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Troglitazone suppresses cell growth of KU812 cells independently of PPARγ

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Abstract

We examined the effects of troglitazone, one of thiazolidinedione derivatives on human basophilic leukemia cell line KU812. Troglitazone caused the suppression of cell growth, which was suggested to result from the decrease in cyclin E and the hyperphosphorylated form of retinoblastoma tumor suppressor gene product (pRb). In addition, troglitazone caused a decrease in histamine secretion due to the reduced expression of histidine decarboxylase mRNA. Peroxisome proliferator-activated receptor (PPAR)-γ mRNA was undetectable by reverse transcription-polymerase chain reaction (RT-PCR) in KU812 cells. These findings suggested that troglitazone suppressed cell growth and histamine synthesis independently of PPARγ. © 2002 Published by Elsevier Science B.V.

Keywords: Thiazolidinedione; PPAR (Peroxisome proliferator-activated receptor); KU812 cell; Cell growth; Histamine secretion

1. Introduction

Thiazolidinedione derivatives improve insulin resistance in patients with non-insulin-dependent diabetes mellitus (Nolan et al., 1994; Suter et al., 1992). Thiazolidinedione derivatives bind to peroxisome proliferator-activated receptor (PPAR)-γ, a member of the nuclear receptor superfamily, and exert their antidiabetic effects (Lehmann et al., 1995). PPARγ is highly expressed in adipose tissue and, to a lesser extent, in other tissues such as the colon and retina (Kersten et al., 2000). PPARγ is also expressed in several myeloid cell lines, monocytes, macrophages and T cells, and it participates in the regulation of inflammatory response and immunoreaction (Asou et al., 1999; Chinetti et al., 1998; Clark et al., 2000; Jiang et al., 1998; Ricote et al., 1998; Sugimura et al., 1999). However, little is known about the effect of thiazolidinedione derivatives on basophils and mast cells. We found that troglitazone, one of the thiazolidinedione derivatives, suppressed cell growth and histamine secretion in a PPARγ-independent manner in human basophilic leukemia cell line KU812. That finding led us to study the suppressive mechanisms of cell growth and histamine secretion induced by troglitazone in KU812 cells.

2. Materials and methods

2.1. Materials

Troglitazone, pioglitazone and rosiglitazone were kindly provided by Sankyo (Tokyo, Japan), Takeda Chemical Industries (Osaka, Japan) and SmithKline Beecham Pharmaceuticals (Welwyn, UK), respectively. They were dissolved in 100% dimethyl sulfoxide (DMSO), and the final DMSO concentration was 0.1% or less. Anti-human retinoblastoma gene product (pRb) monoclonal antibody (IF8), anti-human cyclin D1 polyclonal antibody (H-295), anti-human cyclin E monoclonal antibody (HE12) and anti-human eIF2α polyclonal antibody (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific eIF2α antibody (Rabbit Pan Anti-eIF2α (pS51))

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was purchased from Biosource International (Hopkinton, MA). A horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence blotting detection system were purchased from Amersham Pharmacia Biotech (Tokyo, Japan).

2.2. Cells and cell culture

KU812 cells were maintained in RPMI1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum, 50 μg/ml penicillin G and 100 μg/ml streptomycin. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. The number of living cells was determined by trypan blue exclusion.

