Troglitazone’s rapid and sustained activation of ERK1/2 induces cellular acidosis in LLC-PK1-F+ cells: physiological responses

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Troglitazone’s rapid and sustained activation of ERK1/2 induces cellular acidosis in LLC-PK1-F+ cells: physiological responses. Am J Physiol Renal Physiol 288: F1257–F1266, 2005. First published February 1, 2005; doi:10.1152/ajprenal.00205.2004.—We studied the signal pathway through which troglitazone (TRO) acts in inducing cellular acidosis in LLC-PK1-F+ cells in relation to ammoniagenesis and DNA synthesis. Cells were grown to confluent monolayers in 30-mm chambers and monitored for intracellular pH (pHi) by the BCECF assay and activated ERK by phospho-ERK1/2 antibodies. TRO induces a severe cellular acidosis (pHi, 6.68 ± 0.10 vs. 7.28 ± 0.07 time control at 4 min, P < 0.01), whereas phospho-ERK1/2 to total ERK1/2 ratio increases 3.4-fold (P < 0.01). To determine whether ERK1/2 was activated by cellular acidosis or TRO was acting via MEK1/2 to activate ERK1/2, cells were pretreated with specific inhibitors of MEK1/2 activity, PD-98059 and U-0126, followed by the addition of TRO or vehicle. With MEK1/2 activity inhibited, TRO treatment failed to activate ERK1/2. Preventing ERK1/2 activation abrogated the TRO-induced cellular acidosis and maintained the pHi within the low normal range (7.06 ± 0.11). To determine whether blocking ERK activation prevents TRO’s inhibitory effect on NHE activity, cells were acid-loaded and the recovery response was monitored as ΔpH/i over a 4-min recovery period. TRO inhibited NHE activity by 85% (P < 0.01), whereas blocking ERK activation restored the response. We measured activated ERK levels and pHi after 3- and 18-h exposure to TRO or extracellular acidosis (pHe = 6.95) to determine whether ERK activation was sustained. Whereas both TRO and extracellular acidosis increased activated ERK and decreased pHi after 3 h, only TRO sustained this response at 18 h. Furthermore, both enhanced ammoniagenesis and decreased DNA synthesis reflected the effect of TRO to induce and sustain a cellular acidosis.

Troglitazone inhibits acid extrusion and subsequently elicits the physiological changes remains to be elucidated. This mechanism does not correspond to the traditional peroxisome proliferator-activated receptor-γ (PPARγ) signaling pathway given the rapidity of this response (<4 min) and the relative potency of PPARγ agonists (5, 42). In addition, these responses occur in PPARγ−/− cell lines, whereas a potent PPARγ inhibitor (GW9662) failed to block the responses in PPARγ+/− cells (41). Previous studies established that acidosis rapidly activates ERK1/2 (40) while TRO has also been shown to activate ERK1/2 (12, 16, 20, 21, 39). Because our previous studies showed that TRO negatively influences sodium hydrogen exchanger (NHE) activity and markedly lowers pHi, and, as both cellular acidosis (17, 40) and NHE activity (27) are reported to acutely activate ERK1/2, there exists at least two testable pathways leading from TRO to inhibition of NHE activity and resulting cellular acidosis (Fig. 1). Accordingly, pathway I, TRO could directly inhibit NHE (9) resulting in cellular acidosis and then ERK1/2 activation (via MEK1/2). Alternatively, pathway II, TRO might activate ERK via MEK1/2 resulting in NHE inhibition and cellular acidosis. Therefore, we designed experiments to determine whether ERK activation was associated with the TRO-induced cellular acidosis and, if so, whether ERK activation resulted secondarily from acidosis or primarily from TRO. In addition, we compared the physiological response (ammoniagenesis) elicited by TRO with that elicited by extracellular acidosis. The results to follow are consistent with, and, demonstrative of, a novel action of TRO in inducing cellular acidosis via acute and sustained ERK1/2 activation.

MATERIALS AND METHODS

LLC-PK1-F+ cells (14) were grown to confluency in T150 flasks on DMEM media plus 10% fetal calf serum containing (in mM) 28 sodium bicarbonate, 10 sodium pyruvate, 5 glucose, and 2 l-glutamine at 37°C and 5% CO2 (pH 7.4). Confluent cells were subcultured by detaching using trypsin-EDTA (GIBCO BRL, Rockville, MD) and reseeded onto custom-designed 30-mm chambers (Biotechs, Biological Optical Technologies, Butler, PA) equipped with a heating element. The chambers were placed uncapped inside a 60-mm covered tissue culture dish and incubated at 37°C and 5% CO2. For ammoniagenesis and DNA synthesis studies, cells were grown in 12-well culture plates (Corning Cell Wells, Corning, NY). The cells were fed daily and allowed to gain confluence, usually 1–2 days for cells in chambers and 3–4 days for 12-well plates before use. TRO was used at 25 μM through out these studies based on our previous study showing optimal PPARγ-independent responses at this concentration (45).

