Estrogen receptor α (ESR1) over-expression mediated apoptosis in Hep3B cells by binding with SP1 proteins

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Abstract

Previous studies have reported that estrogen receptors (ERs) are expressed in normal human liver, chronic hepatitis, and benign hepatic tumor tissues. However, decreased expression of ERs can be observed in hepatocellular carcinoma (HCC) and the role of ERs in HCC is not fully understood. Thus, the present study aimed to investigate the molecular mechanism induced by the overexpression of ERα (ERα (ESR1)) in Hep3B cells. We first detected the induction of apoptosis in ERα-negative Hep3B cells using DNA fragmentation assay and flow cytometry. Additionally, western blotting showed increased expression of active caspase 3 and tumor necrosis factor α (TNFα (TNF)) in ERα-transfected cells. To further understand the importance of SP1-binding sites in the TNFα promoter, ERα-negative Hep3B cells were co-transfected with ERα and a wild-type TNFα plasmid or TNFα with deleted SP1 regions. Deletion of both distant and primal SP1 sites abolished the activity of ERα, and similar results were observed by blocking the expression of SP1 protein using mithramycin (MA). This result indicates that SP1 protein is essential for ERα-activated TNFα promoter activity. Co-immunoprecipitation assay further confirmed the binding interaction between ERα and SP1 in a ligand-dependent manner. In general, we demonstrate that the overexpression of ERα mediates apoptosis in ERα-negative Hep3B cells by the binding of ERα to SP1 protein. Additionally, this ERα-SP1 complex binds to the proximal and distal sites of the TNFα gene promoter and further induces the expression of active caspase 3 in a ligand-dependent manner.

Key Words
- ERα
- TNFα
- SP1
- Hep3B cells
- caspase 3
- apoptosis
Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer mortality worldwide with approximately one million fatalities every year (Pisani et al. 1999, El-Serag 2002). The pathogenesis of HCC depends on various factors including environmental, infectious, nutritional, metabolic, and endocrine factors (Rocken & Carl-McGrath 2001). 17β-Estradiol (E2) is the most effective endogenous estrogen that controls the growth, differentiation, and function of various tissues through two distinct intracellular receptors: estrogen receptor α (ERα) and ERβ (Mishkin et al. 1983, Shimizu et al. 1998). Upon ligand binding, ERs dimerize and then bind to specific response elements known as estrogen response elements (EREs) located in the promoters of target genes (Kuiper et al. 1997). Apart from binding to the EREs, ERs directly bind to the transcription factor (TF) complex and regulate the transcription of target genes (Bjornstrom & Sjoberg 2005).

Previous studies have shown that ERα interacts with both SP1 and SP3, and the C-terminal DNA-binding domain (DBD) of SP1 is the major interaction site for ERα (Kim et al. 2005). ERs directly bind to TFs such as activator protein 1 (AP1), nuclear factor κB (NFκB (NFκB1)), and specificity protein 1 (SP1), thereby recruiting their co-activators to activate the transcription of their target genes (Marino et al. 2006). Specificity protein 1 (SP1) is a ubiquitously expressed TF that encompasses several domains, of which the DBD is the most conserved domain in the SP family (Deniaud et al. 2009). Wang et al. (1999) showed that ERα–SP1 protein complexes are required for the transcriptional activation of the E2F1 gene. SP1 binds to GC-rich sequences in the promoters of numerous genes and its binding is abrogated by various cellular stimuli such as cytokines (Li et al. 2004).

Tumor necrosis factor α (TNFα (TNF)) is a major pro-inflammatory cytokine that controls the activation and inhibition of gene transcription, metabolism, and protein synthesis in the liver (Grivennikov et al. 2006, Foka et al. 2009). However, reduced expression of GSK3β (GSK3B), TNFα, TNF-R I, TNF-R II, and IL10 can be observed in HCC and in other liver diseases (Zekri et al. 2009). It is also well accepted that estrogen functions as an immunomodulator with a bidirectional effect on cytokine production: at high concentrations, E2 decreases the production of pro-inflammatory cytokines TNFα and IL1β (IL1B) (Ito et al. 2001, Hsieh et al. 2007), whereas at low concentrations, it increases the production of TNFα and IL1β (Roby & Hunt 1994). In breast cancer cells, TNFα partly controls the growth by downregulating the expression of ERα (ESR1) through PI3K/Akt signaling (Lee & Nam 2008), whereas in ERβ (ESR2)-overexpressing colon cancer cells, E2 treatment induces apoptosis without altering the expression of TNFα (Hsu et al. 2006).