2.3. Cell cycle analysis

Cell cycle analysis was performed as described previously (Sugimura et al., 1999). Briefly, KU812 cells cultured with 0–50 μM troglitazone for 4 days were washed with ice-cold phosphate-buffered saline (PBS) and fixed with 70% ethanol. After washing with PBS, cells were treated with 50 μg/ml RNase A at 37 °C for 30 min and stained with 50 μg/ml propidium iodide. Fluorescence intensity was measured by FACScan, and the cell cycles were analyzed using the Cell Quest and Modfit LT programs (Becton Dickinson, San Jose, CA).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). First strand cDNA was synthesized using a Super Script II RNase H$^-$ Reverse Transcriptase (Life Technologies, Rockville, MD). The cDNA was amplified using the Expand High Fidelity PCR System (Roche Diagnostics, Tokyo, Japan), and 36B4 cDNA was amplified as an internal control (Laborda, 1991). The temperature was 94 °C for denaturation, 55 °C (PPARγ, proliferating cell nuclear antigen (PCNA), B-Myb and histidine decarboxylase), 62 °C (36B4) or 65 °C (PPARα and β) for annealing and 72 °C for extension. The sequences of PCR primers for PPARα were sense 5′-AGCCCACTCTGCCCCCTCTCC-3′ and antisense 5′-GTCCCCGCAGATTCTACATTC-3′; for PPARβ, they were sense 5′-AACTGCAGATGGGCTGTGACG-3′ and antisense 5′-GTCTCGATGTCGTGGATCAC-3′; for PPARγ, they were sense 5′-TCTCCAGCATTTCTACTCCAC-3′ and antisense 5'-GCCAACAGCTTCTCCTTCTCG-3′; for PCNA, they were sense 5′-AGCACCAAACCAGGAGAAAG-3′ and antisense 5'-CGTTGAAGAGAGTGGAGTGG-3′; for B-Myb, they were sense 5′-CGAGGAGGAGGACCGCATCA-3′ and antisense 5'-ACCGATGGGCTCCTGTTCCT-3′; for histidine decarboxylase, they were sense 5'-GCATCCAG2-

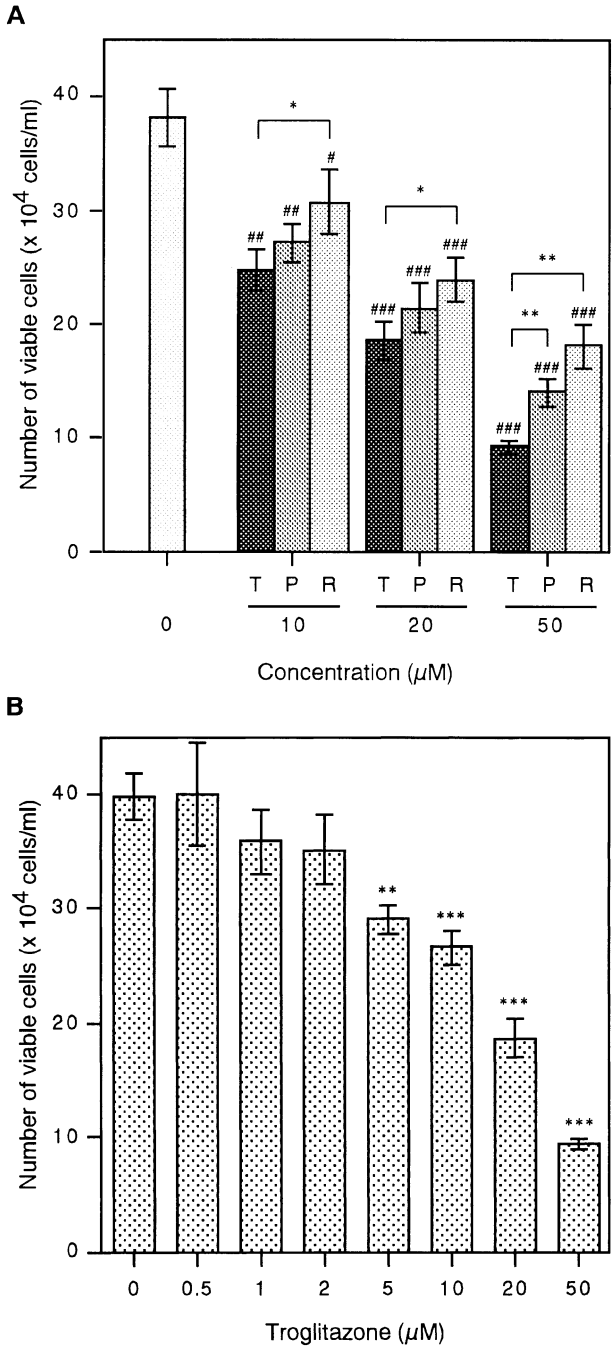

Fig. 1. The inhibitory effects of thiazolidinedione derivatives on cell proliferation of KU812 cells. (A) Cells (5×10^4 cells/ml) were cultured with 0–50 μM troglitazone (T), pioglitazone (P) or rosiglitazone (R) for 4 days. Cells treated with 0.1% DMSO were used as control (0 μM). Number of living cells was determined by trypan blue exclusion. Data are shown as the mean ± S.E.M. of five separate experiments. * $P<0.05$ and ** $P<0.01$ compared with troglitazone. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared with 0 μM. (B) Cells (5×10^4 cells/ml) were cultured with 0–50 μM troglitazone for 4 days. Cells treated with 0.1% DMSO were used as control (0 μM). Data are shown as the mean ± S.E.M. of four to nine separate experiments. ** $P<0.01$ and *** $P<0.001$ compared with 0 μM.

