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ABSTRACT

Purpose: The purpose of this study was to assess whether troglitazone (TRO) would induce cellular acidosis by inhibiting Na+/H+ exchanger (NHE) 1 in breast carcinoma-derived cell lines and, if so, whether cellular acidosis would be associated with a reduction in proliferation.

Experimental Design: Intracellular pH (pH̅) and acid extrusion capacity after an exogenous acid load were assayed using (2, 7)-biscarboxyethyl-5(6)-carboxyfluorescein in MCF-7 and MDA-MB-231 cells treated with TRO. Radiolabeled thymidine incorporation was used to assess DNA synthesis. Peroxisome proliferator-activated receptor (PPAR) γ involvement was assessed using an antagonist and PPARγ−/− NIH3T3 cells.

Results: TRO induced a prompt (<4 minute) and severe cellular acidosis in both MCF-7 (7.54 ± 0.23 to 6.77 ± 0.06; P < 0.001) and MDA-MB-231 cells (7.38 ± 0.18 to 6.89 ± 0.25; P < 0.05) after 12 minutes, without increasing acid production. Acid extrusion as assessed by the response to an exogenous acid load (NH₄Cl pulse) was markedly blunted (MDA-MB-231, P < 0.01) or eliminated (MCF-7, P < 0.001). Chronic exposure to TRO resulted in NHE1 activity reduction (P < 0.05) and a dose-dependent decrease in DNA synthesis (<75% inhibition at 100 μmol/L; P < 0.001 and P < 0.01 for MCF-7 and MDA-MB-231, respectively) associated with a decreased number of viable cells. TRO-mediated inhibition of proliferation was not reversed by the presence of the PPARγ inhibitor GW9662 and was demonstrable in PPARγ−/− NIH3T3 cells, consistent with a PPARγ-independent mechanism.

Conclusions: TRO induces marked cellular acidosis in MCF-7 and MDA-MD-231 cells. Sustained acidosis is consonant with decreased proliferation and growth that is not reversed by a PPARγ antagonist. Our results support a NHE-mediated action of TRO that exerts its effect independent of PPARγ.

INTRODUCTION

The ability to maintain elevated intracellular pH (pH₅) despite growth in a progressively acidic extracellular milieu confers selective advantages to tumor cells for both proliferation and invasiveness (1–3). Recent studies have shown that an alkaline pH₅ is a fundamental step in the acquisition of an induced tumorigenic phenotype in various cells (4, 5). Despite the fact that tumor cells grow in an acidic milieu, they present enhanced glycolysis that enables both endogenous acid production and their reliance on acid extrusion mechanisms (6–8). The “glycolytic phenotype” has been recently associated with acid-mediated tumor invasion in mathematical models in which the tumor cell plays a major role by altering the surrounding microenvironment with acid excretion (9, 10). The acidification of the extracellular environment leads to destruction of the normal tissue and triggers cell death, angiogenesis, extracellular matrix degradation, and inhibition of the immune function (10). Whereas extracellular acidosis favors tumor growth, cellular acidosis leads to p53-dependent induction of apoptosis in various tumor-derived cell lines (10, 11). On the other hand, the permissive role of elevated pH₅ favors DNA synthesis and promotes tumor growth (4, 12, 13). Although the role of Na⁺/H⁺ exchanger (NHE) 1 in tumor growth has been well known for more than a decade, more recently, signaling pathways involved in the activation of NHE1 have been explored in cells with a malignant phenotype (5, 14, 15). In this perspective, the acid–base balance in tumors has been targeted for therapeutic purposes through interference with the function of NHE1 and related biochemical pathways (16–18).