Thus, the present study aimed to investigate the role of ERα in the expression of the pro-inflammatory cytokine TNFα and SP1 in ERα-negative Hep3B cells. Our data clearly suggest that ERα binds to SP1 and induces the complex that forms to bind to the SP1 site of the TNF promoter in the Hep3B cell line.

Materials and methods

Materials

All reagents for cell culture, E2, MA, and anti-ERα antibody were purchased from Sigma–Aldrich. ERα, caspase 3, and α-tubulin antibodies were purchased from Santa Cruz Biotech. TNFα and GAPDH antibodies were purchased from Cell signalling (Beverly, MA, USA) and SP1 antibody was purchased from Abcam (Cambridge, MA, USA). Transfection kit (Lipofectamine 2000 transfection kit) was purchased from Invitrogen. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotech. Luciferase kit was purchased from Promega Corp.

Cell culture

The human hepatoma cell line Hep3B was maintained in MEM (supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and penicillin/streptomycin) at 37 °C in a 5% CO2 atmosphere. Transfections were carried out using liposomes with a phenol red-free and serum-free medium followed by treatment with E2 for 12–16 h. The cells were seeded in 60 mm dishes and were cultured for 24 h in MEM supplemented with 10% FBS, rinsed with serum-free MEM and 1 ml MEM containing 15 μg/ml liposome, and 2 or 4 μg pcDNA. The cells were incubated at 37 °C for 6 h before adding 1 ml MEM supplemented with 10% FBS to the medium. After incubation for 18 h, the medium was replaced with fresh 10% FBS MEM. The concentrations of E2 (10−8 M) and MA (200 ng) used in these experiments have been described previously (Koutsodontis & Kardassis 2004, Lai et al. 2010).
Plasmid constructs

pCMV ERs were kindly provided by Dr Benita S Katzenellenbogen, University of Illinois, Urbana, USA. The promoter region of the hTNFα gene (GenBank accession no. U42625) was prepared by PCR using the 5′ primer-GGTACCGGGAGTGTGAGGGGTATCCT and the 3′ primer-AAGCTTGTCCTTTCCAGGGGAGAG. The PCR product of this gene contained a KpnI restriction site at the 5′ end and a HindIII site at the 3′ end, and it was inserted into a T&A vector (Yeastern Biotech Corp., Taipei, Taiwan). Stable clones were selected by culturing on plates with ampicillin (50 μg/ml). The recombinant gene was then inserted into the pGL3 Basic Vector (Promega Corp.).

Isolation of apoptotic DNA fragments

Isolation of apoptotic DNA fragments was done according to the method of Herrmann et al. (1994). Following treatment, cells were collected and washed with 1× PBS and then lysed using DNA lysis buffer (20 mM EDTA, 100 mM Tris, pH 8.0, and 0.8% SDS) at room temperature for 20 min. After centrifugation for 5 min at ~16 000 g supernatants were collected and treated with RNase A (final concentration 3.33 μg/μl) for 1 h min at 50 °C, followed by digestion with proteinase K (final concentration 2.5 μg/μl) for 2 h at 56 °C. The DNA samples were electrophoresed in 1.5% agarose gel.

Flow cytometry

Apoptosis was determined using annexin V–FITC and propidium iodide staining. The cells were washed with PBS and suspended in annexin V binding buffer, and cell aggregates were filtered through Falcon filter top tubes. The cellular DNA content was determined by FACS analysis using a FACS Calibur flow cytometer (BD PharMingen, SD, USA). Data were analyzed using CellQuest (BD PharMingen) and ModFit (Verity, Topsham, ME, USA).

