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Identification of troglitazone responsive genes: induction of *RTP801* during troglitazone-induced apoptosis in Hep 3B cells

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Troglitazone is an anti-diabetic agent that improves hyperglycemia by reducing peripheral insulin resistance in type II diabetic patients. Troglitazone has been shown to cause growth inhibition of various normal and cancerous cells. However, the molecular mechanism by which troglitazone affects the growth of these cancer cells remains unclear. Here, we report that troglitazone treatment of Hep 3B human hepatocellular carcinoma cells resulted in dose-dependent growth inhibition. Analysis of cell cycle distribution by flow cytometry showed that the number of apoptotic cells was increased in a dose-dependent manner in response to troglitazone treatment. cDNA microarray analysis showed a number of differentially expressed genes in response to troglitazone. Among the upregulated genes, hypoxia-inducible factor 1 (HIF-1)-responsive *RTP801* was induced in a dose-dependent manner. We also observed HIF-1 accumulation by immnocytochemistry after troglitazone treatment. These results strongly suggest that *RTP801* might be involved in troglitazone-induced apoptosis in Hep 3B cells. [BMB reports 2010; 43(9): 599-603]

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant cancers in the world. As such, medical treatments or at least effective chemoprevention methods for HCCs are needed. Recent studies have shown that trogltazone (TGZ), a thiazolidinedione (TZD) derivative containing peroxisome proliferators-activated receptor gamma ligands, causes growth inhibition of HCC cell lines (1-4). Koga *et al* (1) reported that troglitazone induces G1 cell cycle arrest without inducing apoptosis, whereas others have observed induction of apoptosis (2-4). However, the molecular mechanism by which troglitazone affects the growth of these cancer cells remains unclear.

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In this study, we show that troglitazone induced apoptosis in Hep 3B human hepatocellular carcinoma cells. We also identified genes differentially expressed in response to troglitazone by cDNA microarray, and confirmed them by real-time PCR analysis. In addition, we found that troglitazone strongly induced *RTP801*, a gene known to be associated with apoptosis, in a dose- and time-dependent manner.

RESULTS

Growth inhibition by troglitazone

Troglitazone treatment of Hep 3B cells resulted in dose-dependent growth inhibition (Fig. 1A). Cells cultured in the presence of troglitazone were detached from the culture plates, showing the characteristic features of apoptosis (data not shown). Analysis of the cell cycle distribution by flow cytometry showed that the population of cells in sub-G1 phase, apoptotic cells, was increased in a dose-dependent manner in response to troglitazone treatment (Fig. 1B, C).

Identification of differentially expressed genes by troglitazon

To understand the mechanism of troglitazone-mediated cytotoxicity in Hep 3B cells, we performed cDNA microarray analysis. Cells were treated with troglitazon (40 μM) for 2 days. Genes increased in expression over 2-fold were identified as asparagine synthetase, transcript variant 2 (*ASNS*, 3.33-fold), phosphoenolpyruvate carboxykinase 2 (*PCK2*, 3.13-fold), HIF-1 responsive RTP801 (*RTP801*, 2.66-fold) and stanniocalcin 2 (*STC2*, 2.44-fold). Genes that were decreased in expression below 0.5-fold were identified as serum-inducible kinase (*SNK*, 0.46-fold), downregulated in ovarian cancer 1 (*DOC1*, 0.5-fold), chemokine (C-C motif) ligand 4 (*CCL4*, 0.5-fold), vascular endothelial growth factor C (*VEGF*-C, 0.5-fold) and interleukin 8 (*IL*-8, 0.5-fold) (Fig. 2).

Validation of identified genes

To verify the microarray data, we performed real-time PCR analyses of selected genes. Consistent with the microarray data, the expression of *RTP801* was increased by troglitazone, whereas the expression of *DOC1, SNK* and *IL-8* was decreased (Fig. 3). Therefore, we further examined the expression of *RTP801*, a gene associated with apoptosis (5-7). The dose-de-

