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Troglitazone inhibits endothelial cell proliferation through suppression of casein kinase 2 activity

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Abstract

Troglitazone, an agonist of peroxisome proliferator activated receptorγ (PPARγ), has been reported to inhibit endothelial cell proliferation by suppressing Akt activation. Recently, it has been also proposed that phosphatase and tensin homolog deleted from chromosome 10 (PTEN) plays an important role in such effect of troglitazone. However, the mechanism of how troglitazone regulates PTEN remains to be elucidated. We therefore investigated the effects of troglitazone on casein kinase 2 (CK2), which is known to negatively regulate PTEN activity. Troglitazone significantly inhibited serum-induced proliferation of HUVEC in a concentration dependent manner. Serum-induced Akt and its downstream signaling pathway activation was attenuated by troglitazone (10 μM) pretreatment. The phosphorylation of PTEN, which was directly related to Akt activation, was decreased with troglitazone pretreatment and was inversely proportional to CK2 activity. DRB, a CK2 inhibitor, also showed effects similar to that of troglitazone on Akt and its downstream signaling molecules. In conclusion, our results suggest that troglitazone inhibits proliferation of HUVECs through suppression of CK2 activity rendering PTEN to remain activated, and this effect of troglitazone in HUVECs seems to be PPARγ independent.

Keywords: PPARγ; Troglitazone; HUVEC; Proliferation; Akt; PTEN; CK2

The ligand-activated transcription factor peroxisome proliferator activated receptor gamma (PPARγ) is a key factor in adipogenesis and plays an important role in insulin sensitivity, cell cycle regulation, and cell differentiation. Studies have shown that PPARγ ligands also exert antiproliferative and anti-migrative effects in vascular cells, including endothelial cells and vascular smooth muscle cells [1,2]. Proliferation of endothelial cell is a crucial component of angiogenesis which is defined as the formation of new blood vessel from pre-existing ones. Depending on the situation, this formation of new blood vessel can be beneficial or detrimental. Several pathological conditions such as atherosclerosis and tumor growth are associated with an excessive angiogenesis in which vessels develop in an uncontrolled or disorganized manner [3,4].

Regarding the signal pathways with which PPARγ agonist troglitazone inhibits endothelial cell proliferation, PI3K-Akt signaling pathway has been proposed to be one of them [5]. PI3K-Akt signaling pathway is activated by a variety of stimuli in endothelial cells and regulates critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation [6]. One of the upstream factors believed to play an important role in Akt signaling is the phosphatase and tensin homolog deleted from chromosome 10 (PTEN). PTEN has both protein phosphatase (phosphoserine/threonine and phospho-tyrosine) and phosphoinositide 3-phosphatase activities [7,8], and when dephosphorylated it becomes more active, thus antagonizes the phosphoinositide 3-kinase pathway [9]. It has been also proposed

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that PTEN plays an important role in such effect of troglitazone [2].

One of the factors regulating this phosphorylation status of PTEN is the casein kinase 2 (CK2). CK2 has been extensively studied in recent years for its potential role in multiple functional activities, including regulation of cell growth, survival, and proliferation. It is a protein Ser/Thr kinase complex that forms a heterotetramer mainly consisting of two catalytic subunits (α and/or α′) and two regulatory β subunits [10]. CK2 catalyzes the phosphorylation of a large number of proteins modulating their activities [11,12]. Nevertheless, little information is known pertaining to the role of CK2 in the signaling pathways that include PTEN and Akt, in relation to cell survival.

In the present study, we report that PPARγ agonist troglitazone inhibits serum-induced proliferation of human umbilical vascular endothelial cell (HUVEC) by suppressing CK2 activity, subsequently allowing PTEN, a negative regulator of Akt pathway, to remain activated.

Materials and methods

Cell culture. Stock HUVEC was purchased from Clonetics (San Diego, USA). The cells were cultured in EGM-2 (Clonetics). Cells at passages between 2 and 10 were used for the study.

