Troglitazone Induced Cytosolic Acidification via Extracellular Signal-Response Kinase Activation and Mitochondrial Depolarization: Complex I Proton Pumping Regulates Ammoniogenesis in Proximal Tubule-like LLC-PK₁ Cells

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Key Words

PPAR γ agonists • Mitogen Activated Protein Kinase Kinase/Extracellular Signal-Regulated Kinase (MEKK/ERK) inhibitors • Cytosolic pH • Sodium/Hydrogen ion exchanger (NHE) • Acid production • 5-(N-ethyl-N-isopropyl) amiloride • EIPA • Glutamine metabolism

Abstract

Purpose: To determine the mechanism(s) through which troglitazone induces cytosolic acidification and glutamine-dependent ammoniogenesis in pig kidney derived LLC-PK₁ cells. **Experimental Design:** Acute experiments measured acid extrusion, acid production and simultaneous Extracellular Signal-Regulated Kinase activation. TRO-enhanced acid production was correlated with mitochondrial membrane potential and rotenone and 5-(N-ethyl-N-isopropyl) amiloride, were employed to test specifically the role of Complex I proton pumping. Chronic experiments correlated inhibitors of Complex I with prevention of TRO-increased ammoniogenesis and effects on glutamine metabolism. **Results:** Exposure to TRO acutely activated Extracellular Signal-Regulated Kinase in a dose dependent manner associated with a fall in

spontaneous cytosolic pH. Cytosolic acidosis was associated with both an increase in acid production and inhibition of sodium/hydrogen ion exchanger - mediated acid extrusion. Preventing TRO-induced Extracellular Signal-Regulated Kinase activation with Mitogen Activated Protein Kinase Kinase inhibitors blocked the increase in acid production, restored sodium/hydrogen ion exchanger-activity and prevented cytosolic acidification. Mechanistically, increased acid production was associated with a rapid mitochondrial depolarization and Complex I proton pumping. Blocking Extracellular Signal-Regulated Kinase activation prevented both the fall in Ψ_m and the increased acid production suggesting that the former underlies the accelerated mitochondrial "acid production". Mitochondrial Complex I inhibitors EIPA and rotenone prevented increased acid production despite Extracellular Response Kinase activation and reduced sodium/hydrogen ion activity. Inhibition of Complex I prevented TRO's effects on glutamine metabolism. **Conclusion:** TRO induces cellular acidosis through Extracellular Signal-Regulated Kinase activation-associated acid production and impaired acid extrusion. Acutely, increased acid production

reflects mitochondrial Complex I proton pumping into the cytosol while chronically Complex I activity appears coupled to mitochondrial glutamate uptake and oxidation to ammonium at the expense of cytosolic transamination and alanine formation in these proximal tubule-like cells.

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Introduction

Peroxisome proliferator-activated receptor gamma (PPAR γ) agonists have pleiotrophic actions on cellular function that involve the classical PPAR γ mediated pathway [1] as well as less well recognized pathways involving Mitogen Activated Protein Kinase Kinase (MEKK) [2] and mitochondrial [3] signaling. We have previously shown [4] in proximal tubule-like LLC-PK₁ cells that all 3 pathways can be demonstrated and proposed that physiological responses may reflect the interaction of more than one of these pathways. Because a primary function of proximal tubule cells is in acid-base regulation we have focused on cellular acid-base homeostasis and specifically ammoniagenesis from glutamine.

Extracellular Signal-Regulated Kinase (ERK) activation occurs in kidney cells in response to metabolic acidosis in vivo as well as in cultured kidney cells [5-7] suggesting ERK activation plays a role in physiological responses. However, the underlying mechanisms relating TRO-induced ERK activation and cellular acidosis requires further clarification. Our previous studies [8-11] focused on NHE-mediated acid extrusion and lactic acid production as the determinants for the TRO-induced cytosolic acidification observed in a variety of renal and tumorigenic cells. These studies clearly showed that while NHE-mediated acid extrusion was impaired [8, 9, 11], lactic acid production was not increased leading to the conclusion that decreased acid extrusion rather than increased production accounted for the cytosolic acidification. Realization of ERK activated acid production [12] led to consideration of other sources of protons potentially able to acutely acidify the cytosol, including NADPH oxidase activated pentose shunt [13] and mitochondrial "acid production" [9] leading to a reevaluation of the relative roles for acid production vs. acid extrusion in the cytosolic acidification. To evaluate cellular acid-base balance in the context of ERK activation, the model depicted in Figure 1 was deployed. Accordingly, cytosolic pH reflects the balance between acid production, intracellular acid buffering capacity, and under these

conditions, NHE-mediated acid extrusion. Acid production can be assessed from 1] that titrated in the media at the end of the study plus 2] that retained within the cell based on the buffering capacity of the cell ($\beta = \Delta \text{ acid load} / \Delta \text{ pHi}$) and the decrease in pHi. The NHE activity as well as the spontaneous pHi at which acid production and extrusion reach a near steady-state following an exogenous acid load (recovery pHi) can also be measured in these same cells. Since the effect of TRO on cellular acid-base balance is mediated via ERK activation [6], p-ERK can also be simultaneously measured providing an integrative perspective of acid-base regulation via this pathway.

