Pathol. Oncol. Res. (2017) 23:477–485
DOI 10.1007/s12253-016-0136-8

ORIGINAL ARTICLE

E_2/ERβ Inhibits PPARα to Regulate Cell-Proliferation and Enhance Apoptosis in Hep3B-Hepatocellular Carcinoma

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Received: 24 June 2016 / Accepted: 12 October 2016 / Published online: 18 October 2016

Abstract Peroxisome proliferator-activated receptor-α (PPARα) is a member of the nuclear receptor superfamily involved in hepatocarcinogenesis in rodents. In previous studies on liver tumor tissues, PPARα mRNA expression was found to be significantly higher and overexpression of ERα inhibited the PPARα expression, cell-proliferation and also induced apoptosis in Hep3B cell. However, the role of ERβ is not known yet. Therefore, the aim of this study is to define the role of ERβ on PPARα in Hep3B cells. The effect of PPARα signaling cascade were monitored by inducing Hep3B cells by fenofibrate. Further the cells were transfected with pCMV-ERβ and the consequences of ERβ-overexpression on the PPARα induced changes such as enhanced cell-proliferation and suppressed apoptosis were determined using western blot analysis and TUNEL assay. The EMSA was used to identify whether ERβ modulates PPARα expression by binding to PPARα promoter region to repress PPARα promoter activity. In addition, the direct interaction between ERβ and PPARα proteins was verified by co-immunoprecipitation assay. Our results show that the overexpressed ERβ not only attenuated the effects of fenofibrate to induce the levels of apoptosis protein such as Cyt.c, Caspase 9 and Caspase 3 but also inhibited the levels of survival protein such Bcl-xL, p-Bad, cyclin A and cyclin E. All these effects of E_2/ERβ resulted in the enhancement of mitochondria dependent apoptotic pathway and the attenuation of cell proliferation. Moreover, the overexpressed ERβ reduced the mRNA and protein levels of PPARα and its downstream Acyl-CoA oxidase (ACO). EMSA results show that ERβ directly binds to PPRE and inhibit PPARα gene expression and according to immunoprecipitation assay ERβ also binds strongly with PPARα. The E_2/ERβ further inhibited the fenofibrate-induced nuclear translocation of PPARα. Taken together, ERβ might directly downregulate PPARα gene expression and inhibit the nuclear translocation to suppress the proliferation and induce the apoptosis of Hep3B cells.

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Keywords Estrogen · Estrogen receptor, peroxisome proliferator-activated receptor · Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) has become the fifth most prevalent Cancers in the world [1]. HCC is an aggressive cancer and its prominent risk factors include chronic hepatitis B (HBV) and C (HCV) viral infection, chronic alcohol consumption and aflatoxin-B1-contaminated food [2–4]. Hepatocarcinogenesis is widely reported to be induced by peroxisome proliferators that are capable of inducing proliferation of peroxisomes in liver cells [2, 3]. Proliferator-activated receptor-α (PPARα), is responsible for the peroxisome proliferator-induced phenotypic responses that include peroxisome proliferation, enhanced fatty acid oxidation resulting in excess energy burning in liver, and hepatocarcinogenesis [5]. Long-term administration of PPARα agonists have also been reported to cause liver cancer in rodents [6]. Males are affected by HCC more often than females and the worldwide male to female ratio of HCC incidence is around 4:1 [7]. In addition to the difference in living conditions, the hormone might distinction of HCC incidence of men from women. Therefore, estrogen (E_2) is suggested to play a protective role against HCC. Estrogen and its receptors are known to promote apoptosis, suppress the malignant transformation and inhibit the growth of various cancer cells [8–10]. Over-expression of ERβ prevents establishment and growth of breast tumors in a subcutaneous xenograft mouse model [11]. In breast cancer cells the inhibitory effect of ERβ is associated with its ability to decrease the expression of c-myc, cyclin A, cyclin D1 and cyclin E and to increase the levels of p21 (cip1) and p27 (Kip1) [12, 13]. Restoration of ERβ in ovarian cancer cells results in enhancement of apoptosis of tumor cells, and a strong inhibition of their proliferation and invasion [9]. In prostate cancer, E_2-mediated activation of ERα is associated with aberrant proliferation, inflammation and the development of malignancy, whereas E_2-mediated activation of ERβ is associated with anti-proliferation, differentiation and apoptosis [14–16].

These reports show that ER-β play anti-tumor roles on cancers. ER-β promotes apoptosis, suppresses transformation and inhibits the growth of cancer cells [17]. Recently, some reporter show estrogen through estrogen receptor α and β decrease the PPARα reporter gene expression [18]. Moreover, our previous studies indicate that estrogen/ERα down-regulate PPARα expression and β-estradiol (E_2) further induces apoptosis pathway in Hep3B cells, that mediated apoptotic effect was estrogen receptor dependent [19]. In this study, we aim to further determine the role of PPARα in hepatocarcinogenesis, and define how ERβ regulates the PPARα expression in Hep3B cells. To identify whether ERβ could downregulate the level of PPARα, ERβ was overexpressed by transfecting pCMV-ERβ in Hep3B cell line to identify whether ERβ could downregulate the level of PPARα.

