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# Ciglitazone-induced cellular anti-proliferation increases $p27^{kip1}$ protein levels through both increased transcriptional activity and inhibition of proteasome degradation

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**Abstract**

While it is well established that PPARγ ligands inhibit cell growth and induce apoptosis in colon cancer cells, the mechanism of these effects of PPARγ ligands is unclear. In this report, we demonstrate that the PPARγ ligand, ciglitazone, exhibits an anti-proliferative effect and blocks G1/S cell cycle progression through regulation of $p27^{kip1}$ protein levels and inhibition of Cdk2 activity in HT-29 colon cancer cells. The ciglitazone-induced G1/S cell cycle arrest was noted only after 72 h of exposure, corresponding to elevated protein levels of $p27^{kip1}$. However, an increase in $p27^{kip1}$ protein synthesis as evidenced by increased $p27^{kip1}$ gene promoter activity and mRNA abundance was observed as early as 24 h after exposure to ciglitazone. Proteasome activity, an additional mechanism of $p27^{kip1}$ regulation, was dramatically inhibited after ciglitazone exposure, but only after 72 h of exposure. We also note that the effects of ciglitazone on $p27^{kip1}$ gene regulation are PPRE independent. These data suggest that ciglitazone-induced G1/S arrest is through Cdk2 inhibition and an increase of $p27^{kip1}$ protein levels which in turn is due a balance of ciglitazone's affect on new protein synthesis and degradation.


*Keywords:* PPARγ; $p27^{kip1}$; Cdk2; Proteasome

## 1. Introduction

Peroxisome proliferator-activated receptors (PPAR) are a group of nuclear receptors which act as ligand-sensitive transcription factors [1,2]. PPARγ, one of three isoforms of PPAR, is activated by an array of compounds, including fibric acid derivatives, thiazolidinediones (TZD), arachadonic acid metabolites and fatty acids. Once ligand binding occurs, activation of transcription requires PPAR to heterodimerize with retinoid X receptor α (RXRα), which then bind to PPARγ response elements (PPRE) in the target gene promoter [3]. Alternatively, there is a growing body of evidence that PPAR ligands have a more rapid effect through so called non-genomic pathways in which the nuclear receptor ligand activates additional cell signaling and growth pathways [4,5]. While PPARγ appears to play a key role in control of lipid metabolism and energy homeostasis, recent data suggest that these nuclear receptors have also been shown to be involved in carcinogenesis. PPARγ ligands have been reported to have anti-proliferative effects, induce cellular differentiation, and/or apoptosis in various types of tumors, including breast cancer, prostate cancer, lung cancer, and colon cancer [6]. However, the mechanisms through which these phenotypic changes occur remain unknown.

In mammalian cells, the process of cell cycle passage is tightly regulated by several cyclin-dependent kinases (Cdk) which are positively regulated by their corresponding regulatory cyclin subunit(s), and negatively regulated by Cdk inhibitors (CKI). There are two main groups of CKIs based on the sequence homology and targets of inhibition:

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the INK4 family ($p14^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, $p19^{INK4d}$) and the CIP/KIP family ($p21^{WAF/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) [7]. $p27^{Kip1}$ was first identified in cells arrested by transforming growth factor-β, and is a potent inhibitor of the cyclin-Cdk complexes required for entry into S phase of the cell [8]. The expression of $p27^{Kip1}$ is highest during the quiescent (G0) and pre-replicate (G1) phase and decreases upon re-entry into cell cycle, and protein levels are in part regulated through post-transcriptional mechanisms, including proteasome-mediated degradation [9,10]. During the G1 to S phase transition, $p27^{Kip1}$ is phosphorylated by Cdk2/cyclin E complex at its Thr187 residue which is recognized by $SCF^{skp2}$ (Skp2), a protein complex that targets $p27^{Kip1}$ for ubiquitination and subsequent degradation [11,12].

PPARγ ligand exposure in a variety of cell lines in vitro has been shown to lead to cell cycle arrest, and this cell cycle arrest may be in part be regulated through regulation of CKI, including $p27^{Kip1}$ [13]. In this report, we demonstrate that the anti-proliferative effects of the PPARγ ligand, ciglitazone, are associated with a G1/S cell cycle arrest in HT-29 colon cancer cells after 72 h of exposure. This G1/S block correlated with a significant increase in protein and mRNA levels of $p27^{Kip1}$ and was associated with decreased expression of Skp2, Cdk2 and proteasome activity. Conversely, early (24 h) ciglitazone exposure also induced $p27^{Kip1}$ gene transcription, however, $p27^{Kip1}$ protein levels were unaltered nor was any cell cycle block noted. We conclude that the increase in $p27^{Kip1}$ protein levels and subsequent G1/S block is controlled through ciglitazone's inhibition of proteasome activity, which is not an early event and is only diminished after 72 h of exposure. Inhibition of Cdk2 also appears to contribute to the ciglitazone-induced G1/S cell cycle arrest.

## 2. Materials and methods

### 2.1. Cell culture and biological reagents

HeLa cells and HT-29 colon cancer cells (ATCC, Manassa, VA) were maintained in Dulbecco's Modified Eagle Medium and McCoy's media (Gibco, Rockville, Maryland) respectively, supplemented with 10% complement-inactivated fetal bovine serum (FBS) (Gibco) at 37 °C in an atmosphere of 5% $CO_2$. MG-115, MG-132, ciglitazone and 15-deoxy-Δ12,14-prostagladin J2 (PGJ2) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and dissolve in DMSO (Sigma, St. Louis, MO). Antibodies for p27 (sc-1641), phospho-p27 (sc-16324-R), p21WAF/Cip1 (sc-6246), cyclin E (sc-481), Cdk2 (sc-H298), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for β-actin was purchased from Sigma (cat #A1978). Cell viability was determined by trypan blue exclusion.