Employing this model our first goal was to determine the mechanism of the profound cytosolic acidification and the relationship to ERK activation induced by TRO. The results to follow show that an acute increased acid production and a decreased NHE-mediated acid extrusion both contributed to the cytosolic acidification and that these responses are coordinately regulated via TRO-induced ERK activation. Surprisingly, increased acid production rather than decreased extrusion is the dominant factor in the acute cytosolic acidification. Furthermore both were coordinately controlled by ERK since blocking activation in the presence of TRO prevented cytosolic acidification. ERK activated acid production was dependent upon mitochondrial depolarization and could be prevented with MEKK inhibition consistent with an important interaction between ERK and mitochondrial signaling pathways as proposed [4, 6].

Materials and Methods

Experimental Design

Experiments were designed to allow measurements of acid production, cytosolic and media pH as well as ERK activation and NHE activity as described in the model above (Figure 1). LLC-PK₁ cells obtained from American Type Culture Collection (Manassas, VA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum, (in mM) 28 NaHCO₃, 120 NaCl and 5 mM D-glucose plus 2 mM L-glutamine at 37°C and 5% CO₂ (pH 7.47). The confluent cells were detached with trypsin-EDTA and reseeded onto customized designed 30 mm glass chambers (Biopetechs, Biological Optical Technologies Butler, PA) previously layered with DMEM. Cells grown on chambers were studied within 1-2 days of seeding being confluent and exhibiting domes. The cells were loaded with 5 μ M BCECF-AM(2,7-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein BCECF-AM Molecular Probes Eugene, OR) for 10 minutes at 37°C in HEPES(15 mM) buffered Krebs - Henseleit (KHH) media containing 5 mM D-glucose and pH 7.4

Fig. 1. Experimental model designed to measure spontaneous cytosolic pH (pHi) as a function of acid production and NHE mediated acid extrusion regulated by TRO-induced ERK activation. Acid production in nmoles/12 min was measured as the sum of acid produced and 1] buffered within the cell and that 2] extruded into the media. NHE activity was assayed following application of, 20 mM NH₄Cl, and removal, 0 mM NH₄Cl, acid pulse as the rapid upswing following replacement of 140 mM Choline with 140 mM Na⁺ and as the recovery plateau pHi (recovery point). ERK was activated by TRO and assayed at the end of 12 minutes; ERK activation affects on acid production and NHE activity were ablated by inhibiting MEKK using preincubation with PD98059+UO126 in combination. Dotted lines indicated steps not yet elucidated in the TRO-activated pathways.

and then promptly transferred to the stage of an Olympus IMT-2 microscope equipped with a heated stage insert. Fluorescent measurements were made of regions not expressing domes and at 37°C through an inverted epifluorescence microscope as previously described [8, 9]. After washing the cells 2xs with weakly buffered KHH media containing 1 mM HEPES pH 7.4 and 5 mM glucose they were incubated for 12 minutes in 0.5 mL of 1 mM HEPES buffered KHH. This media was then removed for analysis of titratable acid and lactate and then either lysis buffer for western blot analysis of ERK activation, or, normal KHH containing 20 mM NH₄Cl (substituted for an equivalent mM NaCl) then added for NHE activity assay; for Western blot analysis of p-ERK the cells were immediately scraped into the lysis buffer and processed the same day [6]; for NHE activity the recovery response following removal of 20 mM NH₄Cl, 1 minute of minus sodium chloride (choline chloride), KH media titrated to 7.4 with choline bicarbonate and then normal KHH (15 mM HEPES, pH 7.4) media for 8 minutes recovery. The NHE activity was assessed from the rapid recovery upswing (Figure 1) while the plateau pHi attained was observed as the “recovery point” pHi (Figure 1). Thus all 3 parameters, acid production, acid extrusion rate/recovery point pHi and ERK activation could be related to the spontaneous cytosolic pH observed after 12 minutes.

Total acid production

Acid production over the 12 minute time course was measured as the sum of acid titrated in the weakly buffered KH media using 0.02 N NaOH to return the media pH to 7.4. Acid produced and retained intracellularly was determined from β , the intracellular buffer capacity, times the Δ pHi. Media pH measure immediately at 37°C fell approximately 0.1-0.2 units (total volume 500 μ l) whereas the cytosolic pH ranged from 7.0- 7.3 for controls to 6.6 to 6.9 for TRO-treated with an