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TROGLITAZONE IS AN ANTHYPERGLYCEMIA drug that acts as an insulin sensitizer (19). We previously showed that troglitazone (TRO) induces a marked cellular acidosis in a variety of cell types including rat mesangial cells (47); distal tubule-like Madin-Darby canine kidney (MDCK) cells derive from dog kidney (6) and proximal tubule-like LLC-PK1-F+ cells derived from porcine kidney (45). This rapid fall in intracellular pH (pHi) occurring within minutes and persisting for at least 18 h (45, 46) was associated with impaired acid extrusion. Two important consequences of the TRO-induced cellular acidosis are accelerated ammoniagenesis (6, 45) and reduced DNA synthesis (41). However, the signal pathway(s) through which TRO inhibits acid extrusion and subsequently elicits the physiological changes remains to be elucidated. This mechanism does not correspond to the traditional peroxisome proliferator activated receptor-γ (PPARγ) signaling pathway given the rapidity of this response (<4 min) and the relative potency of PPARγ agonists (5, 42). In addition, these responses occur in PPARγ−/− cell lines, whereas a potent PPARγ inhibitor (GW9662) failed to block the responses in PPARγ+/− cells (41). Previous studies established that acidosis rapidly activates ERK1/2 (40) while TRO has also been shown to activate ERK1/2 (12, 16, 20, 21, 39). Because our previous studies showed that TRO negatively influences sodium hydrogen exchanger (NHE) activity and markedly lowers pHi, and, as both cellular acidosis (17, 40) and NHE activity (27) are reported to acutely activate ERK1/2, there exists at least two testable pathways leading from TRO to inhibition of NHE activity and resulting cellular acidosis (Fig. 1). Accordingly, pathway I, TRO could directly inhibit NHE (9) resulting in cellular acidosis and then ERK1/2 activation (via MEK1/2). Alternatively, pathway II, TRO might activate ERK via MEK1/2 resulting in NHE inhibition and cellular acidosis. Therefore, we designed experiments to determine whether ERK activation was associated with the TRO-induced cellular acidosis and, if so, whether ERK activation resulted secondarily from acidosis or primarily from TRO. In addition, we compared the physiological response (ammoniagenesis) elicited by TRO with that elicited by extracellular acidosis. The results to follow are consistent with, and, demonstrative of, a novel action of TRO in inducing cellular acidosis via acute and sustained ERK1/2 activation.
**Fig. 1.** Potential pathways leading from troglitazone (TRO) to cellular acidosis. **I.** TRO is proposed to act to inhibit sodium hydrogen exchange (\(\downarrow\) NHE) activity resulting in cellular acidosis (\(\downarrow\) intracellular pH (pHi)), which then activates MEK1/2 and ERK1/2 followed by increased ammonium production (\(\downarrow\) NH\(_4\)\(^+\)) and decreased DNA synthesis (\(\downarrow\) DNA). **II.** TRO is proposed to act by activating MEK1/2 and ERK1/2 followed by inhibition of NHE and cellular acidosis resulting in \(\downarrow\) NHi\(^+\) and \(\downarrow\) DNA. **III.** Chronic acidosis is divided into an acute phase (0–3 h) and chronic phase (3–18 h) based on results. Note the steps denoted by arrows do not imply direct activation or direct inhibition.

**Cell pHi measurements.** The intracellular pH was assayed using the pH-sensitive fluorescent dye (2,7')-biscarboxyethyl-5-(6)-carboxyfluorescein (BCECF) as described previously (47). Cells were loaded for 10 min with 10 \(\mu\)M BCECF-AM (Molecular Probes, Eugene, OR) dissolved in DMSO and added to HEPES-buffered Krebs Henseleit (KHH) media (pH 7.4) containing 140 mM NaCl, 3 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 20 mM HEPES plus 10 mM glucose, washed once with KHH and promptly studied over a 4-min time course in KHH media; in some experiments, 100 \(\mu\)M amiloride was included in the KHH media to limit the response to the apical NHE3 isomorph (28). TRO, specific MEK1/2 inhibitors (1, 8) PD-098059 (30 \(\mu\)M) and U-0126 (10 \(\mu\)M), or vehicle were added to the KHH and prewarmed to 37\(^\circ\)C; where required, MEK1/2 inhibitors were also present in the 10-min BCECF loading period. For determination of pHi, after 3- and 18-h exposure to TRO and extracellular acidosis, the chambers were incubated with DMEM, DMEM containing TRO, or DMEM, pH 6.95, (DMEM made up with 7 mM bicarbonate instead of 14 mM as previously used) (26) after which the BCECF was loaded in KHH media alone at 7.4 or 6.95 or in KHH containing TRO; pHi was then determined over a 4-min time course in the same KHH media minus the BCECF. The cells were then collected in lysis buffer and P-ERK and total ERK were determined by Western blot analysis.