Treatment with PPARγ ligand. Subconfluent HUVECs were made quiescent by serum starvation (EBM-2 containing 0.1% FBS) for 24 h. The cells were treated with PPARγ ligand troglitazone 30 min prior to medium changing to EGM-2 containing 2% FBS.

Adenoviral infection of HUVECs. Mouse mutated PPARγ was used for this study. Plasmids were constructed using protocol described previously [13]. Briefly, a single point mutant of PPARγ in the ligand binding domain, L466A, was generated by PCR site-directed mutagenesis.

Cells were transfected with 3–100 MOI (multiplicity of infection) of adenovirus. After 5 h, medium was changed to EBM-2 containing 0.1% FBS. The cells were incubated for 2 days and then cells were treated with PPARγ ligand troglitazone 30 min prior to medium changing to EGM-2 containing 2% FBS.

Cellular proliferation assay. Cells were seeded at 2×10^4 cells/well in 48-well plates. After the cells were treated with troglitazone and cultured for additional 24 h, proliferation was measured by using the thiazolyl blue tetrazolium bromide (MTT) colorimetric assay according to the manufacturer's recommendation (Sigma). The absorbance at 570 nm was determined using a microplate reader with SOFTmax PRO software (Molecular Devices). DNA synthesis was determined with the BrdU-incorporation assay using commercial ELISA kit in accordance with the manufacturer's instructions (Roche, Penzberg, Germany).

Wound healing assay. Wound healing assay was done as previously described [14]. The wounds were produced by a 200 μl pipet tip. Cellular migration was induced by EGM-2 containing 2% FBS. Cells were cultured additional 2 days before measuring the distance migrated.

Western blot analysis. Cells were starved for 24 h in EBM-2 containing 0.1% FBS before any treatment. Cells were treated with EGM-2 containing 2% FBS with or without 30 min of troglitazone pretreatment. Equal amounts of cell lysates were separated by SDS–PAGE gel. Membranes were incubated with appropriate primary antibodies, and the immunopositive bands were visualized by ECL (enhanced chemiluminescent labeling) system (Amersham Biosciences, Buckinghamshire, England). Each experiment was triplicated at least.

CK2 activity measurement. CK2 activity was measured by using casein kinase 2 assay kit as per the manufacturer's recommendation (Upstate Biotechnology, Lake Placid, NY). For the assay, 10 μg of each protein sample was used. For in vitro CK2 activity assay, we used 50 ng of purified active CK2, its substrate and troglitazone.

Statistical analysis. The values are means ± SE. Statistical analyses were performed by ANOVA, followed by the Student–Newman–Keuls test. Differences with a value of $p < 0.05$ were considered statistically significant.

Results

Troglitazone inhibits HUVEC proliferation and migration

To examine the effect of PPARγ agonist on serum-induced endothelial cell proliferation, BrdU incorporation was performed after 24 h of troglitazone treatment. EGM-2 contained 2% FBS increased proliferation of HUVECs, and this serum-induced proliferation was abrogated by troglitazone (5–20 μM) in a dose-dependent manner (Fig. 1A and B). GW 9662 (0.5–10 μM), a specific

