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Cancer Letters 179 (2002) 185–195

CANCER Letters

www.elsevier.com/locate/canlet

Troglitazone activates p21$^{Cip/WAF1}$ through the ERK pathway in HCT15 human colorectal cancer cells

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Received 1 December 2001; received in revised form 11 December 2001; accepted 12 December 2001

Abstract

In this study, we identified a new mechanism for the anti-proliferation of HCT15 colorectal cancer cells by troglitazone (TRO). Treating HCT15 cells with 20 μM of TRO transiently increased extracellular signal regulated kinase (ERK) activity within 15 min, and this subsequently induced p21$^{Cip/WAF1}$ cell cycle regulator and localized in the nucleus. Raf-1 modification and MEK activation also occurred after TRO treatment, and Elk-1-dependent trans-reporter gene expression was concomitantly induced. The induction and nuclear localization of p21$^{Cip/WAF1}$ by TRO were blocked by PD98059 pre-treatment, which suggested a role for the ERK pathway in p21$^{Cip/WAF1}$ activation. TRO inhibited BrdU incorporation and no BrdU incorporation was observed in most p21$^{Cip/WAF1}$-activated cells. Therefore, TRO regulates the proliferation of HCT15 cells at least partly by a mechanism involving the activation of p21$^{Cip/WAF1}$.

Keywords: Troglitazone; ERK; p21$^{Cip/WAF1}$; Cell cycle; Anti-cancer

1. Introduction

Troglitazone (TRO) is a member of the family of thiazolidinediones (TZDs), which are orally active drugs that improve insulin sensitivity in both animals and humans with insulin resistance [1,2]. TRO binds to and activates peroxisome proliferator-activated receptor γ (PPARγ), a member of the ligand-activated nuclear receptor family [3,4]. Activated PPARγ regulates transcription of target genes, which are mainly involved in fatty acid and lipid metabolism of fat cells, and are associated with the differentiation of fat cells [5]. However, recent studies found that TRO also inhibits growth of human breast and prostate cancers, myeloid leukemia, and vascular smooth muscle cells [6–9]. A role of TRO in the growth regulation of colorectal cancer cells was also identified and related to the activation of the PPARγ [10]. However, a no-regulatory mechanism of cell growth by TRO independent of PPARγ has been identified. A recent study also found that TRO induced cyclin-dependent kinase inhibitor p21$^{Cip/WAF1}$, and this resulted in the suppression of the growth of a myeloid leukemia cell line [8]. However, the p21$^{Cip/WAF1}$ induction by TRO has not been reported in solid tumors, and the mechanism of p21$^{Cip/WAF1}$ induction has not been determined. The p21$^{Cip/WAF1}$ induction is closely related to the growth arrest of many different cell types, including colorectal cancer cells [11,12], and this is dependent on

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0304-3835/02/$ - see front matter
PII: S0304-3835(01)00869-2

the activation of either p53 tumor suppressor [13] or the extracellular signal regulated kinase (ERK) pathway [14–16]. In addition, high aberrant ERK activities were observed in normal colonic epithelial cells, as compared with the paired tumors, and this might be involved in growth regulation [17]. Therefore, the ERK pathway is likely to be involved in the growth arrest, as well as the proliferation of cells [14–16].

In this study, we found that TRO induces $p21^{Cip/WAF1}$ cell cycle regulator in HCT15 human colorectal cancer cells, and further identified a mechanism for the induction and nuclear localization of $p21^{Cip/WAF1}$ by TRO. The $p21^{Cip/WAF1}$ activation is related to the anti-proliferation of colorectal cancer cells, and this provides an alternative mechanism for the growth regulation of HCT15 colorectal cancer cells by TRO, which is independent of PPARγ.

2. Materials and methods

2.1. Materials

HCT15 (CCL-225) and HT29 (HTB-38) human colorectal cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), McCoy's 5A medium, fetal bovine serum (FBS), antibiotics and Lipofectamin plus reagent were purchased from Life Technologies, Inc. (Grand Island, NY, USA). Phospho-ERK and phospho-MEK antibodies were obtained from New England Biolabs Inc. (Beverly, MA, USA), and ERK antibody from Stratagene (La Jolla, CA, USA). The $p21^{Cip/WAF1}$ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and Raf-1 antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG antibodies were from Transduction Laboratories (Lexington, KY, USA). The BlotMate enhanced chemiluminescence (ECL) system was purchased from Genepia (Seoul, South Korea). The Elk-1-dependent pathdetect plasmids (pFR-Luc and pFA2-Elk1) were from Stratagene (La Jolla, CA, USA), the luciferase assay kit and U0126 were from Promega Co. (Madison, WI, USA), PD98059 from Calbiochem (La Jolla, CA, USA), the protein assay solution was from Bio-Rad Laboratories (Hercules, CA, USA), 4′,6′-diamidine-2′-phenylindole dihydrochloride (DAPI) was from Boehringer Mannheim (Mannheim, Germany), the goat anti-mouse-Cy™2- or goat anti-rabbit Rhodamin Red™-X-conjugated secondary antibodies were from Jackson Immuno Research Laboratories Inc. (West Grove, PA, USA), the anti-BrdU monoclonal antibody was purchased from DAKO Co. (Carpinteria, CA, USA), and finally TRO was a gift from Sankyo, Japan. All other chemicals described in this study were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

The HCT15 [18] cell line was maintained in DMEM, and the HT29 cell line was maintained in McCoy's 5A media in 5% CO_2 at 37°C. Both media were supplemented with 10% (v/v) FBS, 100 units/ml of penicillin and 250 ng/ml of streptomycin. Experiments were performed on cells at 70% confluence, unless otherwise stated. To observe the effects of TRO, most cells were serum starved by being grown in a medium containing 1% FBS for 18–20 h and treated with TRO to 20 μM. TRO (200 μM) was treated for HCT15 cells to observe TRO effects in 10% medium. In cases requiring MEK inhibitor, 30 μM PD98059 or 0–2 μM U0126 was added 1 h prior to TRO treatment.

