Activation of estrogen receptors with E2 downregulates peroxisome proliferator-activated receptor γ in hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality and occurs more often in men than in women; however, little is known about its underlying molecular mechanisms. The present study investigated the effect of estrogen receptor (ER)α and ERβ on peroxisome proliferator-activated receptor γ (PPARγ) expression in Hep3B cells. We examined PPARγ, ERα and ERβ mRNA and protein expression by RT-PCR and western blotting. In order to determine whether PPARγ plays a central role in HCC, we screened for PPARγ expression in liver cancer patient tissues and differentially differentiated HCC cell lines (HA22T, Huh-7, Hep3B and HepG2). We found that PPARγ expression was highly expressed in liver cancer tissues and in Hep3B cells. Furthermore, overexpression of ERα and ERβ was found to decrease PPARγ expression at the transcriptional as well as at the translational level in a ligand-dependent manner. In summary, the present study demonstrated that both ERα and β were sufficient to inhibit PPARγ and provide a valuable therapeutic option for the treatment of HCC patients.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide (1) and its frequency is increasing in Southeast Asia, Africa and Western countries. In particular, the mortality rate of HCC in Taiwan has not decreased due to limited treatment options (2,3). HCC occurs more often in men than in women; in addition, males have a poorer prognosis in comparison with females (4). However, little is known about the underlying molecular mechanisms of HCC.

The role of nuclear receptors in HCC development has drawn considerable attention (5). One such example is peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated transcription factor that is involved in tumor promotion, cellular differentiation and apoptosis (6,7). Several other studies also focused on PPARγ as their target gene to treat various types of cancer, such as colon, thyroid, lung, breast, prostate and liver cancer (8). For example, troglitazone inhibited the growth of human liver cancer cells by inducing apoptosis through caspase-3 activation (9). In breast cancer cells, estrogen receptor (ER)α binds to peroxisome proliferator-activated receptor response element and negatively interferes with PPARγ signaling (10). Similarly, in preadipocytes cells, ERβ overexpression inhibits ligand-mediated PPARγ activity, which further results in a blockade of PPARγ-induced adipocytic gene expression (11).

Expression ratio of ERα and ERβ apparently changes during hepatocarcinogenesis (12). A large body of evidence has shown decreased ERα in HCC patients (13,14); similarly, loss of ERβ expression has been indicated as a common step in the development of colorectal cancer (15). Activation of these ERs controls several biological processes, including cell growth,

differentiation and apoptosis. However, the effect of ERα or ERβ on PPARγ expression in HCC is not well studied. In the present study, either ERα or ERβ is overexpressed by transient transfection and then receptor is activated by 17β-estradiol. At the same time, we conducted the assay with 17β-estradiol alone to elucidate whether ligand alone can induce ERα or ERβ expression in ER-negative Hep3B cells. These results showed that ERα or ERβ may act as a tumor suppressor in downregulating PPARγ expression in Hep3B cells and were further accelerated by ligand addition.

Materials and methods

Specimen collection and immunohistochemistry. Written consent was obtained from all patients. Surgical specimens of human liver cancer tissues were obtained by mastectomy from the operating rooms of the Changhua Christian Hospital in Changhua and the China Medical University Hospital in Taichung, Taiwan. Following resection, these specimens were stored at -70˚C before being used for the analysis. The tissue biopsy was dried at 58˚C overnight, dewaxed in xylene for 40 min and rehydrated in ethanol. Blocking with 3% H_20_2 in 50% methanol/50% phosphate-buffered saline (PBS) and incubated with 5% cosmic calf serum to reduce non-specific staining of the secondary antibody. Tissue sections were incubated overnight at 4˚C with PPARγ (1:100). The sections were washed with PBS and incubated for 1 h at room temperature with the peroxidase-conjugated secondary antibody. Immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB) substrate (Roche Diagnostics, Mannheim, Germany). After coloring and rinsing with distilled water, the sections were counterstained slightly with Mayer's hematoxylin, dehydrated in graded alcohols, cleared in xylene and detected using microscopy (Olympus, Tokyo, Japan).