### 2.2. Cell proliferation and cell cycle measurement

Cell proliferation assay was performed by BrdU incorporation using standard kit, following manufacture's protocol (Roche, Penzberg, Germany). Cellular DNA content was determined following the protocol: HT-29 cells without or with either ciglitazone or PGJ2 treatment were washed with cold 1× PBS twice and digested with trypsin–EDTA. Amount of cells ($2\times10^6$) was washed with cold 1× PBS again and fixed in 80% ethanol at −20 °C overnight. The cells were then rinsed with 1× PBS, and stained with 0.5 ml of 10 μg/ml propidium iodide (PI) in the presence of RNase A (100 μg/ml, BMB, indianapolis, IN) for 2 h. The cellular DNA content was determined using the FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) and Mod Fit LT software (Verity Software House).

### 2.3. RNA isolation and RT-PCR

HT-29 cells, controls or treated with either ciglitazone or PGJ2, were lysed using 1 ml of TRIzol Reagent (GIBCO, Grand Island, NY) containing 0.2 ml of chloroform (Sigma). Total RNA was then purified by isopropanol (Sigma) precipitation and washed with 70% of ethanol for three times. An aliquot of 1 μg of total RNA from each sample was reverse-transcribed to cDNA using GeneAmp kit (Roche) and following the manufacturer's instructions, with oligo(dT) primer. To detect p27kip1 mRNA and β-actin mRNA (an inner control), the PCR reaction was carried out in 50 μl mixture containing 1 μl of the above cDNA product, 0.15 μM each of the sense and antisense primers of each gene, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1 mM MgCl2, 200 μM dNTPs, and 2.5 units of Tag DNA polymerase (Roche). The reaction conditions were as follows: initial denaturation at 95 °C for 5 min and 35 cycles of amplification (95 °C for 40s, 54 °C for 50 s, 72 °C for 50 s) and then followed by a final extension step of 7 min at 72 °C. The PCR products were separated in a 2% agarose gel and stained with ethidium bromide. The intensities of the bands corresponding to p27kip1 and β-actin were measured by Image QuaNT Program (Molecular Dynamics). The primers used for RT-PCR were as follows: p27kip1: 5′-CCTCTTCGGCCCGGTGGAC-3′ (sense), 5′-TCTGCTCCACAGAACCGGC-3′ (antisense); β-actin: 5′-TGACGGGGTCACCCACACTGT GCCCAGCTA-3′(sense),5′-CTAGAAGCATTTGCCGGTGGACGATG-GAGGG-3′ (antisense).

### 2.4. Western blotting

Cells were lysed with 1× cell lysis buffer (20 mM tris–HCl, [pH7.5], 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 μg/ml leupeptin, 1 mM PMSF, Cell Signaling Technology, cat #9803). Protein concentrations were determined by using the Bio-Rad

protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein extracts were mixed with 3× SDS sample buffer (150 mM Tris [pH 6.8], 30% glycerol, 3% SDS, bromophenol blue dye 1.5 mg/100 ml, 100 mM DTT) and denatured by heating to 100 °C. Proteins were then separated by SDS-PAGE (Bio-Rad Laboratories) and transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL). The membrane was subjected to immunoblot analysis and proteins were visualized by the enhanced chemiluminescence method of detection (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was then stripped and reprobed with an antibody against β-actin as loading control.

### 2.5. In vitro Cdk2 associated kinase assay

Protein extracts (200 μg) were incubated with an antibody against Cdk2 for 2 h at 4 °C, followed by an incubation with protein A plus A/G agarose beads for 1 h. Samples were washed in protein lysis buffer (Cell Signaling, cat #9803) and 1× kinase reaction buffer (Cell signaling, cat #9802). The kinase reaction was carried out at 30 °C for 15 min in 30 μl of kinase reaction buffer containing 10 μM ATP, 10 μCi of [γ-$^{32}$P]ATP (sp act 6000 Ci/mmol, NEN, Boston, MA), and 2 μg of histone H1 substrate (BMB), and then stopped by the addition of 3× SDS sample buffer. After boiling, the samples were resolved in a 12% SDS-PAGE gel electrophoresis and the phosphorylated histone H1 was visualized by autoradiography. The amounts of histone H1 were detected by transferring protein to a nitrocellulose membrane and stained with Ponceau S solution (Sigma).

### 2.6. Transient transfections and luciferase assay

The p27$^{kip1}$ promoter-luciferase fusion plasmid, p27PF-luc, and a control plasmid, pGVB2, were kindly provided by Dr. Toshiyuki Sakai (Department of Preventive Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan). For transient transfections, HT-29 cells were seeded in 10% FBS McCoy's media in 24 well plates to 60–80% confluence 24 h before transfection. Fugene 6 transfection reagent (Roche) was used for transfections following the manufacturer's protocol. To each well, 0.8 μg of p27PF-luc, or pGVB2 plasmid and 0.2 μg of pCMV-β Gal expression plasmid were used. After a 4-h transfection, ciglitazone, PGJ2, or DMSO (vehicle) were added and incubated for another 24 or 72 h. Cells were washed twice with cold PBS and lysed with 150 μl of cell culture lysis reagent (Promega, Madison, WI,). Thirty microliters of cell extract was used for luciferase and 10 μl of cell extract was used for β-Gal assays. LumiCount was used to quantitate luciferase activity (Luciferase Assay System, Promega) and the β-Gal assay kit (Invitrogen, cat #45-0449) was used for β-Gal activities following the kit protocol. The luciferase activity was normalized to β-Gal activity.

