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Short communication

Troglitazone but not rosiglitazone induces G1 cell cycle arrest and apoptosis in human and rat hepatoma cell lines

Myung-Ae Bae [a], Herman Rhee [b], Byoung J. Song [a,*]

[a] *Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, NIH, 12420 Parklawn Drive, Rockville, MD 20852, USA*
[b] *Center for Drug Evaluation and Research, Food and Drug Administration, Rockville, MD 20852, USA*

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Abstract

Rosiglitazone (RSG), an agonist of peroxisome proliferator-activated receptor γ (PPARγ), induces minor toxicity in humans relative to another PPARγ agonist, troglitazone (TRO). In contrast, recent reports suggest that RSG causes growth arrest and apoptosis of normal and cancerous cells. Therefore, in this study, we investigated the relative toxicities of TRO and RSG on three different hepatoma cell lines, and observed that TRO, but not RSG, was cytotoxic. Additionally, we studied the mechanism by which TRO induced damage to HepG2 hepatoma cells. Our results indicated that TRO increased the levels of p53, p27, and p21, while it reduced the levels of cyclin D1 and phospho-Rb in a time-dependent manner. Increased p27 and p21 levels coincided with reduced activities of cell cycle dependent kinases (cdk) such as cdk2- and cyclin A-protein kinases 24 h after TRO treatment. These results demonstrate that TRO, but not RSG, causes G1 arrest of hepatoma cells, most likely through changing the levels of cell cycle regulators. Furthermore, because RSG did not affect the levels of cell cycle regulators, TRO-mediated growth inhibition appears independent of PPARγ activation.
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Keywords: Troglitazone; Rosiglitazone; p53; p21; Cell cycle arrest; Cyclin-dependent kinases; Apoptosis

1. Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the steroid/thyroid nuclear hormone receptor superfamily (Evans, 1988) and plays an important role in cellular physiology and metabolism. Because of its physiological role in glucose and lipid metabolism, many natural and synthetic agonists of PPARγ are used to treat adult onset non-insulin dependent diabetic patients (Day, 1999; Levovitz et al., 2002). A naturally occurring lipid analog, 15-deoxy-Δ12,14-prostaglandin J2 is an endogenous agonist to PPARγ (Forman et al., 1995). In contrast, troglitazone (TRO), rosiglitazone (RSG) and pioglitazone are synthetic thiazolidinedione ligands for PPARγ (Day, 1999). These PPARγ agonists are known to sensitize the target cells to insulin,

* Corresponding author. Tel.: +1-301-496-3985; fax: +1-301-594-3113.
E-mail address: bjs@mail.nih.gov (B.J. Song).

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thus improving the impaired metabolic conditions associated with adult onset diabetes. Although the precise mechanism of action of PPARγ agonists is still not fully understood, the rank order of agonist binding affinities to PPARγ closely matches the order of their anti-diabetic potencies (Wilson et al., 1996; Adams et al., 1997; Levovitz et al., 2002).

Besides sensitizing cells to insulin, some of the PPARγ agonists have been shown to cause growth arrest or apoptosis in cultured cells and in animal models (Hirase et al., 1999; Ohta et al., 2001; Toyoda et al., 2001). During the preclinical testing phase of TRO efficacy, approximately 1.9% of patients receiving TRO developed severe hepatic problems with elevated serum transaminase activities (Watkins and Whitcomb, 1997). In the severe cases, TRO caused fulminant hepatic failures, leading to multiple human deaths. Due to the severity of TRO-induced hepatotoxicity and the availability of its structural derivatives such as RSG and pioglitazone (Day, 1999; Levovitz et al., 2002), TRO was removed from the market in 2000.

Recently, a report has also suggested that RSG may cause hepatotoxicity in humans (Forman et al., 2000), although the incidence of RSG-related hepatotoxicity is considered extremely rare (Levovitz et al., 2002). Additional studies have revealed that RSG also causes cell growth arrest or death in normal and cancerous cells: vascular smooth muscle cells (Gouni-Berthold et al., 2001; Wakino et al., 2000), myeloid leukemia cells (Sugimura et al., 1999), differentiated human macrophages (Chinetti et al., 1998), intestinal epithelial cells (Kitamura et al., 2001), and human papillary thyroid carcinoma cells (Ohta et al., 2001). In addition, pioglitazone affects various cancer cells similarly (Dubey et al., 1993; Sugimura et al., 1999; Goke et al., 2001). Together, these results suggest the possibility that RSG and pioglitazone may also damage the cells of hepatic origin.

