Role of Epidermal Growth Factor Receptor (EGFR)-Signaling Versus Cellular Acidosis Via Na^+/H^+ Exchanger1(NHE1)-Inhibition in Troglitazone-Induced Growth Arrest of Breast Cancer-Derived Cells MCF-7

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Key Words

EGFR • pH_i • Troglitazone • NHE1 • EGF • Cell Growth

Abstract

Purpose: We previously showed that troglitazone (TRO) induces a profound cellular acidosis in MCF-7 cells as a result of inhibiting Na^+/H^+ exchanger (NHE)1-mediated acid extrusion and this was associated with a marked reduction in cellular proliferation. The present study focuses on TRO-activated signaling pathways versus TRO-mediated NHE1-inhibition in reducing DNA synthesis. **Experimental Design:** TRO activation of the signaling pathway involving epidermal growth factor receptor (EGFR)/MAPK/ERK kinase (MEK) 1/2/extracellular signal-regulated kinase (ERK) 1/2 was studied by Western blotting and phospho-specific antibodies. TRO induction of cellular acido-

sis and inhibition of NHE1 activity were measured using (2, 7)-biscarboxyethyl-5 (6)-carboxyfluorescein (BCECF) assay and $\text{NH}_4^+/\text{NH}_3$ pulsing. Cellular proliferation was assessed as DNA synthesis by ^3H -thymidine incorporation. **Results:** TRO simultaneously reduces pH_i and elevates phosphorylated-extracellular signal-regulated kinase (p-ERK). These responses reflected inhibition of acid extrusion and EGFR activation respectively and were sustained over 18h associated with a large decrease in DNA synthesis. Preventing TRO-induced ERK activation did not restore DNA synthesis or cellular pH. **Conclusions:** TRO activates two parallel pathways: I) EGFR/MEK1/2/ERK1/2 and II) NHE1 inhibition/cellular acidosis. Elimination of I) did not prevent the inhibition of DNA synthesis consistent with TRO-induced growth arrest dependent upon II) in tumorigenic non-metastatic breast cancer derived MCF-7 cells.

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Introduction

We have recently shown that troglitazone (TRO), a member of the thiazolidinedione (TZD) class of antihyperglycemic agents, and peroxisome proliferator-activated receptor γ (PPAR γ)-ligand family, induces a prompt and severe cellular acidosis in breast cancer-derived cell lines MCF-7 and MDA-MB-231, respectively [1]. The cellular acidosis occurs acutely within 4 minutes (min) of incubation with TRO, and is related to decreased activity of the Na⁺/H⁺ exchanger 1 (NHE1)-mediated acid extrusion rather than to increased nonvolatile acid (lactic acid) production [1]. Furthermore, chronic exposure to TRO (18 h) resulted in persistent NHE1 activity reduction-mediated cellular acidosis, and a dose-dependent inhibition of DNA synthesis associated with a decreased number of viable cells [1]. TRO-mediated inhibition of proliferation associated with cellular acidosis and reduced NHE1 activity was not prevented by the presence of the PPAR γ -inhibitor GW9662, and was demonstrable in PPAR γ ^{-/-} NIH3T3 cells, consistent with a PPAR γ -independent mechanism [1]. Although the molecular mechanism(s) of the action of TRO on intracellular pH (pH_i) has not been elucidated yet, we have recently demonstrated that TRO activates the extracellular signal-regulated kinase (ERK) 1/2 signaling pathway and directly inhibits NHE3 in porcine renal proximal tubule-like LLC-PK1-F⁺ cells [2, 3].

Traditionally, receptor activation-mediated induction of transcription of target genes has been considered the predominant mechanism of action of PPAR γ ligands [4]. More recently, it has become clearer that their actions are pleiospecific reflecting multiple pathways dependent upon the glitazone, the concentration, and cell type studied amongst other factors [5-8]. Particularly, we, as well as earlier studies by others [9-12], have shown that TRO induces a "non genomic" rapid and sustained ERK activation through phosphorylation (p-ERK) that, in our study, was associated with increased ammoniogenesis and inhibition of DNA synthesis in pig kidney-derived epithelium [2, 3]. Noteworthy, ERK activation and NHE3 inhibition were TRO-specific, since neither occurred with another PPAR γ ligand, pioglitazone (PIO) [3].