### 2.7. Proteasome activity assay

HT-29 cells, without or with treatment by ciglitazone for 24 or 72 h, were washed and lysed with 1× cell lysis buffer. To measure the proteasome chymotrypsin peptidase activity, 100 μg of proteins was mixed with 2 ml of a reaction buffer containing 20 mM Hepes, 0.5 mM EDTA pH8.0, 0.035% SDS, and 10 μM succinyl-Leu-Leu-Val-Try-7-amido-4-methylcoumarin at 37 °C for 30 min. Substrate hydrolysis was measured by monitoring the release of Try-7-amido-4-methylcoumarin using a spectrofluorometer (excitation at 370 nm, emission at 460 nm).

### 2.8. Statistics

All experiments were performed in triplicate except where noted. Data are expressed as mean±S.E.M. Analysis of continuous data was by Analysis of Variance (ANOVA, post hoc testing: Bonferroni) or by Student's *t*-test. A $P$ value <0.05 is defined as significant.

## 3. Results

To determine the effects of PPARγ ligand exposure on the cell growth characteristics of the colon cancer cell line, HT-29, cells were treated with ciglitazone and PGJ2 (1, 10, 30 μM) for 24, 48 and 72 h and cell proliferation was measured by BrdU incorporation. As shown in Fig. 1, exposure of HT-29 cells to both ciglitazone and PGJ2 results in increase of cell proliferation early after exposure (24 h). While neither PPARγ ligand had any effect on cell proliferation at the 48-h time point, there was a significant decrease in cell proliferation at 72 h of exposure of HT-29 cells with ciglitazone and this was not noted with PGJ2. To determine whether the decrease in cell proliferation at 72 h by ciglitazone was associated with cell cycle arrest, HT-29 cells were treated without or with either ciglitazone or PGJ2 for 72 h and cell cycle was analyzed by propidium iodide staining. After 72 h of exposure, cells treated with ciglitazone resulted in a G1/S cell cycle arrest. No significant changes in cell cycle distribution were noted after PGJ2 exposure at any dose. These data suggest that the anti-proliferation effect of ciglitazone is through G1/S cell cycle arrest.

Based on the observation that ciglitazone-induced G1/S cell cycle arrest, we next explored the effects of ciglitazone and PGJ2 on the CDKIs, p21WAF/$^{Cip1}$ and p27$^{Kip1}$ protein levels by immunoblotting. Ciglitazone exposure resulted in an increase of p21$^{WAF/Cip1}$ protein levels after 24 h, which persisted through 72 h of treatment. These results were in contrast to the effect of PGJ2, where the increase in p21$^{WAF/Cip1}$ was transiently increased after 24 h of exposure, with normalization of protein levels by 72 h (Fig. 2A). Based on the observation that ciglitazone increases p21$^{WAF/Cip1}$ protein levels by 24 h of exposure,

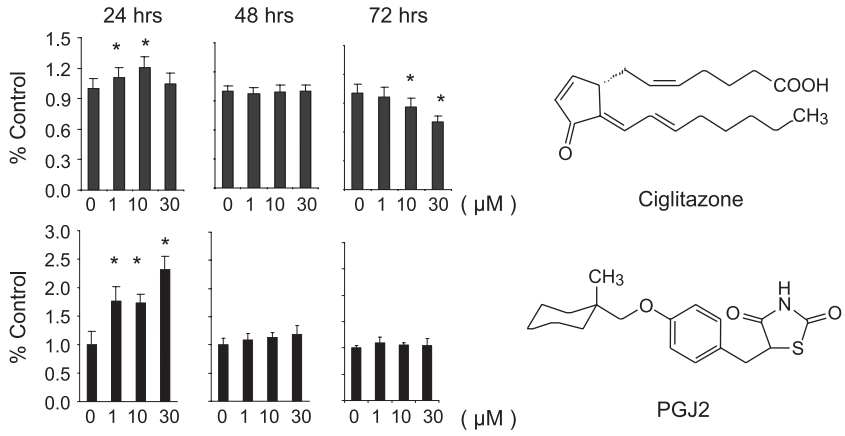


Fig. 1. Effect of ciglitazone and PGJ2 on cell proliferation. HT-29 cells were treated with vehicle (DMSO), ciglitazone or PGJ2 at the indicated doses (1, 10, 30 μM) and times (24, 48, 72 h). Cell proliferation was determined by BrdU incorporation. While both ciglitazone and PGJ2 enhance cell proliferation at 24 h, ciglitazone but not PGJ2 significantly inhibited cell proliferation at 72 h. Data are expressed as the percent control. Shown are the results of triplicate experiments and presented as mean±S.E.M. *$P$<0.05.

without any corresponding cell cycle arrest at 24 h suggests that $p21^{WAF/Cip1}$ is not a key regulator in ciglitazone-induced cell cycle arrest in our system. $p27^{Kip1}$ protein levels were unaffected after 24 h of ciglitazone or PGJ2 exposure. Corresponding to the cell cycle arrest observed in Table 1, $p27^{Kip1}$ protein levels were increased in a dose-dependent fashion after 72 h with ciglitazone, while PGJ2 had no effect on $p27^{Kip1}$ protein levels at any dose or time point.