Cyclin dependent kinases (CDKs) are serine-threonine protein kinases that regulate cell cycle progression. CDKs are activated by various cyclins and inhibited by natural inhibitors such as p21, p27, and p18 (for review, see Sherr and Roberts, 1999). These CDKs, cyclins, and CDK inhibitory proteins are tightly controlled by complex mechanisms of transcriptional and post-translational modifications. Due to the critical roles CDK and CDK suppressor proteins play in cell cycle progression and arrest, the effect of PPARγ agonists on CDKs and growth arrest has been studied. Results from these studies varied, however, depending on target cell type, particular PPARγ agonist used, exposure time, and the presence of other mitogenic factors. For instance, Motomura et al. (2000) demonstrated that TRO inhibited human pancreatic carcinoma cell growth by selective up-regulation of p27 without elevation of p21 and p18. In contrast, Koga et al. (2001) showed that TRO treatment for 24 h increased the levels of p21, p27, and p18 in human hepatoma cells, although the effect of RSG was not studied. Other studies showed that both TRO and RSG selectively inhibited the expression of the cyclin D1 gene (Kitamura et al., 2001), while both of these compounds prevented the induction of p21 in a PPARγ dependent manner (Wakino et al., 2000). On the other hand, TRO was shown to stimulate growth of osteosarcoma cells (Lucarelli et al., 2002). Based on these different results, the mechanism for TRO- or RSG-mediated change in growth arrest or stimulation is still unclear. Furthermore, the direct effect of RSG on hepatocytes or hepatoma cells had not been adequately investigated compared to that of TRO. Therefore, the time-dependent comparative effects of TRO and RSG on growth rate of HepG2 and Chang liver human hepatoma cells and McA-RH7777 rat hepatoma cells were investigated in this study. In addition, to study the mechanism of toxicity by TRO and RSG, levels of various cell cycle regulators in HepG2 hepatoma cells were determined.

2. Materials and methods

2.1. Materials

Propidium iodide, dimethyl sulfoxide (DMSO, tissue culture grade), and other chemicals were purchased from Sigma Chemicals (St. Louis, MO). TRO was kindly provided by the Parke-Davis Company (Ann Arbor, MI). RSG was kindly provided by Dr Joong-Kwon Choi at the Korea

Research Institute of Chemical Technologies (Daejon, Korea). All tissue culture media and other agents including fetal bovine serum were procured from InVitrogen (Carlsbad, CA). Specific antibodies to the protein analyzed were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

2.2. Cell culture and determination of cell viability

HepG2 and Chang liver human hepatoma cells and McA-RH7777 rat hepatoma cells were maintained in minimal essential medium with Earl's salts, 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator under 5% CO_2/95% air at 37 °C. Approximately 70% confluent hepatoma cells were used for most of our experiments, unless otherwise stated. HepG2 and McA-RH7777 hepatoma cells, grown in 96-well microtiter plates (1×10^4 cells/well) for 2 days, were incubated with varying concentrations of TRO or RSG (diluted in DMSO at 0.05% final concentration) for different times. Cell viability was then measured by using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) as a substrate, as described previously (Bae et al., 2001).

2.3. Immunoblot analyses

Immunoblot analyses were performed as described previously to determine the amount of each target protein (Bae et al., 2001). Proteins in the cell lysates (100 μg per lane) were separated on 12 or 14% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and then incubated with the respective polyclonal antibody against the target protein. Enhanced chemiluminescence was used to finally visualize the target protein.