In studies by Yin et al. [13, 14], TRO was shown to reduce proliferation and induce apoptosis in MCF-7 breast cancer cells whereas PIO did not, and, this effect of TRO was associated with mitogen-activated protein kinase kinase (MEK) 1/2 [14]. These authors concluded that the action of TRO was independent of PPAR γ activation because of the failure of PIO to mimic this response and

was likely mediated by an otherwise undefined intracellular stress signal. One possible, but by no means only [14, 15], intracellular stressor might be the TRO-induced intracellular acidosis that we previously reported in this as well as in other tumor cell lines [1]. Therefore in order to test this hypothesis, we first elucidated the pathway through which TRO activates mitogen-activated protein kinase (MAPK) in MCF-7 cells and then determined its role in TRO-induced cellular acidosis. Using DNA synthesis as the end point, we then demonstrated that blocking MEK/MAPK activation did not prevent either the TRO inhibition of DNA synthesis or the sustained and profound intracellular acidosis.

Materials and Methods

Cell lines and tissue culture

Breast cancer-derived MCF-7 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS) containing 28 mM/L sodium bicarbonate, 10 mM/L sodium pyruvate, 5 mM/L D-glucose, and 2 mM/L L-glutamine at 37°C (pH 7.4). TRO (Cayman Chemicals, Ann Arbor, MI) was dissolved in dimethyl sulfoxide (DMSO), added to the media at 100 μ M/L, and diluted to desired concentration vs DMSO vehicle in controls.

Cellular pH_i and NHE1 activity measurements

The pH_i was assayed using the pH-sensitive fluorescent dye (2, 7)-biscarboxyethyl-5 (6)-carboxyfluorescein (BCECF) as previously described [1, 2]. Briefly, cells were seeded onto specially designed 30-mm chambers (Biopetechs, Biological Optical Technologies, Butler, PA) and loaded for 10-20 min with 10 μ M BCECF-AM (Molecular Probes, Eugene, OR) dissolved in DMSO and added to Krebs Henseleit HEPES-buffered (KHH) media (pH 7.40) containing 140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 20 mM HEPES plus 10 mM glucose, washed once with KHH and studied over a 4-10 min time course in KHH media. To inhibit EFGR ligand activation, transactivation, and ERK activation 10 μ M AG1478 (Sigma, St Louis, MO), 50 μ M PD153035 (Calbiochem, LaJolla, CA) and 30 μ M PD098059 (Calbiochem) plus 10 μ M U0126 (Calbiochem), respectively, were present in the 20-min BCECF loading period; inhibitors were also present at these concentrations during the subsequent 4 min and 18 h studies. The cells were consequently collected in lysis buffer, and the levels of MEK/p-MEK/ERK/p-ERK were determined by Western blot analysis.

To assess volatile acid production a method similar to that described by Grinstein and Furuya [16] was followed; the above KHH media was modified so as to be lightly buffered (0.1 mM HEPES, pH 7.2), and monitored for pHo (Orion pH electrode) after 10 min from the same chambers in which control and TRO-treated cells were monitored for pH_i. To test for acid

Fig. 1. TRO acutely activates p-ERK and induces cellular acidosis. **A.** Typical response to 25 μ M TRO added to KH media. Cells were exposed to KH while pH_i stabilized and then 4 min of KH alone, lane 1, or KH + TRO, lane 2, after which the cells were immediately collected in lysis buffer and probed for p-ERK; insert shows the response to TRO and TRO plus PD98059+UO126 over a 12 min time course. To prevent ERK activation cells were pre-treated with 30 μ M PD98059 plus 10 μ M UO126 for 20 min and then exposed to TRO for 4 min, lane 3, or pre-treated with 30 μ M PD98059 plus 10 μ M UO126 and KH alone, lane 4. **B.** pH_i measured before (PRE) and after 4 min (POST) following BCECF loading for 20 min or BCECF plus 30 μ M PD98059 plus 10 μ M UO126. Results are means $\pm$ sem from 4 experiments with statistical analysis as described in Materials and methods and significance as indicated, and **C.** p-ERK to total ERK levels in cells harvested after 4 min exposure to KH or TRO (POST-treatment). Results are means $\pm$ sem from the same 4 POST-treated experiments shown in B. Inserts shows typical blots from 4 min, 1C, and 12 min, 1A, responses.

extrusion capability (acid load), the same cells were acid loaded with a 4-min exposure to KHH in which 20 mM/L NaCl had been replaced with 20 mM/L NH₄Cl. After returning the KHH media, the pH_i response was continuously monitored for 4 min, and the recovery response was taken as the change in ΔpH_i /t (min) and compared to the response with 25 μ M EIPA.