**Western blot analysis.** The level of phospho-ERK1/2 protein as an index of activation was assayed in the same cells in which the pHi was monitored over the 4-min time course. At the appropriate time points, the KHH was rapidly withdrawn and replaced with an aliquot of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.2, 0.1% Triton X-100, 10 mM EDTA, 5 mM benzamid, and 0.2 mM PMSF plus phosphatase inhibitor cocktails 1 and 2, which contain inhibitors of both threonine and tyrosine protein phosphatases; Sigma, St. Louis, MO). Aliquots of the lysates were promptly analyzed for total protein by a dye-binding assay (34). Thirty-microliter aliquots were mixed with an equal volume of sample buffer (63 mM Tris-HCl, pH 7.2, 2% SDS, 10% glycerol, and 50 mM dithiothreitol) and boiled for 3–5 min. Fifteen micrograms of protein were then resolved by SDS-PAGE on a 10% acrylamide gel with a thickness of 0.75 mm using a Mighty Small Dual Gel Caster (Hoefer, San Francisco, CA). The transfer of proteins to a nitrocellulose membrane (13) was performed with a buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol, pH 8.3. Phosphorylated ERK1/2 at Thr202 and Tyr204 (activated form) (23) and total ERK were detected using a chemiluminescence Western blot system (Immum-Star, Bio-Rad, Hercules, CA) using a phospho-specific ERK1/2 mouse monoclonal IgG primary antibody that recognizes phosphorylated Thr202 and Tyr204 (no. 9106, Cell Signaling Technology, Beverly, MA) and a secondary mouse antibody conjugated to horseradish peroxidase (HRP); total ERK1/2 were measured on the same blot after stripping the P-ERK antibody and using a rabbit polyclonal antibody (phosphorylation site independent, no. 9102, Cell Signaling Technology) and a secondary antibody conjugated to HRP. As an additional measure for protein loading, tubulin was quantitated using mouse dual monoclonal antibody cocktail (Lab Vision, Fremont, CA) and detection by a secondary antibody conjugated to HRP. Blots were scanned and quantitatively analyzed using NIH Image software.

**Measurement of Na\(^+\)/H\(^+\) exchanger activity.** The activity of NHE was determined as the rate of pHi recovery after an NH\(_4\)Cl load by a modification of the previously described method (47). In the present study, BCECF-acetoxyxymethyl ester (10 \(\mu\)M) was loaded into cells in Krebs-Henseleit HEPES media containing 20 mM NH\(_4\)Cl (substituted for an equal molar NaCl). After being washed with the same media minus BCECF, minus sodium media KHH (choline chloride in place of sodium chloride) was added for 1 min (which by inactivating NHE provided a deeper pHi trough) followed by reintroduction of KHH for a 4-min recovery period.

**Metabolic studies.** Studies were performed on confluent LLC-PK\(_1\)-F\(^{\text{3}}\) cells grown in the 12-well plates over 3 and 18 h in DMEM media (pH 7.4) containing DMSO (vehicle), DMSO plus TRO, or in acidic DMEM (pH 6.95). Media ammonium concentration was determined by the microdiffusion method (26) and formation rate was determined as above by subtracting the media blank and expressed on the basis of milligrams of protein. Nonvolatile acid production (26) was measured as the difference in media HCO\(_3\)\(^-\) content before and after 3-h incubation and expressed in micromoles of H\(^+\) per milligram of protein. Cells were washed 3× with ice-cold PBS in rapid fashion and harvested in lysis buffer and analyzed for P-ERK and total ERK as described above, or when tritiated thymidine (1 \(\mu\)Ci/ml) was added to the media, in 5% trichloroacetic acid and analyzed for DNA synthesis as previously described (41).

**Statistical analysis.** Differences between control and groups of treated monolayers were analyzed using ANOVA and a corrected Student’s t-test (Bonferroni); comparisons between time controls and their corresponding treated group were analyzed using the Student’s t-test. Differences were considered significant at \(P < 0.05\) using either a one- or two-tailed t-test depending on the a priori hypothesis as depicted in Fig. 1.

**RESULTS**

Figure 2A shows a representative experiment in which confluent LLC-PK\(_1\)-F\(^{\text{3}}\) cells were harvested at time 0 and at 1, 2, and 4 min of exposure to TRO and analyzed for activated ERK. These results show that TRO can effect ERK activation within 2 min of exposure and sustain the activation for at least 4 min. Figure 2B shows the pHi measured in these cells and from four similar experiments. From an initial pHi of 7.20 ± 0.04, TRO exposure depresses the pHi after 2 min (6.92 ± 0.05 vs. 7.27 ± 0.07 for DMSO-treated time control, \(P < 0.05\)) and even more significantly (\(P < 0.01\)) after 4 min (6.68 ± 0.10 vs. 7.28 ± 0.07, respectively). Figure 2C shows that activated ERK is associated with the fall in pHi increasing from 3 (\(P < 0.05\)) to 3.4 (\(P < 0.01\))-fold greater than control (media change alone) at 2 and 4 min, respectively. Figure 2D shows a representative experiment of the acute (4 min) increase in P-ERK in response to extracellular acidosis (pHe 6.95) and the prevention of this response by blocking MEK1/2 activation; results from three similar experiments show that the P-ERK to total ERK increased 2.3-fold (\(P < 0.01\)) after 4-min exposure and that specific MEK1/2 inhibitors prevented this increase (0.43 ±
0.08 to 0.98 ± 0.12 and 0.21 ± 0.05 for control (media change alone), acidosis, and acidosis plus PD-98056 and U-0126, respectively]. These results confirm findings in another proximal tubule-like cell line that acidosis is able to acutely activate ERK (40) providing the basis for pathway III shown in Fig. 1.