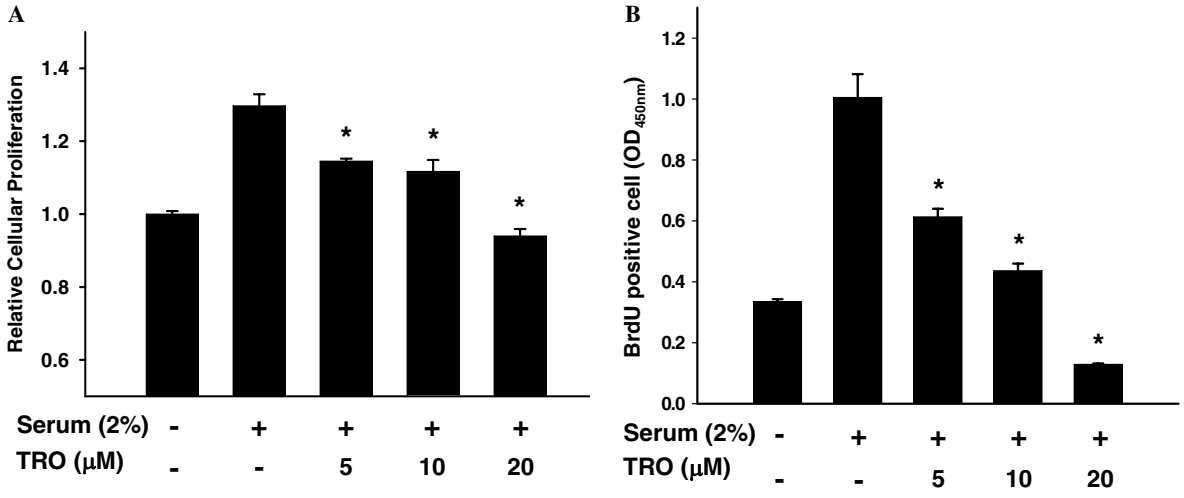

Fig. 1. Troglitazone inhibits HUVEC proliferation and migration. Relative cellular proliferation was measured by MTT assay (A) and BrdU assay (B). HUVECs were treated with serum and troglitazone as indicated. Results are expressed as means ± SE Significance levels are represented by asterisks ($p < 0.05$).