Western blot analysis

The level of pospho-MEK (p-MEK) or phospho-ERK1/2 (p-ERK) proteins were analyzed as an index of activation in the same cells in which the pH_i 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 EDTA, 5 mM benzamide, and 0.2 mM phenylmethanesulfonyl fluoride (PMFS)] with phosphatase inhibitor cocktails, which contain both inhibitors of both threonine and tyrosine protein phosphatases (Sigma, St. Louis, MO). Aliquots of the lysates were analyzed for total protein by a dye-binding assay [1]. 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. An aliquot (15 μ g) of protein were then resolved by SDS-PAGE on 10% polyacrylamide gel (Hoefer, San Francisco, CA). The transfer of proteins to a nitrocellulose membrane 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) and total ERK were detected using a chemiluminescence Western blot system (Immuno-Star, Bio-Rad, Hercules, CA) using a phosphor-specific ERK1/2 mouse monoclonal IgG primary antibody that recognizes phosphorylated Thr202 and Tyr204 (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 (phosphoryla-

tion site independent, Cell Signaling Technology) and a secondary antibody conjugated to HRP. As an additional measure for protein loading, tubulin was quantified 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.

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 as previously described [2].

Results

Troglitazone acutely activates ERK1/2 associated with a spontaneous cellular acidosis

Fig. 1A shows a typical response to TRO (25 μ M) in MCF-7 cells. Compared to cells incubated in KH alone (first lane), exposure to TRO (second lane) resulted in a rapid fall in pH_i over the 4 min time course. Typical pH_i responses to TRO and TRO+PD98059+UO126 over a longer 12 min time are shown in the insert. p-ERK measured at the end of the 4 min time course (and 12 min time course in insert Fig 1A) was increased with TRO compared to the control without any change in the total ERK level, Fig. 1C. This observation demonstrates that TRO acutely induces a profound spontaneous intracellular acidosis and, at the same time, activates ERK. To assess the contribution of acid production to this rapid acidification response, extracellular pH was measured in the chamber media after 10 min exposure to TRO in lightly buffered KHH (0.1 mM HEPES) and compared to the control cells media. After 10 min, pH_i had fallen to 6.70 ± 0.06 in TRO-treated cells compared to 7.07 ± 0.06 in controls ($p < 0.001$, $n = 9$) whereas the fall in pH_o tended to be less in the TRO-treated vs. control groups, -0.40 ± 0.06 vs. -0.48 ± 0.07 pH units respectively ($p < 0.10$, one-tailed paired t test, $n = 5$). Acid extrusion rate in the TRO treated cells measured at the end of the 10 min period was reduced by 51% compared to control (0.026 ± 0.010 vs. 0.053 ± 0.008 $\Delta pH_i/\text{min}$, $n = 5$, $p < 0.01$). Similar to TRO, 25 μ M EIPA reduced NHE activity by 55% but in marked contrast did not lower the pH_i below that of the control (7.02 ± 0.03); however when TRO and EIPA were used in combination (both at 25 μ M) the pH_i fell (6.74 ± 0.08) to the level with TRO alone (6.70 ± 0.06). These results confirm an action of TRO in inhibiting NHE(1) and also suggest an action of TRO in activating an intracellular acidification process from some source other than glycolysis [16, 17] since our previous study showed lactate production did not increase over this time course [1].

To assess the relationship, if any, between ERK activation and the cellular acidosis, cells were incubated with 30 μ M PD98059 in combination with 10 μ M UO126 for a 20 min pre-treatment prior to the addition of TRO with the results shown in the third lane of Fig. 1A. Interestingly, pre-treatment with PD98059 plus UO126 alone appears to decrease the spontaneous pH_i prior to the addition of TRO and abrogates the subsequent acidification induced by TRO. Fig. 1B confirms these results from this with 4 additional experiments; spontaneous pH_i prior (PRE = pre-treatment with PD98059 + UO126) to the