These results showing that both TRO and acidosis acutely activate ERK suggest, in line with Fig. 1, either that TRO-induced cellular acidosis activates ERK (pathways I and III) or TRO-induced ERK activation leads to the cellular acidosis (pathway II).

To dissect this, we prevented MEK1/2-induced ERK1/2 activation by preincubating with the specific inhibitors of upstream MEK1/2 kinase activation (1, 8), PD-098059 (30 μM) and U-0126 (10 μM), and then determined whether TRO would still effect the cellular acidosis (pathway II, Fig. 1). As depicted in the representative experiment presented in Fig. 3, bottom, incubation with TRO (Fig. 3B) over 4 min results in an increase in ERK phosphorylation compared with the 4-min time control (Fig. 3A). Associated with ERK activation was a marked fall in pH$_i$ (6.44 vs. time control 7.29) after 4 min. Preincubation for 10 min with the blockers prevented TRO-induced P-ERK activation (0.36 ± 0.15). The pH$_i$ measured at the end of the 4-min period showed the expected cellular acidosis with TRO (7.32 ± 0.07 vs. 7.06 ± 0.11) and the abrogation of the acidosis when ERK activation was prevented (7.06 ± 0.11 vs. 6.60 ± 0.08) and only marginally (P < 0.10) lower than the control (7.06 ± 0.11 vs. 7.32 ± 0.07). In terms of acidification rates over the 0- to 4-min time course, TRO converts the steady-state balance between acid production and acid extrusion ($\Delta$pH$_i$/t) to a marked acidification (−0.16 ± 0.03 ΔpH$_i$/t, P < 0.001 vs. control), whereas preventing ERK1/2 activation markedly reduces the TRO effect (−0.035 ± 0.020 ΔpH$_i$/t, P > 0.10 vs. control, P < 0.05 vs. TRO alone).

We previously showed that TRO induced cellular acidosis by inhibiting acid extrusion and, therefore, suggesting that one effect of acute ERK activation could be to exert an inhibitory effect on acid extrusion. To test this, confluent monolayers were loaded with NH$_3$/NH$_4^+$ (20 mM NH$_4$Cl) and then exposed to minus sodium KHH media for 1 min followed by recovery in KHH (Fig. 5A). As we previously showed (41, 45, 47), TRO

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Fig. 2. TRO activates ERK associate with intracellular acidosis. A: representative experiment of activated ERK before and at 1, 2, and 4 min following exposure to 25 μM TRO. B: pH$_i$ associated with the ERK shown in C, n = 4. *P < 0.05. **P < 0.01. D: extracellular acidosis (A), pH 6.95, activates ERK within 4 min and this action can be blocked with PD-98059 (PD; 30 μM) plus U-0126 (U; 10 μM). CTL, control.
(25 µM) blunts the recovery response to an NH₄Cl acid load despite attaining an initial cellular acidosis (trough) at least as severe as the controls; preincubating with PD-098059 and U-0126 and then measuring the recovery response in the presence of TRO shows a restoration of acid extrusion activity. These results as well as those from three additional experiments are presented in Fig. 5B. TRO inhibits the recovery response by 85% (0.02/0.13 pHi/n, P < 0.01), whereas blocking ERK activation restored the response to that equal to the control (0.19 ± 0.08 vs. 0.13 ± 0.04). Noteworthy is the pHi attained after the 4-min recovery, 7.13 ± 0.10, 6.51 ± 0.12, and 7.05 ± 0.03 for control, TRO, and TRO plus PD-098059 and U-0126, which are similar to the spontaneous pHi with an endogenous acid load. These results are consistent with TRO-induced acute ERK activation playing an inhibitory role on NHE-mediated acid extrusion in inducing the cellular acidosis (pathway II, Fig. 1).

Prolonged exposure (3 h) to TRO results in a sustained cellular acidosis as shown for a representative experiment in Fig. 6A. TRO exposure for 3 h reduced pHi from 7.20 ± 0.12 to 6.86 ± 0.15 (P < 0.01, n = 3), whereas exposure to TRO plus PD-098059 and U-0126 prevented the cellular acidosis (pHi 7.11 ± 0.12). P-ERK was elevated twofold (P < 0.01) after 3-h exposure to TRO (2.34 ± 0.38 vs. 1.14 ± 0.18) and reduced (P < 0.05) after 3-h exposure to TRO plus PD-098059 and U-0126 (0.52 ± 0.12). Noteworthy, TRO induces this sustained cellular acidosis despite a 32% decrease (P < 0.02) in acid production (3.45 ± 0.4 vs. 5.1 ± 0.3 µmol·mg protein⁻¹·3 h⁻¹) for TRO and control, respectively, n = 6); curiously, with prevention of ERK activation, acid production increased (5.7 ± 0.3 vs. 3.45 ± 0.4 µmol·mg protein⁻¹·3 h⁻¹, P < 0.01, TRO plus PD-098059 and U-0126 vs. TRO alone). Figure 6B shows that 3-h exposure to TRO increased (P < 0.001) ammoniagenesis 2.75-fold (242 ± 57 to 665 ± 144 nmol·mg protein⁻¹·3 h⁻¹) while preventing ERK activation eliminated the TRO-induced increase (282 ± 72 nmol·mg protein⁻¹·3 h⁻¹) as expected from pathway II (Fig. 1).