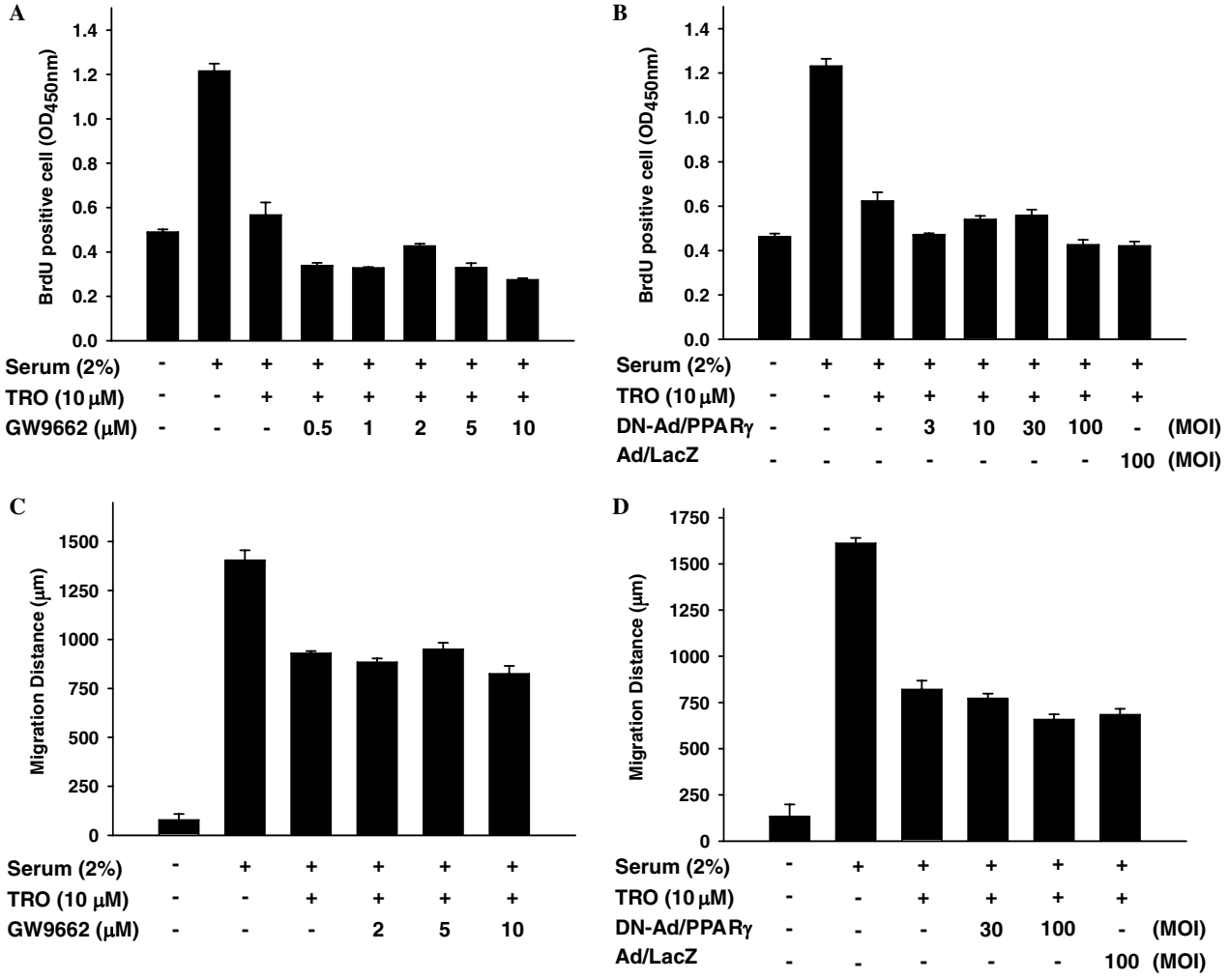

Fig. 2. Anti-proliferative and -migratory effects of troglitazone are PPARγ independent. (A) Cellular proliferation was induced by 2% serum and the cells were pretreated with troglitazone (10 μM) and increasing doses of PPARγ inhibitor GW9662 as indicated. (B) Cells were transfected with various MOIs of DN-Ad/PPARγ with or without troglitazone as indicated. Cellular proliferation was induced by 2% serum. (C) Cells were starved for 24 h before the wound was made by pipette tip. Cells were treated with serum, troglitazone, and GW9662 as indicated. The distance migrated is quantified in μm. (D) Cellular migration was determined after DN-Ad/PPARγ transfection as indicated. Results are expressed as means ± SE.

PPARγ antagonist, treatment failed to reverse the anti-proliferative effect of 10 μM troglitazone treatment (Fig. 2A). Furthermore, dominant negative PPARγ adenoviral infection (DN-Ad/PPARγ) (3–100 MOI) also failed to reverse the anti-proliferative effect of troglitazone (Fig. 2B).

We also assessed the effect of troglitazone on HUVEC migration using a wound healing assay. EGM-2 significantly induced HUVEC migration, and troglitazone treatment suppressed it by approximately 40%. Again, neither GW 9662 treatment nor dominant negative PPARγ adenoviral infection did reverse the effect of troglitazone (Fig. 2C and D).

Troglitazone attenuates serum-induced Akt and p70S6K activation

A recent study reported PPARγ agonist troglitazone inhibits endothelial cell proliferation, possibly, by blocking the PI3K-Akt signaling pathway [5]. We examined whether the troglitazone does inhibit endothelial cell proliferation and migration by targeting the PI3K-Akt signaling pathway. HUVECs were stimulated with 2% serum for 10–120 min. Serum-induced phosphorylation of both Akt and its downstream molecule p70S6K was significantly attenuated by troglitazone treatment (Fig. 3). In contrast, troglitazone did not affect serum-induced ERK-MAPK activation (data not shown).