Co-immunoprecipitation assay

The cells were lysed in 50 mM Tris–HCl, pH 8, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 10% glycerol was done using the FACS analysis. **P<0.01 values were calculated between the ERα-overexpressing cells and vector control using Student’s t-test. (D) Hep3B cells were transfected with pcDNA3 (2 μg) or wild-type ERα (2 μg) and then treated with LPS/or E2 (10^–8 M). Cell lysates were collected and analyzed for the expression of caspase 3 using western blot analysis. α-Tubulin was used as a loading control for western blotting experiments.
with protease and phosphatase inhibitors. Cell lysates were centrifuged at 12,000 g for 20 min, and 100 µg of total protein were mixed with 2 µg of anti-ERα and SP1 antibodies and 20 µl of protein G beads, and the samples were rotated at 4°C for overnight. The samples were then pelleted, washed, and suspended in 50 µl of 2× Laemmli buffer for immunoblotting.

**Western blot analysis**

Total proteins were extracted and separated using 10% SDS–PAGE. The proteins were further transferred onto PVDFs (Amersham). The membranes were incubated with specific primary antibodies and washed with Tris-buffered saline containing Tween-20 (TBST) and then incubated with peroxidase-conjugated secondary antibodies. Finally, signals were detected using the ECL detection kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**RT**

A total amount of 5 µg of RNA was used for the RT reaction. The RT reaction was performed at 37°C for 60 min using 55.5 µl DEPC H₂O, 4 µg total RNA, 0.5 µl RNase inhibitor (40 U/µl; Promega Corp.), 20 µl 5× RT buffer, 8 µl dNTP (2.5 mM), 10 µl oligo-dT (5 µM/ml) (Misson Biotech, Taipei, Taiwan), and 2 µl MMLV reverse transcriptase (200 U/µl; Promega Corp.). The resultant cDNA was added to the PCR mixture containing 9.5 µl DEPC water, 2.5 µl 10× PCR buffer (MD Bio, Taipei, Taiwan), 2.5 µl dNTP (10 mM; Promega Corp.), 0.5 µl each primer (5 µM/ml) (MD Bio, Taipei, Taiwan), and 2.5 µl of each primer (5 µM), 0.5 µl Taq (2 U/µl) (MD Bio), and 4 µl of 2.5 mM DNT buffer. Aliquots of the PCR products were electrophoresed on 1.5% (w/v) agarose gel.

**Luciferase assay**

Transient transfections of pGal–hTNFa–Luc or hTNFa–Luc mutant 1 (deleted 13mer CCCCGCCCCGCCG) or mutant 2 (deleted 8mer CCCCCGCC) were performed using the Qiagen plasmid Maxi Kit (Invitrogen). The transfected cells were treated with 10⁻⁸ M E₂, 200 ng MA (a SP1 agonist), or an ethanol vehicle as indicated. β-Galactosidase activity was used to normalize for transfection efficiency. The transfections were repeated at least twice to ensure the reproducibility of the results. The control pGL3 Basic Vector was used as an internal control for transfection and pGal–ERE–Luc was used as a positive control.

**Statistical analysis**

All values are expressed as means±s.d., and they were evaluated for statistically significant differences using one-way ANOVA. P<0.05 was considered statistically significant.
Results

ERα induced apoptosis in the Hep3B cells

Previous studies have indicated that E2 binds to its receptors (ERs) and modulates gene expression either in a classical or a non-classical manner (Marino et al. 2006) in myocardial cells (Liu et al. 2009), HepG2 cells (Marino et al. 2002), and HeLa cells (Acconcia et al. 2005). Thus, we induced the overexpression of ERα and then detected for the expression of ERα in the presence of E2 to determine whether E2 can induce the expression of ERα in Hep3B cells. Figure 1A shows that the protein levels of ERα in the empty plasmid-transfected Hep3B cells were not affected by E2; however, increased expression of protein was observed after E2 stimulation in the ERα-transfected cells.