We next studied the response of HT-29 cells transiently transfected with pCMV-αp27, a mammilla expression plasmid with p27 cDNA sequence in a reverse orientation under control by CMV promoter [14,15] to confirm that $p27^{Kip1}$ accumulation is required for ciglitazone-induced anti-proliferation of HT-29 cells. As shown in Fig. 2B, ciglitazone exposure (72 h) decreased cell proliferation as measured by BrdU incorporation assay (lane 1 vs. 2, $P$<0.05) and the anti-proliferative effects of ciglitazone were partially restored after transfection with the pCMV-αp27 plasmid (lane 3 vs. 4, $P$>0.05). Corresponding to the BrdU data, the increased $p27^{Kip1}$ protein levels after ciglitazone exposure (lane 2) were also inhibited by transfection with the pCMV-αp27 plasmid (Fig. 2C, lane 4),

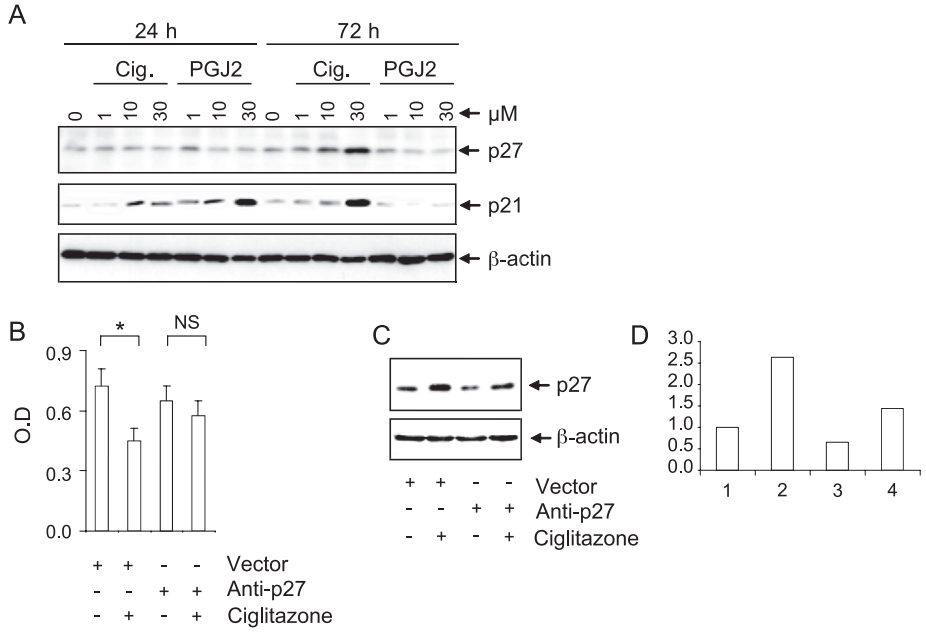


Fig. 2. Effects of ciglitazone and PGJ2 on $p21^{WAF/Cip1}$ and $p27^{Kip1}$ protein levels and antisense p27 effect on ciglitazone-induced cell growth inhibition. (A) HT-29 cells were treated with vehicle, ciglitazone or PGJ2 and p21WAF/Cip1 and $p27^{Kip1}$ proteins levels were detected by immunoblotting. The $p27^{Kip1}$ blot was reprobed for β-actin as a loading control. (B) HT-29 cells were transiently transfected with either empty vector or antisense $p27^{Kip1}$ gene (details in Materials and methods) and cell proliferation was measured by BrdU labeling. Ciglitazone exposure inhibited cell proliferation after 72 h (lane 1 vs. lane 2), which was partially restored using antisense $p27^{Kip1}$ (lane 4 vs. lane 3). Shown are the results of triplicate experiments and presented as mean±S.E.M. *$P$<0.05. (C) Effects of transiently transfected antisense $p27^{Kip1}$ on the protein levels of $p27^{Kip1}$. (D) Densometric quantization of C. Data are represented as percent control. NS=not significant.

Table 1
Cell cycle analysis after ciglitazone and PGJ2 treatment

| | G1 (%) | S (%) | G2 (%) |
|---|---|---|---|
| Vehicle | 60.8±6.1* | 35.8±4.2* | 3.4±0.4 |
| *Cig (μM)* | | | |
| 1 | 59.4±5.8 | 36.0±3.2 | 4.6±0.4 |
| 5 | 64.0±6.2 | 30.8±2.9 | 5.2±0.5 |
| 15 | 79.4±6.5* | 15.4±7* | 5.2±0.3 |
| *PGJ2 (μM)* | | | |
| 1 | 62.8±5.9 | 33.1±3.2 | 4.0±0.4 |
| 5 | 65.0±5.7 | 30.0±3.4 | 5.0±0.3 |
| 15 | 62.5±5.6 | 30.7±3.5 | 6.7±0.6 |
| | *$P$<0.05 | *$P$<0.05 | |

Values represent mean±S.E.M. of three determinations. Asterisks indicated groups which are compared and $P$ values are shown.

suggesting that $p27^{Kip1}$ plays a critical role in ciglitazone-induced G1 phase arrest of HT-29 cells.