addition of TRO was reduced ($p < 0.05$) with PD98059 + UO126 (7.25 ± 0.04 vs 7.50 ± 0.05 for control). Addition of TRO (POST = treatment with TRO or KH for 4 min) reduced ($p < 0.01$) the spontaneous pH_i compared to the KH time control (7.18 ± 0.06 vs 7.56 ± 0.07 respectively). The fall in pH_i was reflected in the change in $\Delta pH_i/\text{min}$ to acidification (-0.101 ± 0.008 vs 0.0009 ± 0.00013 for control, $p < 0.001$). Addition of TRO to the PD98059 + UO126 pre-treated cells also decreased ($p < 0.05$) the pH_i further from 7.25 ± 0.04 to 7.14 ± 0.03 , but at a markedly reduced acidification rate compared to TRO treated cells (-0.034 ± 0.0125 vs -0.101 ± 0.0075 , $p < 0.01$). Fig. 1C shows that TRO acutely increases p-ERK 3 fold over control ($p < 0.05$) and that pre-treatment with PD98059 + UO126 virtually eliminates this rise in p-ERK and actually reduces p-ERK below the control level ($p < 0.01$) associated with the reduced pH_i shown in Fig. 1B above (pre-treatment PD + U vs pre-treatment CTL). In addition, upstream MEK activation also occurred within 4 min (0.95 ± 0.31 vs 0.15 ± 0.01 TRO vs control, $n = 3$; $p < 0.05$) indicating TRO activates the ERK signaling pathway at a further upstream site. These results demonstrate: 1) TRO acutely induces both ERK activation and cellular acidification, 2) the latter reflects reduced acid extrusion and, perhaps, accelerated acid production, and, 3) that p-ERK is necessary to defend the elevated pH_i in this tumor cell line.

Preventing EGFR transactivation eliminates the TRO-induced p-ERK activation

In order to elucidate upstream sites involved in the activation of MEK/ERK signaling pathways and their role in maintaining pH_i , we focused on the well established EGFR activation of ERK. Inhibiting EGFR transactivation by pre-incubating with PD153035 (Fig. 2A), resulted in the elimination of the TRO-induced ERK activation and the abrogation of TRO's acidification effect. Unlike PD98059 + UO126 pretreatment, PD153035 pretreatment did not induce a spontaneous acidosis nor was the p-ERK level reduced compared to the control, (Fig. 2B). However, the TRO-induced acidification ($\Delta pH_i/\text{min}$, -0.115 ± 0.0125 vs 0.0063 ± 0.0005 , $p < 0.01$ control, Fig. 2C) was abrogated with the prevention of ERK activation (-0.058 ± 0.0125 vs -0.115 ± 0.125 , $p < 0.05$ vs TRO alone, Fig. 2C) just as it was with MEKK inhibition. To confirm the involvement of this site, AG1478 was used to prevent ligand mediated EGFR activation with a typical result presented in Fig. 2D and the pH_i averages from 5 similar experiments shown in Fig. 2E. Surprisingly, 10 μ M AG1478 pretreatment (Fig. 2D), results in

Fig. 2. Blocking EGFR activation prevents TRO's activation of ERK and abrogates the cellular acidosis. A. Typical response showing abrogation of TRO-induced cellular acidosis in cells pre-treated with 50 μ M PD153035 for 0.5 h. B. TRO-activation of p-ERK is prevented by inhibiting EGFR activation with PD153035; results are means $\pm$ sem from 4 experiments. C. Inhibiting EGFR activation abrogates TRO-induced cellular acidification; results are means $\pm$ sem. D. Typical response to blocking EGFR with AG1478 abrogating TRO-induced acidification; cells were pre-treated for 0.5 h with KH or KH plus 10 μ M AG1478 and then exposed to KH, KH + TRO alone, TRO + 10 μ M AG1478 or AG1478 alone for 4 min. E. pH_i measured after 4 min in KH following BCECF loading for 20 min or BCECF plus 10 μ M AG1478 (PRE-treated) and pH_i after subsequent exposure of pre-treated cells to KH or KH plus 25 μ M TRO (POST-treated). Results as means $\pm$ sem from 6 experiments with statistical analysis as described in Materials and Methods and significance as indicated, and F. p-ERK to total ERK levels in cells harvested after 4 min exposure to KH or TRO (POST-treatment). Results are means $\pm$ sem from the same 6 POST-treated experiments shown in D.

a spontaneous intracellular acidosis and a reduction in p-ERK below the control level while TRO accelerates the acidification rate and this acidification rate is abrogated with AG1478 pretreatment. As shown in Fig. 2E, pre-treatment with AG1478 reduces the control pH_i from