Cell culture. The Chang liver cell line, HepG2, Hep3B, Huh-7 and HA22T cells were purchased from ATCC. Chang liver cells were grown in DMEM, HepG2 and Hep3B were grown in MEM (Gibco, Grand Island, NY, USA) and Huh-7 and HA22T cells were grown in DMEM. All media were supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and 1% penicillin streptomycin (Gibco).

Establishment of the double-stable Tet-On/ERα and ERβ Hep3B cell line. The double-stable Tet-On/ERα or ERβ Hep3B cell line, which grows well in the presence of both G418 and hygromycin, was established by plasmid transfection using the Lipofectamine method. Briefly, the primary Tet-On Hep3B cell line was generated by transfecting Hep3B cells with 10 μg Tet-On (Clontech Laboratories, Worcester, MA, USA), a regulator plasmid encoding the G418 resistance gene. The primary Tet-On Hep3B cells were then transfected with 10 μg of pTRE2/ERα or ERβ plasmid encoding the hygromycin resistance gene. Double-stable cells were selected with 700 μg/ml G418 and 100 μg/ml hygromycin and further screened for ERα mRNA using DNA sequencing.

Transfection. Hep3B cells were transfected with a plasmid carrying the ERα and ERβ gene using 10 and 100 μM of Lipofectamine (Invitrogen, Auckland, New Zealand) according to the manufacturer's guidelines. After 6 h of transfection, MEM supplemented with 10% charcoal/dextran (CD)-FBS (Sigma, St. Louis, MO, USA) was added for 12 h, and MEM containing 1% FBS and antibiotics were added for 6 h. Prior to treatment, the cells were starved in MEM (no phenol red) with 1% antibiotics for 6 h and then replaced with phenol red-free MEM containing 1% FBS and vehicle or 17β-estradiol (E2) (Sigma), doxycycline or fenofibrate (Clontech, Mountain View, CA, USA) for different times. These transfection experiments were repeated three times with consistent results.

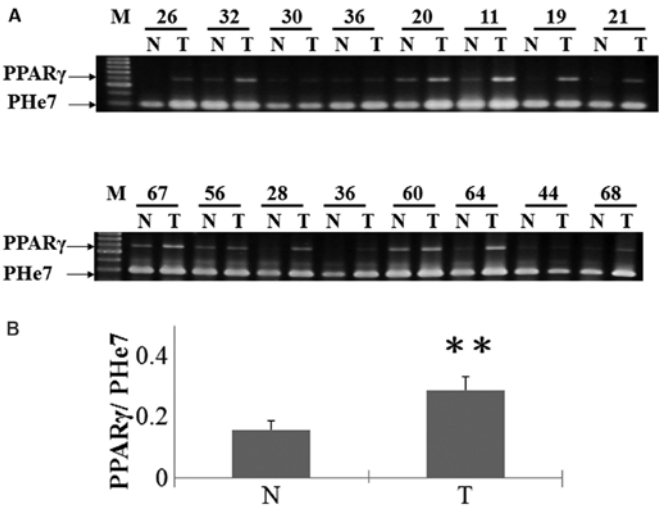

Figure 1. PPARγ expression compared between normal and tumor tissues. The expression of PPARγ in HCC patients was determined by (A) RT-PCR analysis and (B) densitometry analysis of the mRNA expression. M, marker; N, normal tissue; and T, tumor tissue. The data represent the mean ± SE of 16 patient specimens ($^{**}P \leq 0.005$).