approximate intracellular volume of $2 \pm 0.4 \mu$ l determined for confluent monolayer's on 30 mm chambers as the difference between simultaneously measured ³H water volume minus ¹⁴C-methoxy inulin volume. Intracellular buffering capacity was assessed using progressively decreasing concentrations of NH₃/NH₄⁺ and measuring $\beta = \Delta [\text{NH}_4^+]_i / \Delta \text{pHi}$ [14]; $[\text{NH}_4^+]_i$ was determined from $[\text{NH}_3]_i$, pK and pH with $[\text{NH}_3]_i$ assumed to equal $[\text{NH}_3]_o$. Taking $\Delta [\text{NH}_4^+]_i = \Delta [\text{H}^+]_i$ gave β an average of 16 nmol H⁺/0.1 pHunit/2 μ L cell volume over the pHi range 6.8-6.4. Although this value for β may overestimate the intracellular retained acid in the controls where pHi remained in the 7.0-7.3 range, this overestimate would in effect account for only a small error because most of the acid produced in control cells was extruded to the media. Lactic acid production was analyzed in the media using lactate oxidase (Pointe Scientific Canton, MI) and expressed as nmoles lactate released per 12 minutes. To test the effect of the hydrophobic amiloride derivative 5-(N-ethyl-N-isopropyl)amiloride (EIPA, SIGMA, St. Louis) on acid production, cells were pre-incubated with 40 μ M EIPA immediately after BCECF loading followed by 40 μ M EIPA added to the media for the 12 minute study where appropriate.

Intracellular pH, p-ERK and NHE activity measurements

Spontaneous cytosolic pH was monitored continuously over 12 minutes as the BCECF emission ratio and calibrated using high K⁺/nigericin calibration method as previously described [8, 9]. At the end of 12 minutes cells were either analyzed for ERK activation (phosphorylation) or for NHE activity. To prevent ERK activation, cells were pre-incubated for 5 minutes with KHH plus the MEKK inhibitors 30 μ M PD98059 plus 10 μ M UO126 after which the cells were studied for an additional 12 minutes in the presence of these inhibitors. To assess the effect of EIPA on NHE activity, 40 μ M EIPA was present in the minus sodium and KHH recovery media.

In contrast TRO was not present in the media used to assess NHE activity after 12 minutes with TRO in the 1 mM HEPES KH media.

Mitochondrial $\Delta\Psi_m$ measurement and Mitotracker Green Imaging

Cells were grown to confluence in chambers in DMEM and then loaded with the potentiometric dye tetramethylrhodamine ethyl ester (TMRE, Molecular Probes) at 25 nM in KHH media plus 1 $\mu\text{g}/\text{ml}$ Hoechst 33342 (nuclear stain) for 10 min at 37°C. After loading, the cells were immediately washed and then treated for 12 minutes at 37°C exactly as in the above pHi measurements. Immediately at 12 minutes the cells were viewed on a Nikon Eclipse TE300 inverted microscope utilizing IPLab3.7 software. TMRE is a nonfixable cationic dye that is taken up and retained in energized mitochondria according to $\Delta\Psi_m$; increased inner membrane proton permeability will deenergize the mitochondrial membrane resulting in a release of the dye from the mitochondrial matrix resulting in reduced fluorescence. MitotrackerGreen (Molecular Probes), which accumulates in the mitochondria regardless of membrane potential, was used to monitor mitochondrial localization and matrix volume.

Mitochondrial Isolation and Complex I Assay

LLC-PK₁ cells were collected using trypsin and centrifugation at 1000 xg for 5 minutes at 4°C. The pelleted cells were resuspended in ice cold DMEM and immediately repelleted followed by resuspension in ice cold wash buffer (ApoAlert, Cell Fractionation Kit, Clontech) and promptly pelleted as above. The cells were then resuspended in ice cold fractionation buffer and mitochondria and cytosolic fractions obtained as per protocol (ApoAlert, Clontech) with the efficiency of homogenization checked by microscopic examination as recommended. The resulting mitochondrial pellet was resuspended in 0.1 mL of 10 mM Tris/pH 7.8 and frozen at -80°. Aliquots of both cytosolic and mitochondrial fractions were analyzed by dye binding assay for protein (Bio-Rad) and by Western blotting for intrinsic mitochondrial membrane cytochrome oxidase IV and diffusible cytochrome c protein using antibodies provided (ApoAlert, Clontech) as indices of mitochondrial distribution in the two fractions and mitochondrial integrity respectively. Mitochondria suspended in 10 mM Tris, pH 7.6, and frozen at -80° were thawed just prior to assaying Complex I activity.