2.4. Measurement of cyclin-dependent protein kinase activity

HepG2 cells grown in culture flasks (150-mm diameter) treated with TRO for indicated times were harvested and homogenized in ice-cold lysis buffer (Bae et al., 2001). Untreated control was designated as time 0. Cell debris and particulate fractions were removed by centrifugation at 14 000 rpm for 10 min at 4 °C. Activities of specific cyclin-dependent protein kinase (cdk) in the soluble extracts were determined by the published method of using histone H1 as a substrate (Levkau et al., 1998). Soluble fractions (300 μg per reaction) were incubated with their respective polyclonal antibodies to cdk2, cdc2, cyclin A, or cyclin E for 2 h followed by incubation with protein A-agarose beads (0.2 ml/reaction) for an additional 2 h at 4 °C. The immunoprecipitated proteins were washed with the lysis buffer three times before washing with the kinase buffer twice. The cdk reaction buffer contained: 20 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 5 mM EGTA, 0.5 mM dithiothreitol, 0.1 μg histone H1 (Calbiochem), 30 μM ATP and 10 μCi [γ-^{32}P]ATP. After incubation for 15 min at 37 °C, 2 × SDS sample buffer was added to terminate the enzymatic reaction. Proteins were then separated by 12% SDS-PAGE and visualized by autoradiography.

2.5. Flow cytometry cell cycle analysis

HepG2 hepatoma cells were grown in culture dishes (150 mm diameter at 2×10^6 cells/dish) for 2 days to allow attachment and growth. Under our experimental conditions, HepG2 cells were approximately 70% confluent at the beginning of treatment with TRO or RSG. HepG2 cells were then treated with 50 μM TRO or RSG up to 48 h, as indicated. Both adherent and floating hepatoma cells were combined, washed twice with cold PBS, then fixed in 70% ice-cold ethanol with vortexing and finally stored at −20 °C for at least 4 h. Following two more washes with PBS, the cell pellets were stained with the fluorescent probe solution containing 50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.5 mg/ml RNaseA in PBS for 1 h at room temperature in the dark. The cell suspension was filtered through a 60-μm Specrta/Mesh nylon filter (Spectrum Medical Industries) to remove the aggregated cells. Approximately 10 000 cells from each group were then analyzed for DNA histograms and cell cycle phase distributions using a FACS Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ) with excitation at 488 nm and

emission at 620 nm. The percentage of cells undergoing apoptosis was obtained from the percentage of cells in the distinct sub-diploid region of the DNA distribution histograms analyzed by a CELLQUEST software program (Becton Dickinson). The flow cytometry analysis was performed three times.

2.6. Statistical analysis

Experimental results shown were repeated twice or three times, unless otherwise indicated. Results are expressed as means ± SEM. The mean values were compared using Student's *t*-test. $P < 0.05$ values were considered statistically significant.

3. Results

3.1. Differential effects of TRO and RSG on apoptosis of cultured hepatoma cells

To evaluate the cytotoxic effects of TRO and RSG on cultured hepatoma cells, we treated HepG2 human hepatoma and McA-RH7777 rat hepatoma cells with varying concentrations (up to 100 μM) of TRO or RSG for different times and then measured the cell viability by the MTT cell proliferation assay. TRO caused cell death of HepG2 and McA-RH7777 hepatoma cells in a time- and TRO concentration-dependent manner (Fig. 1). At 12.5 and 25 μM TRO, less than 10% of HepG2 and McA-RH7777 hepatoma cells died at 24 h post-treatment with TRO. As expected, only a small number of cells (less than 5%) died in the DMSO-treated control. Approximately 32 and 41% of HepG2 cells died after exposure to 50 and 100 μM TRO, respectively, for 24 h. TRO also caused damage to 28 and 36% of McA-RH7777 hepatoma cells after 24 h of treatment. In contrast, less than 8% cells of HepG2 or McA-RH7777 hepatoma cells died after exposure to 100 μM RSG for 24 h. Similar results of TRO-induced damage, but not by RSG, were also observed by the MTT reduction assay in Chang liver hepatoma cells (data not shown). Morphology of TRO-treated hepatoma cells changed considerably into the round and lobulated appearances of apoptotic cells, while the DMSO-treated control cells remained the same (data not shown). In addition, treatment with 50 μM TRO for 24 h caused marked DNA fragmentation and fluorescent staining with Hoechst 33258 (data not shown), demonstrating that TRO damages these hepatoma cells mainly by apoptosis.

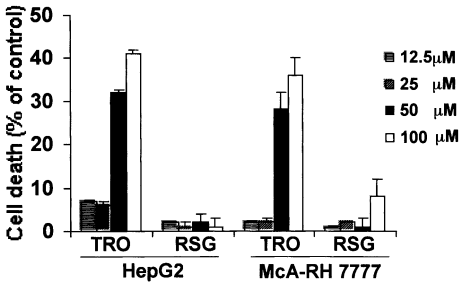

Fig. 1. Differential effects of TRO and RSG on the rate of damage to hepatoma cells. HepG2 and McA-RH7777 hepatoma cells, grown in 96-well microplates, were treated with different concentrations of TRO or RSG, as indicated. Cell viability was determined 24 h after TRO treatment by the reduction of MTT. Results represent the average ± SEM from three to five experiments.