2.3. Extract preparation and Western blot analysis

Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and harvested by scraping in 500 μl of ice-cold PBS. They were then centrifuged and resuspended in 200 μl of lysis buffer (70 mM β-glycerophosphate pH 7.2, and 0.1 mM each of *meta*- and *ortho*-vanadate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.5% Triton X-100, 0.2 mM phenylmethylsulphonyl fluoride and 5 μg/ml each of pepstatin A, chymostatin, leupeptin and peptin). After incubation on ice for 30 min, the lysate was sonicated for 20 s on ice and unbroken cell debris was removed by centrifugation at $23{,}000 \times g$ for 15 min. Samples were immediately aliquoted and stored frozen at −70°C. Protein concentrations were determined using a Bio-Rad protein assay kit.

The lysates containing 40–50 μg of protein were separated by 8–12% sodium dodecyl sulfate polyacrylamide gel (acrylamide: bis-acrylamide at a ratio of 29:1). Western blot analysis was performed, as

described previously [17], using anti-ERK rabbit polyclonal antibody, phospho-mitogen-activated protein kinase (MAPK), phospho-MEK antibody, anti-c-Raf-1 antibody or anti-p21 antibody followed by HRP-conjugated secondary antibodies at a dilution of 1:3000. The blots were developed using a BlotMate ECL kit.

2.4. PathDetect trans-reporting system and luciferase assay

To determine the activation status of the MAPK pathway in vivo, we adapted the Elk-1-dependent [19] trans-reporting system. For transfection, HCT15 cells were plated into 6-well plates at 5×10^5 cells/well using DMEM supplemented with 10% FBS, 100 units of penicillin and 250 ng/ml of streptomycin. After cultivating for 24 h, the cells were transiently transfected with 0.5 μg of pFR-Luc trans-reporter vector together with 25 ng of pFA2-Elk-1 trans-activator plasmids using Lipofectamin plus reagent according to the manufacturer's instructions. When required, 0.5 μg of pCMV-MEK-2A (a gift from Dr G. Johnson, National Jewish Medical and Research Center, Denver, CO, USA), pSV-Sport1-Raf-1-dn (a gift from Dr J.H. Kim, Korea University, Seoul, South Korea) or the empty vector was co-transfected. For normalization purposes, the cells were co-transfected with 0.1 μg of plasmid containing the gene for β-galactosidase under the control of a CMV promoter. TRO treatment was performed after 18–20 h of serum starvation in DMEM containing 1% FBS. One hour prior to the TRO treatment, 30 μM of PD98059 was added in the required cases. The cell extracts were prepared 7 h after TRO treatment. Luciferase activity was measured using a luciferase assay kit, and normalized using the β-galactosidase level as an internal control.

2.5. Cell counting

HCT15 cells were seeded into 12-well plates at 0.8×10^5 cells/well. After growing for 24 h in DMEM, the cells were placed in the same medium containing 1% FBS for 18–20 h. TRO was then added at a concentration of 20 μM. The cells were incubated in a CO_2 incubator for the indicated time at 37°C, and the attached cells were washed with PBS and harvested using trypsin (0.05% trypsin, 0.5 mM EDTA in PBS). Cell numbers were counted by mounting 10 μl of the cell mixture (1:1 mixture of 0.4% trypan blue and cell suspension) onto a Tiefe Depth Profondeur 0.0025 mm^2 cell counting plate (Superior Co., Germany) and observed under the microscope.

2.6. Immunocytochemistry and BrdU incorporation

For immunocytochemistry, HCT15 cells were plated onto coverslips at a density of 2×10^5 cells/coverslip into 6-well plates, grown in DMEM containing 1% FBS for 18–20 h and treated with 20 μM TRO for 9 h before being subjected to immunocytochemistry. If required, 30 μM PD98059 was added 1 h before TRO treatment. The cells were then washed twice with PBS, fixed in methanol–formaldehyde (99:1) mixture at −20°C for 15 min, permeabilized with PBS containing 0.2% Triton-X-100 and finally washed gently five times with PBS for 5 min. Cells were then treated for 30 min with blocking solution (PBS containing 1% BSA, 0.1% gelatin and 5% goat serum), the cover slips were further incubated with anti-rabbit-p21 antibody for 2 h, and washed five times with PBS containing 1% BSA and 0.1% gelatin for 5 min. They were then further incubated with goat anti-rabbit-Rhodamin Red™-X-conjugated secondary antibody at a dilution of 1:100 for 1 h, and washed five times with PBS for 5 min.