Troglitazone (TRO) is a thiazolidinedione that exhibits antihyperglycemic and antiproliferative actions (19–22). In growth factor-induced cell growth, the antiproliferative activity of TRO can be related to signaling via peroxisome proliferator-activated receptor (PPAR) γ pathway and down-regulation of cyclins and cyclin-related kinases as well as hypophosphorylation of the negative regulatory retinoblastoma protein (19, 23–26). However, in serum-independent tumor cell growth, TRO reduces proliferation by both PPARγ-dependent and -independent pathways (15, 22, 27–34). Recent studies of PPARγ-negative cancer cell lines have shown that TRO reduces proliferation in a dose-dependent manner by partial intracellular Ca²⁺ depletion and Ca²⁺-mediated inhibition of translation initiation, cy-
clin D and E expression, or expression of the hyperphosphorylated form of the retinoblastoma tumor suppressor gene product as well as cyclin E formation (31–34). Therefore, depending on the concentration and physiologic conditions, the antitumorigenic action of TRO appears to involve both PPARγ and PPARγ-independent pathways.

Another apparent PPARγ-independent action that may play an anticancer role is the ability of TRO to induce cellular acidosis, which we have demonstrated previously in normal cells (35–37). Our previous findings are consistent with glutamines inducing a spontaneous cellular acidosis associated with a shift in glutamine amino nitrogen metabolism from a predominantly anabolic to a catabolic pathway in canine mesangial cells (36). Consequently, we have shown that TRO induces a spontaneous cellular acidosis resulting from a reduction in cellular acid extrusion in murine mesangial cells (35). Consequently, we have shown that TRO induces a spontaneous cellular acidosis associated with a shift in glutamine amino nitrogen metabolism from a predominantly anabolic to a catabolic pathway in canine mesangial cells (36). Consequently, we have shown that TRO induces a spontaneous cellular acidosis resulting from a reduction in cellular acid extrusion in murine mesangial cells (36).

MATERIALS AND METHODS

Cell Lines and Tissue Culture. MCF-7 and MDA-MB-231 breast carcinoma-derived cell lines and murine NIH3T3 fibroblasts 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 bovine serum (FBS) and 1% antibiotic/antimycotic (PAA Laboratories, Cincinnati, OH) in an incubator set at 37°C in 5% CO2 atmosphere.

Cellular pH and NHE Activity Measurements. Cells were seeded on specially designed 30-mm chambers (Biotechnics, Biological Optical Technologies, Butler, PA). The chambers were placed uncapped inside a 60-mm covered tissue culture dish, incubated at 37°C in 5% CO2 atmosphere, and allowed to gain confluence. The pH was determined with the pH-sensitive fluorescent dye (2,7)-biscarboxyethyl-5(6)-carboxyfluorescein [BCECF (Molecular Probes, Eugene, OR)] assay performed at 37°C by spectrofluorometry as described previously (35, 36). Changes in the emission ratio (490 nm/440 nm) were taken as an index of changes in pH. After each experiment, the high K+ / nigericin technique was used to clamp the pH, to media standards of known pH (confirmed on a Corning 240 pH meter at 37°C after withdrawing the sample from the chamber) obtaining a pH calibration of the 490 nm/440 nm signal ratio. To monitor the spontaneous pH response to TRO, the DMEM/10% FCS was replaced with fresh Krebs-Henseleit-HEPES (KHH) media in which 24 mmol/L HEPES buffer replaced bicarbonate, and the 490 nm/440 nm signal ratio was followed continuously for 12 minutes. Fresh KHH media minus TRO was then added to determine the reversibility of any TRO effect on pH. Cells were tested for pH, in short-term (acute ΔpH) and long-term incubation with and without TRO (chronic ΔpH). To test for acid extrusion capability (acid load), the cells were incubated in KHH media (pH 7.4) and then acid loaded with a 4-minute exposure to KHH in which 20 mmol/L NaCl had been replaced with 20 mmol/L NH4Cl. After returning the KHH media, the pH response was continuously monitored for 4 minutes. The recovery response was taken as the change in pH per time interval. Time control experiments for a repetitive acid load were also performed, establishing that the recovery rates were not different for the repetitive loadings.