CCCTGCGTGTAC-3′ (Kuramasu et al., 1998) and antisense 5′ -CACAAGGGAAATCAAACCAG-3′ ; and for 36B4, they were sense 5′ -TGTTTCATTGTGGGAGCAGAC-3′ and antisense 5′ -AAGCACTTCAGGGTTGTAGAT-3′ . The amplified PCR products were resolved on 2% agarose gel and visualized with ethidium bromide under UV light illumination.

2.5. Western blot analysis

The cells were lysed with lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.5% Triton X-100, 0.25% sodium cholate, 1 mM phenylmethanesulfonyl fluoride, 1 mM NaF, 1 mM Na_3VO_4, 30 U/ml aprotinin, 10 μg/ml leupeptin). The cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene difluoride membranes. The membranes were immunoblotted and stained with antibodies and chemiluminescence system. Densitometric analysis was performed using NIH Image 1.62 software on a Macintosh PC.

2.6. Histamine measurement

Culture medium or cells (1×10^6 cells) were deproteinized with 2.1% perchloric acid at final concentration. After neutralization with KOH, histamine in the supernatant was measured by high-performance liquid chromatography (HPLC) with postcolumn fluorescence detection using *o*-phthalaldehyde (OPA) (Tateda et al., submitted for publication). The separation was performed on Develosil ODS UG-3 (4.6 × 100 mm I.D., Nomura Chemical, Seto, Japan), which was protected by a guard column (10 × 4.0 mm I.D. ODS UG-5), with a 14:86 (v/v) mixture of acetonitrile and 50 mM phosphate buffer (pH 6.0) containing 10 mM sodium octane sulfonate as an eluent. OPA reagent was a 1:1 (v/v) mixture of 50 mM phosphate buffer (pH 8.0) and 6.3 mM OPA in acetonitrile. The eluent was delivered to the column at 0.5 ml/min through a preheater tube (stainless steel tube, 10 m × 1.0 mm I.D.). The eluate from the column was added with OPA reagent delivered at 0.5 ml/min to a mixing T-joint through a preheater tube (stainless steel tube, 10 m × 1.0 mm I.D.). The mixture was passed through a reactor tube (polytetrafluoroethylene tube, 2.5 m × 0.5 mm I.D.), and the generated fluorescence was detected at 425 nm with excitation at 350 nm. All columns, preheater- and reactor tubes were placed in the column oven maintained at 40 °C. Histamine was detected at 9.3 min without the interference