Materials and Methods

Cell Culture

Hep3B cells were grown in Dulbecco's modified Eagle's medium (MEM) supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin, and 0.1 mM Non-essential amino acids, 1.5 g/L sodium bicarbonate and 1.0 mM sodium pyruvate at 37 °C in 5 % CO_2.

Plasmid DNA Purification

*E. coli c*ontaining plasmid DNA carrying ERβ gene was grown in 250 mL LB medium overnight, after centrifuge at 3000 g for 10 min to pellet the bacteria, a maxi-prep kit (Invitrogen, Carlsbad, CA) was used to perform plasmid DNA purification.

MTT Assay

MTT assay was used to detect the changes in the cell viability. The Hep3B cells were inoculated into 24-well plate 24 h before the treatment. After treatment, the medium was removed and MTT 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (0.5 mg/mL) was added to each well followed by 4 h incubation. MTT solution was replaced by isopropanol to dissolve blue formazan crystals, and absorbance was measured at 570 nm using a microplate reader.

DAPI Staining and TUNEL Assay

After various treatments, Hep3B cells grown on 6 mm plate were fixed with 4 % paraformaldehyde solution for 30 min at room temperature. After a rinse with PBS, the samples were first incubated with phalloidin rhodamine for 1 h and with Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling (TUNEL) reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP (Roche Applied Science, Indianapolis, IN, USA). The cells were also stained with 1 μg/mL 4-, 6-diamidine-2-phenylindole dihydrochloride (DAPI, Roche) for 30 min to detect cell nucleus by UV light microscopic observations (blue). Samples were analyzed in a drop of PBS under a fluorescence microscope using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green). The number of TUNEL-positive Hep3B cells and number of apoptotic bodies were determined by counting

3×10^5 Hep3B cells. All morphometric measurements were performed by at least two independent individuals in a blinded manner.

Transfection

Cells were seeded into 100-mm dishes in minimum essential medium (MEM) containing 10 % FBS and, on the next day, medium was replaced with MEM without antibodies, and plasmid DNA with ERβ gene was introduced into cells using Lipofectamine according to the manufacturer's guidelines. Six hours later, the cells were fed with fresh MEM medium containing 10 % CCS, 12 h thereafter, medium was replaced with fresh phenol red-free MEM medium containing 1 % FBS and vehicle or 17-β-estradiol (10^{-8} M).

Semi-Quantitative RT-PCR (Polymerase Chain Reaction)

Cells grown in monolayer were lysed by adding Ultraspec™ RNA kit (Amsbio, Milton Park, Abingdon, UK) following the manufacturers instruction. The PCR cocktail consisted of DEPC- H_2O (9.5 μL), cDNA (5 μL), 10X PCR buffer (2.5 μL), 2.5 mM dNTP (2.5 μL), 5 μM/mL 5′ primer (2.5 μL), 5 μM/mL 3′ primer (2.5 μL), Taq (0.5 μL). Samples were incubated in a Techne PHC-3 thermal cycler for a total of 18 or 25 cycles with appropriate primers (Table 1). Each cycle was carried out for 1 min at 94 °C followed by 1 min at 50 ~ 55 °C and 2 min at 72 °C. PCR products were visualized on a 1.2 % TBE agarose gel containing GelStar stain (Lonza, NJ, USA) under UV illumination.

Whole Cell and Nuclear Extraction

For whole cell extraction, the cells were extracted in a cell lysis buffer (50 mM Tris-base, 0.5 M NaCl, 1.0 mM EDTA, 1 % NP40, 1 % Glycerol, 1 mM -Mercaptoethanol, proteinase k inhibitor). The extracts were collected by centrifugation. For nuclear extraction, the cells were washed with PBS and 1 mL ice-cold PBS was added and the cells were scraped gently and collected into a 1.5 mL tube. The contents were centrifuged at 4 °C for 5 min at 500 rpm, and carefully supernatant was aspirated with pipet. The pellet was suspended in 200 μL ice cold buffer I (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, Proteinase inhibitor cocktail tablet in 10 mL) and incubated for 15 min on ice to allow cells to swell. To the cell suspension added Igepal-CA630 to 1 % (100 μl of a 10 % stock solution), vortexed for 10 s and centrifuged for 2–3 min at maximum speed (~15 K RPM). The pellet was suspended in 100 μL ice cold buffer II (20 mM Hepes pH 7.9, 25 % glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, Proteinase inhibitor cocktail tablets in 10 mL) and vortexed for 30 s; rotated vigorously at 4 °C for 30 min. The nuclear fraction was centrifuged for 15 min at maximum speed; the supernatant was collected in a fresh, chilled tube.