Troglitazone inhibits serum-induced phosphorylation of PTEN and casein kinase 2 activity

According to our data, serum treatment increased the phosphorylation of PTEN, allowing Akt signaling pathway to be activated, but this serum-induced phosphorylation of PTEN was significantly inhibited by troglitazone treatment (Fig. 4A). The activity of PTEN is regulated by its

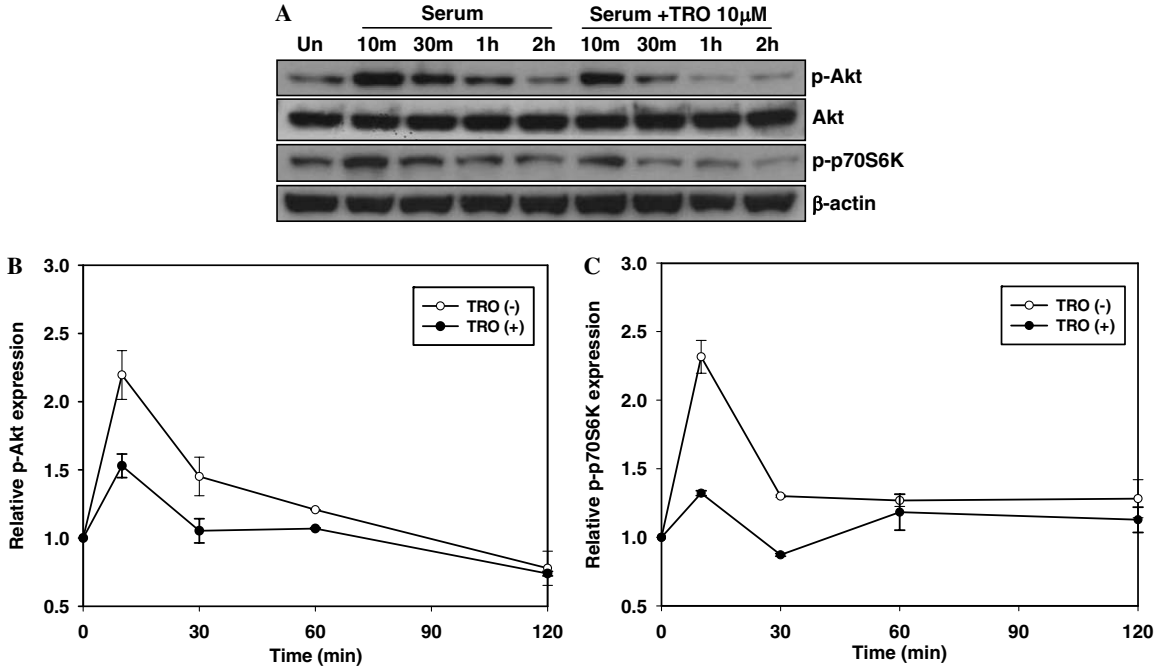

Fig. 3. Troglitazone inhibits phosphorylation of both Akt and its downstream signaling molecule p70S6K. (A) Western blot of phosphor Akt and p70S6K. Cells were treated with serum and troglitazone as indicated. Relative activation of Akt and p70S6K is quantified in (B) and (C), respectively. Results are expressed as means ± SE.