7.45 $\pm$ 0.04 to 7.04 $\pm$ 0.08, respectively ($p < 0.01$ vs control). After pre-treatment with KH, TRO reduces the pH_i from 7.39 $\pm$ 0.04 to 6.95 $\pm$ 0.04 ($p < 0.01$), while after AG1478 pretreatment TRO still reduces pH_i (6.84 $\pm$ 0.07, $p < 0.05$ vs AG1478-PRE). Although the decrement is far

Fig. 3. EGF activates p-ERK and augments TRO induced acidification. A. Typical response to EGF(20 ng/ml) alone and EGF in combination with TRO (25 μ M) over a 4minute time course. B. p-ERK results from 3 experiments carried out as described above in 3A.

less than TRO alone (0.20 ± 0.02 vs 0.49 ± 0.03), preventing ERK activation slowed the TRO-induced acidification by more than half ($p < 0.01$). Associated with the spontaneous intracellular acidosis was a reduction in p-ERK with AG1478 pretreatment (Fig. 2F), and although TRO induces a large 4 fold elevation in p-ERK, this effect is virtually eliminated with AG1478. Thus both inhibition of EGFR activation and of ligand binding to EGFR eliminate TRO's action to increase p-ERK which in turn abrogates the cellular acidification rate.

TRO abrogates EGF-induced ERK activation

The effects of EGF alone and of EGF in the presence of TRO on ERK activation is shown in Fig. 3A. EGF, 20ng/mL, shown in fourth lane elicits a large increase in the p-ERK/ total ERK ratio within 4 min associated with a 42% reduction in the spontaneous acidification rate (0.043 to $0.025 \Delta pH_i/\text{min}$). TRO alone, second lane, results in an increase in p-ERK/total ERK but less than occurs with EGF, and, in contrast to EGF, induces a marked acidification. In combination with EGF, TRO abrogates the effect of EGF on p-ERK, third lane, but enhances the acidification rate. Fig. 3B shows the p-ERK/ total ERK ratio results from 3 additional studies; TRO alone increased p-ERK/ total ERK 3.8 fold while EGF alone increased p-ERK/ total ERK 16.1 fold ($p < 0.01$ vs control) while in combination TRO abrogates the EGF-induced increase in the p-ERK/ total ERK (8.4 fold $p < 0.05$ vs EGF alone).

EGFR via p-ERK enhances NHE1 and facilitates TRO-induced NHE1 inhibition

The effect of ERK activation on NHE1 activity as reflected in acid extrusion following an acid pulse (NH_4Cl) is shown in Fig. 4A, 4B and 4C. In cells pre-treated with 20 ng/ml EGF, (Fig. 4A) NHE1 activity and p-ERK were both elevated compared to control cells. In cells pre-treated with PD153035 (Fig. 4B, I), NHE1 activity was much lower than in the EGF-pretreated cells (Fig. 4B, I vs 4A, and 4C, V). Adding TRO to the recovery KH media containing 140 mM Na^+ slowed the recovery in the control cells (Fig. 4C, II) and PD153035 pre-treated cells (Fig. 4B, II). Adding 25 μ M TRO to the low Na^+ recovery media (10 mM Na^+) and as well to the 140 mM Na^+ virtually eliminated NHE1 activity in control cells (Fig. 4C, IV). When TRO was added to the low sodium recovery media in the PD153035 treated cells (Fig. 4B, III), NHE1 activity also was eliminated, but upon adding 140 mM sodium plus TRO, NHE1 activity returned. In marked contrast, in cells pre-treated with EGF (Fig. 4C, VI), 25 μ M TRO added to the 140 mM Na^+ recovery media blunted the recovery occurring with the low Na^+ media and then completely blocked acid extrusion, reversing to acid loading. Fig. 4D shows the composite of these 3 experiments. Basal NHE1 activity (recovery with 140 mM Na^+) was faster ($p < 0.001$) with EGF pre-treatment and much slower with EGFR activation blocked with PD153035 (0.17 ± 0.01 vs $0.068 \pm 0.015 \Delta pH_i/\text{min}$). TRO at 25 μ M reduced the NHE1 activity by