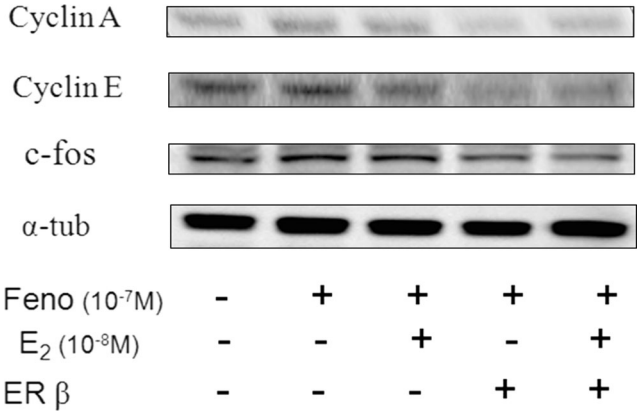

Fig. 1 E_2 and/or ERβ inhibited the effects of PPARα-activated cell proliferation related proteins Hep3B cells were transfected with pcDNA3 or ERβ (5 μg), and treated with or without E_2 (10^{-8} M) and fenofibrate (10^{-7} M) for 16 h. Lane 1, 2 and 3 were transfected with pcDNA3; Lane4 and 5 were transfected with ERβ. Lane1 and 2 treated by 100 % etOH; Lane3 and 5 treated by E_2 (10^{-8} M). Lane1 treated with DMSO and Lane 2–5 were treated by fenofibrate

Immunoprecipitation

The cells were Lysed with cold IP buffer 40 and incubated at 4 °C for 30 min and vortexed for six times. The suspension was centrifuged at 4 °C for 10 min to the supernatant was collected as the total protein. To 100 μg of total protein, 10 μL of protein G (for IgG1)-agarose and 500 μL of IP buffer

Table 1 Primers used for semi-quantitative RT-PCR analysis for mRNA expression

Gene	Primer sequence(5 → 3)	Length (bp)	T_m (°C)	Cycles
ACO	5′-GTGGGCGCATACATGAAGGAGACC-3′ 3′-GTGGCTGGATGCGCTGACTGG-5′	367	50	30
PPARα	5′-TCCTGCAAGAAATGGGAAAC-3′ 3′-GTTGTGTGACATCCCGACAG-5’	428	50	30
ERβ	5′-GGAGACAGAGAAGTGCCGAC-3’ 3′-TCTACGCATTTCCCCTCATC-5’	424	55	30
pHe7	5′-CTTCGAAAGGCAAGGAGGAA-3’ 3′-TGGCTCTACAATCCTCAGCA-5’	291	53	25

were added and then incubated with agitation at 4 °C for 1 h. The agarose was pelleted by brief centrifugation and the supernatant was transferred to a new eppendorf tube. To each sample 2 μg of antibody was mixed and incubated with agitation at 4 °C overnight. Next day, 20 μL of protein G (for IgG1)-agarose was mixed and incubated with agitation at 4 °C for 2 h. The protein G (for IgG1)-agarose was pelleted by brief centrifugation. The pellet was washed 5 times with IP buffer by repeating centrifugation step as above. The complex of protein, antibody and protein G-agarose was analyzed by western blot.