We determined what effect ERK activation might play in extracellular acidosis-induced ammoniagenesis in this pH-sensitive proximal tubule-like cell line (pathway III, Fig. 1). As shown in Fig. 6C, extracellular acidosis (pHe 6.95) over 3 h increased ammoniagenesis by 49% (450 ± 75 vs. 302 ± 64 nmol·mg protein⁻¹·3 h⁻¹, P < 0.05) associated with a 2.7-fold elevation of P-ERK1/2 (3.90 ± 1.31 vs. 1.45 ± 0.31 for acidic and control, respectively, P < 0.05). Similar to the effect of TRO, extracellular acidosis, pHe 6.95, and the ERK

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**Fig. 3.** Top: pH, traces from 3 chambers, A, B, and C containing confluent monolayers. A: control loaded with BCECF, B: TRO treated and loaded with BCECF followed by KHH plus TRO (25 µM) for 4 min. C: TRO treated and MEK1/2 inhibited loaded with BCECF and PD-98059 (30 µM) plus U-0126 (10 µM) for 10 min followed by TRO (25 µM) and the inhibitors for 4 min. Bottom: ERK activation corresponding to chambers A, B, and C analyzed at the end of each 4-min experiment.

**Fig. 4.** Inhibition of ERK activation prevents cellular acidosis. A: activated ERK over total ERK from 4 experiments in which monolayers were treated with KHH alone for 4 min, TRO (25 µM) for 4 min, pretreated with PD-98059 (30 µM) and U-0126 (10 µM) for 10 min followed by TRO plus inhibitors for 4 min, or pretreated with PD-98059 plus U-0126 followed by both inhibitors for 4 min. B: pHi measured at 4 min in the respective groups; results are from 4 chambers per group. *P < 0.01.
activation were associated with decreased endogenous nonvolatile acid production compared with control, pH $7.48$ (2.9 ± 0.4 vs. 5.1 ± 0.3 μmol/mg protein, $P < 0.01$). Preventing extracellular acidosis-induced ERK activation with PD-098056 plus U-0126 reduced the acidosis-increased ammoniagenesis by 85% (450 ± 95 to 324 ± 85 nmol/mg protein, $P < 0.05$) and increased the endogenous nonvolatile acid production to

Fig. 5. TRO inhibits NHE activity and inhibitors of ERK activation restore NHE activity. A: representative response to an NH$_4$Cl pulse in control (KHH alone), TRO (KHH plus 25 μM TRO), and monolayers pretreated with PD-98059 (30 μM) and U-0126 (10 μM) and recovery with KHH plus TRO and inhibitors. B: results from 3 additional experiments with *$P < 0.01$.

Fig. 6. A: TRO-enhanced ammoniagenesis is abrogated by preventing ERK activation. Results are from 4 separate 12-well plates with triplicate controls, DMEM plus DMSO, TRO (25 μM), TRO plus PD-98059 (30 μM), and U-0126 (10 μM). A: TRO exposure for 3 h sustains intracellular acidosis and elevated P-ERK and both are prevented by PD-98059 plus U-0126 (PD+U). B: ammoniagenesis is increased over 3 h in the presence of TRO and this increase is prevented by PD+U. C: extracellular acidosis pH 6.95 for 3 h enhances ammoniagenesis and this increase is prevented by PD+U.
Because the physiological responses to TRO and to extracellular acidosis may reflect the duration of ERK activation (23, 33), we measured P-ERK levels after 18-h exposure to TRO and to extracellular acidosis (pH 6.95). A representative experiment is shown in Fig. 7. Compared with control, TRO exposure for 18 h results in a cellular acidosis and a sustained increase in P-ERK (P-MEK measured in these same lysates was also increased). In marked contrast, cells exposed to a severe extracellular acidosis (pH, 6.95) restored their pH to the control level with a return of P-ERK (and P-MEK) to the control level. Results from five additional experiments are presented in Fig. 8. TRO sustained P-ERK (Fig. 8A) at a level that was 329% (1.33 ± 0.26 vs. 0.31 ± 0.07, P < 0.01) above control, whereas the extracellular acidosis failed to maintain P-ERK activation above the control level. Associated with the sustained P-ERK activation was a chronic cellular acidosis (Fig. 8C) in the TRO-treated cells (6.67 ± 0.09 vs. 7.26 ± 0.07, P < 0.01) despite an extracellular pH that was not different from control (7.26 ± 0.04 vs. 7.30 ± 0.02); cells exposed to the extracellular acidosis (pH, 6.93 ± 0.03) were not chronically acidotic and showed a tendency (P < 0.10) to be alkalotic (pH, 7.38 ± 0.07). An important physiological response to chronic acidosis is ammoniagenesis. As shown in Fig. 8B, TRO-induced chronic cellular acidosis sustained over the 3- to 18-h time course increased ammoniagenesis by 2.3-fold (3,884 ± 216 vs. 1,701 ± 177 nmol/mg, P < 0.001); in contrast, extracellular acidosis over the time course 3–18 h did not sustain the increased ammoniagenesis (2,090 ± 157 vs. 1,701 ± 177 nmol/mg). Another important response to cellular acidosis is the rate of DNA synthesis (4, 22, 32). As shown in Fig. 8D, TRO-treated cells showed a marked reduction (P < 0.001) in DNA synthesis (9,557 ± 498 vs. 15,526 ± 405 cpm/mg), whereas cells exposed to extracellular acidosis for...
18 h showed a tendency ($P < 0.10$) to increase DNA synthesis (20,868 ± 2,769 vs. 15,526 ± 405 cpm/mg).