For the BrdU incorporation study, HCT15 cells were grown in DMEM containing 20 μM of BrdU for 5 h before performing the immunocytochemistry, as described earlier. The cells were then fixed in 3.7% formaldehyde for 30 min at room temperature and rinsed once with PBS for 5 min before being incubated for 10 min in 2 N HCl. They were then washed three times with PBS for 5 min. After blocking, the cells were incubated with anti-BrdU moncoclonal antibody at a dilution of 1:20 for 2 h and washed five times with PBS containing 1% BSA and 0.1% gelatin for 5 min. The goat anti-mouse-Cy™2-conjugated secondary antibody at a dilution of 1:100 was incubated for 1 h and the cells were washed five times with PBS for 5 min. Each experiment was performed at least three times. DAPI was then treated at a final concentration of 1 μM in PBS for 10 min, and the cells were extensively washed five times with PBS and three times with distilled water. The samples were mounted for photography, which was conducted

using a Radiance 2000/MP, multi-photon imaging system (Bio-Rad, UK).

3. Results

3.1. TRO transiently increases ERK activity and subsequently induces p21$^{Cip/WAF1}$ cell cycle regulator

The ERK pathway, which is also often called the MAPK pathway, is an important signaling route in cell proliferation, and aberrant activation of the MAPK pathway by genetic alterations of the signaling components often results in the development of cancers [20–22]. However, recent studies have also shown that the activation of the MAPK pathway plays a role in the growth arrest of the cells, and that this is related to the activation of the cell cycle regulator, p21$^{Cip/WAF1}$ [14,15,23]. Because TRO is known to regulate colorectal cancer cell growth [10], we were interested in determining whether the TRO is also involved in the activation of the ERK pathway.

When minimal serum starved HCT15 human colorectal cancer cells were stimulated by 20 μM of TRO, ERK activities transiently increased within 15 min, peaked at 30 min and thereafter decreased (Fig. 1A). Treatment with TRO, also significantly increased the cell cycle regulator p21$^{Cip/WAF1}$ from 2 h after treatment (Fig. 1A). The increased ERK activity and the subsequent p21$^{Cip/WAF1}$ induction suggested that the

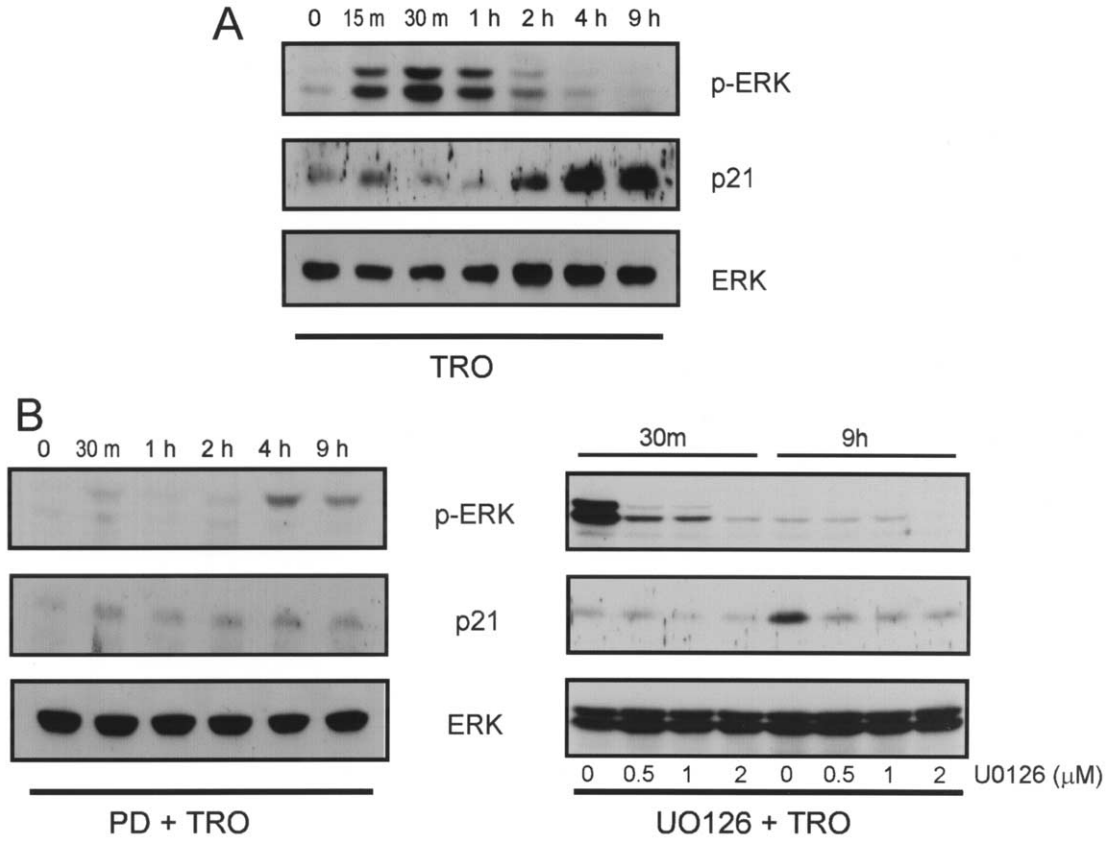


Fig. 1. Transient activation of ERKs and the induction of p21$^{Cip/WAF1}$ by TRO. (A) HCT15 colorectal cells were grown in DMEM, as described in Section 2, and then serum starved for 18 h by growing them in identical medium containing 1% FBS before treatment with 20 μM TRO. (B) The HCT15 cells were grown as shown in Fig. 1A except that 30 μM of PD98059 or 0–2 μM of U0126 was added 1 h before TRO treatment. The activities of ERKs were measured by Western blot analysis using anti-phospho-ERK antibody, and the levels of p21$^{Cip/WAF1}$ and ERK proteins were detected by Western blot analysis using anti-p21$^{Cip/WAF1}$ or anti-ERK antibody.