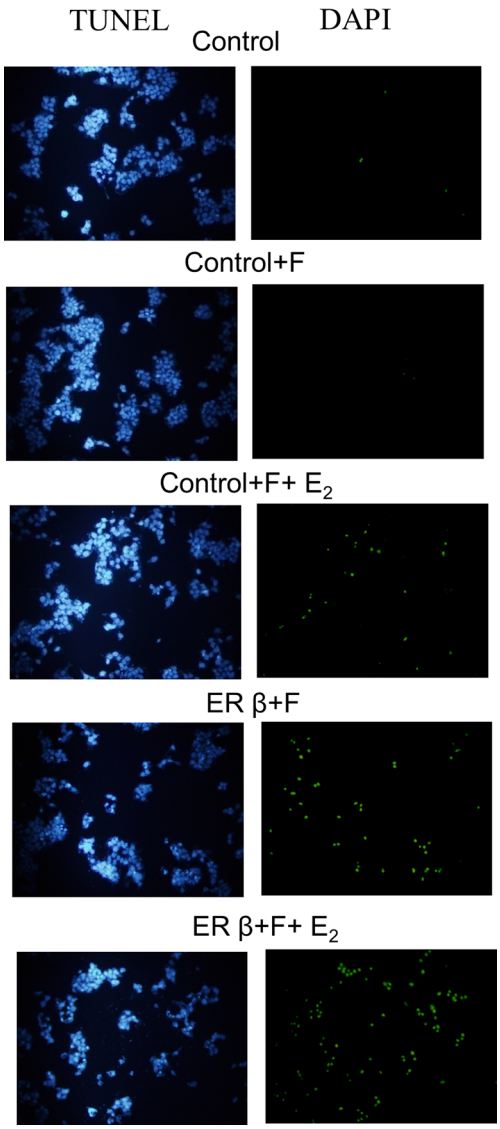

Fig. 2 Effect of E_2 and/or ER β on the cell apoptosis by TUNEL The Hep3B cells which were transfected PADNA3 or ERβ (5 μg) treated with or without E_2 (10^{-8} M) and fenofibrate (10^{-7} M) for 16 h. The control group was transfected with pcDNA3 and treated with DMSO and 100 % etOH, Control + F group was transfected with pcDNA3 and treated with fenofibrate and 100 % etOH. Control + F + E_2 group was transfected with pcDNA3 and treated with fenofibrate and E_2 (10^{-8} M). ERβ + F group was transfected with ERβ and treated with fenofibrate. ERβ + F + E_2 group was transfected with ERβ and treated with fenofibrate and E_2 (10^{-8} M). TUNEL assay was performed after these treatments. The upper panel showed DAPI stain nucleus in blue and the lower panel represent TUNEL stained apoptotic nucleus in green

Western Blotting

The proteins of cell lysates were analyzed by 8 % or 12 % SDS-PAGE, and proteins were transferred to nitrocellulose (or PVDF) by electrophoresis. Residual protein sites were blocked in Tween/Tris-buffer saline (TBS) containing 5 % skimmed milk. The filters were incubated with primary antibodies in TBS plus 2.5 % skimmed milk at recommended concentrations for 2 h or at 4 °C overnight and incubated with secondary antibodies for 1 h at room temperature. Antibody reaction was visualized with enhanced chemiluminescence (ECL) reagent for Western blotting.

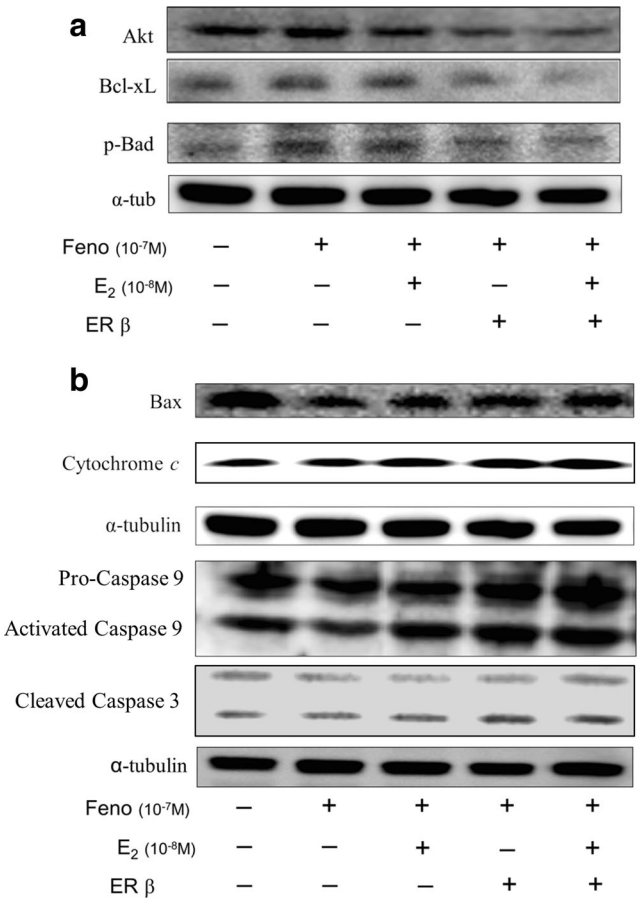

Fig. 3 Effect of E_2 and/or ER β on the expression of apoptotic and survival proteins. The Hep3B cells were transfected pcDNA3 or ERβ (5 μg) treated with or without E_2 (10^{-8} M) and fenofibrate (10^{-7} M) for 16 h. Lane 1, 2 and 3 were transfected pcDNA3; Lane4 and 5 were transfected ERβ. Lane1 and 2 were treated 100 % etOH; Lane3 and 5 were treated E_2 (10^{-8} M). Lane1 was treated DMSO and Lane 2–5 were treated fenofibrate. After these treatments, the cells were collected and applied to the following assays. (A) E_2 and/or ERβ overcome the effect of fenofibrate on the protein levels of Bcl-xL, p-Bad and Akt of survival pathway. (B) E_2 and/or ERβ overcome the effect of fenofibrate on the apoptotic protein levels of Bax, Cytochrome *c*, Caspase 9 and Caspase3

Electrophoretic Mobility Shift Assay (EMSA)