Our previous studies (41, 45, 46) showed that TRO induced a chronic cellular acidosis, which resulted from impaired acid extrusion. The present study supports an important role for P-ERK activation in negatively modulating acid extrusion. Because extracellular acidosis activates P-ERK at 3 h but not at 18 h, the question arises as to potential influences of P-ERK activation in the acid extrusion response to extracellular acidosis. To test this, we exposed cells to an extracellular acidosis for 3 and 18 h and then challenged them with the NH$_4$Cl acid load and monitored the recovery response in KH at pH 7.4. As shown in Fig. 9 for representative experiments, the recovery response in cells exposed for 3 h to extracellular acidosis (Fig. 9B) was slower than the control (Fig. 9A); on the other hand, exposure to extracellular acidosis for 18 h (Fig. 9D) gave a recovery response that was faster than the control (Fig. 9C); results from three additional experiments gave a 32% reduction in acid extrusion at 3 h (0.25 ± 0.04 vs. 0.37 ± 0.05 pH/l, $P < 0.05$) and a 44% increase at 18 h (0.52 ± 0.06 vs. 0.36 ± 0.05 pH/l, $P < 0.05$).

DISCUSSION

Our previous studies showed that TRO induces a cellular acidosis in a wide variety of kidney-derived cell lines associated with the inhibition of NHE-mediated acid extrusion (6, 45–47). The present study was designed to elucidate signal pathways involved in mediating this novel action of TRO. Identifying such putative pathways would be important for an understanding of acid-base regulation as well as for elucidating affects on processes modulated by cellular acidosis such as proliferation (4, 22, 32), apoptosis (30), and protein synthesis (34, 48) as well as affects mediated through the better recognized PPARγ-dependent pathway.

Surprisingly, TRO treatment resulted in a rapid activation of ERK1/2 associated with the large drop in pH$_i$ (Fig. 2). Although previous studies reported TRO induced ERK activation in nonepithelial cell lines (12, 21, 39), the present study shows a remarkably fast (<2 min) response in a renal epithelial cell line that was inversely related to pH$_i$. The LLC-PK$_1$-F$^+$ cell line is known to be pH responsive (7, 15), and because ERK activation can be induced by low pH in this (see RESULTS and Fig. 2D) and other pH-sensitive proximal tubule cell lines (40) as well as other nonepithelial lines (17, 38), we asked whether ERK activation resulted from the an acidosis secondary to TRO inhibiting NHE directly pathway I or whether TRO activation of ERK effects the acidosis pathway II (Fig. 1). The test was to prevent ERK1/2 activation by blocking the MAP kinase pathway (MEK1/2) using pretreatment with PD-098059 (1) in combination with U-0126 (8). We confirmed that this prevented TRO-induced ERK1/2 activation, which demonstrated that TRO was acting via MEK1/2 to activate ERK1/2 (Figs. 3 and 7). We then looked at the effect of preventing TRO-induced ERK1/2 activation on the cellular acidosis and found 1) the cellular acidosis was largely, but not entirely, abrogated (Figs. 3 and 6 despite an increased nonvolatile acid production); 2) NHE-mediated acid extrusion was restored (Fig. 5, A and B); and 3) the increased ammoniagenesis was largely suppressed (Fig. 6B). Taken together, these results are consistent with, and supportive of, the hypothesis that ERK1/2 activation plays an important role in the observed changes in acid-base metabolism following TRO (6, 34, 45, 46).

