mitochondria, then methyl pyruvate should by-pass that block and restore alanine production while restoring the keto-acid pool and relieving the "pull" mechanism. On the other hand, if TRO's affect was specifically to inhibit PDH, then pyruvate delivery would have no affect on ammoniagenesis, although it might further increase the alanine production. The results show that methyl pyruvate suppressed TRO's induced increase in ammoniagenesis, as well as returning alanine production to the control level, Fig. 5A and C. Taken together our results are more consistent with TRO's effect being that of limiting mitochondrial pyruvate uptake rather than inhibiting PDH activity as the mechanism underlying the "pull" affect. Although the effectiveness of methyl pyruvate in restoring the apparent control GDH/ALT fluxes in TRO treated LLC-PK_1 cells was in line with the "pull" mechanism, the magnitude of the GDH suppression was unexpected because cellular acidosis, the "push" mechanism, would not be expected to be affected by intramitochondrial pyruvate delivery/metabolism. Surprisingly, both cytosolic pH and NHE activity were also nearly restored (Fig. 6B) with keto acid pool homeostasis raising the possibility of some functional link between anaplerosis, alanine production and cellular acid-base balance. Given the dependence of cancer cell proliferation on a relatively alkaline cytosolic pH [25], regulation of anaplerosis may offer strategies as well as diagnostic insights into therapeutic treatments.

The ability of another glitazone, rosiglitazone to mimic troglitazone's effect on apparent GDH/ALT fluxes over a therapeutic plasma concentration range raises the possibility of a mitochondrial receptor for thiazolidindiones that regulates pyruvate entrance, oxidation and, or, PDH activity such as the recently discovered mito-NEET protein [19, 26]. Interestingly, pioglitazone was shown to be a

high affinity ligand for mitochondrial mito-NEET [26] and pioglitazone activates GDH flux [27] and also induces a chronic–cytosolic acidosis [27] consistent with PPARgamma dependent down regulation of NHE expression [24] suggesting an affect on anaploresis via the "push" mechanism. Noteworthy the PPARgamma antagonist, GW9662, was ineffective in blocking either the "pull" or "push" mechanisms activated by TRO consistent with a site other than PPARgamma and one possibly associated with the mitochondria at which TRO and RGZ bind and affect anaplerosis.

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References

1 McKeehan WL: Glycolysis, glutaminolysis and cell proliferation. Cell Biol Intern Reports 1982;6:635-650.

2 DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB: Beyond aerobic glycolysis: Transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci 2007;104:19345-19350.

3 Owen OE, Kalhan SC, Hanson RW: The key role of anaplerosis and cataplerosis for citric acid cycle function. J Biol Chem 2002;277:30409-30412.

4 Rossignol R, Gilkerson R, Aggeler R, Yamagata K, Remington SJ, Capaldi RA: Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer Res 2004;64:985-993.

5 Regan DH, Lavietes BB, Regan MG, Demopoulos HB, Morris HP: Glutamate-mediated respiration in tumors. J Natl Cancer Inst 1973;51:1013-1017.

6 Warburg O: On the origin of cancer cells. Science 1956;123:309-314.

7 Yang C, Sudderth J, Dang T, Bachoo RG, McDonald JG, DeBerardinis RJ: Glioblastoma cells require glutamate dehydrogenase to survive impairments of glucose metabolism or Akt signaling. Cancer Res 2009;69:7986-7993.

8 Busch H: Studies on the metabolism of pyruvate-2C14 in tissues of tumor bearing rats. Cancer Res 1955;15:365-374.

9 Nyhan WL, Busch H: Metabolic patterns for glutamate-2-C14 in tissues of tumor bearing rats. Cancer Res 1958;18:385-393.

10 Christofk HK , Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wie R, Fleming MD, Schreiber SL, Cantley LC: The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 2008;452:230-233.