Whether TRO or RSG also affects cell cycle arrest was determined by flow cytometric analysis after staining the cells with propidium iodide (Fig. 2). The percentage of HepG2 cells in the sub-G1 (apoptotic cells) phase significantly elevated as the concentration of TRO increased. For instance, 20.9 ± 3.4% and 40.5 ± 5.2% of HepG2 cells existed in the sub-G1 area after treatment with 50 and 100 μM TRO for 24 h, respectively (data not shown). Cell population under sub-G1 stage from three independent analyses increased from 1.5 ± 0.3% to 7.4 ± 3.2%, 19.6 ± 4.4%, and 34.4 ± 6.4% after treatment with 50 μM TRO for 12, 24, and 48 h, respectively. However, the best histogram was presented in Fig. 2. Sub-G1 cell populations after TRO exposure are significantly different from that of control ($P < 0.01$). In contrast, 50 μM RSG did not change the G1 cell population differently from the DMSO-treated control (Fig. 2).

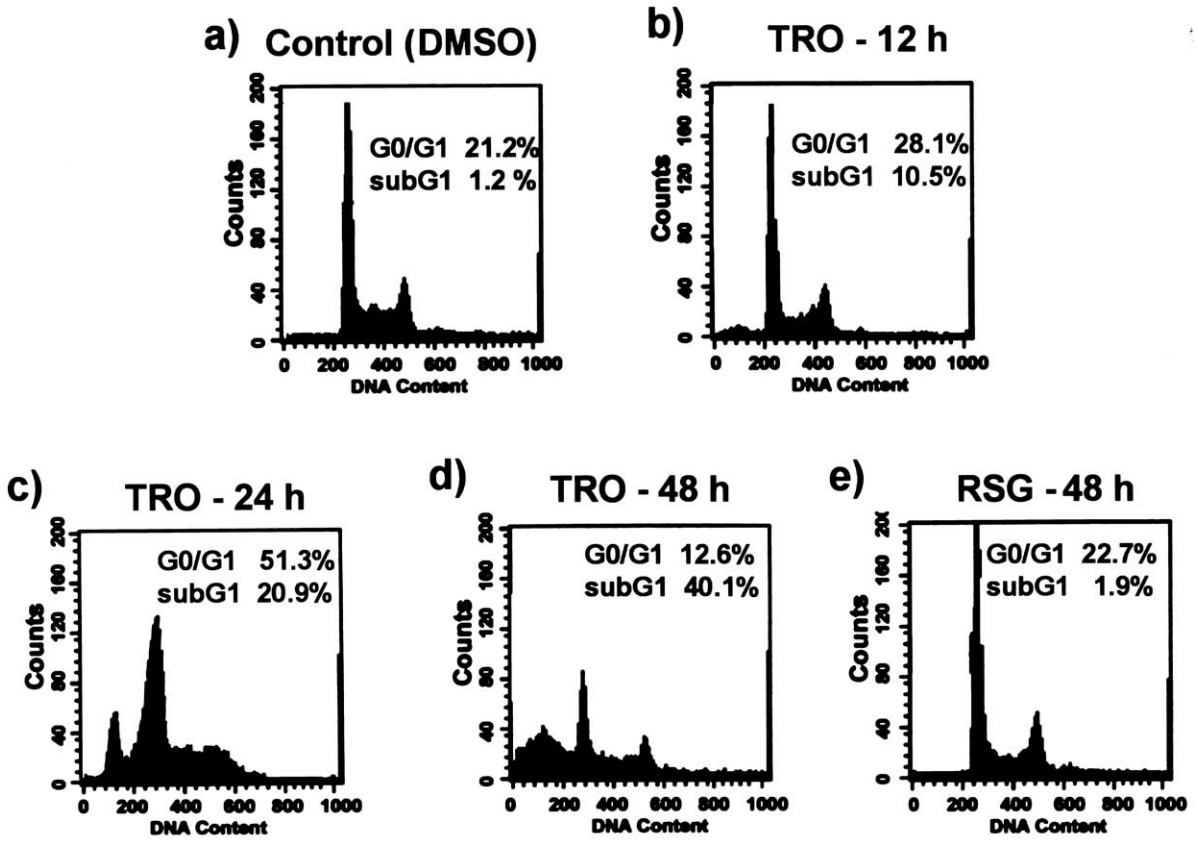

Fig. 2. Flow cytometry analysis following treatment with TRO or RSG. HepG2 cells were treated with different concentrations of TRO or RSG as indicated. Both attached and detached cells were collected 12, 24, or 48 h after treatment with TRO or RSG, fixed, stained with propidium iodide, and subjected to flow cytometry cell cycle analysis.

3.2. TRO-induced changes in the levels of cell cycle regulatory proteins

It is well established that p53 tumor suppressor protein can cause G1 arrest and apoptosis (Vogelstein et al., 2000; Vousden, 2000). Because of the observed increase in G1 population after exposure to TRO, but not RSG, we determined the levels of p53 and its downstream proteins such as Gadd45, $p21^{Waf1/cif1}$ (p21), and $p27^{kip1}$ (p27) in HepG2 hepatoma cells using the specific antibodies against each target protein. Immunoblot