$p21^{Cip/WAF1}$ levels could be increased by activating ERKs [14,15,23,24]. Therefore, we investigated whether $p21^{Cip/WAF1}$ induction is dependent of ERK activation by checking the effect of an MEK inhibitor, PD98059 [25] or U0126 [26]. Both transient activation of ERKs and subsequent induction of $p21^{Cip/WAF1}$ were mostly abolished by PD98059 or U0126 pre-treatment (Fig. 1B). Therefore, the induction of $p21^{Cip/WAF1}$ by TRO is dependent on the activation of ERKs.

The transient ERK activation and subsequent $p21^{Cip/WAF1}$ induction by TRO were also observed in HCT15 cells grown in 10% FBS at a higher TRO concentration (200 μM) (Fig. 2A). In addition, TRO-induced ERK activation and $p21^{Cip/WAF1}$ induction were also observed in HT29 colorectal cancer cells which were treated with 20 μM TRO in media containing either 1 or 10% FBS (Fig. 2B). Furthermore, the TRO effects were blocked by pre-treatment with PD98059 (Fig. 2B).

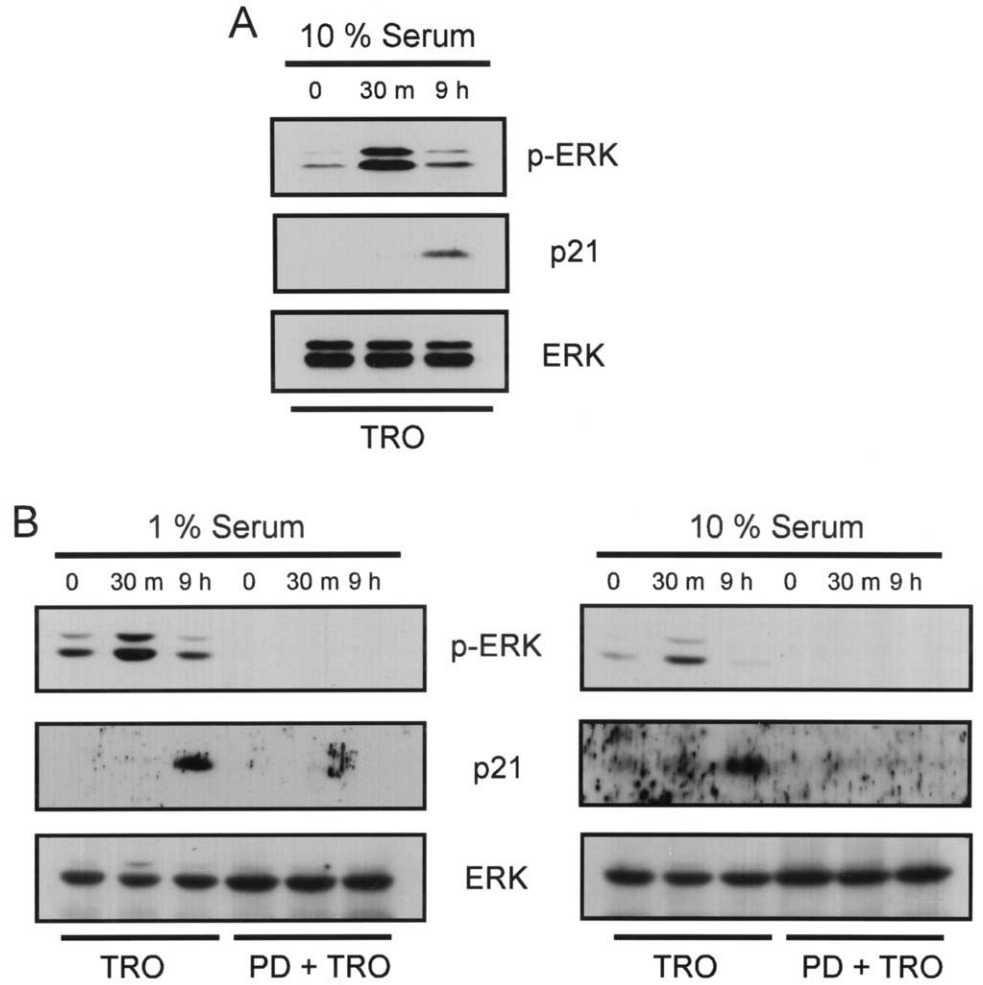
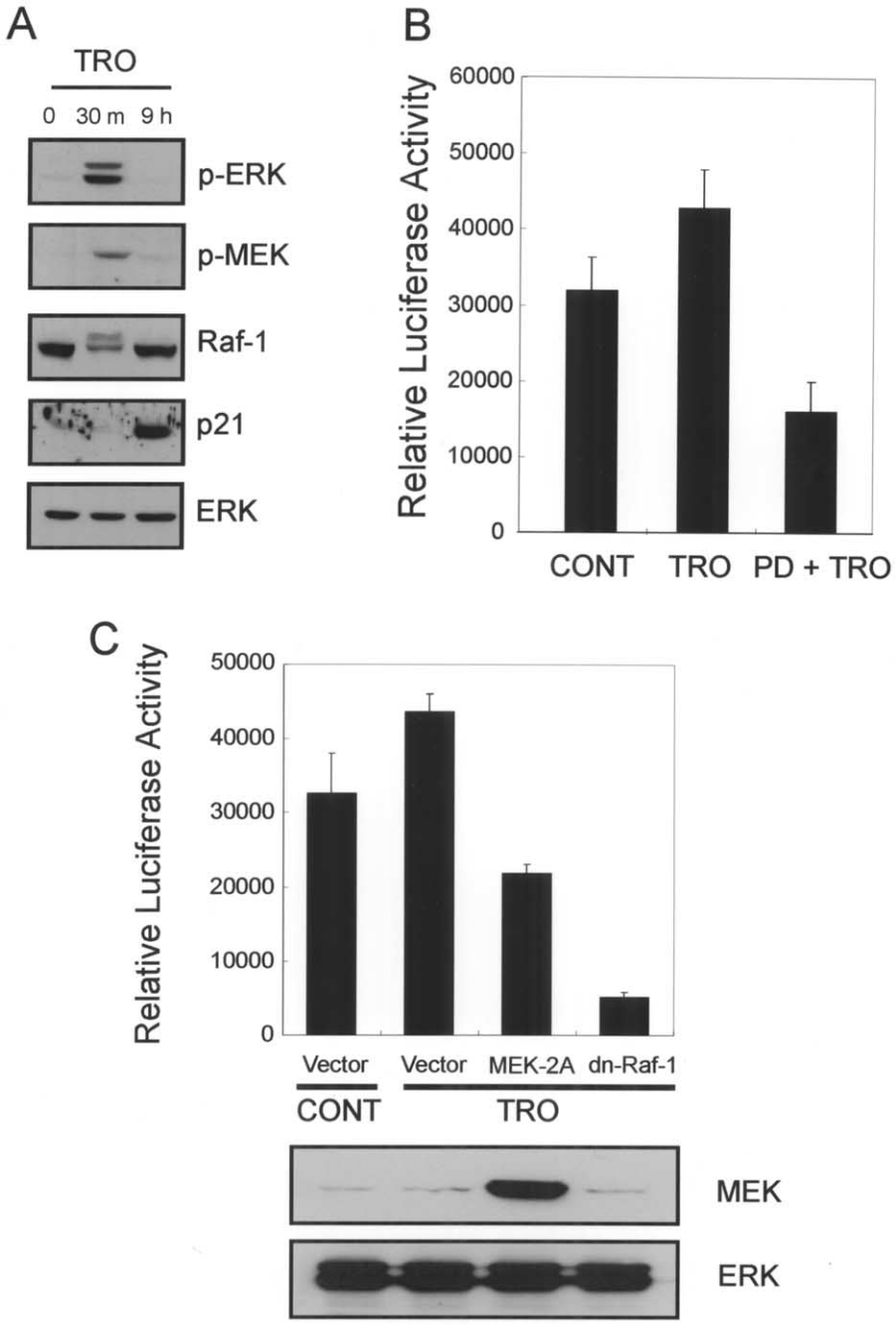