Complex I activity

Mitochondria frozen suspended in hypotonic 10 mM Tris buffer were thawed and assayed for activity in reducing 2,6-dichloroindophenol (DCIP) following the addition of NADH+H⁺. The advantage to using DCIP reduction as opposed to NADH+H⁺ oxidation is the 3 fold greater sensitivity of the former as an electron acceptor [15]. Mitochondria (0.5 μg to 5 μg) were incubated at 37°C in 1 mL assay media containing 25 mM potassium phosphate (pH 7.5), 3.5 g/L bovine serum albumin (fatty acid free), 60 μM DCIP, 70 μM decylubiquinone and 1 μM antimycin-A. DMSO (control) or DMSO containing 5 μM rotenone or DMSO containing 20 μM EIPA were added

Fig. 2. A shows a representative response for pHi from chambers over a 10 fold TRO concentration range (TRO present only for the 12 minute spontaneous pHi time course). pHi values for each are from the end of the 12 minutes time course and recovery point. B shows results from 24 chambers used for the acid production values (n=6), and 12 chambers for ERK and 12 for NHE activity measurements (n=3). Results are means $\pm$ sem with * and ** representing $p < 0.05$ and $p < 0.01$ respectively.

for 10 min prior to adding NADH+H⁺ (20 μL 10 mM NADH+H⁺). DCIP reduction was monitored at 600 nm over 8 minutes at 1 min intervals. The assay was linear with time and mitochondrial protein over the 8 min time course.

Metabolic studies

Cells were grown to confluency in 24 well dishes and incubated in 0.5 ml DMEM containing 5mM D-Glucose and 2 mM L-glutamine for 18 h after which the media was taken for analysis of ammonium (microdiffusion) and alanine/glutamate/glutamine production by HPLC as previously described [11] Acid production over 18 h was determined from the decrease in media bicarbonate content measured as total CO₂ by microgasometric analysis [6] and compared to media incubated in the absence of cells; lactic acid production was measured

enzymatically using lactate oxidase as described above. The effect of EIPA and rotenone on the 18 h ammonium or alanine production as well as glutamine utilization was expressed as net production/utilization per 18 h per mg protein.

Statistical analysis

Differences between control and TRO and TRO plus treatments responses were analyzed by ANOVA and a corrected t-test (Dunnett) with the nul hypothesis rejected at $p < 0.05$.

Results

To test the model presented in Figure 1 for measuring total acid production (sum of acid buffered within the cell plus acid titrated in the media), ERK activation and NHE activity (following exogenous NH_4Cl acid load), LLC-PK₁ cells were exposed to a 10 fold TRO concentration range (5–50 μM). As shown in a representative experiment in Figure 2A and for 5 additional experiments in 2B, p-ERK activation occurs at the lowest concentration (5 μM TRO, 1.1 ± 0.1 vs. 0.4 ± 0.1 , $p < 0.01$) and increases to 5 fold higher than the control at 25 μM (2.23 ± 0.43 vs. 0.4 ± 0.1 , $p < 0.01$) and to 10 fold at 50 μM TRO. Total acid production was enhanced 2 fold at 5 μM (118 ± 30 vs. 54 ± 9 nmoles, $p < 0.05$) and increased to 3 fold at 50 μM with 49, 42, and 36% of the acid produced retained within the cell at 50, 25, and 5 μM respectively in contrast to only 7% in the controls. NHE activity assayed at the end of the 12 min time course and without TRO in the media was reduced by 67, 65, and 15% at 50 ($p < 0.01$), 25 ($p < 0.01$), and 5 ($p < 0.10$) μM respectively compared to the control. The recovery pH_i following the exogenous NH_4Cl acid load was depressed at 50, 25, and 5 μM , 6.74 ± 0.06 ($p < 0.01$), 6.87 ± 0.08 ($p < 0.01$) and 7.03 ± 0.09 ($p < 0.10$) respectively compared to the control (7.20 ± 0.09) and not different from their respective spontaneous pH_i measured at the end of 12 min. Consequently, spontaneous pH_i falls because of both increased acid production and decreased NHE activity (recovery pH_i) resulting in retention of the acid load within the cytosol.

To assess the role of ERK activation on both acid production and acid extrusion, MEKK inhibitors (PD98059 plus UO126) were used in combination (see Methods) to block ERK activation and these same responses monitored. Figure 3A presents a representative experiment showing that these two MEKK inhibitors largely prevents both the decrease in spontaneous pH_i measured at the end of 12 mins and restores NHE activity. Figure 3B shows results from 5 additional experiments.

Fig. 3. A shows a representative response for pHi for control and 25 μM TRO-treated chamber and after ERK inhibition by combined 30 μM PD98059+10 μM UO126. B shows results from 24 chambers for acid production values ($n=6$) and 12 for ERK and NHE respectively ($n=3$). Results are means $\pm$ sem with * and ** representing $p < 0.05$ and $p < 0.01$ respectively.