Metabolic Studies. Studies were performed on confluent cells previously grown in DMEM/10% FCS in which the media were replaced in the 12-well plates with KHH or KHH + TRO over a 12-minute period. Lactate concentration was determined enzymatically using lactate dehydrogenase (Sigma, St. Louis, MO) coupled to the reduction of NAD to NADH monitored as absorbance at 340 nm as described previously (38). The assay conditions included a hydrazine trap for pyruvate to force the reaction to completion. The production of lactate was expressed as pmol of proteins.

DNA Synthesis and Cell Viability Studies. DNA synthesis was determined by following the incorporation of [methyl-3H]thymidine (70–86 Ci/mmol; Amersham, Piscataway, NJ). Briefly, the cells were incubated in 12-well plates in fresh DMEM/10% FCS plus 1 μCi/mL radiolabeled thymidine for 18 hours. The media were then withdrawn, and the cells washed three times with PBS followed by the addition of 5% trichloroacetic acid (TCA). After scraping TCA-treated cells, the cells were exposed to three freeze-thaw cycles and then pelleted by centrifugation. TCA supernatant was dissolved in dimethyl sulfoxide (DMSO), added to the media at 100 μg/mL, and diluted to desired concentration; controls received the DMSO vehicle.

Immunocytochemistry and Western Blot Analysis. Cells were seeded on two-chamber permanox slides (Nalge-Nunc, Naperville, IL) and grown to approximately 70% confluency. The media were removed, and the cells washed with PBS to remove unattached cells. Media [DMEM/10% FCS (control) or DMEM/10% FCS + TRO] were added, and cells were harvested at different time points as specified. The viable cells were counted by trypan blue exclusion.

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Slides were mounted with Slowfade antifade reagent (Molecular Probes) and visualized.

For Western blot analysis, cells were seeded in a 6-well culture dish in duplicate at 2.5 × 10^5 cells per well and allowed to attach and grow for 24 hours. Cells were lysed by the addition of 100 μL of ice-cold radioimmunoprecipitation assay buffer supplemented with Complete mini-protease inhibitor tablet (Roche Biochemicals, Indianapolis, IN). Protein concentration was determined by the BCA assay (Pierce, Rockford, IL). Sixty micromgrams of total protein were loaded on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose filter and probed with primary antibody to PPARγ (Santa Cruz Biotechnology). The blots were stripped and reprobed for β-actin (Sigma) to serve as a loading control. Bands were developed using Immunostar-horseradish peroxidase chemiluminescent detection reagent (Bio-Rad, Hercules, CA).

**Statistical Analysis.** Comparisons between multiple groups (nonrepeated and repeated measurements) were made using analysis of variance and a corrected Student’s t test, and comparisons between two groups were made using Student’s t test. A one-tailed t table was consulted when an a priori hypothesis was tested; otherwise a two-tailed table was used.

**RESULTS**

**Troglitazone Induces Acute Cellular Acidosis in MCF-7 and MDA-MB-231 Cells.** We have shown previously that TRO induces cellular acidosis in nontumorigenic cells expressing either NHE1 or NHE3 (35–37). Based on these observations, we asked whether TRO would decrease pH in breast carcinoma-derived cell lines MCF-7 and MDA-MB-231. As shown in Fig. 1A and B, the steady-state pH decreased promptly after the replacement of KHH with KHH + TRO (25 μmol/L). The pH fell from the pretreatment average of 7.49 ± 0.10 to 6.77 ± 0.03 for MCF-7 cells (P < 0.001; n = 4), and from 7.38 ± 0.07 to 6.89 ± 0.09 for MDA-MB-231 cells (P < 0.05; n = 7) after 12 minutes, whereas pH for DMSO-treated time controls after 12 minutes was 7.72 ± 0.12 for MCF-7 cells (n = 3) and 7.27 ± 0.03 for MDA-MB-231 cells (n = 4), respectively (Fig. 1C). Although the values of pH for DMSO-treated time controls were different compared with controls at time 0 for both cell lines, this difference was not statistically significant and was reproducible (data not shown). It is noteworthy that a significant (P < 0.01) decrease in the steady-state pH could be observed at 4 minutes after the addition of TRO in the MCF-7 cells. After 12 minutes, pH tended (P = 0.06) to be lower in the