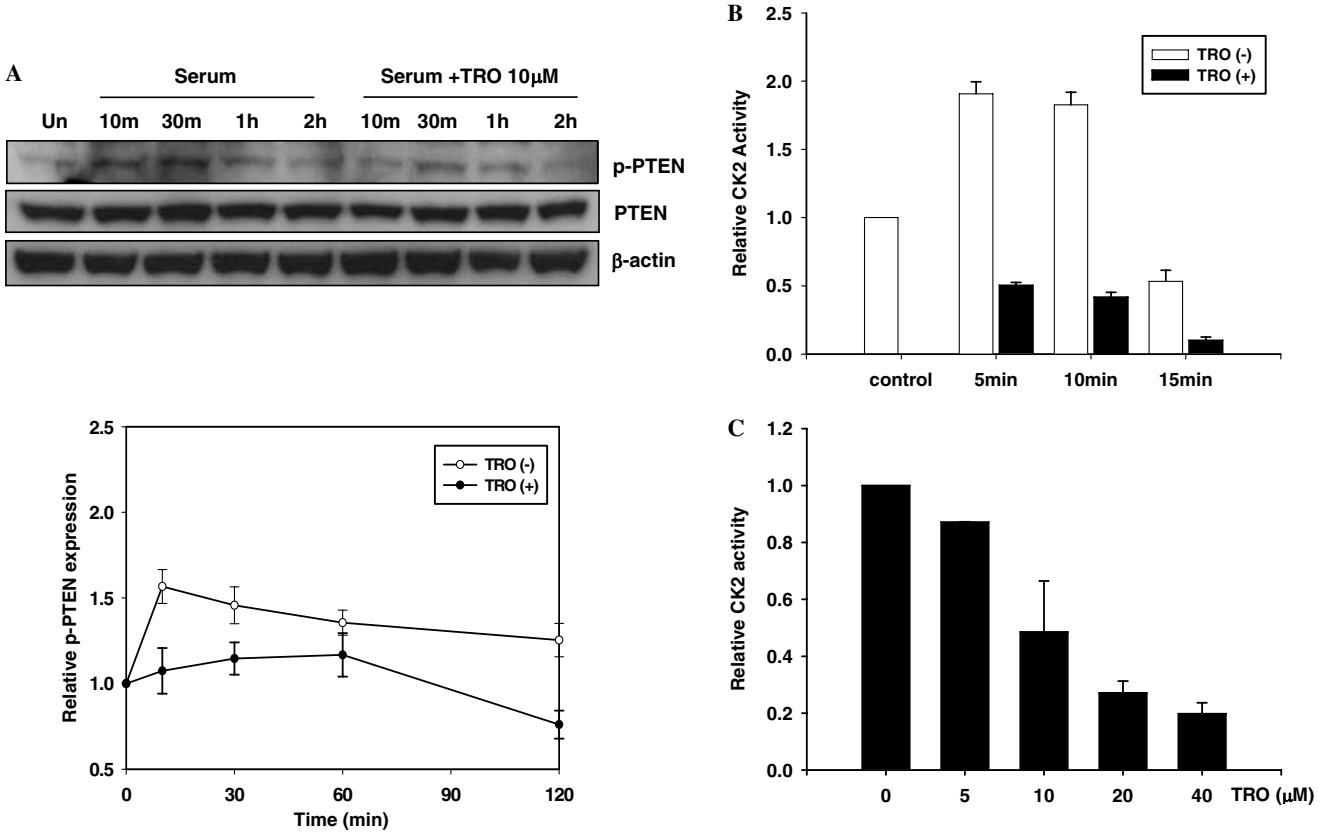

Fig. 4. Troglitazone suppressed phosphorylation of PTEN and CK2 activity. (A) HUVECs were stimulated with EGM-2 containing 2% serum with or without 10 μM of troglitazone treatment for the indicated time periods for Western blot analysis. Relative intensity of each band is quantified in the panel below. (B) Serum activated HUVECs were treated with or without 10 μM of troglitazone. Samples were collected at the times indicated then subjected to CK2 activity assay. (C) CK2 activity was measured using purified CK2 (50 ng), its substrate, and increasing dose of troglitazone as indicated. Results are expressed as means ± SE.

phosphorylation status which can be regulated by casein kinase 2 (CK2). Therefore, to further examine the possibility of troglitazone targeting a certain factor upstream of PTEN, we assessed the effect of troglitazone on CK2 activity. As shown in Fig. 4B, troglitazone treatment significantly decreased serum-induced CK2 activity as early as 5 min. Further, we performed in vitro CK2 activity assay using troglitazone and purified CK2 to examine whether this observed effect of troglitazone was due to a direct interaction with CK2. According to our data, troglitazone decreased activity of purified CK2 in a dose-dependent manner (Fig. 4C).