At 25 μM , TRO-induced ERK activation 5.1 fold ($p < 0.01$), increased total acid production by 1.5 fold ($p < 0.05$) of which 46% was buffered intracellularly and reduced NHE activity by 61% ($p < 0.01$). As a consequence, both the spontaneous pH_i and the recovery pH_i measured following the exogenous NH_4Cl acid load were reduced (6.76 ± 0.04 vs. 7.04 ± 0.03 , $p < 0.01$ for spontaneous pH_i and 6.65 ± 0.06 vs. 7.07 ± 0.04 , $p < 0.01$ for recovery pH_i). Treatment with MEKK inhibitors prevented ERK activation (3.8 ± 1.0 vs. 0.11 ± 0.07 , $p < 0.01$), eliminated the increased total acid production (111 ± 10 vs. 178 ± 20 nmoles, $p < 0.01$ vs. 118 ± 22 nmoles CTL) and restored NHE activity resulting in spontaneous (end of 12 min) and recovery pH_i not different from control (6.92 ± 0.05 vs. 7.04 ± 0.04 and 7.03 ± 0.06 vs. 7.07 ± 0.04 respectively). These results are consistent with ERK activation being largely responsible for the coordinated increase in acid production and the

Fig. 4. A shows Western blot for mitochondrial cytochrome c oxidase IV subunit and cytochrome c present in subcellular fractions labeled mitochondria and cytosol; fractions isolated from two cultures. B shows NADH+H dependent DCIP reduction as an index of Complex I activity and sensitivity to 5 μ M rotenone or 40 μ M EIPA; results from 6 different experiments. No DICP reduction occurred in the absence of NADH or mitochondrial fraction.

decrease in NHE activity.

To assess whether increased lactic acid production contributes the enhanced acid production, lactate was measured after the 12 min time course. Interestingly, ERK activation induced by 25 μ M TRO was associated with reduced (39 ± 3 vs. 56 ± 5 nmoles, $n=6$, $p<0.05$), rather than increased, lactic acid production and the ratio of total acid produced to lactic acid actually increased with ERK activation (2.2 ± 0.6 to 4.6 ± 0.8 , $p<0.05$); preventing ERK activation restored lactic acid production (51 ± 4 nmoles) as the acid production decreased with the total acid to lactic acid ratio returned to control (2.0 ± 0.4). This inverse relationship between enhanced acid production and decreased glycolysis suggests that p-ERK activated acid production results from either the pentose shunt or mitochondrial pathways. To test for the former we employed an inhibitor (apocynin) of NADPH oxidase

Fig. 5. A shows representative experiment of 40 μ M EIPA preventing cytosolic acidification in the presence of 25 μ M TRO; neither NHE activity nor ERK activation were affected by EIPA. B shows results from 24 chambers for acid production and 12 each for NHE and ERK measurements. Results are means $\pm$ sem with * and ** representing $p<0.05$ and $p<0.01$ respectively.

activity which is presumed to enhance the pentose shunt pathway by accelerating NADP availability [13]. However, the potent NADPH oxidase inhibitor apocynin (10 μ M) failed to prevent TRO-induced intracellular acidification (6.79 vs. 6.80 for TRO +APO and TRO alone, $n=2$) and failed to prevent enhanced total acid production (144 vs. 135 vs. 76 for TRO+APO, TRO and control respectively) suggesting that NADPH oxidase activation and subsequent increased pentose shunt activity does not account for the increased acid production.

We had previously shown [4, 8, 11] utilizing ¹⁵N-amino labeled glutamine that TRO-induced ERK activation accelerated glutamate flux through the mitochondrial-matrix glutamate dehydrogenase (GDH) while the NADH+H⁺ produced in this reaction is coupled to the mitochondrial inner membrane Complex I. Complex I activity mediates the movement of electrons and loosely

Fig. 6. TRO reduces mitochondrial membrane potential, Ψ_{mit} , as reflected by tetramethylrhodamine ethyl ester, TRME, retention for control, 5, 25, and 50 μ M TRO after 12 minute incubation at 37°C in KHH media, top panel. Bottom panel shows nuclei (stained with Hoechst 33342) distribution in the same field. Inserts show mitochondria outlined using Mitotracker Green for control and 25 μ M TRO.

coupled protons [16] with the latter extruded into the intermembrane compartment between the inner (IM) and outer (OM) membranes and contiguous with the cytosol. Previous studies in mitochondria isolated from rat liver [17] demonstrated that EIPA, like rotenone, was a potent inhibitor of Complex I. To confirm that EIPA would inhibit Complex I in our cells, we isolated mitochondria (Figure 4A) and assayed Complex I activity following 10 minute preincubation with 40 μ M EIPA and compared the response to that seen with 5 μ M rotenone (Figure 4B). As shown in 4A the mitochondrial fraction contained virtually all of the inner membrane cytochrome c oxidase IV protein while cytochrome c remained and had not leaked out into the cytosol consistent with enrichment of intact mitochondria in this fraction. To assess whether EIPA would inhibit Complex I activity, we compared the EIPA response to that of the known inhibitor rotenone.

Figure 4 B shows that $68 \pm 5\%$ of the measured DCIP reduction was prevented by 5 μ M rotenone and that 40 μ M EIPA was almost as effective in inhibiting $60 \pm 8\%$ (both $p < 0.001$ vs. control).