analyses revealed that TRO treatment gradually elevated the levels of p53, Gadd45, p21, and p27 in a time-dependent manner during the 24 h incubation (Fig. 3, left panel). The levels of these proteins at 24 h after TRO treatment were markedly increased over those of the untreated control (time 0) or DMSO-treated samples (data not shown).

Phosphorylation of Rb by CDK plays an important role in regulating G1/S progress, leading to cell proliferation or cell death (Sherr and Roberts, 1999). Therefore, we also determined the levels of cyclin D1 and phospho-Rb in TRO-treated HepG2 cells. TRO markedly reduced the levels of cyclin D1 and phospho-Rb in a time-dependent manner, further supporting our findings that TRO-induced G1 arrest. However, TRO did not affect the levels of proliferating cell nuclear antigen (PCNA) and actin (Fig. 3, left panel). Furthermore, the levels of these proteins did not change upon treatment with 50 μM RSG (Fig. 3, right panel), although RSG slightly elevated the content of PI 3-kinase (110 kDa subunit), consis-

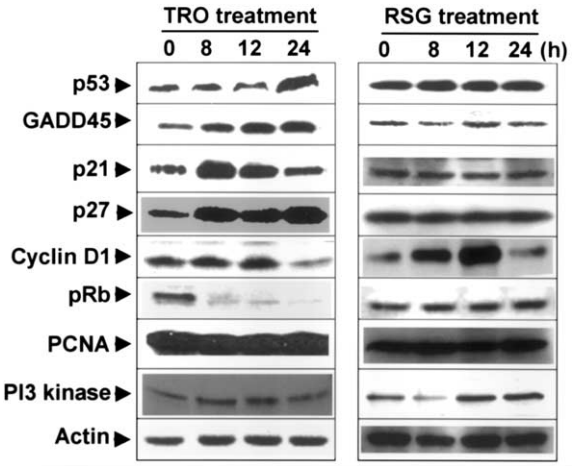

Fig. 3. Immunoblot analysis for the levels of cell cycle regulatory proteins. HepG2 cells were treated with 50 μM TRO or RSG for different times as indicated. The soluble fraction from each sample was separated by 14% SDS-PAGE, followed by immunoblot analysis. Each antigenic protein was detected by using the respective antibody against p53, Gadd45, p21, p27, cyclin D1, phospho-Rb (pRb), PCNA, PI 3-kinase, or actin.

tent with the earlier results (Rieusset et al., 1999). RSG transiently increased the level of cyclin D1 before returning to the untreated control levels (time 0). These results demonstrate that TRO, but not RSG, causes growth arrest by changing the levels of cell cycle related proteins in hepatoma cells.