To determine whether ERα mediates apoptosis through an E2-dependent pathway, we transfected cells with a vector/ERα and evaluated apoptosis using DNA fragmentation assay following incubation with or without E2. E2 treatment increased the apoptotic ladder in the ERα-transfected cells than in the pcDNA3-transfected cells (Fig. 1B). However, E2 treatment did not reproduce the yield of apoptotic DNA fragments in the ERα-overexpressing cells. This was consistent with the data obtained from the annexin V–FITC/propidium iodide double staining. As shown in Fig. 1C, E2 treatment greatly increased the level of apoptosis in the ERα-transfected cells than in the vector-transfected cells, whereas this increase was not greatly altered by E2 treatment as that observed with transfection with ERα alone.

As caspase 3 is a crucial factor for hepatitis, we further examined the effects of ERα and E2 on the expression of caspase 3. Pro-caspase 3 was detected in the ethanol- or ERα- or LPS-treated vector-transfected Hep3B cells and in the ethanol- or E2-treated ERα-transfected cells. In contrast, the active form was only detected in the ERα-transfected cells and in the ERα plus E2-treated cells. As shown in Fig. 1D, the expression of caspase 3 was not significantly altered in both the vector- and ERα-transfected cells. From these data, we can speculate that ERα functions in an E2-independent manner.

ERα overexpression induced TNFα expression

E2 and ERα negatively regulate the secretion of TNFα in various systems (Lambert et al. 2004). To further validate the effects of ERα on the expression of TNFα, Hep3B cells were transfected with a vector for 6 h and then stimulated with or without LPS to upregulate the release of cytokines (Yang et al. 1998). In another group, Hep3B cells were transiently transfected with ERα and further incubated with E2 for 16 h. As shown in Fig. 2A and B, a small increase in the secretion of TNFα was observed with E2 or

![Figure 3](https://jme.endocrinology-journals.org/assets/figures/00853107.jpg)

**Figure 3**

Apoptotic effect detected in the Hep3B cells. The cells were transfected with ERα and treated with E2 (10⁻⁸ M) or TNFα (50 ng/ml) or with anti-TNFα (0.1 ng/ml) for 16 h. (A) The cells were collected and analyzed for DNA fragmentation using 1.5% agarose gel. (B) The x-axis (FL1) indicates annexin V–FITC fluorescence; the y-axis (FL3) indicates PI fluorescence. Late apoptotic (annexin V–FITC-positive) cells are shown in the upper right quadrant of the dot plot. A typical result from three independent experiments is shown. (C) The vertical bars represent means ± s.e.m. from five independent experiments. In the pcDNA3 transfectants, **P<0.01 vs TNFα. In the ERα transfectants, anti-TNFα vs ethanol or E2, ***P<0.01.
LPS treatment; however, a significant stimulatory response was observed in the ERα and E2 plus ERα-treated Hep3B cells. E2 treatment did not affect the secretion of TNFα in response to the overexpression of ERα.

To further examine the role of ERs and estrogen in the induction of the secretion of TNFα, we transfected Hep3B cells with a reporter construct containing the TNF promoter in front of the luciferase gene (TNFα–Luc) and co-transfected with an ERα expression vector. This transactivation property of ERα was further compared using the ERE–Luc plasmid. In the pcDNA3-transfected cells, either EtOH or E2 treatment increased the basal levels of TNFα; however, after transfection with ERα, luciferase activity was highly increased and there was no significant increase in the latter transfectants treated with E2. Compared with the vector-transfected cells, the ERα-transfected cells exhibited increased expression of TNFα. Our result shows that the overexpression of ERα stimulated TNFα promoter activity in an E2-independent manner. However, ERα strongly bound to the ERE promoter region and the luciferase signal was increased twice in an E2-dependent manner (Fig. 2C).