We then employed EIPA to test a putative role of Complex I TRO-induced cytosolic acidification in cells exposed to 25 μ M TRO as shown in Figure 5A. Noteworthy, EIPA prevented the fall in cytosolic pH_i that otherwise occurs despite TRO-induced p-ERK activation and reduced NHE activity (Figure 5B), emphasizing the importance of the increased acid production. EIPA alone at 40 μ M had no affect on NHE activity or on spontaneous pH_i after 12 minutes consistent with its effect being inhibition of Complex I proton pumping into the cytosol. As shown in Figure 5B acid production was reduced ($p < 0.01$ vs. TRO) to the control level (166 ± 36 to 45 ± 9 nmoles compared to control, 63 ± 6 nmoles), despite

Fig. 7. Preventing ERK activation abrogates the fall in $\Delta\Psi_{mit}$ induced by 25 μM TRO. Top panel shows TRME staining for control, TRO-treated and TRO-treated following MEKK inhibition with 30 μM PD98059+10 μM UO126 and 12 minute incubation in KHH at 37°C. Bottom panel shows nuclei distribution in the same field.

the 3 fold p-ERK activation with the spontaneous pHi measured after 12 min higher ($p < 0.01$) than TRO (7.12 ± 0.03 vs. 6.78 ± 0.05) and not different from the control (7.09 ± 0.04); in contrast, the NHE activity remained reduced with TRO+EIPA at the level observed with TRO alone (0.062 ± 0.010 vs. 0.086 ± 0.013 vs. 0.14 ± 0.01 $\Delta\text{pHi}/\text{min}$ for control) demonstrating increased acid flux into, rather than out of, the cytosol was responsible for the acute acidification. Similar results were obtained using 5 μM rotenone in combination with TRO (TRO-induced acid production decreased ($p < 0.01$) from 122 ± 7 to 70 ± 3 nmoles, while increased ERK activation and decreased NHE activity were both maintained, data not shown). Together, these results are consistent with cytosolic acidification dependent upon p-ERK activated acid production channeled through Complex I proton pumping.

Cytosolic acidification resulting from Complex I proton pumping necessitates that the inner mitochondrial membrane become permeable to protons so as to prevent limiting alkalization of the matrix space. This putative back leak of positively charged protons into the matrix

would have the effect of reducing both the proton as well as the charge gradient ($\Delta\Psi_{mit}$) across the inner membrane. To test whether in fact mitochondria become depolarized under these conditions, cells were loaded with the mitochondrion $\Delta\Psi$ sensitive stain (TRME) and then exposed for 12 min to 50, 25, and 5 μM TRO or media alone (same dose-response experiment as shown in Figure 2A for cytosolic acidification). As shown in Figure 6, in control cells the mitochondrion $\Delta\Psi$ sensitive dye appears brightly in punctuate particles ringing the nuclei and threading out into the cytosol; at 5 μM TRO the dye intensity decreases although the perinuclear localization of the dye remains distinct; at 25 μM TRO dye intensity is further reduced and no longer distinct showing a faded halo effect surrounding the nuclei and at 50 μM TRO the dye appears diffuse throughout the cytosol consistent with an inability to retain the mitochondrial membrane sensitive dye within the mitochondria. The mitochondria stained with Mitotracker Green and shown as inserts for control and 25 μM TRO confirm that the TRO-induced diffusion of the membrane-sensitive dye occurs with a similar

Table 1. Effect of TRO vs. TRO plus Complex I inhibitors on 18 h Metabolic Fluxes(nmol/mg protein)

	CTL	TRO (25uM)	EIPA (20uM) + TRO	ROTENONE (5uM) + TRO
NH ₄ ⁺	1195±170	2087±208**	1219±75	876±292
ALA	1022±61	565±77*	919±83	1368±185
Glu ⁻	15±10	33±1	125±41*	62±8*
Gln	2235±191	1467±258	2037±483	2189±502
H ⁺	6890±408	6880±238	7315±408	7761±321
Lactate	4592±394	4337±532	6715±1536	7242±842

*p<0.05, **p<0.01, N=6

mitochondrial volume as observed in the control field. Since the increased acid production and cytosolic acidification could be largely prevented by blocking p-ERK activation, cells were pre-incubated for 5 min with MEKK inhibitors and then exposed to 25 μ M TRO+PD98059 plus UO126, Figure 7 (essentially the same experimental protocol as above in Figure 3A). Prevention of ERK activation largely restored the mitochondrial retention of the $\Delta\Psi$ m sensitive TRME. These findings (Figures 6, 7 and 2A, 3A) are consistent with enhanced H⁺ fluxes across both the inner and outer mitochondrial membranes (inward flux reducing $\Delta\Psi$ m and enhanced outward flux resulting in cytosolic acidification). Experiments were performed over the 10 fold TRO dose range(5-50 μ M) with prevention of ERK activation resulting in enhanced dye retention within the mitochondria at all TRO concentrations (data not shown). These findings are consistent with mitochondrial depolarization and cytosolic acidification as closely linked responses largely dependent upon TRO-induced ERK activation.