![Fig. 1](image-url) Effects of TRO on pH, in MCF-7 and MDA-MB-231 cells. pH was determined with the pH-sensitive fluorescent dye BCECF loaded into monolayers of MCF-7 (A) and MDA-MB-231 (B) cells. Representative tracing of spontaneous pH was measured at 37°C in KHH media before and for 12 minutes after the addition of KHH media containing 25 μmol/L TRO. C, the fall in spontaneous pH monitored over 12 minutes after the addition of TRO in MCF-7 (n = 4) and MDA-MB-231 cells (n = 7). *, P < 0.05; **, P < 0.01; ***, P < 0.001. Calibration of pH was by the nigericin/high K⁺ technique in the presence of 25 μmol/L TRO. D, lactate production over the 12-minute time course shown in C.
Fig. 2  NHE1 expression and activity. A, expression of NHE1 in MCF-7 and MDA-MB-231 cells. Cells were seeded on slides and incubated with primary monoclonal antibody anti-NHE1 and a secondary fluorescent antibody. Hoechst 33342 was used for nuclear counterstain. Fluorescent microscope images are shown (×100). B and C, representative pH re recovery response to NH4Cl acid load in MCF-7 and MDA-MB-231 cells treated with TRO (left panels). BCECF-loaded monolayers of MCF-7 and MDA-MB-231 cells were exposed to an NH4Cl (20 mmol/L) pulse for 4 minutes, followed by KHH for an 8-minute recovery period. The ΔpH/Δt measured over the fast rising portion of the recovery curve is taken as the RR, with the subsequent rise taken as the slow response (secondary response). TRO was added to the recovery media after the second NH4Cl pulse and compared with the first response as presented in the right panels for (B) MCF-7 (n = 15) and (C) MDA-MB-231 (n = 7).
MCF-7 cells as compared with the MDA-MB-231 cells (6.66 ± 0.04 versus 6.89 ± 0.09, respectively).

TRO-induced cellular acidosis could result from an increased production of acid associated with accelerated glycolysis, particularly in these markedly acidogenic tumor cell lines (1, 2). To assess whether the spontaneous cellular acidosis observed in Fig. 1C was associated with enhanced acid production, lactate appearance in the media was determined over the 12-minute time course. As shown in Fig. 1D, the rate of lactate appearance was not increased by TRO in either cell line (4.44 ± 2.31 versus 5.89 ± 2.9 nmol/mg protein for MCF-7 cells and 15.72 ± 6.64 versus 15.59 ± 4.61 nmol/mg protein for MDA-MB-231 cells). These results point to the TRO-induced cellular acidosis resulting from decreased H⁺ extrusion in these tumorigenic cells.