$p27^{Kip1}$ protein levels are regulated at multiple levels including transcription, translation, and post-translational modifications. Based on the observation that ciglitazone increases $p27^{Kip1}$ protein levels, we examined whether ciglitazone directly regulated $p27^{Kip1}$ gene transcriptional activity. HT-29 cells were transiently transfected with the empty vector or the $p27^{Kip1}$ reporter plasmid, p27PF-luc. Ciglitazone exposure induced luciferase activity in cells transfected with the $p27^{Kip1}$ reported plasmid in a dose- and time-dependent manner as compared to vehicle-treated control, suggesting that ciglitazone induced $p27^{Kip1}$ transcriptional activity. In contrast, PGJ2 exposure did not significantly increase luciferase activity at either time point or dose (Fig. 3A,B). We also observed that $p27^{Kip1}$ mRNA by RT-PCR was increased after treatment of HT-29 cells with ciglitazone but not PGJ2 at 24 and 72 h (Fig. 3C,D). These data suggested that ciglitazone but not PGJ2 regulates $p27^{Kip1}$ gene transcriptional activity.

Previous studies showed that 26S proteasome activity plays an important role in ubiquitination-mediated degradation of $p27^{Kip1}$ protein during cell cycle progression from G0/G1 to S phase [10]. We have observed that although $p27^{Kip1}$ mRNA was elevated after ciglitazone exposure, the corresponding increase of $p27^{Kip1}$ protein levels with subsequent cell cycle arrest did not occur after a 24-h exposure of HT-29 cells to ciglitazone. We hypothesized that $p27^{Kip1}$ protein levels were maintained at constitutive levels at the 24-h time point by an intact proteasome mechanism, despite an increase in $p27^{Kip1}$ transcriptional activity (Fig. 3).

To confirm that $p27^{Kip1}$ protein levels are regulated in part through ubiquitination, HT-29 cells were exposed to two specific 26S proteasome activity inhibitors, MG115, and MG132, and $p27^{Kip1}$ protein levels measured by immunoblotting. As shown in Fig. 4A, $p27^{Kip1}$ protein levels increased in a dose-dependent manner after exposure to both inhibitors. We next evaluated the effects of ciglitazone on 26S proteasome activity. After a 24-h exposure of HT-29 cells to ciglitazone, proteasome activity was not affected. However, after a 72-h exposure to ciglitazone, the 26S proteasome activity was inhibited in a dose-dependent manner, with greater than 90% inhibition (30 uM) compared with untreated control (Fig. 4B). When cells pretreated with MG132 were subsequently treated with ciglitazone for 24 h, protein levels were dramatically

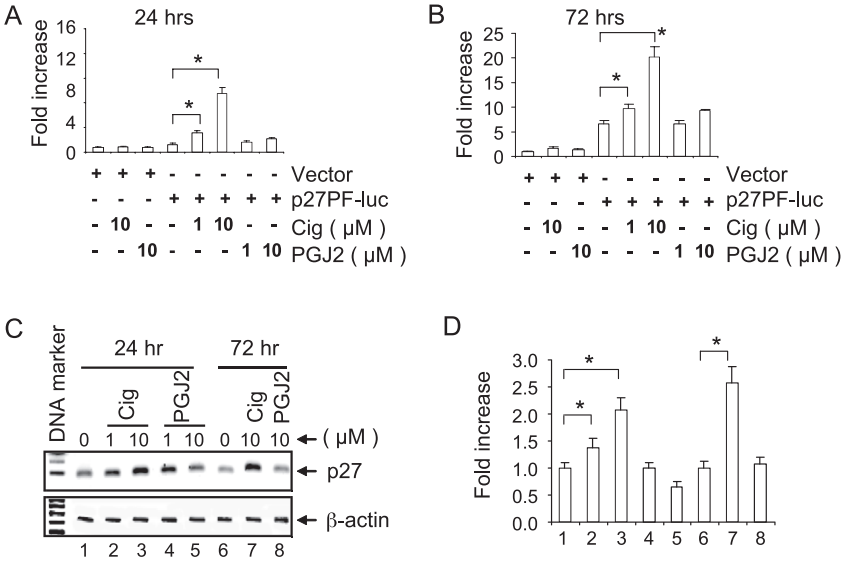


Fig. 3. Ciglitazone but not PGJ2 enhances $p27^{Kip1}$ gene transcriptional activity. (A, B) HT-29 cells were transfected with either empty vector or p27PF-luc reporter construct, following treatment with vehicle, ciglitazone or PGJ2 at indicated concentrations for 24 and 72 h. Luciferase activity was measured in the cell lysates and was normalized by β-galactosidase activity. Shown were represent three experiments, each performed in triplicate, and presented as means±S.E.M. (C) RT-PCR for $p27^{Kip1}$ gene and β-actin gene mRNA amounts in HT-29 cells treated either with vehicle, ciglitazone or PGJ2 at the indicated dose for 24 and 72 h. $p27^{Kip1}$ mRNA levels increase after ciglitazone but not PGJ2 treatments. (D) Quantization of RT-PCR analysis. Shown are the results of three experiments and the values shown are means±S.E.M. *$P$<0.05.