**Activation of TNFα by ERα induced apoptosis in an E2-independent manner**

The pcDNA3-transfected cells treated with E2 (10^{-8} M) or TNFα (10 ng/ml) exhibited many apoptotic DNA fragments than the ethanol-treated cells (Fig. 3A, lanes 1, 2 and 3). However, in the ERα-transfected cells, treatment with E2 increased the number of apoptotic fragments, whereas treatment with the hTNFα antibody resulted in a reduced number of apoptotic DNA fragments (Fig. 3A, lanes 4, 5 and 6). Similar results were obtained on interpreting the results using annexin V–FITC/propidium iodide double staining (Fig. 3B). These results suggest that ERα induced the expression of TNFα and further induced apoptosis via an ER-dependent pathway.

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**Figure 4**

Analysis of binding affinity by luciferase activity and TNFα promoter activity after overexpression of ERα and estradiol (E2) treatment. (A) Schematic representation of TNFα gene used in this study. (B) Enhancement of the non-ERE TNFα promoter activity by the overexpression of ERα in the Hep3B cells. Hep3B cells were transiently co-transfected with pcDNA-3 or ERα with pGL3–hTNFα–Luc or ERE–TNFα–Luc and then treated with either ethanol or E2 for 16 h. TNFα promoter gene activity was measured using luciferase assay. All treatments of each bar are described as indicated. The ERE–Luc activities were used as positive control. These results were based on three independent experiments. Experimental group vs control group is represented as ***P < 0.001. (C) TNFα promoter activity was analyzed after E2 and mithramycin A (MA; SP1 inhibitor) treatment in the Hep3B cells. Hep3B cells were transiently co-transfected with TNFα–Luc and ERα. Bar 1, pGL3 transfection. Bars 2 and 3, pGL3 co-transfected with wt ERα. Bars 4 and 5, wt ERα co-transfected with pGL3–TNFα–Luc. Bars 1, 2, and 4, ethanol treatment. Bar 3, E2 (10^{-8} M) treatment. Bar 5, MA (200 ng/ml) treatment. The results were obtained from three independent experiments. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-13-0085
ERα induced TNFα transcription through SP1

To further investigate the mechanism by which ERα and E2 activate TNFα transcription and to identify the promoter-specific effect of the deletion of the SP1 site on TNFα, vectors expressing wild-type TNFα or TNFα with a deletion in the proximal or distant SP1-binding motif (Fig. 4A) were individually co-transfected with ERα into the Hep3B cells. The reporter gene activity was determined in cells that had been either left untreated or stimulated with E2 for 16 h. Stimulation of the ERα-transfected cells with TNFα alone or TNFα plus E2 produced 4.1- and 5.2-fold increase in luciferase activity respectively. In contrast, deletion of the SP1 site in TNFα impaired the trans-activating activity of ERα. These results suggest that the SP1 site of TNFα is critically involved in the interaction of ERα and the TNFα promoter through the SP1-binding sites (Fig. 4B).

MA binds to GC-specific regions of DNA and inhibits the transcription of a number of genes including SP1 (Ryuto et al. 1996). We evaluated the effect of MA on ERα-induced TNFα expression. Initially, Hep3B cells were co-transfected with ERα without TNFα-pLuc expression and then treated with an E2 ligand. We found that E2 treatment was unable to increase the luciferase signal (Fig. 4C); however, in the presence of TNFα-pLuc plus ERα, E2 treatment was able to stimulate luciferase signal significantly (Fig. 4B).

The cells were further treated with or without E2 or MA and relative luciferase activity was determined. The ERα- and TNFα-co-transfected cells exhibited increased luciferase activity up to 25-fold when compared with the control cells. This induction of the TNFα promoter by ERα was further prevented by MA treatment (Fig. 4C). Therefore, we conclude from this set of experiments that SP1 is important for TNFα-mediated promoter activation.

Detection of ERα-SP1 interactions by co-immunoprecipitation assay

Direct interactions between ERα and SP1 proteins were detected using co-immunoprecipitation assay in vitro. Cells transfected with ERα and whole-cell lysates from the transfected cells were treated with EtOH or E2 and then immunoprecipitated with the SP1 or ERα antibodies. Western blot analysis of these lysates with SP1 or ERα antibodies showed that E2 enhanced the binding interaction between ERα and SP1. Similar results were obtained while using ERβ (Fig. 5).