Fig. 4. ERK activation increases NHE1 activity and enhances TRO inhibition. **A.** Typical response showing EGF pre-treatment increases NHE1 activity measured following an acid pulse as described in Materials and Methods. Control and EGF pre-treated cells were exposed to 20 mM NH₄Cl in 120 mM Na⁺ KH for 4 min and then to (-) minus Na⁺ (10 mM Na⁺ plus 130 mM Choline⁺) for 1min replaced by (+) plus Na⁺ (140 mM Na⁺) for 4 min after which the cells were lysed and analyzed for p-ERK. **B.** PD153035 treatment slows recovery to an acid load and blunts TRO-induced inhibition. (B, I) CTL, (B, II) 25 μM TRO added to (+) 140 mM Na⁺, (B, III) 25 μM TRO added to both (-) 10 and (+) 140 mM Na⁺. **C.** (C, I) CTL, (C, II) 25 μM TRO added to (+) 140 mM Na⁺, (C, III) CTL, (C, IV) 25 μM TRO added to both (-) 10 and (+) 140 mM Na⁺, (C, V) EGF pre-treated CTL and (C, VI) EGF pre-treated plus 25 μM TRO added to (+)140 mM Na⁺. **D.** Results from 4-PD153035-treated, 12 control and 13 EGF- treated experiments as depicted in typical responses shown above in A, B, and C. Control activity measured during recovery with 140 mM Na⁺ vs 25 μM TRO. Elimination of p-ERK with PD153035 pre-incubation and enhancement with EGF pre-incubation as described above in A, B, and C.

41%, 78% and 96% for PD153035, control and EGF-treated cells, respectively. These results suggest that TRO acts to induce cellular acidification through direct inhibition of acid extrusion and that this inhibition is facilitated by ERK activation.

TRO induces a prolonged ERK activation and cellular acidosis

Incubation of cells with TRO for 18 h results in a sustained ERK activation and chronic cellular acidosis

as shown in Fig. 5A and 5B, respectively. Fig. 5A illustrates an almost 7 fold increase in p-ERK levels at 24 h (0.97 ± 0.02 for TRO vs 0.14 ± 0.04 for control, respectively, $p < 0.01$). Cytosolic pH in cancer cells is higher than in normal cells, and as shown in Fig. 5B the spontaneously measured cytosolic pH was 7.55 ± 0.06 and reduced to 7.00 ± 0.21 with TRO ($p < 0.05$) despite an unchanged nonvolatile acid production (11.5 ± 0.3 and $13.5 \pm 0.8 \mu\text{mol/mg}$ respectively) over the 18h time course.

Fig. 5. TRO induces a sustained 18 h ERK activation A, and intracellular acidosis B. Results are means $\pm$ sem from 4 pairs of chambers incubated for 18 h in DMEM or DMEM plus 25 μ M TRO. For spontaneous pH_i measurements, cells were loaded with BCECF and after 4 min incubation in KH alone or KH + TRO for determining pH_i , they were then harvested in lysis buffer and promptly probed for p-ERK and total total-ERK plus tubulin.

Fig. 6. TRO inhibition of DNA synthesis is not prevented by blocking EGFR activation A, but is associated with cellular acidosis, B. A. Cells were incubated with DMEM containing 1 μ Ci/ml 3 H-thymidine and treated with AG1478 and exposed to 25 μ M TRO alone, 10 μ M AG1478 alone, or 25 μ M TRO plus 10 μ M AG1478 for 18 h. Cells were then harvested in 10% TCA and thymidine incorporation measured as described; results are means $\pm$ sem from 4 separate experiments with no increase in DNA synthesis in TRO plus AG1478 vs TRO alone (corrected Student t test). B. Cells were grown to confluence on chambers and treated as described in A above. After 18 h the cells were loaded with BCECF and pH_i measured over a 4 min in KH (7.4).

Differentiating TRO's activation of EGFR vs inhibition of NHE1 activity on DNA synthesis