The binding of PPARα to the PPAR-specific oligonucleotide probe was assayed by adding 15 μg of crude nuclear extract from Hep3B cells to each gel shift reaction mixture. An oligonucleotide consensus element was synthesized with the following sequence: 5-GGAACTAGGTCAAA GGTCATCCCCT-3 along with an oligonucleotide of complementary sequence. The oligonucleotides were mixed (50 ng/μL final concentration) and denatured by heating to 95 °C for 10 min in 0.1 M Tris-HCl and 50 mM $MgCl_2$ (pH 7.9) and allowed to anneal by slowly cooling to room temperature. The annealed oligonucleotides were end-labeled with biotin. In a total volume of 20 μL of binding buffer (25 mM Tris-Cl, pH 7.5, 40 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 10 % glycerol), the following components were combined: 1 μg of poly(dI-dC), 15 μg of nuclear extract. For the supershift experiment, 2 μg of PPARα antibody was added to the reaction mixture before addition of the oligonucleotide probe. After 20 min of incubation at room temperature, the biotin labeled oligonucleotide was added, and the incubation was continued for a further 20 min. Samples were analyzed on a 4 % non-denaturing polyacrylamide gel, containing 30 % Acrylamide/Bisacrylamide (2.0 mL); 5xTBE buffer (2.0 mL)100 % Glycerol (1.0 mL), 1 % APS(500 μL), ddH_2O (4.5 mL), TEMED (10 μL)). All the samples were loaded to gel & were run at 120 V in the cold room at 4 °C for 2.5 h; and transferred for 1 h. The complex of probes and protein was crosslinked by UV crosslinking for 5 min. The membrane was blocked in blocking buffer for 30 min at room temperature. The membrane was hybridized at room temperature with streptavidin-HRP for 15 min. The membrane was washed 4 times for 5 min each in washing buffer. And use equilibration buffer to stop the reaction of probe and streptavidin. Probe reaction was visualized with enhanced chemiluminescence (ECL) reagent for EMSA.

Statistical Analyses

All experimental data were expressed as mean ± SD. The significance between experimental groups was measured by using one-way ANOVA. Data with $p < 0.05$ was considered statistically significant.

Results

E2 and/or ERβ Inhibit PPARα-Induced Cell Proliferation by Downregulating the Cell Cycle Regulating Proteins

To investigate whether E2 and/or ERβ inhibit PPARα-induced cell proliferation, the expression of cyclins and c-fos protooncogene were analyzed. The cells were transfected with lipofetamine which carried pcDNA3 (as transfection control) and pCMV-ERβ (5 μg), then the cells were treated with or without 10^{-8} M E2 and 10^{-7} M fenofibrate (activator of PPARα). The fenofibrate treatment caused enhanced expression of c-fos, cyclin A and cyclin E but were downregulated in E2, ERβ or combined E2/ERβ treatment groups. This result implies E2 and/or ERβ inhibit cell proliferation via downregulation of PPARα-induced cyclins and c-Fos expression (Fig. 1).

PPARα Inhibits Apoptosis but E2 and/or ERβ Reverses the Effect in Hep3B Liver Cancer Cells

To identify whether E2 and/or ERβ could inhibit Hep3B cells by superseding the effect of PPARα and by enhancing cell apoptosis, number of TUNEL positive cells among the control cells with pcDNA3 and cells with pcDNA3-pCMV-ERβ was determined. Fenofibrate induced PPARα exhibited anti-apoptotic effect on Hep3B liver cancer cells, but the activity was reversed in cells with pcDNA3-pCMV-ERβ and in cells treated with E2 (Fig. 2). The result therefore reveal that E2/or ERβ suppresses the tumorigenic effect of

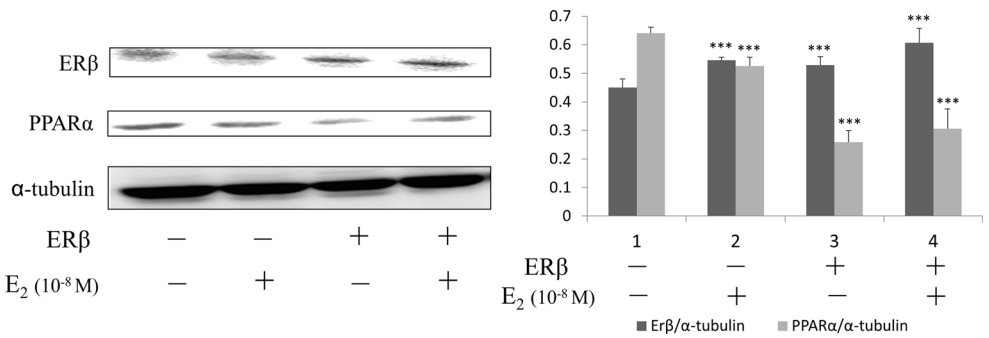

Fig. 4 E_2 and/or ERβ downregulate the protein level of PPARα on Hep3B liver cancer cells The Hep3B cells were transfected with pcDNA3 or ERβ (5 μg) treated with or without E_2 (10^{-8} M) for 16 h. Lane 1 and 2 were transfected with pcDNA3; Lane3 and 4 were transfected with ERβ. Lane 1 and 3 were treated with 100 % etOH; Lane 2 and 4 were treated with E_2 (10^{-8} M). α-tubulin was used as internal control. ***$p < 0.001$ represent significantly different as compared to untreated control group