Fig. 2. ERK activation and induction of $p21^{Cip/WAF1}$ occurs in media containing 10% FBS in the presence of a high concentration of TRO. (A) The HCT15 cells were grown in DMEM medium containing 10% FBS, and treated with TRO (200 μM). (B) HT29 cells were grown in McCoy's 5A medium containing either 1 or 10% FBS, and if required, cells were pre-treated with 30 μM of PD98059 for 1 h before treating with 20 μM of TRO. Cells were harvested at 0 and 30 min, and 9 h after TRO treatment. The ERK activities and levels of $p21^{Cip/WAF1}$ and ERK proteins were detected as described in Fig. 1.

3.2. TRO activates the ERK cascade and activates trans-reporter gene expression

To elucidate the mechanism for the activation of ERKs by TRO, we measured a modification of Raf-1 and the activation status of MEK kinases after stimulation by TRO. Specifically, we checked changes in the kinases 30 min and 9 h after TRO stimulation, to monitor both transient ERK activation and subsequent $p21^{Cip/WAF1}$ induction. In cells with



activated ERKs by TRO, both an increase of phospho-MEK and a modification of Raf-1, to a higher molecular weight form, were also observed within 30 min of TRO treatment (Fig. 3A). Therefore, all the MAPK module kinases (Raf-1, MEK and ERK) were concomitantly affected by TRO.

To determine whether the signal for activating the MAPK cascade is further transmitted into the nucleus or not, we used Elk-1-dependent [19] trans-reporter to determine whether TRO activates Elk-1 trans-reporter transcription. As shown in Fig. 3B, TRO did increase Elk-1 trans-reporter gene expression by around 25%, and this was inhibited by PD98059 pre-treatment. However, the level of Elk-1 trans-reporter gene expression observed after PD98059 pre-treatment was much lower than the level that was observed in resting cells (Fig. 3B). Because basal ERK activity is significantly lower in the resting state (Fig. 1A), high basal Elk-1 trans-reporter gene expression may also be acquired independent of ERK activity. Significantly, Elk-1-dependent trans-reporter gene expression induced by TRO was also significantly decreased by the expression of a catalytically inactive MEK (MEK-2A) or dominant negative Raf-1 (dn-Raf-1) (Fig. 3C). In this case, the levels of expression, which were lowered by either the expression of kinase inactive MEK-2A or dn-Raf-1, were also significantly lower than the levels of Elk-1 trans-reporter gene expression in resting cells.