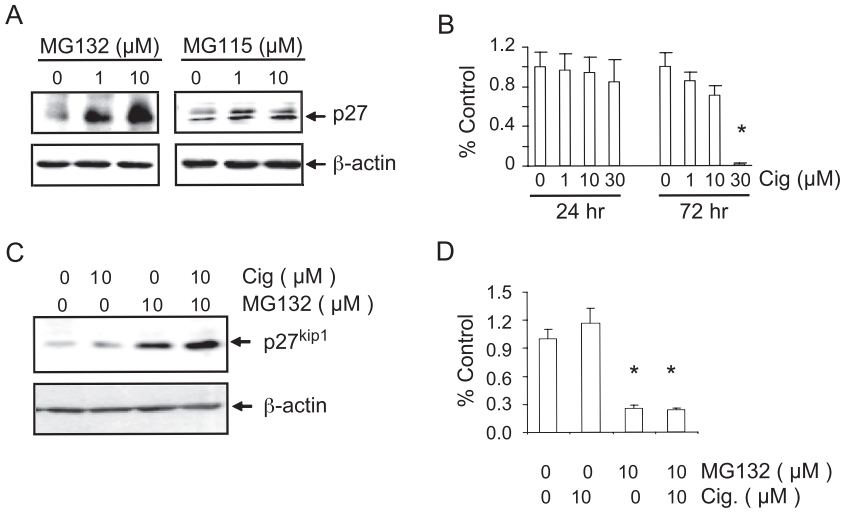


Fig. 4. Ciglitazone exposure inhibits proteasome activity and elevated $p27^{Kip1}$ protein levels. (A) HT-29 cells were treated with vehicle, MG115 or MG132, two specific proteasome activity inhibitors, at the indicated dose for 1 h, and the amounts of $p27^{Kip1}$ proteins were assessed by immunoblotting with β-actin used as loading control. Shown is representative of three experiments with similar results. (B) Cell extracts were prepared form HT-29 cells treated with ciglitazone at indicated dose for 24 and 72 h, and were assay for proteasome activity. Data are represented as mean±S.E.M. of triplicate experiments. *$P$<0.01. (C) HT-29 cells were pretreated with MG132 (10 μM) for 1 h then treated with ciglitazone (10 μM) for 24 h and $p27^{Kip1}$ protein levels were assessed by immunoblotting. While MG132 alone increased $p27^{Kip1}$ protein levels, the increase was further accentuated when combined with ciglitazone. (D) HT-29 cells were treated as described above and cell proliferation was determined by BrdU incorporation assay. Data are expressed as percent control. Shown are the results of triplicate experiments and presented as mean±S.E.M. *$P$<0.05.

increased over those cells treated with only the proteasome inhibitor (Fig. 4C). Corresponding to elevated the $p27^{Kip1}$ protein levels, we observed that ciglitazone alone enhanced cell proliferation at 24-h cell proliferation by BrdU incorporation, while MG132 with or without ciglitazone significantly inhibited cell proliferation (Fig. 4D).

Since the degradation of $p27^{Kip1}$ via the ubiquitin-proteasome pathway is dependent on its phosphorylation at Thr187 and mediated by skp2, we next investigated the effect of ciglitazone on $p27^{Kip1}$ phosphorylation and skp2 protein levels. Ciglitazone exposure inhibited skp2 expression at 24 and 72 h, which paralleled an increase in $p27^{Kip1}$ phosphorylation at Thr187 (Fig. 5A). Phosphorylation of $p27^{Kip1}$ has been reported to be mediated through Cdk2-cyclin E associated kinase activity in some systems [10]. Since we observed that phosphorylation of $p27^{Kip1}$ at Thr187 was increased after exposure of HT-29 cells to ciglitazone, we treated HT-29 cells without or with ciglitazone for 72 h, and measured Cdk2-associated kinase activity using Histone H1 as substrate. Surprisingly, we found that ciglitazone induced a decrease in Cdk2-associated kinase activity as well as decreased the protein levels of Cdk2 but not affected the protein levels of cyclin E (Fig. 5B,C). These data suggested that $p27^{Kip1}$ phosphorylation at Thyr187 is not mediated through Cdk2 kinase activity.

Since ciglitazone enhances p27PF-luc reporter's luciferase activity, and no PPRE element was found from the DNA sequence in this promoter area, we hypothesized that ciglitazone regulated p27 gene expression through a PPRE-independent pathway. To test this, we transiently transfected HeLa cells (PPARγ negative) with either empty vector or p27PF-luc reporter and following treatment of these cells with either vehicle or ciglitazone for 24 h. As shown in Fig. 6A, ciglitazone increased luciferase activity in a dose-dependent manner. PPARγ protein levels was not detected in HeLa cells and expression was not induced by ciglitazone (Fig. 6B).

## 4. Discussion

Extended exposure to PPARγ ligands has been shown to inhibit cell growth, induce differentiation and trigger apoptosis in a variety of cancers using in vitro and animal models [3,6,16]. The exact mechanism of action of PPARγ ligands remains unclear, but some data suggest that PPARγ ligands exert their anti-proliferative effects through cell cycle regulation [13,17]. The present report demonstrates that after prolonged exposure (72 h), ciglitazone, but not PGJ2, induces a G1/S cell cycle block in a colon cancer cell model, which appears to be regulated through two potential pathways. First, ciglitazone exposure increases $p27^{Kip1}$ protein levels, through both induction of $p27^{Kip1}$ gene transcription, as well as inhibition $p27^{Kip1}$ protein degradation. A second mechanism of G1/S arrest is also through ciglitazone's direct effect on decreasing the expression and the associated kinase activity of Cdk2 (Fig. 7).