Troglitazone Reduces the Response to NH₄Cl Load-Induced Cellular Acidosis in MCF-7 and MDA-MB-231 Cells. We first confirmed the exchanger activity present in these two cell lines by immunostaining for NHE1, an important acid-extruding mechanism for maintaining the steady-state pH, as well as for responding to a superimposed exogenous acid load (1, 2). NHE1 staining appeared highly expressed and uniformly distributed on the cell membrane of both cell lines (Fig. 2A). To assess the effect of TRO on the ability of these cells to extrude acid, cells were exposed to an NH₄Cl acid pulse, and their recovery response was monitored. Representative responses for MCF-7 and MDA-MB-231 cells are presented in the left panels of Fig. 2B and C. As shown in Fig. 2B and C, left panels, addition of 20 mmol/L NH₄Cl to the media results in an initial alkalinization of the cells resulting from diffusion of NH₃ and intracellular formation of acid (NH₄⁺); removal of NH₄Cl from the media results in the rapid outward diffusion of NH₃ and a rapid cellular acidification. The lowest pH represents the "trough," where the rates of acid loading and extrusion are equivalent, and the maximal stimulus for NHE activation. Thereafter, the activated acid extrusion exhibits a rapid response (RR) and a slower secondary response returning pH to the steady state. Addition of TRO to the recovery media virtually eliminated both the rapid and the low acid extrusion responses to the NH₄Cl load in the MCF-7 cells (ΔpH/Δt = 0.13 ± 0.01 to −0.012 ± 0.027 RR; P < 0.001) and 0.082 ± 0.009 to 0.001 ± 0.009 (secondary response; P < 0.001)). It is noteworthy that the trough obtained was no different between the first and second loadings (6.82 ± 0.09 and 6.74 ± 0.09, respectively), whereas the steady-state pH achieved was markedly reduced (P < 0.0001) in the presence of TRO (6.69 ± 0.06 versus 7.24 ± 0.08). The MDA-MB-231 acid extrusion response to the acid load is characterized by a more robust RR than that seen in the MCF-7 cells (ΔpH/Δt = 0.52 ± 0.30 versus 0.12 ± 0.01; P < 0.01), despite a similar trough pH, consistent with the higher rate of acid production in MDA-MB-231 cells (Fig. 1D). Nevertheless, TRO blunted the RR (0.52 ± 0.30 to 0.37 ± 0.09, 29; P < 0.01) and virtually eliminated the slow response (0.06 ± 0.02 to −0.002 ± 0.001; P < 0.01; n = 7). The steady-state pH, achieved after the recovery from the NH₄Cl acid load was markedly reduced (P < 0.05) in the presence of TRO (7.73 ± 0.31 versus 7.10 ± 0.29).

To determine the effect of chronic TRO exposure on the ability of both cell lines to extrude acid (NHE activity), TRO or DMSO was added to the media for 18 hours, after which NHE activity was assayed in the absence of TRO. As shown in Fig. 3A and B for representative experiments, 18-hour exposure to TRO (treatment) decreased assayable NHE activity (measured as the RR after NH₄Cl pulse) as compared with the DMSO-treated control. For both the MCF-7 and MDA-MB-231 cells, NHE activity was decreased (P < 0.05) approximately 70% (ΔpH/Δt = 0.21 ± 0.03 to 0.07 ± 0.01 and 0.33 ± 0.08 to 0.10 ± 0.02, respectively). The chronic reduction in the capacity of Fig. 3 NHE1 activity in chronic TRO-treated cells. A and B show representative assays of NHE activity in TRO-treated (A) MCF-7 and (B) MDA-MB-231 cells compared with time controls. NHE activity was assessed as the RR after NH₄Cl pulse; minus sodium (choline) was used to ensure maximal acidification stimulus. C, assayable NHE activity as ΔpH/Δt measured over the RR during recovery from the acid pulse from control and TRO-treated MCF-7 cells (n = 3) and MDA-MB-231 cells (n = 4).
to extrude acid for both cell lines is consistent with a sustained cellular acidosis over the 18-hour time course during which DNA synthesis is measured.

**Chronic Troglitazone Exposure Inhibits \[^3H\]Thymidine Incorporation into DNA.** Recent studies have shown that alkaline pH plays a major role in DNA synthesis, activation of tumor promoters, tumor transformation, and growth (5, 13). To determine whether TRO-induced acidosis inhibited DNA synthesis, we measured radiolabeled thymidine incorporation in both MCF-7 and MDA-MB-231 cells after 18 hours of incubation with TRO. As shown in Fig. 4A, TRO inhibited \[^3H\]thymidine incorporation in a dose-dependent manner in both the MCF-7 and MDA-MB-231 cell lines, although the 14% and 26% decrease at 10 μmol/L TRO did not achieve statistical significance. At 25 μmol/L TRO, both cell lines exhibited a marked reduction in DNA synthesis to 70 ± 3% (MCF-7; \(P < 0.05\)) and 43 ± 5% (MDA-MB-231; \(P < 0.01\)) of the control. At the highest concentration of TRO, 100 μmol/L, the synthesis of DNA was reduced >75% in both the cell lines (to 23 ± 2% and 22 ± 2% of control for MCF-7 and MDA-MB-231 cells).