Discussion

In order to better understand the consequences of high expression of ERα in ERα-negative Hep3B cells, we induced the overexpression of ERα and detected for the induction of apoptosis by analyzing the expression of caspase 3. Interestingly, transient overexpression of ERα increased the expression of caspase 3 and induced apoptosis in an estrogen-dependent manner. Similar results were observed while inducing the overexpression of ERα in ERα-negative breast cancer cells (Tolhurst et al. 2011). It has been reported that in ER-negative MDA-MB-231 breast cancer cells re-expression of ERα results in the suppression of proliferation by E2 (Moggis et al. 2005).

ERα and ERβ belong to a large superfamily of nuclear hormone receptors, comprising a DBD along with a
carboxy-terminal hormone-binding domain and a N-terminal domain required for recognizing GC-rich (GGGCGGGGG) promoter sequences (Kuiper et al. 1996). ERs form homo- and heterodimers to interact either directly with genomic targets encoded by EREs (5’-GGTCAnnnTGACC-3’; Katzenellenbogen & Katzenellenbogen 2002) or indirectly by binding to nuclear proteins, such as AP1 and SP1, through the zinc finger domain (Shang et al. 2000, Marino et al. 2006, Lin et al. 2007). SP1 and SP3 are the members of the SP family of TFs that are involved in the expression and regulation of many genes. Although SP1 has been described as a transcriptional activator, it may also function as a repressor based on the promoter or co-regulators it binds to (Kaczynski et al. 2003). Wilson et al. (2010) have shown that histone deacetylase inhibitors induce apoptosis in colon cancer by activating the SP1 protein in a non-classical manner. Consistent with these findings, we observed that E2 enhanced ERα-SP1 interactions with GC-rich motifs in the ERα-transfected Hep3B cells.

Using a reporter gene construct linked to TNFα (GC rich) or ERE, we proved that ERα binds to TNFα and induces its transactivation in an E2-dependent manner. SP1-binding sites in the promoter are the critical regulatory cis-acting elements that mediate the activation of LDLR transcription by ERα (Li et al. 2001). Consistent with this study, we observed two SP1-binding sites on TNFα (351–343 and 63–51) regulating the gene expression by binding to ERα. Thus, deletion of these two functional regions blocked ERα from binding to the TNFα promoter, indicating that both SP1-binding sites are required for basal promoter activities to enhance transcriptional activity. Based on the previous observation that MA could effectively prevent the binding of SP1 to the CAR promoter (Chung et al. 2011), we blocked SP1 using MA and then observed for TNFα core promoter activity in the ERα-transfected cells. Ectopic expression of ERα enhanced TNFα promoter activity, and this promoter activity was abolished by the SP1 inhibitor (Sleiman et al. 2011). These observations suggest that ERα is able to bind to TNFα in the presence of estrogen stimulation and enhance its transcription; however, deletion of the SP1 site in the TNFα promoter region blocked its transcription. Therefore, we identified the SP1 site as an important E2/ERα regulatory element in the human TNFα gene promoter in Hep3B cells.

In this study, we also investigated the expression of TNFα in hepatocarcinogenesis (Chae et al. 2012), and we found that the overexpression of ERα induced apoptosis in Hep3B cells by increasing the expression of the TNFα gene in a ligand-independent manner. Altogether, we demonstrated that apoptosis induced by the overexpression of ERα is mediated through the expression of the TNFα gene. The association of ERα with SP1 protein to form a complex that binds to the SP1 site of the TNF promoter is essential for the action of ERα in an E2-dependent manner (Fig. 6).

In conclusion, estrogens suppress the proliferation of ER-negative Hep3B cells by inducing the re-expression of ERα. Targeting this mechanism may lead to the development of estrogen-like therapeutic agents to control the proliferation of ER-negative liver cancer cells.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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