We also included the ERK activation responses to an extracellular acidosis as shown in Fig. 1, pathway III for comparing the responses to the ERK activation elicited by TRO. These findings confirmed the acidosis induced acute ERK activation first reported by Alpern and associates (40) in opossum kidney (OKP) cells and in vivo following an acute acid load. Here, we show that P-ERK was elevated for 3 h and that this acute activation was associated with cellular acidosis and an increase in ammoniagenesis (Fig. 6C). Noteworthy, we show that acid extrusion activity measured at this time was reduced (Fig. 9B) consistent with decreased acid extrusion contributing to the acute cellular acidosis and enhanced ammoniagenesis. However, there is a clear-cut difference in the ERK activation response to extracellular acidosis as opposed to TRO. With extracellular acidosis, the ERK activation is acute but not sustained chronically (Fig. 8A) in contrast to the response to TRO, which sustains the ERK activation and the cellular acidosis (Fig. 8C). These results, in turn, are consistent with impaired acid extrusion with TRO (45, 46) at 18 h and the return to control, and above, with chronic extracellular acidosis (Fig. 9). Previous studies in pH-sensitive OKP cells showed that extracellular acidosis increases NHE3 expression and activity when measured after 24-h exposure (50), whereas assayable NHE3 activity was not increased at 3 h despite

Fig. 9. Acute exposure to extracellular acidosis slows the recovery to an NH$_4$Cl acid load in contrast to accelerated recovery following chronic exposure to extracellular acidosis. Acute (3 h) exposure to pH$_o$ 6.95 (acidotic) with representative tracing shown in B vs. control, pH$_o$ 7.40 in A. Chronic (18 h) exposure to acidosis with representative tracing presented in D vs. C control.
increased amounts of NHE protein on the plasma membrane (50); notably, the increase in chronic NHE3 expression at 24 h could be blocked by preventing ERK activation with PD-098059 (40) consistent with ERK activation coupled to immediate early gene response (2). In the present study, in pH-sensitive LLC-PK1-F" cells, acid extrusion activity was acutely reduced and ERK was activated by 3-h exposure to extracellular acidosis; chronically, NHE activity was elevated and ERK activity returned to the control level, results not necessarily inconsistent with the NHE activity findings in the OKP cell line.

Our results suggest that the differential responses to TRO and extracellular acidosis may reflect a transient activation of ERK in the case of the latter vs. the sustained ERK activation in the former. For example, ammonium production directly followed the cellular acidosis in increasing acutely with both extracellular acidosis and TRO while sustained over 3–18 h only with TRO and the associated cellular acidosis. We (26) and others (15) noted that the ammoniagenic response to a severe extracellular acidosis (initial pH 6.90) was a surprisingly modest 20–30% increase over 18–24 h; subsequent increases in ammoniagenesis corresponded with adaptive increases in glutaminase activity and gene expression (15). The enhanced ammoniagenesis associated with TRO-induced cellular acidosis was previously shown using 15N-labeled glutamine to be accelerated flux via glutamate dehydrogenase and release of the amino nitrogen as ammonium (45).

Another response also dependent on pHi, DNA synthesis (4, 32) was markedly suppressed after 18 h of TRO but attained the control, and above, level with chronic extracellular acidosis. In mesangial cells, Ghosh et al. (11) confirmed our finding (47) that TRO inhibits NHE exchange-mediated acid extrusion and that TRO is a far more potent inhibitor of proliferation than rosiglitazone despite the latter being a far better PPARß and that TRO is a far more potent inhibitor of proliferation than rosiglitazone despite the latter being a far better PPAR interference. In mesangial cells, Ghosh et al. (11) confirmed our finding (47) that TRO inhibits NHE exchange-mediated acid extrusion and that TRO is a far more potent inhibitor of proliferation than rosiglitazone despite the latter being a far better PPAR antagonist than TRO (5, 42); similar results were found in an earlier study of endothelial cells (9). Although both of these studies attributed the effect of TRO, at least in part, to inhibition of NHE activity, e.g., cellular acidosis, neither study demonstrated a spontaneous intracellular acidosis with TRO nor provided a mechanism. Here, we propose that ERK1/2 activation may be inhibitory for acid extrusion and thereby lead to a cellular acidosis with the duration dependent on sustained ERK activation; noteworthy duration of ERK activation has been proposed as a basis for selective proliferative vs. differentiation cellular responses (23, 33). Lotspeich (22), in his pioneering studies, was the first to show that the response to metabolic acidosis was more catholic than recognized despite lacking an understanding of underlying signaling pathways (2); in this regard, NH4Cl-induced metabolic acidosis and transient ERK activation may induce renal hyperplasia (22), whereas TRO-induced sustained ERK activation may inhibit proliferation (20, 35, 36, 41) and even in PPARγ-negative cells (35, 41).

Such a model may also explain how extracellular acidosis (transient MAPK activation) upregulates tumorigenic cyclooxygenase-2 expression (38) while sustained intracellular acidosis (although difficult to achieve by reducing extracellular pH in normal (18) and, especially, in more alkaline tumor cells (41)) can inhibit expression of cyclooxygenase-2 in colon cancer cells (31). These studies, as well as the present findings, suggest potentially important but ill-defined relationships between acute and sustained MAPK activation and pHi in regulating growth in normal as well as malignant cells.