3.3. TRO stimulates the induction and nuclear localization of $p21^{Cip/WAF1}$

To further confirm the role of TRO in the induction of $p21^{Cip/WAF1}$, HCT15 cells were treated with TRO and the numbers of cells inducing $p21^{Cip/WAF1}$ were determined by immunocytochemistry. When HCT15 cells were grown without stimulating media containing 1% FBS, only 3% of the cells retained high $p21^{Cip/WAF1}$ levels in their nuclei (Fig. 4; representative data are shown in Fig. 6B). The percentage of cells inducing $p21^{Cip/WAF1}$ protein was increased to around 12% by TRO treatment, and this was significantly reduced by PD98059 pre-treatment (Fig. 4).

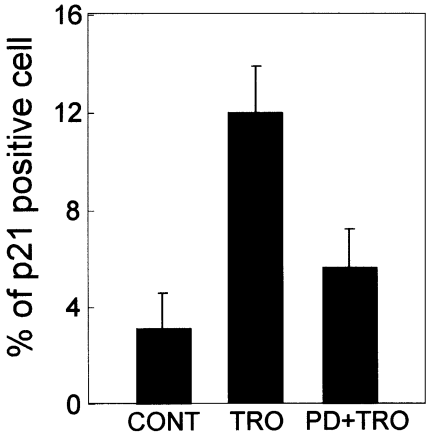


Fig. 4. Activation $p21^{Cip/WAF1}$ by TRO and its inhibition by PD98059. HCT15 cells were grown in DMEM and treated with 20 μM TRO for 9 h. When required, 30 μM of PD98059 was treated before TRO treatment. Cells containing induced and nuclear localized $p21^{Cip/WAF1}$ proteins were recorded as $p21^{Cip/WAF1}$ positive cells.

3.4. TRO inhibits HCT15 cell growth

To determine the role of TRO in the regulation of HCT15 colorectal cancer cell growth, we measured

Fig. 3. Activation of the ERK pathway and the subsequent induction of $p21^{Cip/WAF1}$ by TRO. (A) HCT15 cells were grown in DMEM as described in Fig. 1A, and the cells harvested at 0 and 30 min, and 9 h after 20 μM TRO treatment. ERK and MEK activity were detected by Western blot using anti-phospho-ERK and anti-phospho-MEK antibodies, respectively. Raf-1, $p21^{Cip/WAF1}$ and ERK proteins were also detected by Western blot analysis using anti-Raf-1, -$p21^{Cip/WAF1}$, and -ERK antibodies, respectively. (B) HCT15 cells were grown in DMEM, and the cells were co-transfected with reporter plasmid pFR-Luc and trans-activator pFA2-Elk-1. For normalization, the cells were co-transfected with a plasmid containing the gene for β-galactosidase under the control of CMV promoter. The cells were transferred into medium containing 1% FBS for an additional 18 h and replaced with fresh medium containing 1% FBS containing 20 μM of TRO. When required, 30 μM of PD98059 was also treated for 1 h before TRO treatment. Extracts were made 7 h after TRO treatment. Luciferase activities were measured and normalized using β-galactosidase levels as a control. (C) HCT15 cells were grown and transfected as described in Section 2 with pFR-Luc and pFA-2-Elk-1 plasmids together with a vector, or an expression vector for kinase inactive MEK (MEK-2A), or dnRaf-1. When required, TRO was treated as described in Fig. 3B. Each data point represents the average value of three independent experiments. Error bars indicate standard deviations. Expression of MEK-2A and levels of the ERK proteins were measured by Western blot analysis using MEK or ERK antibody.

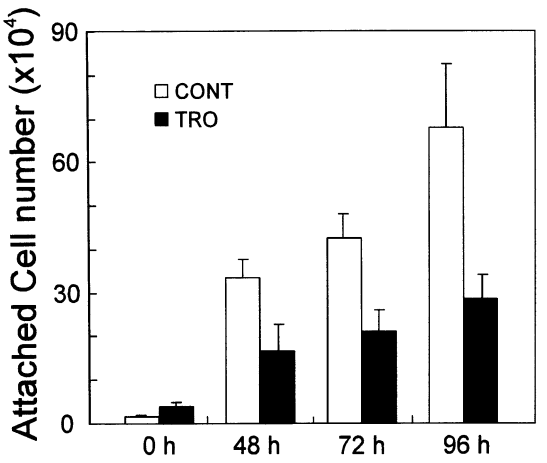


Fig. 5. Inhibition of HCT15 cell growth by TRO. The HCT15 cells were seeded at a density of 0.8×10^5 cells/well in 12-well plates. The cells were grown in the medium containing 1% FBS for 18 h, and were either treated or left untreated with 20 μM TRO. The numbers of attached cells were counted at 0, 48, 72 and 96 h after TRO treatment, as described in Section 2. Each data point represents the average value of three independent experiments. Error bars indicate the standard deviations of three independent experiments.

the cell numbers after TRO treatment. Cell numbers significantly declined over time after TRO treatment compared with the untreated control (Fig. 5). Therefore, TRO is involved in the growth inhibition of HCT15 colorectal cancer cells.