**Fig. 4** TRO decreases DNA synthesis and cell number in a dose-dependent manner in MCF-7 and MDA-MB-231 cells. A. Cells were incubated in fresh DMEM plus 1 μCi/mL \[^3H\]thymidine for 18 hours with and without TRO. Results are the percentage of radiolabel incorporated compared with the time control measured after 18 hours for MCF-7 (\(n = 7\)) and MDA-MB-231 cells (\(n = 5\)). *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\). B and C, time course of cell viability incubation in DMEM/10% FCS with or without TRO (DMSO) and expressed as cell number (\(P < 0.05\) at 18 hours).

**Fig. 5** TRO-induced decreased DNA synthesis is not blocked by GW9662. A. Immunofluorescent images (original magnification, \(\times 60\)) show the predominantly nuclear localization of PPARγ. MDA-MB-231 cells were stained with Hoechst 33342 (nuclear dye, left panel), anti-PPARγ antibody, and secondary fluorescent antibody (right panel). PPARγ colocalizes with the nuclear stain. B, PPARγ Western blot. Total protein from whole cell lysate was separated on a 10% SDS-PAGE gel, transferred to membrane, and labeled with anti-PPARγ monoclonal antibody. Membrane was stripped and reprobed with anti-β-actin for equal load. C, \[^3H\]thymidine labeling in MDA-MB-231 cells. Cells were incubated in fresh DMEM/10% FCS plus 1 μCi/mL \[^3H\]thymidine for 18 hours and treated with either control (DMSO only), 25 μmol/L TRO, 5 μmol/L GW9662, or 25 μmol/L TRO + 5 μmol/L GW9662. Results are radiolabel incorporation as a percentage of time controls (\(n = 3\); *, \(P < 0.05\)).
TRO-Induced Cellular Acidosis in Breast Cancer Cells

To determine the effect of TRO on cell growth, cells were counted over a 48-hour period with the results presented in Fig. 4B and C for MCF-7 and MDA-MB-231 cells, respectively. TRO decreased the cell number in a dose-dependent fashion over the time, in line with the decrease in DNA synthesis at 18 hours shown above. At the highest TRO concentration, 100 μmol/L, the number of MCF-7 and MDA-MB-231 cells was 65% (P < 0.05) and 50% (P < 0.05) of their controls after 18 hours, respectively. These results show that cell numbers are reduced in parallel over a prolonged time with the TRO-induced inhibition of DNA synthesis shown in Fig. 4A.

Reduced DNA Synthesis Associated with Troglitazone-Induced Acidosis Cannot Be Blocked by a Peroxisome Proliferator-Activated Receptor γ Inhibitor. To determine whether the inhibitory effect of TRO-induced acidosis on [3H]thymidine incorporation was PPARγ mediated, we initially assessed the expression and cellular localization of PPARγ in MDA-MB-231 cells. As shown in Fig. 5A and B, MDA-MD-231 cells show staining that is predominantly localized to the nucleus. To demonstrate that TRO reduces DNA synthesis independent of this signaling system, a selective and irreversible PPARγ antagonist, GW9662 (5 μmol/L), was added with TRO (25 μmol/L) or without TRO (DMSO vehicle), and the response was compared with that of TRO (25 μmol/L) alone. As shown in Fig. 5C, TRO-induced inhibition of DNA synthesis (from 100 ± 7% in controls to 62 ± 4% with TRO; P < 0.05; n = 3) was not blocked by GW9662 (51 ± 8% with TRO + GW9662, P < 0.02 versus control). These results are consistent with the inhibitory effect of TRO on DNA synthesis being independent of the nuclear PPARγ signaling system.