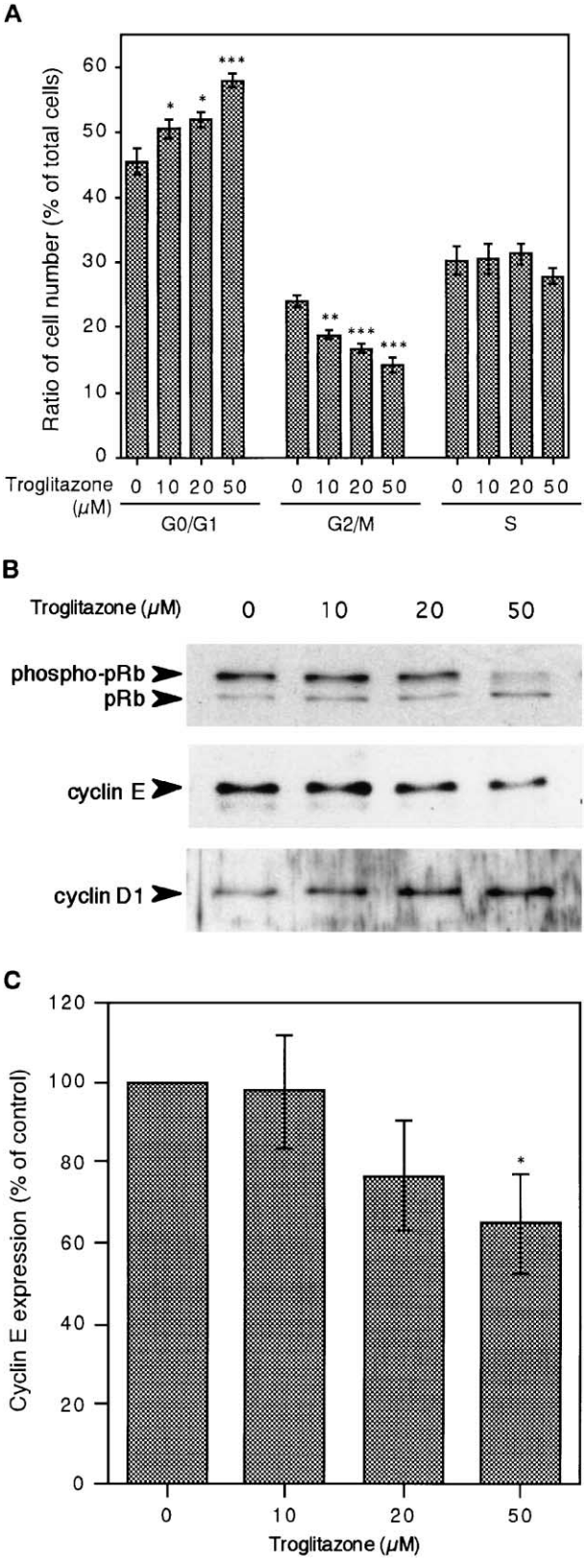

Fig. 2. The effect of troglitazone on cell cycle in KU812 cells. Cells (5×10^4 cells/ml) were cultured with 0–50 μM troglitazone for 4 days. Cells treated with 0.1% DMSO were used as control (0 μM). (A) Cell cycle analysis was performed as described in Materials and methods. Data are shown as the mean ± S.E.M. of four separate experiments. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with 0 μM. (B) The cell lysate was subjected to SDS-PAGE and immunoblotted with antibody as described in Materials and methods. (C) Results of the densitometric analysis of cyclin E. Data are shown as the mean ± S.E.M. of three separate experiments. * $P<0.05$ compared with 0 μM.

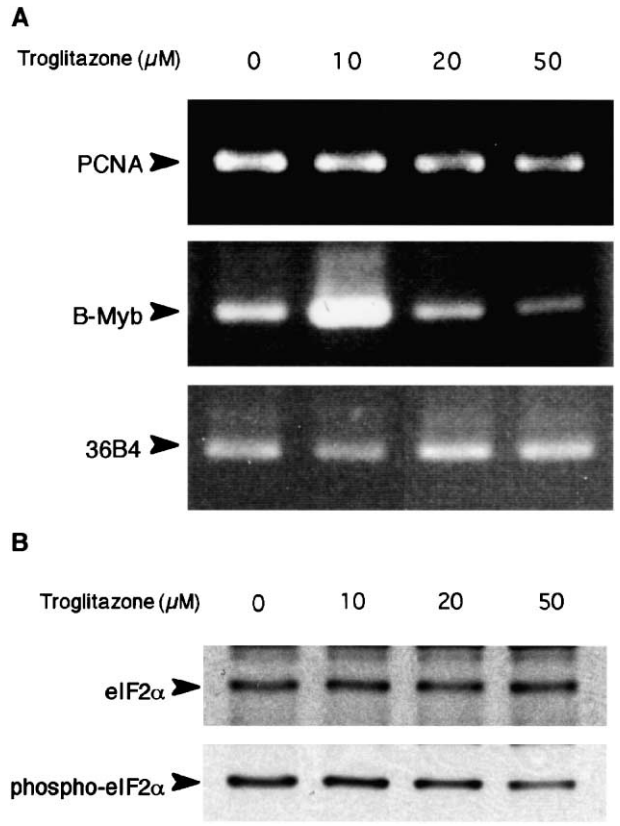

Fig. 3. The effect of troglitazone on E2F transcriptional activity and translation initiation. Cells (5×10^4 cells/ml) were cultured 0–50 μM troglitazone for 4 days. Cells treated with 0.1% DMSO were used as control (0 μM). (A) Total RNA was extracted and analyzed by RT-PCR as described in Materials and methods. (B) The cell lysate was subjected to SDS-PAGE and immunoblotted with antibody as described in Materials and methods.

from other amines and amino acids coexisting in the supernatant.