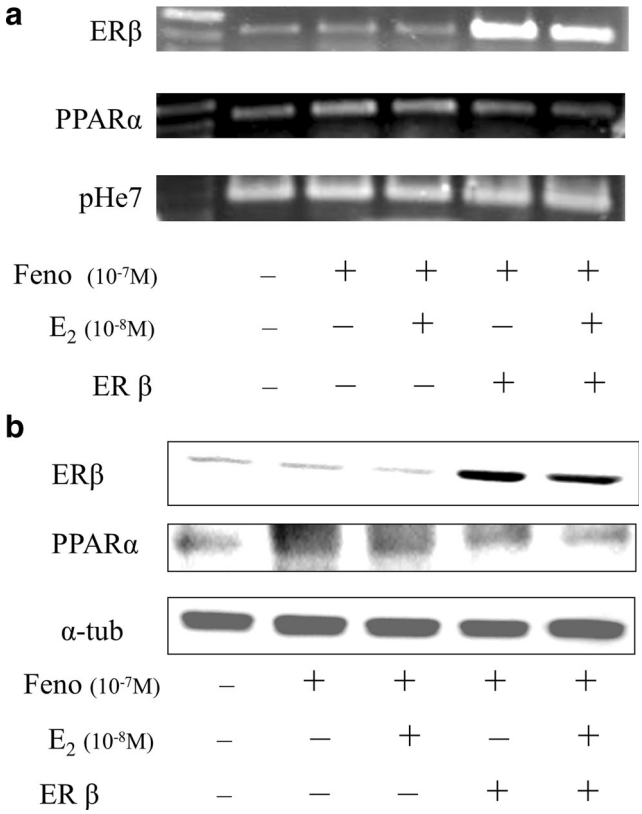

Fig. 5 E_2 and/or ERβ downregulate the fenofibrate-induced the mRNA and protein levels of PPARα on Hep3B liver cancer cells The Hep3B cells which were transfected with pcDNA3 or ERβ (5 μg) treated with or without E_2 (10^{-8} M) and fenofibrate (10^{-7} M) for 16 h. **a** mRNA levels of PPARα in Hep3B cells as determined by reverse transcription-PCR analysis. **b** Protein levels of PPARα in Hep3B cells as determined by western blotting analysis. Lane 1, 2 and 3 were transfected pcDNA3; Lane 4 and 5 were transfected ERβ. Lane 1 and 2 were treated with 100 % etOH; Lane 3 and 5 were treated with E_2 (10^{-8} M). Lane1 was treated DMSO and Lane 2–5 were treated with fenofibrate (10^{-7} M)

PPARα by accelerated apoptosis in Hep3B cells. Fenofibrate induced PPARα also enhanced anti-apoptotic proteins such as Akt, Bcl-xL, p-Bad and suppressed the levels of pro-apoptotic proteins such as Bax, Cytochrome *c,* cleaved Caspase-9, cleaved Caspase-3. However, E2 and/or ER*β* proved to be effective against the effect of PPARα by suppressing the anti-apoptotic proteins and enhancing the pro-apoptotic proteins (Fig. 3).

ERβ Suppresses PPARα with or without E2 in Hep3B Liver Cancer Cells

To clarify whether E2 and/or ER*β* would downregulate PPARα in Hep3B cells, the protein level of PPARα, in the cells with pcDNA3 and cells with pcDNA3-pCMV-ERβ, with respect to the level of ER*β* was determined. The results show that overexpression of ER*β* or treatment with E2 efficiently downregulates the protein level of PPARα (Fig. 4). The treatment of fenofibrate induced the mRNA and protein expression levels of PPARα. E2 and/or ERβ downregulated PPARα expression effectively in Hep3B cells treated with 10^{-7} M fenofibrate (Fig. 5). The suppressive effect of E2 and/or ER*β* on PPARα was reversed when treated with 10^{-8} M of PHTPP.

E2 and/or ERβ Inhibit PPARα Activity and its Downstream Gene ACO Expression

Treatment with fenofibrate enhanced the mRNA expression of ACO and the expression was higher than the control group. However ACO expression was downregulated by E2, ERβ or combined E2/ERβ treatment. This result implies that E2 and/or ERβ inhibit not only PPARα expression but also characteristic PPARα activity (Fig. 6).