CK2 inhibitor, DRB, inhibits HUVEC proliferation and attenuates serum-induced PI3K-Akt signaling pathway

We next examined the effect of CK2 inhibitor 5, 6-dichloro-1-(β-D-ribofuranosyl) benzimidazole (DRB), on HUVEC proliferation. DRB treatment (5–20 μM) inhibited serum-induced endothelial cell proliferation in a dose-dependent manner (Fig. 5A). Furthermore, serum-induced phosphorylation of both Akt and its downstream molecule p70S6K was significantly attenuated by 10 μM DRB treatment. Also, DRB inhibited serum-induced phosphorylation of PTEN in HUVECs (Fig. 5B).

Discussion

For decades, the role of endothelial cells in pathological angiogenesis has been investigated as a possible therapeutic target in cardiovascular disease, diabetes, and cancer. The ligand-activated transcription factor PPARγ has been known as a key factor in adipogenesis and plays an important role in insulin sensitivity, cell cycle regulation, and cell differentiation. Studies also have shown that PPARγ ligands also exert anti-proliferative and anti-migrative effects in vascular cells [1,2]. Therefore, we have investigated the effect of PPARγ agonist troglitazone on serum-induced endothelial cell proliferation and migration along with the signaling pathways involved.

Regarding the signaling pathways by which PPARγ agonist troglitazone inhibits endothelial cell proliferation, PI3K-Akt signaling pathway has been proposed to be one of them [5]. In the present study, we showed that 2% FBS increased proliferation of HUVECs, and this serum-induced proliferation was abrogated by troglitazone (5–20 μM) in a dose-dependent manner. However, a specific PPARγ antagonist, GW 9662, treatment did not reverse this anti-proliferative effect of troglitazone, indicating that the anti-proliferative effect of troglitazone on HUVEC was PPARγ independent. We also assessed the effect of troglitazone on HUVEC migration using a wound healing assay.

Serum treatment significantly induced HUVEC migration, but troglitazone pretreatment suppressed it by approximately 40%. Again, GW 9662 treatment did not reverse the effect of troglitazone, suggesting the anti migrative effect of troglitazone was also PPARγ independent. This interpretation is also substantiated by the observations that dominant negative PPARγ adenoviral transfection failed to reverse the anti-proliferative effect of troglitazone and troglitazone treatment suppressed Akt phosphorylation in a relatively short period of time making it highly unlikely to involve classical PPARγ pathway.

Our western blot data demonstrated that serum-induced HUVEC proliferation and migration by up-regulating phosphorylation of Akt and its downstream molecule p70S6K as early as 10 min, and this was abrogated by troglitazone treatment. If the anti-proliferative effect of troglitazone was PPARγ dependent, so it required mRNA and protein synthesis of factors that would deter cellular proliferation, we would have observed the effect of troglitazone much later than we observed in the present study.

To date, several studies have shown that PPARγ ligands exert anti-proliferative and -migrative effects in endothelial