3. Results

When KU812 cells were cultured with various concentrations of troglitazone, pioglitazone or rosiglitazone for 4 days, all thiazolidinedione derivatives suppressed cell proliferation in a dose-dependent manner (Fig. 1). Troglitazone showed the most potent suppression, and the rank order of the potency was troglitazone>pioglitazone>rosiglitazone (Fig. 1A). The percentage of cells not stained with trypan blue was more than 80% at each concentration (data not shown), suggesting that all of three thiazolidinedione derivatives had little cytotoxicity at least to 50 μM. Troglitazone suppressed cell proliferation significantly at concentrations of 5 μM or higher (Fig. 1B). It was reported that low concentrations of troglitazone (<5 μM) increased cell proliferation (Clay et al., 2001). We found no cell growth enhanced with troglitazone at any concentrations in KU812 cells.

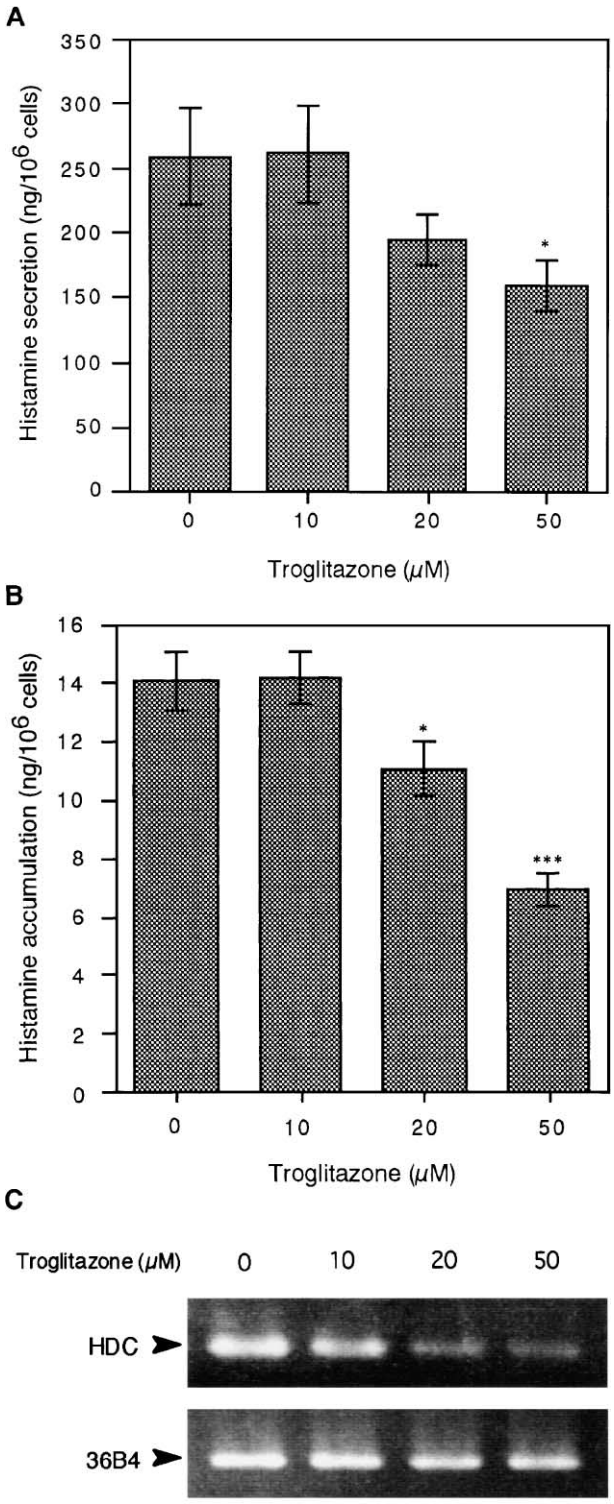

Fig. 4. The effect of troglitazone on histamine secretion and intracellular histamine. Cells (5×10^4 cells/ml) were cultured 0–50 μM troglitazone for 4 days. Cells treated with 0.1% DMSO were used as control (0 μM). (A) Culture medium or (B) cells were collected and deproteinization with perchloric acid. After neutralization with KOH, the amount of histamine in supernatant was measured as described in Materials and methods. Data are shown as the mean ± S.E.M. of four separate experiments. * $P<0.05$ and *** $P<0.001$ compared with 0 μM. (C) Total RNA was extracted and histidine decarboxylase (HDC) m RNA was analyzed by RT-PCR as described in Materials and methods.