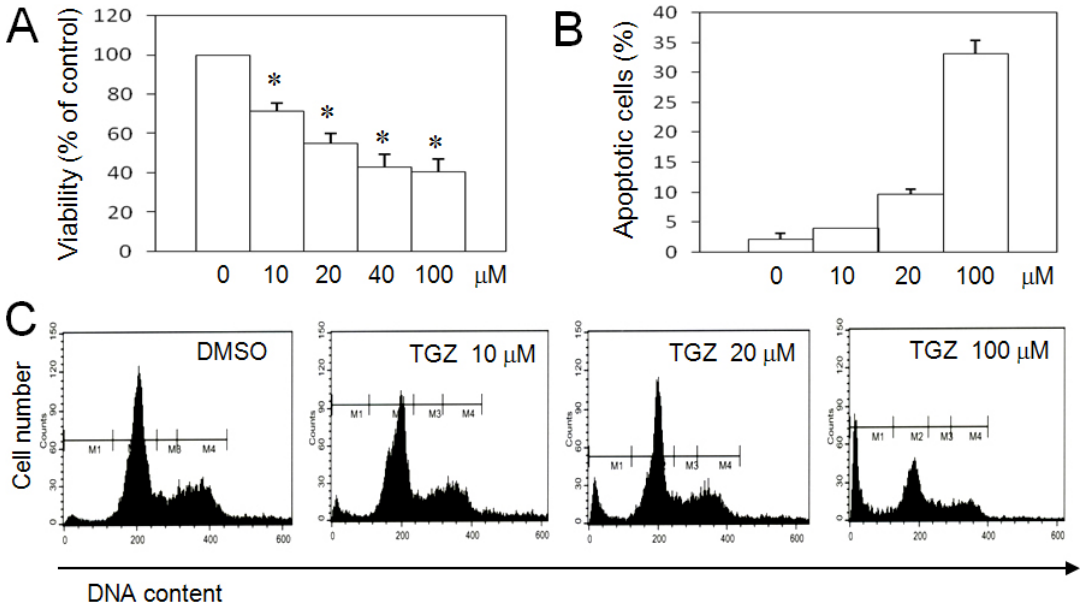

Fig. 1. Effects of troglitazone on growth of Hep 3B cells. (A) Dose-dependent growth inhibition. Cells were treated with the indicated concentrations of troglitazone for 48 h. Data are the means of five determinations per experiment from three independent experiments. (*P < 0.05). (B) Dose-dependent increment of apoptotic cells. The results are the means of two independent flow cytometric analyses. (C) A representative DNA histogram is shown.

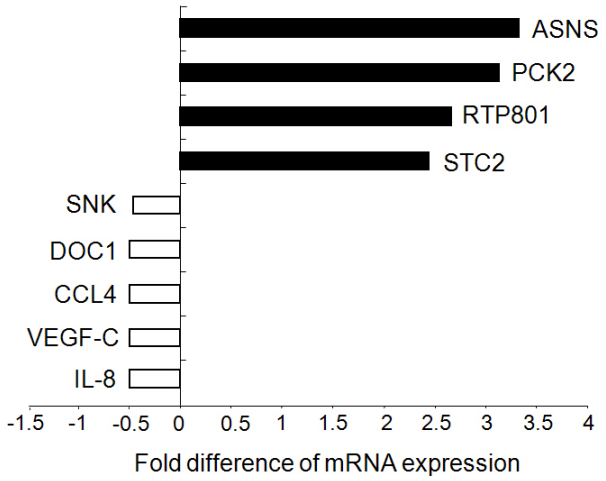

Fig. 2. Differentially expressed genes upon troglitazone treatment. cDNA microarray was performed with mRNA from Hep 3B cells treated with 40 μM troglitazone for 48 h.

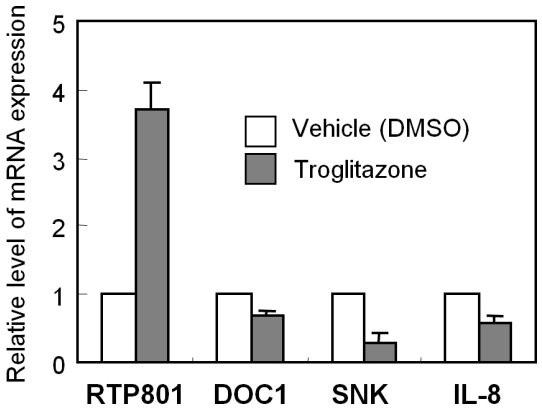

Fig. 3. Verification of differential expression of genes. Cells were treated with 40 μM troglitazone for 48 h, after which the relative expression level of each gene was measured by real-time RT-PCR.

pendent induction of *RTP801* indeed occurred (Fig. 4A), suggesting that troglitazone-mediated apoptosis in Hep 3B cells was associated with the expression of *RTP801*. The induction of *RTP801* was evident as early as 1 h after troglitazone treatment, compared to that of DMSO control (Fig. 4B).