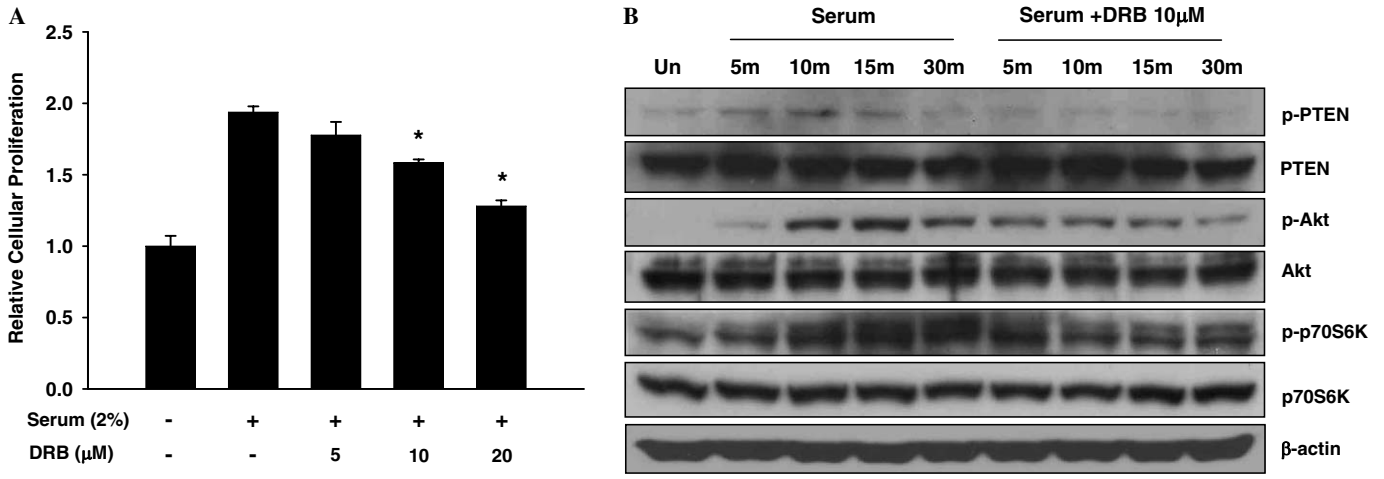

Fig. 5. CK2 inhibitor DRB inhibits serum-induced HUVEC proliferation. (A) HUVECs were treated with various concentrations of CK2 inhibitor DRB as indicated. Relative cellular proliferation was measured by MTT assay. Results are expressed as means ± SE. Significance levels are represented by asterisks ($p < 0.05$). (B) phosphorylation of PTEN, Akt, and p70S6K is visualized by Western blot.

cells by blocking Akt signaling pathway, and one of such studies suggested PTEN as a potential negative regulator of Akt signaling [2]. Numerous studies have demonstrated that PTEN suppresses proliferation and survival of tumor cells by inhibiting PI3K-Akt signaling [15–17]. PTEN was identified originally as a tumor suppressor protein [18] and later it was also found to exhibit both protein phosphatase and inositol 3′-phosphatase activity [7,8], and when dephosphorylated it becomes more active, thus antagonizes the phosphoinositide 3-kinase pathway [9].

In fact, it has been reported that increased PTEN inhibited VEGF induced endothelial cell migration and angiogenesis by suppressing Akt phosphorylation [19]. We further tried to identify which factor was directly affected by troglitazone in the pathway involves PTEN and Akt. Protein kinase CK2 is a highly conserved, ubiquitously expressed Ser/Thr kinase that phosphorylates a variety of substrates involved in essential cell processes, including cell cycle and growth [11,12]. Such substrates include PTEN, thus, CK2 can negatively affect Akt pathway by phosphorylating PTEN which results in inactivation of PTEN [20,21]. Therefore, we assessed the effect of troglitazone on CK2 activity.

Troglitazone treatment significantly decreased serum-induced CK2 activity. Furthermore, CK2 inhibitor 5, 6-dichloro-1-(β-D-ribofuranosyl) benzimidazole (DRB) treatment inhibited serum-induced endothelial cell proliferation in a dose-dependent manner. In addition, serum-induced phosphoryation of both Akt and its downstream molecule p70S6K was significantly attenuated by 10 μM of DRB treatment. Also, DRB inhibited serum-induced phosphorylation of PTEN in HUVECs.

All these data support our hypothesis that troglitazone inhibits endothelial cell proliferation and migration by decreasing CK2 activity, thus inhibiting subsequent Akt signaling. In addition, this effect of troglitazone on CK2 activity seems PPARγ independent considering the response was relatively prompt.

To date, to our knowledge, no study has shown that troglitazone produces such effect by targeting CK2. Considering that many current thiazolidinedione (TZD) drug related studies are focused on finding possible targets and applications of the drugs other than its presumed function such as improving insulin sensitivity, our work will help us to better understand the TZD drug's action mechanism.

In conclusion, we showed that reported anti-proliferative effect of troglitazone in endothelial cell is, at least in part, PPARγ independent, and identified CK2 as the one of important targets of troglitazone in such mechanism.

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