Binding Capacity of ERβ to Peroxisome Proliferator Response Element (PPRE) to Regulate PPARα Gene Regulation

To investigate if ER*β* directly binds to PPRE and prevents PPARα-induced downstream expression the nuclear proteins were extracted and the binding capacity of the respective proteins was observed by Electrophoretic Mobility Shift Assay (EMSA). The shift in PPRE probe band towards the ERβ show that ERβ directly binds to PPRE and might inhibit PPARα gene expression. The appearance of a super shift band

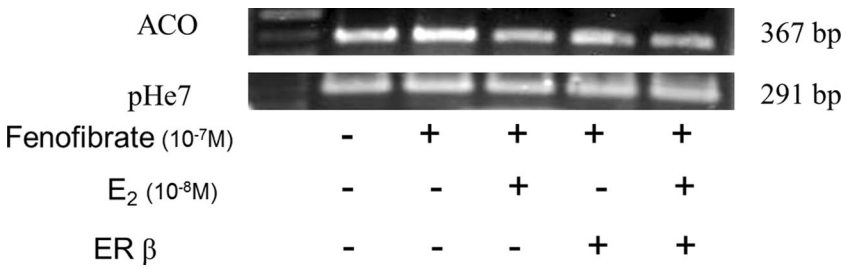

Fig. 6 E_2 and/or ERβ reduced the PPARα downstream gene mRNA expression The Hep3B cells were transfected pcDNA3 or ERβ (5 μg), and treated with or without E_2 (10^{-8} M) and/or fenofibrate (10^{-7} M) for 16 h. Lane 1, 2 and 3 transfected with pcDNA3; Lane 4 and 5 were transfected with ERβ. Lane 1 and 2 were treated by 100 % etOH; Lane 3 and 5 were treated by E_2 (10^{-8} M). Lane 1 treated with DMSO and Lane 2–5 treated with fenofibrate. pHe7 was used as RT-PCR internal control

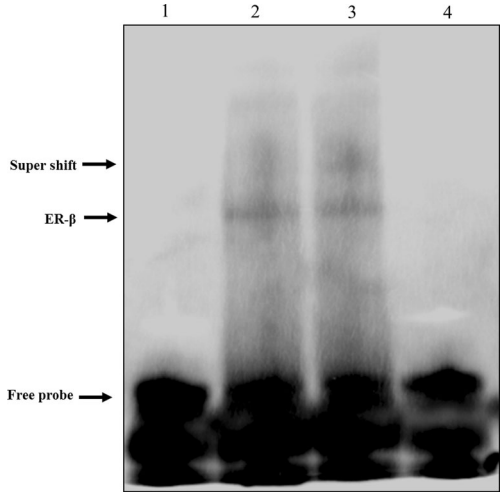

Fig. 7 The binding capacity of ERβ on Perioxisome proliferator response element PPRE The Hep3B cells which were transfected ERβ (5 μg) treated with E_2 (10^{-8} M)and fenofibrate (10^{-7} M) for 16 h. Lane 1 is control including only PPRE probe; Lane 2 is including nuclear protein and PPRE probe; Lane 3 is including nuclear extract proteins, PPRE probe and ERβ antibody; Lane 4 is including PPRE probe and 5× PPRE competitor

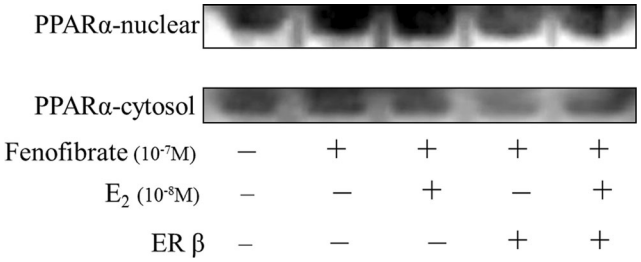

Fig. 9 E_2 and/or ERβ inhibit the fenofibrate-activated the nuclear translocation effect of PPARα Hep3B cells were transfected with pcDNA3 or ERβ (5 μg), and treated with or without E_2 (10^{-8} M) and/or fenofibrate (10^{-7} M) for 16 h. Lane 1, 2 and 3 were transfected with pcDNA3; Lane 4 and 5 were transfected with ERβ. Lane 1 and 2 treated 100 % etOH; Lane 3 and 5 were treated E_2 (10^{-8} M). Lane 1 treated DMSO and Lane 2–5 treated fenofibrate

when the nuclear extract was mixed with ERβ and absence of the band shift when mixed with PPRE competitor further confirms that ERβ binds directly to PPRE and inhibit PPARα gene expression (Fig. 7). However, immunoprecipitation assay to find the binding efficiency of ERβ with PPARα showed that ERβ also binds strongly with PPARα. But the binding capacity drop gradually when treated with different concentrations (10^{-7}, 10^{-6}, 10^{-5} M) of Fenobirate (Fig. 8). Therefore ERβ potentially binds with PPRE to appropriately regulate PPARα gene regulation.

E2 and/or ERβ Inhibit the Translocation of PPARα from Cytosol to Nucleus

To observe whether E2 and/or ERβ could prevent fenofibrate-induced PPARα translocation, the cells were transfected with pCMV-ERβ (5 μg) and then the cells were treated with or without E2 (10^{-8} M) and fenofibrate (10^{-7} M). The treatment with fenofibrate increased PPARα nuclear translocation than in control groups but the effect was reversed in ERβ overexpressed cells. This result implies that ERβ inhibits the translocation of PPARα from cytosol to nucleus (Fig. 9).