Troglitazone-Induced Cellular Acidosis and Inhibition of DNA Synthesis Occurs in the Absence of Peroxisome Proliferator-Activated Receptor γ Signaling. To demonstrate that the above responses can be elicited in a cell line that does not express PPARγ, we performed parallel studies in PPARγ−/− NIH3T3 cells (ref. 19; cells were confirmed to be negative in our laboratory; data not shown). The nontumorigenic NIH3T3 cell line exhibited a significantly (P < 0.05) lower pH, than either tumorigenic cell line (7.18 ± 0.05 versus 7.54 ± 0.08 and 7.38 ± 0.07) as noted previously for nontumorigenic and tumorigenic cell lines (15, 18). Exposing PPARγ−/− NIH3T3 cells to TRO (25 μmol/L) results in a rapid (Fig. 6A) and profound cellular acidosis, with pH, dropping significantly (P < 0.05) lower than either tumorigenic cell line (6.52 ± 0.02 versus 6.89 ± 0.09 and 6.66 ± 0.04 for MDA-MB-231 and MCF-7 cell lines, respectively) at 12 minutes (Fig. 6B). The effect of TRO DNA synthesis as evaluated by tritiated thymidine incorporation is shown in Fig. 6C. Even at the lowest concentration (10 μmol/L), TRO reduces DNA synthesis by >90%, despite the absence of a PPARγ signaling pathway in the NIH3T3 cell line (P < 0.001). These results clearly confirm that the effects of TRO-induced cellular acidosis on DNA synthesis are PPARγ independent.

FIG. 6 TRO induces acidosis and reduces DNA synthesis in NIH3T3 cells (PPARγ−/−). A, representative steady-state pH response in NIH3T3 cells to 25 μmol/L TRO. B, steady-state pH response to 25 μmol/L TRO in NIH3T3 cells (PPARγ−/−) over the 12-minute time course (n = 4). C, TRO reduces DNA synthesis in NIH3T3 cells (PPARγ−/−); results are percentage of time controls (n = 4; P < 0.001).

DISCUSSION

TRO is a well-established antiproliferative and apoptotic agent with potential antitumorigenic activity (31, 32). Previous studies have shown that TRO has the possibility to influence cell proliferation through PPARγ-dependent and/or PPARγ-independent signaling pathways (23, 24, 27–34, 40, 41). Our study is predicated on the observations in normal cells that TRO induces a spontaneous cellular acidosis, that cellular prolifera-
tion is pH dependent, and, that an alkaline pH, is permissive for tumorigenesis (3, 4, 12, 15, 35–37, 41–45). The fact that cellular alkalinization plays this permissive role in transformation and proliferation has lead to therapeutic strategies based on inducing cellular acidosis through inhibiting NHE activity and hence reducing tumor growth and inducing apoptosis (17, 18, 46).

Precisely how a decrease in cytosolic pH acts to block transformation and inhibit the proliferative response is unclear, but there are several possibilities. One possibility is via pH-sensitive metabolic pathways whose contribution is required both to initiate and support cellular proliferation; in this regard, glycolysis is well known to be enhanced in cancer cells and to be acidogenic and thus requires an up-regulated acid-extruding system (10, 12, 15, 18). Another possibility is that acidosis induces down-regulation of retinoblastoma protein hyperphosphorylation resulting in cell cycle arrest. This event has been described to occur in both human colonic adenoma-derived epithelial cell lines and normal tubule-derived cells (11, 47). Interestingly, TRO down-regulates retinoblastoma gene expression and its conversion to the hyperphosphorylated form in both PPARγ+/+ and PPARγ−/− cells (29–31, 34).