3.3. TRO-induced decrease in the activities of cyclin A-cdk2 and cyclin E-cdk2

Cell cycle progression and apoptosis process are tightly controlled by p21 and p27 as well as several CDKs, such as cdk2-cyclin E and cdc2-cyclin A (Morgan, 1996; Sherr and Roberts, 1999). Therefore, we also measured the amounts and the activities of cdk2, cdc2, cyclin A, and cyclin E. TRO (50 μM) significantly altered the cdc2 and cdk2 levels in a time-dependent manner (Fig. 4A). Immunoblot analysis of cdk2 protein revealed two immunoreactive bands (33 and 34 kDa), possibly representing the phosphorylated and dephosphorylated forms of each protein (Morgan, 1996). TRO decreased the levels of dephosphorylated (active) cdk2 (33 kDa), and increased the levels of phosphorylated (inactive) counterparts (34 kDa). The level of cdc2 markedly decreased at 12 and 24 h after TRO treatment (Fig. 4A), compared to the untreated control (time 0) or DMSO-treated samples. Consistent with the immunoblot results, TRO treatment markedly decreased the activities of cdk2, cdc2, and cyclin A kinases at 24 h post-treatment, compared to the untreated control (time 0) (Fig. 4B). Under our experimental conditions, the amount of cyclin A kinase did not change after TRO treatment. The level of cyclin E kinase activity was transiently elevated at 8 and 12 h, before it returned to a level at 24 h after TRO exposure as similar to that of the untreated control (Fig. 4B).

4. Discussion

PPARγ agonists play important roles in fat and glucose metabolism in the liver, muscle, and adipose tissue and differentiation of adipocytes (Forman et al., 1995; Day 1999; Levovitz et al., 2002). Despite the insulin-sensitizing action of PPARγ agonists, TRO and RSG were also shown to cause growth arrest and apoptosis of various normal and cancerous cells. These cells include: hepatocytes (Toyoda et al., 2001, 2002), hepatoma cells (Koga et al., 2001), monocyte-derived macrophages (Chinetti et al., 1998), colon cells (Kitamura et al., 1999), HL60 promyelocytic leukemia cells (Hirase et al., 1999), papillary thyroid carcinoid cells (Ohta et al., 2001), intestinal epithelial cells (Kitamura et al., 2001), and vascular smooth muscle cells (Okura et al., 2000; Gouni-Berthold et al., 2001; Wakino et al., 2000). However, these reports showed different mechanisms of apoptosis, depending on the target cell type, particular PPARγ agonist used, duration of treatment, dosage used, and the presence of other mitogenic factors. For instance, RSG appears more potent in causing apoptosis of vascular smooth muscle cells than TRO (Gouni-Berthold et al., 2001), whereas, TRO is more hepatotoxic than RSG, as observed clinically (Watkins and Whitcomb, 1997; Levovitz et al., 2002). In addition, relative toxicities of TRO and RSG on cultured cells and their toxic mechanism have not been investigated with respect to the

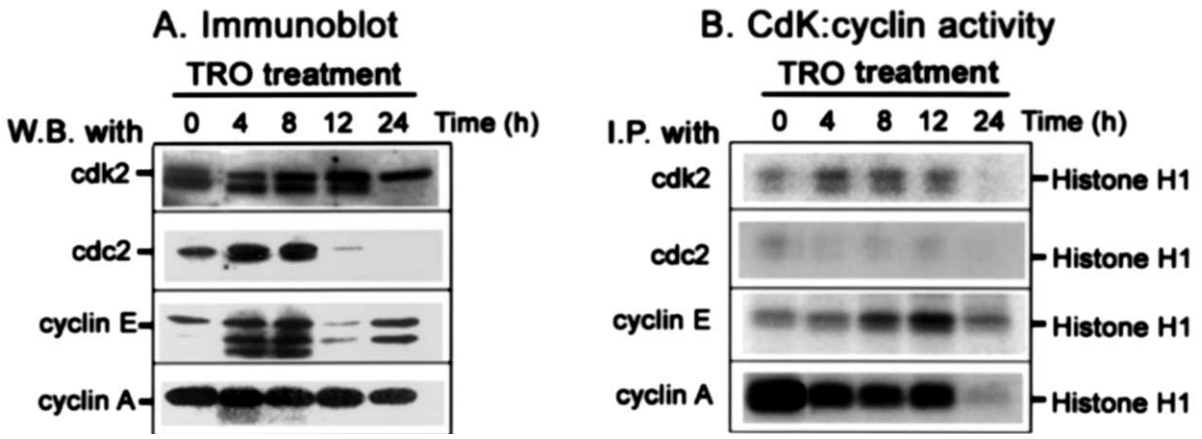

Fig. 4. Change in the levels and the activities of cyclin-dependent protein kinases. (A) The soluble fraction from TRO-treated HepG2 cells for different times were subjected to 14% SDS-PAGE, followed by immunoblot analysis using the specific antibody against each target protein as indicated. (B) Protein kinase activity associated with each immunoprecipitated cdk protein was determined using histone H1 as the substrate.