3.5. TRO inhibits anti-proliferation of HCT15 colorectal cancer cells through the activation of p21$^{Cip/WAF1}$

To identify a role for the TRO in the anti-proliferation of HCT15 cells, we monitored DNA synthesis at the single-cell level by measuring BrdU incorporation into TRO-treated cells. In the resting state, around 53% of the HCT15 cells incorporated BrdU, but this percentage decreased to 33% after TRO treatment (Fig. 6A,B). Therefore, TRO inhibits the proliferation of colorectal cancer cells by inhibiting the G1 to S phase cell cycle transition.

To understand the role of p21$^{Cip/WAF1}$, which was induced by TRO in colorectal cancer cells, we also counted the percentages of those cells that incorporated BrdU among those that induced p21$^{Cip/WAF1}$. Although around 53% cells incorporated BrdU in the total cell population, almost none of the cells that induced p21$^{Cip/WAF1}$ incorporated BrdU (Fig. 6A,B). Therefore, p21$^{Cip/WAF1}$ which were induced by activation of the ERK pathway may play a role in the anti-proliferation of HCT15 cells.

4. Discussion

The p21$^{Cip/WAF1}$ is a cell cycle regulator, which is involved in the anti-proliferation of cells by inhibiting cell cycle progression at the G1 to S phase; moreover, the induction of p21$^{Cip/WAF1}$ is a good indicator of growth arrest in colorectal cancer cells [11,12]. In the present study, we identified a mechanism for the activation (induction and nuclear localization) of p21$^{Cip/WAF1}$ by TRO in HCT15 cells, and related this to cell growth. Transient activation of the ERK pathway appeared to be a route for the induction of p21$^{Cip/WAF1}$, with respect to the inhibition of colorectal cancer cell proliferation. TRO activates ERKs by direct signal transduction, which occurs within 15 min of treatment, and may be acquired by the Raf-1 → MEK → ERK module. TRO further induced Elk-1-dependent trans-reporter gene expression, and these results suggest that the signal that activates the MAPK pathway is further transmitted into the nucleus to induce the target gene(s). The levels of Elk-1-dependent trans-reporter gene expression were decreased by PD98059 pre-treatment or by the expression of catalytically inactive MEK/dn-Raf-1. These results suggest that TRO increases the expression of the Elk-1 trans-reporter through the activation Raf-1, MEK and ERK module kinases.

Together with the activation of the ERK pathway, TRO subsequently activated p21$^{Cip/WAF1}$, and this was inhibited by PD98059 or U0126 pre-treatment, suggesting that the activation of p21$^{Cip/WAF1}$ was caused by the transient activation of ERK by TRO. Induction times of p21$^{Cip/WAF1}$ after TRO treatment in HCT15 and HT29 colorectal cancer cells was similar to that found in a myeloid leukemia cell line [8]. The TRO-induced activation of ERKs and the subsequent induction of p21$^{Cip/WAF1}$ were also observed in the cells grown in media containing 10% FBS at a higher TRO concentration (200 μM) than that used to detect the TRO effects in 1% FBS. In addition, G1 to S phase cell cycle progression was also significantly blocked by treating HCT15 cells grown at 10% FBS with