Because both pathways of TRO's actions, ERK activation and NHE inhibition/cellular acidosis could potentially reduce DNA synthesis, AG1478 was used to selectively block the EGFR activated p-ERK pathway and the incorporation of radio labeled thymidine measured as an index of DNA synthesis. As shown in Fig. 6A, TRO reduced ($p < 0.01$) thymidine incorporation by 66% ($7,727 \pm 1,106$ vs $22,541 \pm 1,234$ cpm/mg protein) and similar to our previous study in MCF-7 cells [1]. Interestingly, pre-

venting EGFR activation with AG1478 alone also tended to reduce DNA synthesis compared to the control ($13,662 \pm 5,450$ cpm/mg protein, $p < 0.10$ vs control). Despite preventing ERK activation, AG1478 + TRO still reduced ($p < 0.05$) thymidine incorporation by the same extent as occurred with TRO alone ($7,568 \pm 1,363$ vs $7,727 \pm 1,234$ cpm/mg protein for TRO alone). The effect of TRO and TRO + AG1478 on pH_i is shown in Fig. 6B. An intracellular acidosis was induced by TRO alone (7.07 ± 0.08 vs 7.54 ± 0.03 , $p < 0.05$) as previously reported [1], or in combination with EGFR blockade (6.98 ± 0.02 , $p < 0.01$

vs CTL) and sustained for at least 18 h. This shows that a similar low steady-state pH_i results from TRO inhibition of acid extrusion whether or not ERK activation occurs.

Noteworthy, EGFR blockade alone tended to result in a sustained intracellular acidosis (7.17 ± 0.12 vs 7.49 ± 0.05 , $p < 0.10$) as well as tended to reduce DNA synthesis (Fig. 6A). These results are consistent with the TRO-induced cellular acidosis/NHE1 inhibition rather than ERK activation as the dominant factor in reducing DNA synthesis in this line of breast cancer cells.

Discussion

The results from the present study may be viewed as depicted in Fig. 7. The two demonstrable pathways we focused on are: I) TRO-activated EGFR leading to an acute and sustained rise in p-ERK via MEK and, II) TRO's direct inhibition of NHE and apparent activation of an acidification process leading to the acute and sustained fall in cytosolic pH. Both responses ERK activation/cellular acidosis to TRO (but not with the more effective PPAR γ -ligand, pioglitazone, and [3]) occur within minutes and therefore rules out a traditional PPAR γ "genomic" signaling pathway.

Although depicted as two separate pathways, there is in fact at least one interaction between p-ERK and NHE1 as first shown by Fliegel and coworkers [18]; ERK activation results in simultaneous dual site phosphorylation of NHE1 increasing activity [19], and elevating steady-state pH_i . Here we demonstrate in line with these findings, that EGF activates ERK and plays an important role in pH homeostasis in this tumor cell line through up-regulation of NHE1 activity and resetting pH_i above the one present in non-tumor cell lines [20]. Thus ERK activation alone should facilitate acid extrusion and maintain the elevated pH_i . Noteworthy, an elevated pH_i [21] and/or increased NHE1 activity [22] appears as a necessary, if not sufficient [23], condition for tumor cell proliferation. In decided contrast to what would be expected from ERK activation alone, there occurs a simultaneous and precipitous fall in pH_i with TRO induced ERK activation. This rapid fall in pH_i is dependent in part upon TRO's ability to activate ERK since ablation of pathway I slows the acidification rate with TRO. Precisely how p-ERK and TRO combined to accelerate acidification was not definitively established, but several possibilities exist: one possibility is an increase of acid production similar to episodes of transient acidosis occurring with tumor-promot-

Fig. 7. Dual pathways hypothesized to explain TRO inhibitory effect on DNA synthesis: I] TRO increases p-ERK via EGFR, and II] TRO inhibits NHE1 activity and induces cellular acidosis. Noteworthy is the association between p-ERK and pH_i ; p-ERK activation in the absence of TRO enhances NHE activity elevating pH_i whereas p-ERK activation in the presence of TRO increases NHE inhibition and possibly also acidogenesis and decreases pH_i . Together the two pathways (I + II) sustain an elevated p-ERK and depressed pH_i associated with impaired DNA synthesis. However, preventing p-ERK activation fails to reverse the inhibitory effect on DNA synthesis. Dotted-lined arrows indicate intermediate steps not as yet elucidated.

ing phorbol ester activation of neutrophils [16]; another possibility might be activation of a potassium/proton exchange as previously reported in rat ileum brush borders [24]; yet another possibility is a facilitating effect of p-ERK enhancing TRO's inhibitory action directly upon NHE1 activity. Although further experiments are required to elucidate the mechanism, it may be significant that the combination of ERK activation and TRO converted the acid extrusion response to acid loading. Nevertheless, the ability to virtually eliminate the TRO-induced ERK activation, pathway I, while preserving the TRO-induced cellular acidosis, pathway II, provides the basis for differentiating the relative roles of the two pathways in TRO's ability to arrest tumor growth.