levels of p53-related cell cycle regulators and CDKs. Subsequently, the mechanism of cell damage by TRO and RSG is poorly understood. Therefore, we compared the effects of TRO and RSG on the growth rates of three hepatoma cell lines. Our data demonstrated that TRO causes G1 growth arrest at similar doses (25–50 μM) used in other studies (Kitamura et al., 1999; Ramachandran et al., 1999). TRO-mediated growth arrest most likely results from the up-regulation of the cell cycle regulatory proteins such as p53, p27, and p21 as well as reductions in phospho-Rb and the activities of various CDKs in a time- and TRO concentration-dependent manner. These results with HepG2 hepatoma cells are in agreement with the elevations in p21, p27, and p18 contents at 24 h after TRO exposure to other kinds of human hepatoma cell lines, although the role of CDKs and the effect of RSG on cell death rate were not investigated (Koga et al., 2001). However, our results differ from the data of other studies, since TRO up to 100 μM did not elevate the levels of p21 and p18 (Motomura et al., 2000), and TRO prevented the induction of p21 in a PPARγ dependent manner (Wakino et al., 2000). Our results reveal that RSG neither caused G1 cell cycle arrest and apoptosis of three different hepatoma cells, nor changed the levels of the cell cycle regulators determined in this study. Relatively little toxicity by RSG observed in the three hepatoma cell lines is consistent with that observed clinically (Levovitz et al., 2002). However, TRO-induced G1 arrest does not appear to be mediated through PPARγ activation since RSG, which binds PPARγ much stronger than TRO, causes very little growth inhibition and apoptosis (Lehmann et al., 1995; Wilson et al., 1996; Levovitz et al., 2002).

Compared to RSG, TRO can cause liver or cell damage in many different ways. For instance, the α-tocopherol moiety of TRO can be metabolized to reactive quinone metabolites or in a ring scission pathway (Yamazaki et al., 1999; Kassahun et al., 2001). It was proposed that accumulation and subsequent binding of TRO or its reactive quinone metabolites to various hepatic proteins may inhibit the protein synthesis and function of the target proteins, leading to reduced cell viability (Kostrubsky et al., 2000). In contrast, RSG, without the α-tocopherol moiety, does not produce the reactive quinone metabolites (Levovitz et al., 2002) which do not interact with the cellular proteins and result in cell damage. In addition, TRO and its metabolites are known to accumulate in the liver through the enterohepatic circulation while RSG neither undergoes the enterohepatic circulation

nor gets accumulated in the liver. Furthermore, TRO may cause an additional problem of drug interaction due to its metabolism via cytochrome P450 3A, which is involved in the metabolism of many clinically used drugs (Yamazaki et al., 1999; Kostrubsky et al., 2000, Levovitz et al., 2002). In parallel to these possibilities of TRO-induced toxicity, early signaling mechanisms may also be involved (Xia et al., 1995). Our unpublished results reveal that TRO can cause apoptosis of hepatoma cells by promoting the c-Jun N-terminal kinase (JNK)-related cell death pathway. In contrast, RSG does not activate the JNK pathway, leading to much reduced cell damage by RSG than TRO (Bae and Song, manuscript in preparation). In addition to the above possibilities, our current data suggests the existence of another mechanism by which TRO, but not RSG, can directly cause G1 cell cycle arrest through the induction of p53 related proteins and the reduction of cyclin D1, phsopho-Rb and CDK activities. Furthermore, it is likely that these possibilities may work in a concerted or synergistic manner toward cell damage.

In conclusion, our results demonstrate that TRO most likely causes G1 arrest and apoptosis of three hepatoma cell lines through up-regulation of the cell cycle regulators while down-regulation of phosphorylated Rb and cell cycle dependent kinases. In contrast, RSG, a potent PPARγ agonist with little hepatotoxicity, does not affect the levels of the cell cycle regulators in HepG2 hepatoma cells. Since RSG causes very little growth arrest, TRO-mediated growth inhibition appears to be independent from PPARγ activation.

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