Classically, once PPARγ ligand binding occurs, activation of transcription requires PPARγ to heterodimerize with retinoid X receptor α (RXRα), followed by binding to specific DNA response elements (PPRE). However, recent data suggest that PPARγ ligands also promote their biologic effects through a non-genomic or PPARγ-independent

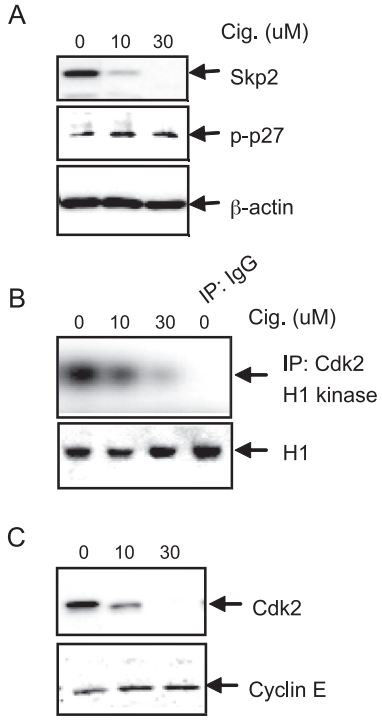


Fig. 5. Effects of ciglitazone on the protein amounts of skp2, phosphorylated $p27^{Kip1}$ and Cdk2 expression and its associated kinase activity. (A) HT-29 cells were treated with ciglitazone at the indicated dose for 72 h. Cells were harvested for immunoblotting. Noted is the dosage-dependent decrease in skp2 expression and increase of phosphorylated $p27^{Kip1}$. (B) HT-29 cells were treated with ciglitazone at the indicated concentrations for 72 h. Total cellular protein was immunoprecipitated with Cdk2 antibody, the dissolved immunoprecipitate was used for the kinase reaction. Total histone H1 added to the kinase reaction was detected by transfer this protein from 12% SDS-PAGE gel to nitrocellulose membrane and stained with Ponceau S solution. (C) Total protein immunoblotting with antibodies against Cdk2 and cyclin E demonstrates the inhibition of protein expression of Cdk2 but not cyclin E. Shown are the results of triplicate experiments with similar results.

pathways. For example, we and others have reported that PPAR ligands activated the MAP kinase signaling pathway through PPRE-independent mechanisms [4,5]. Based on our findings, we conclude that ciglitazone-induced cell cycle effects to be PPRE independent. Evidence for this includes the observation that ciglitazone is a weak PPAR ligand and inhibits PPRE response gene transcription [5]. Secondly, ciglitazone exposure increases $p27^{Kip1}$ reporter gene luciferase activity (Fig. 3), but no PPRE or similar elements are found in the promoter of $p27^{Kip1}$ gene. Finally, $p27^{Kip1}$ reporter gene is also induced by ciglitazone in HeLa cells, in which the PPARγ is not expressed nor is induced by ciglitazone exposure (Fig. 6). It is possible that ciglitazone regulates $p27^{Kip1}$ gene transcription through the Sp1 transcription factor since $p27^{Kip1}$ gene transcription was regulated by 1,25 vitamin $D_3$, not through VDR element, but through sp1 binding elements in the promoter region of $p27^{Kip1}$ gene in U937 cells [18].

In general, the INK4 and CIP/KIP families of CDKIs participate in the regulation of the G1/S cell cycle traverse [7,19]. Specifically, $p21^{WAF/Cip1}$ regulates CDK2-cyclin complexes at the G1 phase of cell cycle following DNA damage or nucleotide pool perturbation [19,20], while $p27^{Kip1}$ is a key regulator of the G1/S phase entry and is involved in the response of the cells to environmental mitogen stimulation [7]. We noted that while ciglitazone increased protein expression of $p21^{WAF/Cip1}$, this increase was observed within 24 h of exposure, which did not correlate with any alterations in cell cycle traverse. On the other hand, protein levels of $p27^{Kip1}$ increased only after 72 h of exposure to ciglitazone, correlating with a G1/S cell cycle block (Fig. 2). These data suggest that $p27^{Kip1}$, not $p21^{WAF/Cip1}$, is a major participant in regulating ciglitazone-induced G1/S arrest in our model. The role for $p27^{Kip1}$ as a major regulatory protein is further supported by the observation that $p27^{Kip1}$ anti-sense abrogated ciglitazone-induced anti-proliferation (Fig. 2B) and increasing $p27^{Kip1}$ levels through proteasome inhibition decreased BrdU incorporation (Fig. 4C,D).

A variety of drugs have been shown to induce G1/S arrest in cancer cells through increasing $p27^{Kip1}$ protein levels.