Because we focused specifically on the heretofore little recognized role of TRO-induced cellular acidosis, our design was to study the effect of TRO on pH, and how the induced acidosis acts on [3H]thymidine incorporation in alkalinized malignant MCF-7 and MDA-MB-231 cells, which express high levels of PPARγ. Therefore, our study did not investigate the solicited signaling pathways responsible for the cellular acidosis and associated reduced DNA synthesis. In fact, we show that TRO exerts a rapid effect on pH, within 1 minute of incubation in both cell lines (Fig. 1A and B). The rapidity of action implies an immediate effect that inevitably bypasses the slower signaling pathways solicited by activation of PPARγ, as shown previously in breast cancer-derived cell lines (27, 28). The decrement in steady-state pH with TRO did not differ between malignant (MCF-7, and MDA-MD-231) and nonmalignant cells (NIH3T3; ref. 37), but because the resting pH, was higher in the malignant cells, the extent of cellular acidosis was less severe in the tumorigenic cells. We also show that TRO-induced cellular acidosis was attributable to inhibition of the active extrusion of acid via NHE, previously as shown for normal cells (35, 37, 38). TRO-induced cellular acidosis in the cell lines tested in this study is shared by another compound of the same family of thiazoladinedione, such as rosiglitazone, although the magnitude of acidosis induced by the latter is less severe than that induced by TRO (data not shown).

Although the mechanism of the effect of TRO to slow acid extrusion by this system has not been elucidated, it does not seem to be at the exchanger’s Na+-binding site because removal of the compound from the media did not restore exchanger activity, as occurs with inhibition by amiloride derivatives (43). Furthermore, the action of TRO on either spontaneous pH, or NHE1 activity is additive rather than competitive with the action of amiloride analog 5-(N,N-dimethyl)amiloride on the exchanger.4 It is noteworthy that previous studies have shown that TRO induces an activation of signaling pathways that could potentially exert an effect on cellular acidosis (22, 34, 40). If TRO acts through an internal regulatory mechanism that maintains steady-state pH+, rather than directly competing at the NHE Na+-binding site, then this compound or compounds modeled after TRO would have the therapeutic effectiveness of being able to act on tumors expressing both NHE1 and NHE3 isoforms, something that may provide a basis for looking at the subsequent acidosis-induced effects on proliferation in terms of the spontaneous acidosis. In fact, for example, a greater inhibition of DNA synthesis might be expected in MCF-7 cells exposed to TRO because, besides the developing cellular acidosis, these cells also express PPARγ. In a previous study of MCF-7 cell growth, TRO slowed growth in a dose-dependent manner, with a nearly 50% reduction after 48 hours with 40 μmol/L TRO; after 96 h, approximately 70% of the cells present at 48 hours had undergone cell death (30). Thus, these findings indicated that TRO not only suppressed cell growth but also apparently activated cell death pathways. The growth-suppressing effect occurring over the first 24 to 48 hours was associated with decreased expression of cyclin D1, retinoblastoma protein dephosphorylation, and G1 cycle arrest in previous studies (30).

In our study, 25 μmol/L TRO reduced the pH, and inhibited DNA synthesis as reflected by reduced [3H]thymidine incorporation after 18 hours (Fig. 4A). It is noteworthy that the nontumorigenic cell line NIH3T3 exhibited a lower pH, with TRO than did the tumorigenic cells MCF-7 and MDA-MD-231, although the activity of NHE-mediated acid extrusion was not different. This relatively higher pH, presumably explains why TRO was less effective in inhibiting DNA synthesis in the tumorigenic cell line than in normal cells (Fig. 5B and C). Alternatively, cancer cells may defend themselves from excessive lowering of pH, by different mechanisms than normal cells in an attempt to protect their proliferation and growth. In this case, a prolonged acidification as observed with TRO may prime the cell for activation of either PPARγ-dependent or -independent mechanisms of cell death occurring at a later time after the initial cellular acidosis. Further work is under way in this perspective.

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4 Unpublished preliminary data.

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