Reverse transcription (RT). Total RNA was extracted using an Ultraspec™ kit (Biotecx, Houston, TX, USA) according to the manufacturer's instructions. A total of 4 μg of RNA was used for the RT reaction. RT was performed at 37˚C for 60 min using 55.5 μl DEPC H_2O, 4 μg total RNA, 0.5 μl of RNase inhibitor (40 U/μl) (Promega, Madison, WI, USA), 20 μl of 5X RT buffer, 8 μl of dNTP (2.5 mM), 10 μl of oligo(dT) (5 μM/ml) (Mission Biotech, Taipei, Taiwan) and 2 μl of MMLV reverse transcriptase (200 U/μl) (Promega). The resulting cDNA was added to the PCR mixture containing 9.5 μl of DEPC water, 2.5 μl of 10X PCR buffer (MD Bio, Taipei, Taiwan), 2.5 μl of dNTP (10 mM) (Promega), 2.5 μl of each primer (5 μM), 0.5 μl of Taq (2 U/μl) (MD Bio) and 4 μl of 2.5 mMdNTP mixture.

Western blotting. Cells were lysed at each time-point with lysis buffer [50 mM Tris base (pH 7.4), 0.5 M NaCl, 1 M ethylenediamine-mercaptoethanol (BME), 1% NP-40%, 10% glycerol, Igepal CA-630] (Sigma) and protease inhibitor cocktail tablets (Roche). Proteins were analyzed and separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies against the following proteins: PPARγ, ERα, ERβ and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were incubated with peroxidase-conjugated secondary antibody for 1 h. Bands were monitored using western blot chemiluminescence reagent (Santa Cruz Biotechnology).

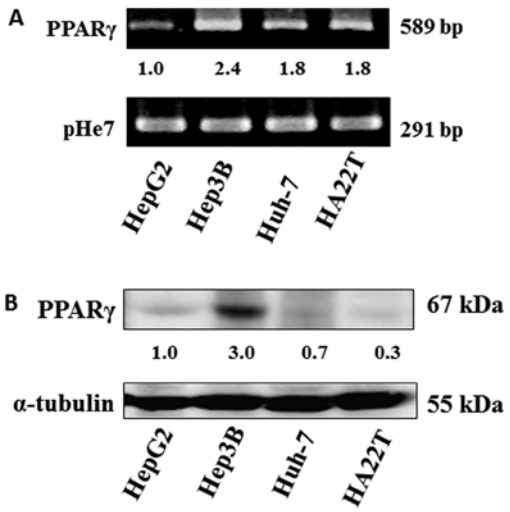

Figure 2. PPARγ expression is screened in Chang liver, HepG2, Hep3B, Huh7 and HA22T cells using (A) RT-PCR and (B) western blotting. For mRNA expression and for protein expression, pHe7 and α-tubulin were used as a loading control individually. All experiments were repeated thrice with similar results.

Statistical analysis. All data are expressed as percentages of the control and mean ± SD. The results are based on three independent experiments. Student's unpaired t-test was used to compare the differences between groups. Experimental group vs. control group: P-value <0.05 was considered to indicate a statistically significant difference; *P<0.05 and **P<0.01.

Results

PPARγ expression in HCC is significantly increased in tumor tissues compared with surrounding non-tumorous liver, particularly in poorly differentiated tumor compared to well-differentiated tumor (16). To assess the role that PPARγ in liver cancer *in vivo*, we analyzed tumor and non-tumor patient tissues for PPARγ expression. The majority of normal liver tissues do not express PPARγ, whereas tumor tissues from the liver cancer patients showed a significant increase in PPARγ expression (Fig. 1).

Next, we analyzed the expression of PPARγ in HCC cells *in vitro* using HepG2, Hep3B, HuH-7 and HA22T cell lines. As shown in Fig. 2A, RT-PCR analysis readily detected the expression of PPARγ mRNA in all cell lines. Western blot analysis did not display exactly same expression pattern when compared with the mRNA expression. Compared with other cell lines used, only Hep3B cells expressed PPARγ protein (Fig. 2B). These results were consistent with the hypothesis that mRNA levels do not necessarily correlate with the protein expression data (17).