11 Welbourne T, Friday E, Fowler R, Turturro F, Nissim I: Troglitazone acts by PPARγ and PPARγ-independent pathways on LLC-PK_1-F^+ acid-base metabolism. Am J Physiol 2004;289:F100-F110

12 Bradford MM: A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.

13 Jijakli H, Nadi AB, Cook L, Best L, Sener A, Malaisse WJ: Insulinotrophic action of methyl pyruvate: enzymatic and metabolic aspects. Arch Biochem Biophys 1996;335:245-257.

14 Nissim I, Sahai A, R. Sandler RS, Tannen RL: The intensity of acidosis differentially alters the pathways of ammoniagenesis in LLC-PK_1 cells. Kidney Int 1994;45:1014-1019.

15 Kvamme E, Torgner IA, Roberg B: Evidence indicating that pig renal phosphate glutaminase has a functional predominant external localization in the inner mitochondrial membrane. J Biol Chem 1991;266:12185-12192.

16 LaNoue KF, Schoolwerth AC: Metabolite transport in mitochondria. Ann Rev Biochem 1979;48:871-922.

17 Lee MK, Olefsky JM: Acute effects of troglitazone on in vivo insulin action in normal rats. Metabolism 1995;44:1166-1169.

18 Fediuc S, Pimenta AS, Gaidhu MP, Ceddia RB: Activation of AMP-activated protein kinase, inhibition of pyruvate dehydrogenase activity, and redistribution of substrate partitioning mediate the acute insulin-sensitizing effects of troglitazone in skeletal muscle cells. J Cell Physiol 2008;215:392-400.

19 Feinstein D, Spagnolo A, Akar C, Weinberg G, Murphy P, Gavrilyuk V, Dello Russo C: Receptor-independent actions of PPAR thiazolidinedione agonists: is mitochondrial function the key. Biochem Pharm 2005;70:177-188.

20 Fürnsinn C, Brunmair B, Neschen S, Roden M, Waldhäusl WJ: Troglitazone directly inhibits CO(2) production from glucose and palmitate in isolated rat skeletal muscle. Pharmacol Exp Ther 2000;293:487-493.

21 Oliver R III, Friday E, Turturro F, Welbourne T: Troglitazone induced cytosolic acidification via ERK activated mitochondrial depolarization: Complex I proton pumping regulates ammoniagenesis in proximal tubule like LLC-PK_1 cells. Cell Physiol Biochem 2008;22:475-486.

22 Coates G, Nissim I, Battarbee H, Welbourne T: Glitazones regulate glutamine metabolism by inducing a cellular acidosis in MDCK cells. Am J Physiol 2002;283:E729-737.

23 Turturro F, Friday E, Fowler R, Surie D, Welbourne T: Troglitazone acts on cellular pH and DNA synthesis through a peroxisome proliferator-activated receptor γ-independent mechanism in breast cancer-derived cell lines. Clin Cancer Res 2004;10:7022-7030.

24 Kumar AP, Quake AL, Chang MK, Zhou T, Kim KS, Singh R, Hewitt RE, Salto-Tellez M, Pervaiz S, Clement MV: Repression of NHE1 expression by PPARgamma activation is a potential new approach for specific inhibition of the growth of tumor cells in vitro and in vivo. Cancer Res 2009;69:8636-8644.

25 Pouyssegur J, Franchi A, L'Allemain G, Paris S: Cytoplasmic pH, a key determinant of growth factor-induced DNA synthesis in quiescent fibroblasts. FEBS 1985;190:115-119.

26 Coca JR, McDonald WG, Weldon DJ, Leone JW, Lull JM, Bannow CA, Lund ET, Mathews WR: Identification of a novel mitochondrial protein ("mitoNEET") cross- linked specifically by a thiazolidinedione photoprobe. AJP Endo Metab 2003;286:E252-E260.

27 Turturro F, Oliver R III, Friday E, Nissim I, Welbourne T: Troglitazone and pioglitazone interactions via PPAR-gamma-independent and -dependent pathways in regulating physiological responses in renal tubule-derived cell lines. Am J Physiol Cell Physiol 2007;292:C1137-1146.