Since *RTP801* expression is regulated by transcription factor HIF-1 (5, 7), we used immunocytochemistry to examine whether or not the nuclear translocation and accumulation of HIF-1α occurs upon troglitazone treatment. We found that HIF-1α was clearly increased in the nucleus by troglitazone, compared to that of control (Fig. 4C). These results suggest that the upregulation of *RTP801* by troglitazone might be dependent on HIF-1α.

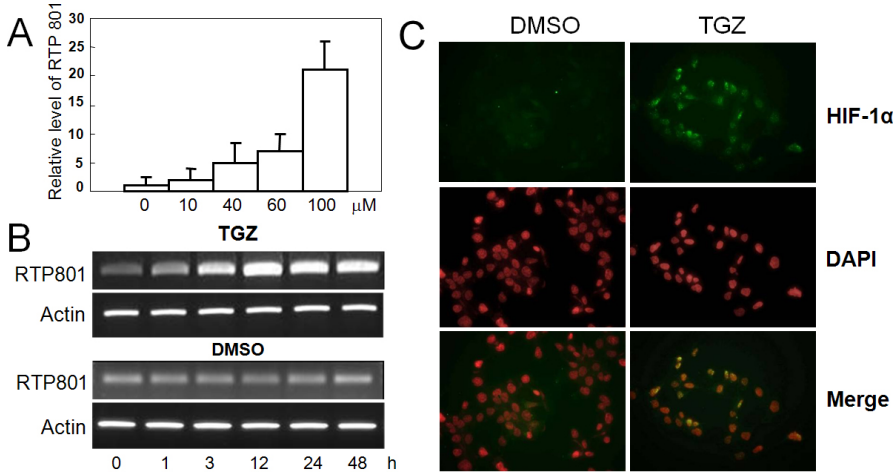

Fig. 4. Upregulation of *RTP801* by troglitazone. (A) Dose-dependent upregulation of *RTP801* as measured by real-time RT-PCR. Cells were treated with the indicated concentrations of troglitazone for 48 h. (B) Time-dependent upregulation of *RTP801*. Cells were treated with 40 μM troglitazone (TGZ, upper) or DMSO (lower) for the indicated times. (C) The nuclear translocation and accumulation of HIF-1α by troglitazone. Cells were treated with troglitazone (TGZ) and DMSO (control) for 1 h and then were subjected to immunostaining with anti-HIF-1α antibody. The nucleus was stained by DAPI as a counterstain.

DISCUSSION

The molecular mechanism by which troglitazone affects the growth of hepatocellular carcinoma cells remains unclear, though troglitazone-mediated growth inhibition of these cancer cells has been reported (1-3). In the present study, we showed that troglitazone, a PPAR-γ agonist, inhibited the growth of Hep 3B human HCC cells by inducing apoptosis in a dose-dependent manner. Our data are in agreement with the finding that troglitazone induces cell cycle arrest and apoptosis of HCC cell lines (2, 3, 8).

We also showed that troglitazone affected gene expression in Hep 3B HCC cells by cDNA microarray analysis. Genes related with inflammation, such as *IL*-8 and *CCL4*, were downregulated by treatment with troglitazone, suggesting an anti-inflammatory role for troglitazone. On the other hand, genes related with angiogenesis, such as *VEGF-C*, were downregulated upon troglitazone treatment, suggesting that troglitazone inhibits angiogenesis by reducing VEGF-C production. A recent report also suggested a regulatory role for troglitazone in VEGF-induced angiogenic signaling (9). Genes implicated in apoptosis and cell cycle regulation are HIF-1 responsive *RTP801*, *DOC1* and *SNK*. *DOC1* is a processivity factor for the anaphase-promoting complex (10) and plays an important role in exiting mitosis. *SNK* is a member of the 'polo' family of serine/threonine protein kinases and plays a role in normal cell division (11). Decreased expression of *DOC1* and *SNK* in the microarray data suggests that cell cycling is also affected by troglitazone treatment.