Cell cycle analysis was performed using cells treated with troglitazone for 4 days by FACScan (Fig. 2A). Troglitazone increased the ratio of the number of cells in the G0/G1 phase and decreased the ratio in the G2/M phase in a dose-dependent manner. No change was found in the S phase. pRb and cyclin D1 and E are involved in cell cycle progression from the G1 to the S phase. We examined the effects of troglitazone on phosphorylation of pRb and the expression of cyclin D1 and E in 4-day-cultured cells by Western blotting. In cells treated with 0.1% DMSO (0 μM), pRb was highly phosphorylated (Fig. 2B, top). Troglitazone greatly decreased the phosphorylated pRb at 50 μM in contrast to the non-phosphorylated form, which was slightly increased by troglitazone. In addition, troglitazone caused a decrease in cyclin E and an increase in cyclin D1 (Fig. 2B, middle and bottom, respectively). Results of the densitometric analysis showed that the decrease of cyclin E with troglitazone was inclined toward dose-dependency (Fig. 2C). We examined the effect of troglitazone on the transactivation of E2F by measuring mRNA expression of PCNA and B-Myb, known E2F target genes, with RT-PCR. Troglitazone decreased mRNA expression of both PCNA and B-Myb (Fig. 3A). It was also examined whether troglitazone inhibits translation initiation via phosphorylation of eIF2α. Troglitazone had no stimulatory effect up to 20 μM and rather suppressed on phosphorylation of eIF2α at 50 μM (Fig. 3B).

Histamine is a feature product of basophils and mast cells. It was reported that basophilic differentiation increased intracellular histamine and histamine release in KU812 cells (Hara et al., 1998). To elucidate the effects of troglitazone on histamine secretion, the amount of histamine was measured using HPLC. Troglitazone decreased both histamine secretion and intracellular histamine concentration in 4 days of culturing (Fig. 4A,B). Histamine is synthesized from histidine via decarboxylation catalyzed by histidine decarboxylase. We examined the effect of troglitazone on the expression of histidine decarboxylase mRNA using RT-PCR. The expression of histidine decarboxylase mRNA was decreased in a dose-dependent manner after 4 days of culturing (Fig. 4C).

Troglitazone binds to PPARγ, resulting in up- or down-regulation of its expression (Camp et al., 1999; Davies et al., 1999). The expression of PPARγ mRNA was measured using RT-PCR in KU812 cells. PPARγ mRNA expression was undetectable and unchanged by troglitazone treatment. On the other hand, PPARα and PPARβ mRNAs were easily detected (Fig. 5).

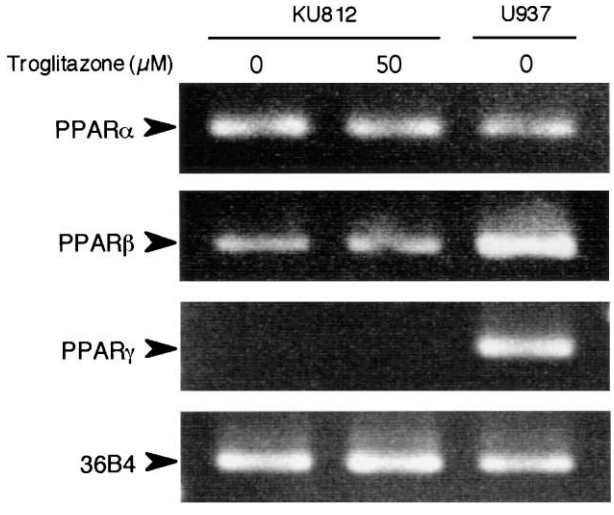

Fig. 5. The expression of PPAR mRNA in KU812 cells. Cells (5×10^4 cells/ml) were cultured with or without 50 μM troglitazone for 4 days. Cells treated with 0.1% DMSO were used as control (0 μM). Total RNA was extracted and analyzed by RT-PCR as described in Materials and methods. RNA extracted from U937 cells was used as a positive control.