An important physiological consequence of mitochondrial Complex I activation within these proximal tubule-like cells could be increased oxidation of glutamate yielding ammonium and alpha ketoglutarate (α KG²⁻ potential base) plus NADH+H⁺. Further, a fall in cytosolic pHi could drive glutamate formed from glutamine in the outer mitochondrial compartment [18, 19] into the matrix compartment and away from cytosolic transamination (ALT) and alanine formation [8]. If glutamate uptake into the mitochondria is coupled to Complex I proton secretion, then blocking Complex I proton secretion could limit glutamate transport into the mitochondrial matrix, and, by default, preserving cytosolic substrate for the transamination pathway and alanine formation. As shown in Table 1, 25 μ M TRO for 18 h increased ammonium production (2087±208 vs. 1195±170 nmol/mg, p<0.01) associated with a reciprocal decrease in alanine production

(567±77 vs. 1022±61 nmol/mg, p<0.05) consistent with ammonium largely produced from glutamine's amino nitrogen [4, 8, 11]. Note that the increased ammonium and decreased alanine production with TRO occur without an increase in glutamine utilization consistent with a shift of glutamate from cytosolic transamination into the mitochondrial oxidative deamination pathway possibly linked to Complex I proton extrusion. If so, inhibiting Complex I should prevent both the increased ammonium derived from glutamate oxidation but also, as a default pathway, restore alanine production. Employing EIPA, after confirming EIPA to be a potent Complex I inhibitor (Figure 4B above and ref. 8), prevented TRO from increasing ammonium production (1219±75 vs. 2087±208 nmol/mg for TRO alone, p<0.01, Table 1) and just as importantly, restored alanine production (919±83 vs. 565±77 nmol/mg for TRO, p<0.01). To confirm that this effect is specifically a result of Complex I inhibition, we used 5 uM rotenone in combination with TRO. Complex I inhibition prevented TRO from increasing ammonium production (for TRO + rotenone, 876±292, p<0.01 vs. TRO alone, 2087±208 and not different from control, 1195±170 nmol/mg) and restored alanine production (for TRO + rotenone, 1368±185, p<0.05 vs. TRO alone, 565±77 and not different from control 1022±61 nmol/mg). Unlike the acute 12 min study, neither acid production nor the acid production to lactate production ratio (1.5±0.2 vs. 1.6±0.2 for control and TRO respectively) were increased over 18 h suggesting that protons pumped via Complex I into the intermembrane space in part reenter the matrix compartment coupled to glutamate [20, 21] and do not contribute to cytosolic acidification at this time.

Discussion

Our earlier studies [4, 8, 11] revealed the rapid onset and sustained cytosolic acidification effect of troglitazone

and concluded that reduced acid extrusion rather than increased lactic acid production best accounted for the cytosolic acidification. Subsequent studies [6] showed that this rapid cytosolic acidification effect required ERK activation and that preventing ERK activation restored NHE activity and, paradoxically, restored lactic acid production. Our present study demonstrates acute cytosolic acidification results primarily through enhanced mitochondrial “acid production” [22], and, surprisingly, that both the accelerated acid influx into the cytosol as well as the reduced NHE-mediated acid extrusion are largely, and coordinately, dependent upon ERK activation. We also demonstrate that cytosolic acidification and mitochondrial depolarization are closely linked through ERK activation. The proposal of a relationship between ERK activation and mitochondrial depolarization/ “acid production” is a novel concept with potential physiological implications.

As depicted in Figure 2, TRO rapidly and in a dose-dependent manner activates ERK [4, 6] via a putative plasma membrane receptor leading to ERK activation detectable in minutes ([4, 6] and Figure 2B) and sustained for at least 18h [6] in LLC-PK₁ proximal tubule-like cells. ERK activation in these cells has two actions relevant to acid-base regulation: 1] transient activation of mitochondrial “acid production”, and, 2] reduction of NHE3 activity. Interestingly, ERK activation in this proximal tubule like cell is associated with NHE3 inhibition, not unlike NHE3 inhibition and reduced bicarbonate absorption by ERK activation in the thick ascending limb of the nephron [23]. This effect plus ERK activation dependent depolarization of mitochondria may contribute to the profound natriuresis observed in vivo with metabolic acidosis [5, 24-26]. Metabolic base generation requires that glutamate be oxidized by glutamate dehydrogenase within the mitochondrial matrix and coupled to Complex I via NADH+H⁺. Since the functional glutaminase, PAG, may be localized to the intermembrane compartment [18, 19], glutamate formed from glutamine requires H⁺ for coupled transport across the inner mitochondrial membrane [20]. Thus one physiological consequence could be the observed shift in glutamate from the cytosolic transamination pathway and alanine formation into the oxidative pathway and ammoniogenesis as observed for proximal tubule-like cells in vitro (Table 1) and as an increased ammoniogenesis and decreased alanine production from glutamate in vivo for pigs receiving an NH₄CL acid load [27].