Moreover, we showed that troglitazone induced *RTP801* in a dose-dependent manner, possibly due to accumulation of HIF-1, which suggests that troglitazone-mediated apoptosis in Hep 3B cells is due to *RTP801* expression. Recent findings support this idea: 1) *RTP801* is known to be related with apoptosis under oxidative stress conditions (5, 12). 2) Troglitazone induces oxidative stress in rat hepatoma cells, accounting for apoptosis (13). 3) Expression of *RTP801* exacerbates amyloid β-peptide and H_2O_2-mediated cytotoxicity in CHP134 human neuroblastoma cells (6).

In conclusion, our data show that a variety of genes are regulated by troglitazone, and suggest that HIF-1 responsive *RTP801* might be involved in troglitazone-mediated apoptosis in Hep 3B cells.

MATERIALS AND METHODS

Cell culture and viability assay

Hep 3B cells were purchased from Korea Cell Line Bank (Seoul, Korea) and routinely maintained in DMEM containing 10% FBS at 37°C under 5% CO_2 atmosphere. For the viability assay, cells were plated overnight at a density of 5,000 cells/well in 96-well plates containing 10% FBS-supplemented medium. The medium was then switched to serum-five medium containing various concentrations of troglitazone. MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) assay was employed to check cell viability as previously described (14). Troglitazone was dissolved in DMSO, such that the final concentration of DMSO was 0.1%.

Analysis of cell cycle distribution

Hep 3B cells were cultured in the presence or absence of various concentrations of troglitazone for 12 h. Cells were collected and washed with PBS and fixed in 100% ethanol at 4°C overnight. Cell cycle distribution was analyzed by flow cytometry as previously described (15).

Microarray analysis

Total RNA was isolated from the cells using TRIzol reagent. Messenger RNA (mRNA) was purified from total RNA using an Oligotex mRNA purification kit (Qiagen, Valencia, CA, USA). Using reverse transcription, two micrograms each of mRNA from cells treated with 40 μM troglitazone and from cells treat-

ed with vehicle (DMSO) was labeled with cy3- and cy5-dCTP, respectively, as previously described (16). Microarray hybridizations were performed as previously described (16) using KNU 4.9K DNA chips (Tricogene, Daegu, Korea). Fluorescence intensities were measured using a Scanarray 4000 with a laser confocal microscope, and the data were normalized and analyzed using Quantarray software as previously described (16).

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA isolation and reverse transcription were performed as previously described (17). RT-PCR analysis for *RTP801* was performed using a PTC-200 thermal cycler. Real-time quantitative RT-PCR analysis was performed using a LightCycler system (Roche Diagnostics, Switzerland). This system uses SYBR Green, a fluorescent dye that only intercalates with double stranded DNA, and the sequences for a set of gene specific primers as follows. RTP801-F: 5′-CAA GAT CCA GGG GCT GTT TA-3′, RTP801-R: CAC CCC AAA AGT TCA GTC GT-3′, DOC1-F: GTC ACC ACC CTG AAA GAG GA-3′, DOC1-R: TCA GCA AGG GCT AGT TTG GT-3′, SNK-F: 5′-GAC ACA GTG GCA AGG GTT CT-3′, SNK-R: 5′-GGC CAA GCT CTG CGT AAT AG-3′, IL-8-F: 5′-TAG CAA AAT TGA GGC CAA GG-3′, IL-8-R: 5′-AGC AGA CTA GGG TTG CCA GA-3′. The relative level of each gene was normalized with the level of β-actin.

Immunocytochemistry

Hep 3B cells were plated in an 8-chamber slide (Nunc Lab-Tek, Roskilde, Denmark) at a density of 50,000 cells per well. Cells were washed with PBS after treatment with 40 μM troglitazone for 1 h, then fixed with cold methanol for 10 min. After blocking with 4% normal donkey serum for 1 h at room temperature, the slide was incubated with anit-HIF-1α antibody (1 : 100 dilution; Abcam, Cambrige, UK) at 4°C overnight, then incubated with Alexa Flour 488 labeled anti-rabbit secondary antibody (1 : 100 dilution; Molecular Probes, Eugene, OR, USA) for 1 h. The slide was then washed with PBS and counterstained with 4, 6 Diamidino-2-phenylindole (DAPI) for 10 min.

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