Because pHi reflects a steady-state balance between acid extrusion and production, we also measured nonvolatile acid production. Curiously, nonvolatile acid production decreased with TRO and extracellular acidosis while preventing ERK1/2 activation returned both pHi and nonvolatile acid production to the normal range. These findings point to a role of ERK1/2 in coordinately regulating both acid extrusion and nonvolatile acid production. Alternatively, TRO may have increased carbonic acid production with the H+ contributing to an intracellular acidosis and generated bicarbonate preventing a fall in the extracellular bicarbonate level. However, in cells exposed to 10% CO2 TRO increased ammonium production 1.8-fold but had no effect on media bicarbonate concentration (data not shown), suggesting that under these conditions, TRO may induce a sustained cellular acidosis without increasing carbonic acid production. If so, activating ERK1/2 was associated with only two mechanisms for defending the extracellular bicarbonate, new bicarbonate produced from glutamate deamination and intramitochondrial oxidation of alpha ketoglutarate (bicarbonate and ATP synthesis) and decreased acid production (ATP hydrolysis coupled to resynthesis via glycolysis and lactate formation) (26). Preventing ERK1/2 activation eliminated both the ammoniagenic base generation and acid production consistent with a coordinated response. Note that in these pH-sensitive proximal tubule-like cells, the reduction in acid production (5.1 to 3.0 or 2.1 μmol/mg protein) was quantitatively more significant than the increased ammoniagenesis (0.2 to 0.6 μmol/mg protein) for TRO as well as for the extracellular acidosis-induced ERK1/2 activation. In other cell lines, chronic TRO treatment enhances delayed glucose uptake presumably via PPARγ signaling (41) and acid production (6, 34). In pH-sensitive epithelial cells such as those comprising solid tumors, TRO might have the dual and dose-dependent affects of accelerating acid production (glucose uptake at low concentrations) while impairing acid extrusion (at higher concentrations), which if coordinated, might be expected to exert the maximal impact in arresting proliferation and promoting cell death. Cellular acidosis has been shown to activate apoptotic pathways (30) and specifically to activate caspase 3 (30); interestingly, caspase 3 inactivates NHE as one of its target substrates (49). Thus TRO’s ability to induce a profound and sustained cellular acidosis provides a basis of a positive feedback promoting delayed apoptosis in heavily angiogenic tumor cells (25). Consequently, elucidating and then coordinating the effects of both the PPARγ-dependent and PPARγ-independent pathways might offer unique insights into therapeutic strategies.

In contrast to the present study showing impaired acid extrusion, ERK activation is generally associated with growth factor stimulation, enhanced acid extrusion, and elevated pHi, at least in pHi-insensitive nonepithelial cell lines (3, 29). In epithelial cells, on the other hand, Watts and Good et al. (43, 44) first showed that in thick ascending limb (TAL) cells of the rat kidney, nerve growth factor-induced ERK activation inhibited both basal (NHE1) and apical surface (NHE3) mediated acid extrusion. In line with this study from the TAL, our results in proximal tubule-like LLC-PK1-F" cells suggest an inhibitory action of activated ERK1/2 on acid extrusion. Indeed, in the present study, we readily detected an association
of ERK activation with a spontaneously reduced pH (Fig. 2) as well as a blunted NHE response to an exogenous acid load (Fig. 5). How TRO acting through ERK activation brings about this inhibition remains an open question. Our previous studies in rat mesangial cells and in MDCK cells derived from dog kidney showed that TRO inhibited acid extrusion in cells expressing only the NHE1 isoform (6, 28, 47) similar to the inhibitory effect of ERK activation on the basolateral NHE1 in the TAL (44). Our results in this proximal tubule-like cell line as well as the OKP cell line which expresses only NHE3 (46) are also consistent with inhibition of apical acid extrusion as well as first shown in situ for the TAL (44). Nevertheless, further studies are required to firmly establish the actual acid extruding systems involved and the precise mechanism(s) through which TRO acts in inducing sustained cellular acidosis in proximal tubule-like LLC-PK1/Fβ cells.

Our previous studies in cultured cell lines showed that although TRO is the most potent inducer of intracellular acidosis and ammoniagenesis, rosiglitazone (a far more potent PPARγ agonist than TRO) (5, 42) also induces cellular acidosis and enhances ammoniagenesis (6). Several in vivo studies suggest such effects may also occur within the functioning kidney. For example, chronic administration of TRO to Zucker diabetic rats not only prevented mesangium expansion (24) but doubled their ammonium excretion (47) in the absence of a systemic acidosis consistent with an intracellular acidosis expressed in vivo within both the renal tubules and mesangial cells; subsequent in vitro studies demonstrated that TRO can induce a cellular acidosis in mesangial cells (47) and reduce collagen and proteoglycan formation (34), not unlike the effect of pH on matrix synthesis previously observed (48); in pH-sensitive proximal tubule-like cells, TRO induced cellular acidosis and accelerated ammoniagenesis via a pH-sensitive ammoniagenic pathway (45). Chronic administration of rosiglitazone to Sprague-Dawley rats also increased ammonium excretion (10), again without a systemic metabolic acidosis but was associated with a 67% reduction in citrate excretion; again without a systemic metabolic acidosis but was associated with a 67% reduction in citrate excretion; and rosiglitazone (5, 42) also induces cellular acidosis and enhances ammoniagenesis (6).

Further studies seem warranted to elucidate receptor or receptor complexes and pathways involved through which TRO acts to modulate acute and chronic acid-base regulation in pH-sensitive cells.

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