Estrogen exerts its biological function by binding to one of two specific ERs, ERα and ERβ. Thus, the level of exogenous ERα and endogenous PPARγ in Hep3B cells transfected with empty vector or ERα expression vectors was examined in the presence of E2. As shown in Fig. 3A, ERα containing Hep3B cells induced ERα mRNA expression and further decreased PPARγ expression. On the other hand, E2 treatment altered the expression level of ERα and PPARγ in ERα overexpressing Hep3B cells. However, in empty vector transfected Hep3B

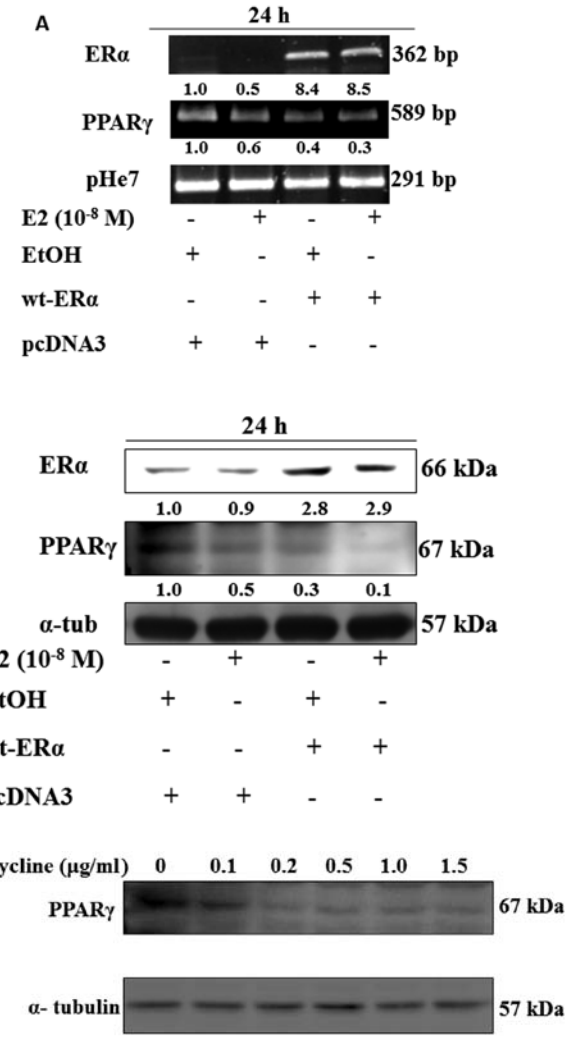

Figure 3. Hep3B cell lines were transfected with empty vector (4 μg), or with wild-type estrogen receptor α (wt-ERα) plasmid DNA (4 μg) and then treated with or without E2 (10^{-8} M) and EtOH (10 μl) into cells and cultured for 24 h at 37˚C. The cells were collected and analyzed for PPARγ expression at both the (A) mRNA and (B) protein level. All experiments were repeated thrice with similar results. pHe7 and α-tubulin were used as internal control. (C) Tet on ERα expressing Hep3B cells were treated with doxycycline (0.1, 0.2, 0.5, 1.0 and 1.5 μg/ml) for 6 h. Proteins were collected and observed for ERα and PPARγ expression using western blotting. α-tubulin was used as an internal control.

cells, E2 treatment reduced PPARγ mRNA levels without increasing ERα expression. This was further confirmed by western blot analysis (Fig. 3B). We then verified the possibility of ERα in inhibiting PPARγ expression using a stable cell line that expresses ERα. Tet-On/ERα Hep3B cells were treated with a range (0-1.5 μg/ml) of Dox for 24 h and then analyzed for PPARγ expression. As shown in Fig. 3C, a dose-dependent decrease in PPARγ was observed in response to Dox. This was more noticeable at the 0.2 μg/ml concentration.

In order to elucidate whether exogenous expression of ERβ inhibits PPARγ expression, vector or ERβ transfected Hep3B cells were exposed to E2 treatment. No activation of ERβ and PPARγ was observed in cells transfected with empty vector and in E2 exposed cells. Fig. 4 shows that ERβ overexpression plus E2 treatment effectively inhibited PPARγ mRNA and protein expression.