Having elucidated the upstream EGFR site for TRO's activation of ERK, we then employed the EGFR

ligand site inhibitor AG1478 to demonstrate that preventing activation of MEK (as opposed to only blocking p-ERK) did not further enhance TRO's inhibitory effect on DNA synthesis. Note that a high dose (10 μ M) of this blocker was used to prevent TRO (25 μ M) from activating EGFR, apparently by blocking binding at the receptor. However the high AG 1478 used to ensure that ERK activation did not occur with TRO could also have non-specific effects. Nevertheless, the results clearly showed that preventing this pathway's activation through inhibiting binding, and, or, transactivation neither reversed TRO-suppressed proliferation nor TRO-induced sustained cellular acidosis. Furthermore, in the absence of TRO, blocking EGFR activation with AG1478 (but not with a high PD153035 dose) alone reduced p-ERK below control and tended to lower both the spontaneous pH_i and DNA synthesis in line with the recent study [25] suggesting a positive action of ERK activation on DNA synthesis.

Indeed the present results are consistent with and support the earlier therapeutic strategies utilizing amiloride and derivatives to block NHE1 and induce growth arrest [26]. Amiloride derivatives act at the exchanger's sodium-binding site and are therefore susceptible to being displaced unlike TRO whose effectiveness appears to be enhanced by ERK activation. The recent study [25] employing both the amiloride derivative cariporide and ERK inhibitor (PD98059) to effect a cellular acidosis also provoked tumor cell growth arrest. In agreement with this approach, we show that TRO's effectiveness also results from the ability to inhibit NHE1 (apparently similar to amiloride derivatives, which do not activate ERK, data not shown) and at the same time activate ERK which in turn enhances the TRO-induced cellular acidosis. These results therefore point to NHE1 inhibition and an associated cellular acidosis as critical in the unique TRO effect to arrest growth in this cancer cell line.

The TRO-induced fall in pH_i could in turn reduce cellular proliferation by itself as previously shown [27], or by increasing p53 levels, also concurrently elevated by acidosis [28]. The profound intracellular acidosis induced by TRO's might also contribute to the observed apoptosis through activating caspase 3, a pH-dependent mechanism, as previously shown [29], or alternatively since MCF-7 cells lack a functional caspase 3 other mechanisms. Thus cellular acidosis induced by TRO may explain both the growth arrest as well as reduction in cell number previously reported [1]. Nevertheless, TRO's

antiproliferative effect may not be solely pH-related, but, might also reflect regulation of the cell cycle/cellular cytoskeleton through the NHE1's function other than exchange activity [30]. Further studies are required to determine the relative roles of cellular acidosis vs NHE inactivity-mediated cytoskeleton regulation in the promotion of growth arrest in breast cancer tumors, and whether this pathway plays a role in other cancers as well.

Our findings also suggest that both limbs of the model proposed in Figure 7 be monitored when testing the glitazone's (troglitazone, rosiglitazone, ciglitazone, pioglitazone) effectiveness in cancer therapy since activation of EGFR/MEK/ERK alone, and therefore enhanced NHE1 activity, could likely promote tumor growth. Indeed, numerous reports of glitazone's dual effect of stimulation-inhibition on tumor cell growth have appeared [31-33]. In general at low concentrations glitazones may have a tumor growth promoting effect whereas at higher concentrations they arrest tumor cell growth, a duality perhaps reflecting the two pathways shown in Fig. 7. Ambiguous results from animal studies [33] and clinical trials [34] may also reflect differential activation of these two pathways. Accordingly, the effectiveness of TRO in tumor therapy would likely depend upon the extent to which NHE1 inhibition occurs relative to ERK activation. If, for example TRO's activation of EGFR occurs without sufficient TRO concentration to inhibit the NHE1 activity, then TRO might paradoxically enhance tumor cell growth through the consequences of EGFR activation, including an increase in pH_i. In addition, sustained activation of ERK with loss of the NHE1-inhibitory site might result in rebound proliferation [35-37] and enhanced growth. If so, TRO's actions on tumor growth may be biphasic depending upon the delivery and concentration employed, e.g., stimulatory at low levels and inhibitory at the higher levels [31-37]. Whether differential activation of pathway I or II occurs over the therapeutic concentration ranges employed for a given tumor cell is of some importance and will require further study.

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