Normally, acid pumped out of the mitochondrial matrix via Complex I (and Complexes III and IV) would

diffuse back down the Δ pH into the matrix space, with this potential energy utilized for ATP synthesis [28] and substrate, e.g. glutamate, delivery [20] to reunite with electrons forming water: in other words, no net “acid production” should occur. Although it is clear that the mitochondrial electron transport proton pumping does indeed lower the intermembrane space pH (and by extension, potentially the cytosol as well providing favorable H⁺ diffusion gradients) proton pumping ceases as the Δ pH reaches a maximum. For acid pumping to continue, the pH gradient would have to be modulated downward via a leak in the inner membrane e.g. uncoupling proteins allowing back-flow of protons and reduction of Ψ m, which, the latter in fact we observed (Figure 6). Thus a reduction in mitochondrial membrane potential appears as a prerequisite for enhanced Complex I proton pumping. Our ability to prevent enhanced acid production with TRO-induced ERK activation using EIPA or rotenone is consistent with our studies in isolated mitochondria showing that both EIPA and rotenone inhibit Complex I activity (Figure 4B) and suggests that Complex I with “loose” electron and proton coupling [16, 17] plays the major role in the acute cytosolic acidification response to TRO. In contrast, the prolonged 18h cytosolic acidification is also ERK dependent [6] but unlike the acute increase in mitochondrial “acid production”, the chronic cytosolic acidification appears related to the inhibition of NHE-mediated extrusion of acid produced largely from glycolysis (Table 1) with Complex I acid pumping coupled to glutamate transport into the matrix space. Inhibition of Complex I proton pumping with EIPA and/or rotenone blocks the increased ammoniogenesis derived from the amino nitrogen of glutamine [4, 8, 11] consistent with coupling of Complex I proton pumping to glutamate uptake into the mitochondrion matrix [20]. Because of this blockage, glutamate formed outside the mitochondria would be obligated to enter the cytosolic transamination pathway restoring alanine production as was observed (Table 1) or be released into the media.

EIPA, of course, is a potent inhibitor of other membrane proton pumps e.g. NHE1 which pump acid out of the cytosol into the media, and, presumably, would also inhibit the mitochondrial NHE activity which also pumps protons out of the cytosol and into the matrix space. Therefore inhibition of these proton fluxes, even if they were active under these conditions, would not account for EIPA's action in preventing cytosolic acidification, more likely the opposite. Rotenone, on the other hand, would not face this criticism and acts similarly to EIPA to block both acute acidification and chronic ammoniogenesis

and restore alanine production (Table 1). Others [22] have shown that acute cytosolic acidification can result from enhanced “mitochondrial proton production” and that this acidification of the cytosol can be completely prevented by blocking Complexes I, II, and IV in contrast to our study in which TRO appears to partially uncouple (depolarize) mitochondria (ERK activation dependent) and accelerate Complex I proton pumping.

Protons pumped out of the matrix space and into the intermembrane space contribute to both the pH gradient [28] and electrical potential across the inner mitochondrial membrane (Ψ_m). Because TRO-induced cytosolic acidification rapidly enhances the proton gradient, it would seem unlikely to occur via Complex I unless a significant back leak also occurred to prevent the build up of the limiting proton gradient. If so, a consequence of this putative back flux would be a fall in the Ψ_m which we looked for and, indeed, found. The results show that the greater the ERK activation and increase in acid produced (Figure 2A), the more depolarized the mitochondria appeared (Figure 6); conversely, blocking ERK activation prevented increased acid production (Figure 3B) and also prevented the fall in Ψ_m (Figure 7) suggesting that the two are somehow related. Noteworthy, EIPA blocked the cytosolic acidification but neither the ERK activation nor the fall in Ψ_m (data not shown) suggesting that the Complex I acid secretion requires a proton back leak but whether the proton back leak by itself [4, 24, 29] is sufficient for cytosolic acidification and ammoniogenesis from glutamine’s amino nitrogen requires further study.

Others have previously shown that troglitazone depolarizes the mitochondrial membrane in mitochondria isolated from liver cells as well as in intact cells [30-32]. In general, studies of isolated mitochondria show that at high concentrations (50-100 μ M) and after significant time delays (hours) TRO elicits a decline in Ψ_m ; in live cells [30], on the other hand TRO elicits a fall in Ψ_m within minutes at concentrations as low as 25 μ M, similar to those used in the present study. Noteworthy, in our studies both cytosolic acidification and mitochondrial depolarization could be observed within 12 minutes even at 5 μ M TRO, a concentration within the therapeutic plasma range for troglitazone [31, 33]. Significantly, our studies show the effect of TRO on both the mitochondrial depolarization and cytosolic acidification could be blocked by preventing ERK activation in the presence of TRO ruling out a direct effect of troglitazone on membrane permeability to protons in the present study. Whether, in addition, TRO exerts a direct affect on mitochondrial depolarization and Complex I activity is an important question requiring further studies.

Acknowledgements

This work was supported by the Southern Arizona Foundation (T.W.) and, the Feist-Weiller Research Fund and Louisiana Gene Therapy Research Consortium, Inc(F.T.) and LA Board of Regents Support Fund, LEQSF (2007-09) RD-A 19 (F.T.).

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