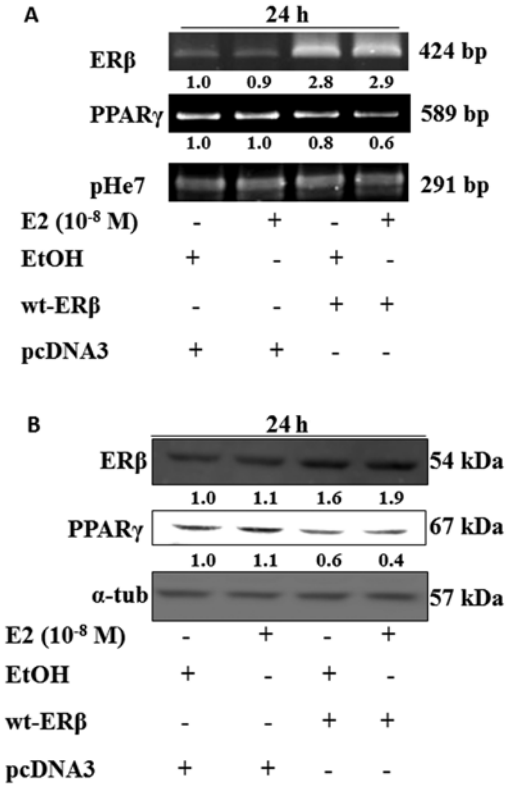

Figure 4. Hep3B cell lines were transfected with empty vector (4 μg), or with wild-type estrogen receptor β (wt-ERβ) plasmid DNA (4 μg) and then treated with or without E2 (10^{-8}M) and EtOH (10 μl) into cells and cultured for 24 h at 37˚C. The cells were collected and analyzed for PPARγ expression at both the mRNA (A) and protein (B) level. All experiments were repeated thrice with similar results. pHe7 and α-tubulin were used as internal control.

Discussion

The nuclear receptor superfamily (estrogen, thyroid, glucocorticoid receptors and peroxisome proliferator-activated receptors) plays an important role in controlling cellular homeostasis, and administration of its ligand has been effectively used in cancer treatment (18-21). The role of PPARγ in tumor development is controversial as fewer studies showed ligand activated PPARγ promotes growth inhibition and apoptosis in human esophageal (22) breast (23) ovarian and liver cancer (24) and other reports showed PPARγ ligand could inhibit growth and metastasis of PPARγ positive cancer cells (25). In the present study, we found a significant increase in PPARγ mRNA expression in HCC tissues compared with non-cancerous tissues. Similarly, in human lung cancer tissues, increased PPARγ expression was observed compared with non-tumor tissues (25). These findings suggest that PPARγ was involved in hepatocarcinogenesis. However, decreased PPARγ expression in tumor tissue has been observed in human esophageal, breast and ovarian cancer (22,26,27). Therefore, a better understanding of the PPARγ mechanism in different cancer tissues is required.

PPARγ expression has been demonstrated *in vitro* in several cell lines; particularly in liver cancer cell lines, PPARγ mRNA was expressed at various levels (9). Similarly, we analyzed the effects of PPARγ activation in four human HCC cell lines, compared with the HepG2, Huh-7 and HA22T cells, Hep3B cells constitutively express PPARγ expression at the RNA and protein levels. Having observed significant upregulation of PPARγ expression in Hep3B cells, we next conducted experiments to test the potential role of ERs in inhibiting PPARγ expression in Hep3B cells.

Previous studies showed overexpression of ERα inhibits growth of ECV304 and the Ishikawa cell line by decreasing endothelin-1 and VEGF expression (28). Our results, consistent with a previous report (29), showed that ERα binds with PPARγ and functionally interferes with PPARγ signaling in a ligand-dependent manner. Compared with ERα expression, decreased ERβ was found in patients with chronic hepatitis or cirrhosis and in those with HCC. In normal breast cells, ERβ was found to negatively regulate cellular proliferation. Our data are in agreement with these results, showing ERβ overexpression decreased PPARγ expression in an E2-dependent manner. Collectively, the present study provided a basic understanding of ERα and ERβ in PPARγ expression; further studies using these ERs are currently being conducted to elucidate how these ERs control Hep3B cell molecular mechanisms.

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