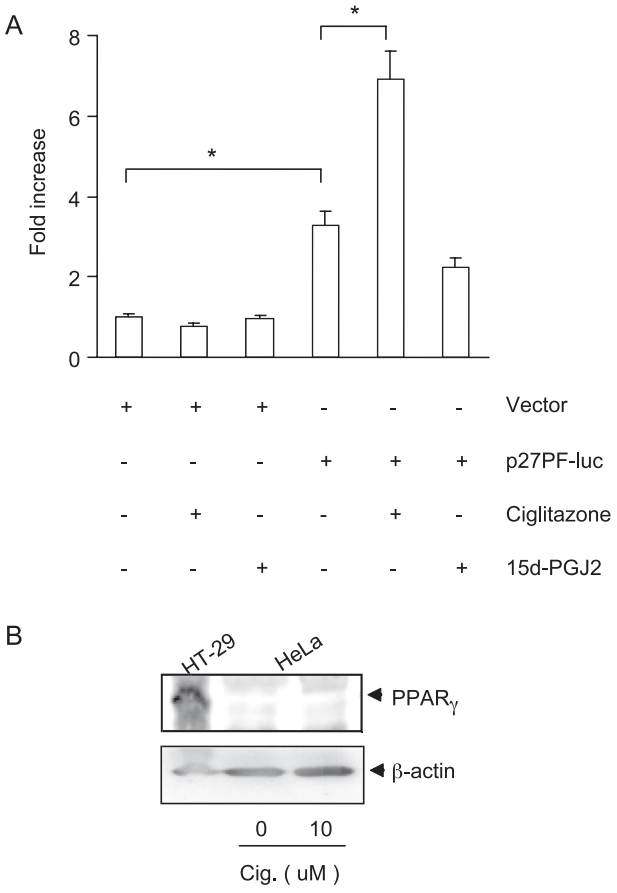


Fig. 6. Effects of ciglitazone on $p27^{Kip1}$ gene transcription are PPRγ independent. (A) P27PF-luc reporter plasmid was transfected into HeLa cells and subsequently exposed to ciglitazone for 24 h, similar to the experiments designed as in Fig. 3A. Shown are results of three experiments, and presented as means±S.E.M., *$P$<0.05. (B) Immunoblotting for PPARγ in HeLa cells in the absence and presence of ciglitazone (10 μM for 24 h).

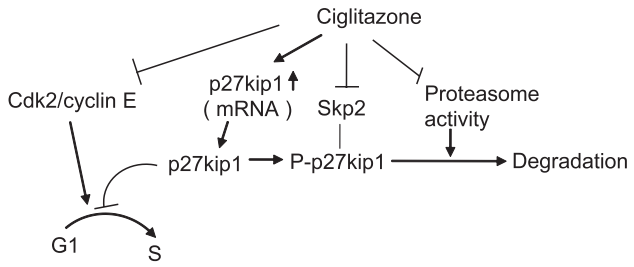


Fig. 7. Summary of ciglitazone's effects on regulating $p27^{Kip1}$ expression and G1/S transition in HT-29 cells. Ciglitazone enhanced $p27^{Kip1}$ gene transcriptional activity and inhibited its degradation by inhibiting proteasome activity and skp2 expression. Ciglitazone also inhibited Cdk2 and its kinase activity.

Similar to these models, we also observed that $p27^{Kip1}$ protein levels were increased after ciglitazone exposure. However, we noted that the increase in $p27^{Kip1}$ gene transcriptional activity and increased mRNA levels were detected within 24 h, which persisted through 72 h of treatment. $p27^{Kip1}$ protein levels, however, did not increase until after 72 h, suggesting that while increased protein production plays a role in ciglitazone-induced increase of $p27^{Kip1}$ protein levels, other mechanisms of $p27^{Kip1}$ regulation may also be important. In addition to the regulation of gene transcription, $p27^{Kip1}$ expression is also controlled through the ubiquitin-proteasome pathway [11]. Consistent with the previous reports [13,17], we found that $p27^{Kip1}$ was degraded in HT-29 cells by 26S proteasome activity, as evidenced by the use of two specific proteasome activity inhibitor, MG115 and MG132 (Fig. 4). Moreover, phosphorylation of $p27^{Kip1}$ at Thr187 by the Cdk2/cyclin E complex, with subsequent binding to skp2 has been shown to trigger ubiquitin-mediated proteolysis of $p27^{Kip1}$ [12]. We observed that $p27^{Kip1}$ was phosphorylated at Thr187 and the amount of phosphorylated $p27^{Kip1}$ protein was increased while skp2 was decreased after ciglitazone exposure, implying that degradation of $p27^{Kip1}$ was dependent on its binding with skp2. The Cdk2/cyclin E kinase complex does not phosphorylate $p27^{Kip1}$ after ciglitazone exposure in our system, since Cdk2 and its associated kinase activity were inhibited directly by ciglitazone (Fig. 5). Alternative pathways leading to $p27^{Kip1}$ phosphorylation have been reported, including the PI3K/AKT and MAP kinase signaling pathways, and it is possible that these signaling pathways were involved in the phosphorylation of $p27^{Kip1}$, rather than Cdk2 [21,22].

The interplay of $p27^{Kip1}$ and CDK2 inhibition may be important in the ultimate fate of cells after ciglitazone exposure. We hypothesized that the combination of inhibited Cdk2 kinase activity, in conjunction with elevated $p27^{Kip1}$ protein levels by ciglitazone, permanently arrests HT-29 cells in the G1 phase of cell cycle. Indeed, when HT-29 cells were exposed to ciglitazone (10 uM) for 72 h, a dose at which Cdk2 kinase was inhibited, and $p27^{Kip1}$ was only slightly elevated, the G1 arrest of HT-29 cells could be reversed after washing away ciglitazone. However, when HT-29 cells were exposed to ciglitazone at a higher concentration (30 uM) for 72 h, at which Cdk2 kinase was inhibited and p27 was strongly elevated, the cell cycle traverse could not be restored by releasing ciglitazone exposure(unpublished data).

In summary, we have provided evidence for a novel mechanism by ciglitazone effects on anti-proliferation of HT-29 cells through up-regulation of $p27^{Kip1}$ gene transcription, inhibition of proteasome activity, and inhibition of Cdk2 kinase activity. This finding may have important clinical implications for the design of PPARγ antagonists as drug for the treatment of malignancies.

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