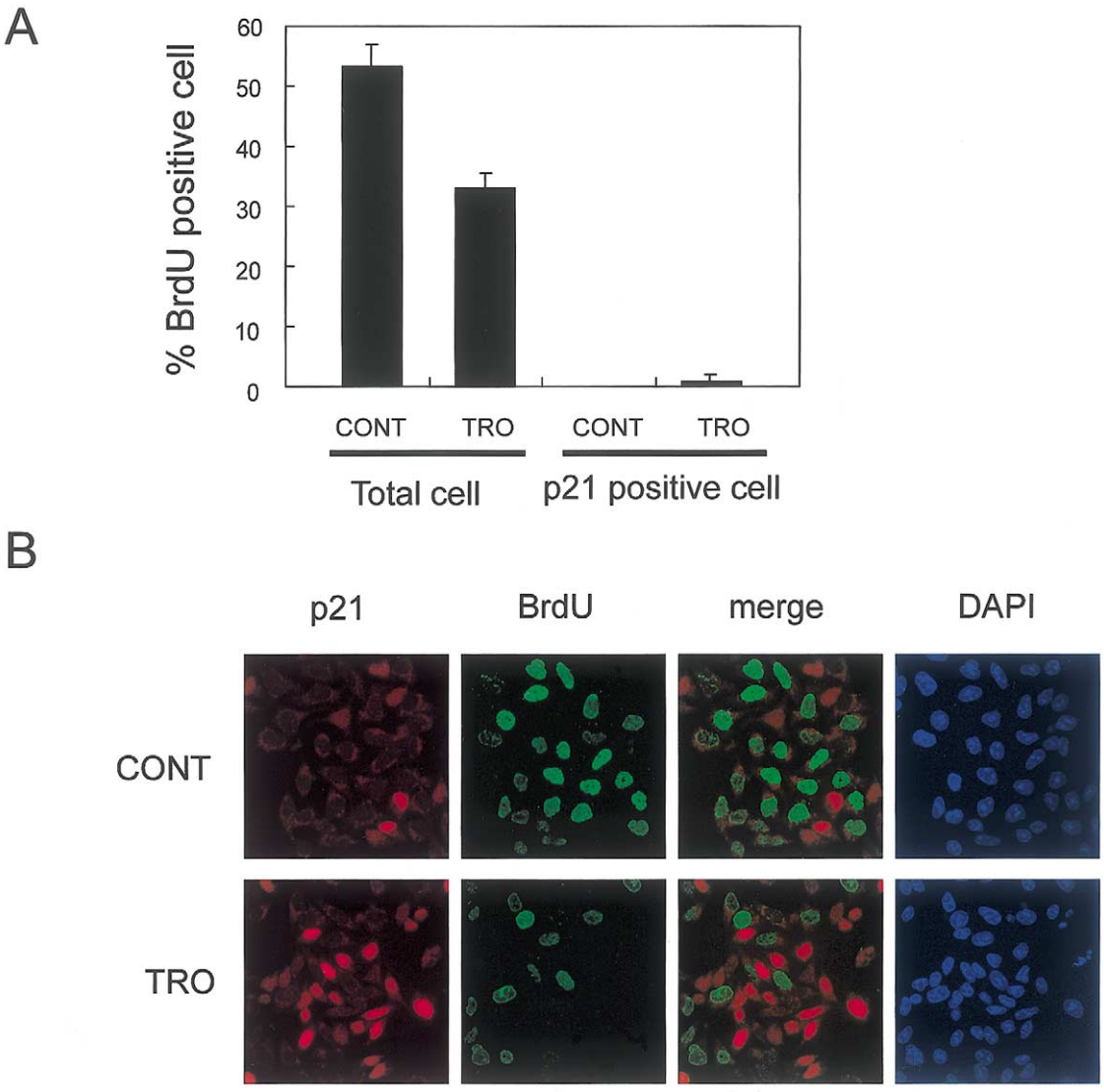


Fig. 6. Inhibition of BrdU incorporation by TRO and the effect of $p21^{Cip/WAF1}$ activation. HCT15 cells were grown in DMEM, and starved for 18 h by growing the cells in medium containing 1% FBS and treated for 9 h with 20 μM TRO. The cells were labeled with 20 μM of BrdU for the last 5 h before assay, and $p21^{Cip/WAF1}$ was visualized using anti-$p21^{Cip/WAF1}$ antibody followed by incubation with Rhodamin-Red-conjugated goat anti-rabbit IgG, and BrdU incorporated into nuclei was observed as a green color by using anti-BrdU antibody followed by Cy2-conjugated goat anti-mouse IgG. Cell nuclei were stained with DAPI. The cells containing induced and nuclear localized $p21^{Cip/WAF1}$ were scored as $p21^{Cip/WAF1}$ positive cells. The experiments were repeated at least three times. (A) Inhibition of BrdU incorporation by TRO among total or $p21^{Cip/WAF1}$ positive cells. (B) Representative data for the inhibition of BrdU incorporation by TRO, and the inhibition of BrdU incorporation by $p21^{Cip/WAF1}$ activation.

100 μM TRO (data not shown). These results suggest that these TRO effects also occur in synchronously growing cells with different drug sensitivities. We also observed similar TRO effects (ERK activation and $p21^{Cip/WAF1}$ induction) when using HT29 colorectal cancer cells even at 20 μM of TRO. Therefore, the TRO effects could also occur in other types of colorectal cancer cells such as HT29.

The percentage of cells incorporating BrdU was decreased by around 20% by TRO among the total cell population, suggesting a role for TRO in the anti-proliferation of cells. Less than 1% of the cells inducing $p21^{Cip/WAF1}$ incorporated BrdU compared with 50% of the total cells that incorporated BrdU. These results show that the $p21^{Cip/WAF1}$ protein, which is induced by the ERK pathway, is involved in the anti-proliferation of several colorectal cancer cells. We did not observe any significant changes in the nuclei or in the accumulation of the sub-G1 fraction by immunocytochemistry and fluorescent activating cell sorting analysis, respectively, in HCT15 cells (data not shown). Therefore, growth arrest of cells by TRO may not be significantly attributed to apoptosis.

We noticed that many cells blocked BrdU incorporation independently of $p21^{Cip/WAF1}$ induction (Fig. 6B). Although $p21^{Cip/WAF1}$ positive cells were increased by around 9%, BrdU positive cells were reduced by around 20% by TRO (compare Figs. 4 and 6A). These results suggest that TRO also inhibits the proliferation of colorectal cancer cells via mechanism(s) independent of the induction of $p21^{Cip/WAF1}$. It is known that TRO regulates the growth of colorectal cancer cells via a PPARγ-dependent mechanism ([10]), and this could provide an alternative route for the growth regulation of colorectal cancer cells by TRO.

In conclusion, this study identifies a new mechanism for the induction of $p21^{Cip/WAF1}$ by TRO, which may be involved in the regulation of the G1 to S phase of cell cycle progression.

Acknowledgements

We would like to thank Drs G. Johnson and J.H. Kim for providing kinase inactive MEK and dominant inhibitory Raf-1 kinase construct, respectively. This work was supported by the following grants to K.-Y.C.: the 1999 Korean National Cancer Control Program, Ministry of Health and Welfare, Korea; grant 1999-1-212-001-5 from the basic research program, and Research Center for Biomedical Resources of the Korean Science and Engineering Foundation. J.-A.K., K.-S.P. and H.-I.K. are recipients of a scholarship from the Brain Korea 21 Project for Medical Science, Ministry of Education, Korea.

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