4. Discussion

In this study, we showed that troglitazone suppressed cell growth and histamine secretion in human basophilic leukemia cell line KU812. Troglitazone inhibited cell cycle progression at the G1 phase (Fig. 2A). pRb is known as the tumor suppresser gene product. Non-phosphorylated form of pRb can bind to transcription factor E2F, which plays a pivotal role in cell cycle progression from the G1 to the S phase, and inhibit transactivation of E2F during the G1 phase. Recent studies showed that troglitazone suppressed cell growth accompanied with reduction of phosphorylation of pRb (Fujimura et al., 1998; Wakino et al., 2000). In addition to pRb, cyclins regulate cell cycle progression. In this study, troglitazone decreased hyperphosphorylated pRb, cyclin E and activation of E2F target genes, PCNA and B-Myb (Figs. 2B,C and 3A), suggesting that they are responsible for the suppression of cell proliferation in KU812 cells. It also has been shown that troglitazone inhibits translation initiation and cell proliferation via phosphorylation of eIF2α (Palakurthi et al., 2001). Troglitazone did not, however, induce phosphorylation of eIF2α, suggesting that the suppression of cell proliferation with troglitazone was not mediated by inhibition of translation initiation in KU812 cells (Fig. 3B). It was suggested that troglitazone might inhibit cell cycle progression from the S phase to the G2/M phase because of the reduced ratio of the number of cells in the G2/M phase and no change in the S phase. It is paradoxical that troglitazone inhibited cell growth, although cyclin D1 was up-regulated with troglitazone. Although it was reported that cyclin D1 played stimulative role in differentiation (Li et al., 1997), further studies are required to reveal the relationship between growth inhibition and cyclin D1 up-regulation.

Histamine is a characteristic product of basophils and mast cells. We showed that troglitazone decreased both histamine secretion and accumulation in the cells (Fig. 4A,B). In addition, troglitazone decreased the expression of histidine decarboxylase mRNA determined using RT-PCR (Fig. 4C), indicating that troglitazone inhibited histamine production,

at least in part, through the decreased expression of histidine decarboxylase mRNA. Histamine is one of the potent mediators of inflammation and allergic response and also regulates cytokine production as an immunomodulator (Kohka et al., 2000). Troglitazone may be available for the regulation of immunoreaction and inflammatory and allergy response in addition to the suppression of tumors.

The expression of PPARγ mRNA was undetectable by RT-PCR in KU812 cells, while other subtypes of PPARs (α and β) were easily detected (Fig. 5). It has been reported that rosiglitazone binds to PPARγ with the highest affinity among the three thiazolidinedione derivatives used in this study and that the rank order of potency for activation of PPARγ is rosiglitazone>pioglitazone>troglitazone (Young et al., 1998), which is inconsistent with our results (Fig. 1A). Recent studies showed troglitazone signal transduction pathways independent of PPARγ (Hattori et al., 2000; Inoue et al., 1997; Palakurthi et al., 2001; Wang et al., 1999; Yasunari et al., 1997). In agreement with our findings and these studies, we indicate that there is a signal transduction pathway of troglitazone independent of any action by PPARγ at least in KU812 cells. Only troglitazone has a chroman structure of vitamin E among the three thiazolidinedione derivatives. It also has been reported that vitamin E regulates signal transduction pathways through some mechanisms other than its scavenging effect (Boscoboinik et al., 1994; Brigelius-Flohe and Traber, 1999; Sen et al., 2000). Considering these reports, we conclude that it is possible that troglitazone may exerts its effects by antioxidant property according to structural similarity to vitamin E (Inoue et al., 1997) and/or via the signaling pathway mimicking vitamin E. We have found that both PPARα and PPARβ are expressed and related to the regulation of cell proliferation by troglitazone in KU812 cells, suggesting that they may participate in the signal transduction pathway of troglitazone, even if indirectly (in preparation).

In conclusion, we found that troglitazone suppressed cell growth via the decrease in cyclin E and the phosphorylated form of pRb independently of PPARγ in KU812 cells. Troglitazone also decreased histamine secretion by suppressing histidine decarboxylase mRNA expression, which might help to prevent allergic reactions.

Further studies are required to identify the direct target of troglitazone without PPARγ.

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