Discussion

Long-term administration of hypolipidemic drugs, peroxisome proliferators, induced hepatic disorders and hepatocarcinomas [20]. The PPARα subtype of PPARs is predominantly expressed in liver, kidney, heart, and vascular tissues [20–22]. PPARα is activated by various ligands, including the fibrate class of hypolipidemic drugs such as clofibrate; fenofibrate, fatty acids, and eicosanoids, to induce the transcription of genes containing a PPRE in its promoter region. PPARα work as a transcription factor that translocate to nucleus and binds to PPREs as heterodimers with retinoid X receptors (RXRs) to form PPARα:RXRα heterodimers [23]. PPARα-induced genes are involved in peroxisomal and mitochondrial fatty acid β-oxidation such as L-type carnitinpalmitoyl transferase I, ACO, cytochrome P450 4 A1, medium-chain acyl-CoA dehydrogenase and long-chain acyl-CoA dehydrogenase [24, 25]. The ACO expression usually reflects the PPARα activity.

PPARα increases the ACO which is a H_2O_2-generating enzyme and causes detrimental effects on cells through oxidative DNA damage [26]. The results show that increase

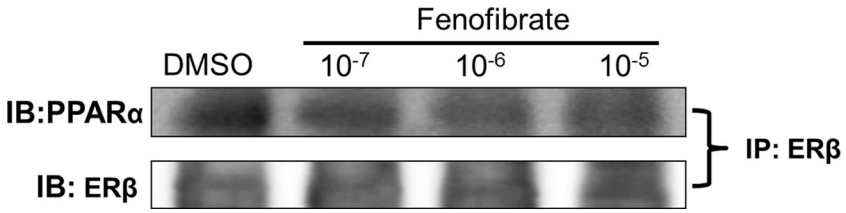

Fig. 8 The binding capacity between PPARα and ERβ was reduced by fenofibrate The Hep3B cells which were transfected ERβ (5 μg) treated with E_2 (10^{-8} M) and different concentrations of fenofibrate (0, 10^{-7}, 10^{-6}, 10^{-5} M). After 16 h, the cell lysate were applied into co-immunoprecipitation and western blotting assay

in PPARα expression induced by fenofibrate increases the expression of ACO. Therefore PPARα expression adversely affects Hep3B cells. Impairment in cell cycle regulation plays a central role in promoting hepatocarcinogenesis through evasion of growth suppressors, sustaining proliferative signaling, and resistance to cell death [27]. PPARα increases expression levels of several proteins, such as proto-oncogenes (c-Fos and c-Myc), cell-cycle regulators such as cyclin D1, cyclin-dependent kinase [CDK 4], and PCNA, and phosphorylated ERK 1 and 2, all of which are associated with cell proliferation [25]. While cell cycle progression is regulated through cdks, passage through G1 into S phase is regulated by the activities of cyclin D–, cyclin E–, and cyclin A–associated cdks [28].

Our present results clearly show that PPARα expression is correlated with expression of cell cycle regulators such cyclin A, Cyclin E and proto-oncogenes such as c-Fos. Cyclin E remains stably expressed in HCC and can accelerate cell-cycle progression from the G1 to S phase and reinforce the loss of growth control [27, 29]. Activation of PPARα has been shown to increase proliferation in cancers of the breast, brain, colon and in rodent livers [30]. PPARα mediated gene transcription is also known to suppress hepatocyte apoptosis in response to peroxisome proliferators [31]. Increase in PPARα expression induced by fenofibrate also reduced the number of TUNEL positive apoptosis cells in Hep3B cells. Inhibition of apoptosis by peroxisome proliferators, PPARα could be responsible for the carcinogenic effects [32–35].

Estrogen (E_2) is suggested to play a crucial role in protecting the liver tissues through ERα and β to decrease the PPARα reporter gene expression [18]. Interestingly, the results reveal that the protein level of PPARα was downregulated by E_2, ERβ or by combined E_2/ERβ treatment. Further the enhanced proliferation and decreased apoptosis following fenofibrate administration were also reversed when Hep3B cells were treated with E_2, ERβ or by combined E_2/ERβ treatment. E_2 and/or ERβ prevented PPARα translocation from cytosol to nucleus and reduced the PPARα activity on transcription. Taken together, ERβ obviously downregulate PPARα gene expression to suppress the proliferation and induce the apoptosis of Hep3B cells. However, as the experiments were done in an HCC cell line the possibility of tumor related gene dysregulation might have an impact on the result. But with the available evidence we conclude that ERβ is a co-repressor which directly binds to PPARα promoter sequence and suppresses PPARα associated hepatocarcinognesis.

Compliance with Ethical Standards

Financial Statement This study is supported in part by Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence (MOHW105-TDU-B-212-133,019).

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