The mitogenic effectors of isoproterenol in human hepatocellular carcinoma cells

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Abstract. Increasing data indicate that stress hormones and their corresponding receptors play an important role in the carcinogenesis and progression of hepatocellular carcinoma (HCC). However, there is presently no study investigating the influence of stress hormones in correlation with ß2-AR on human HCC cells. We examined the expression of α1- and ß-ARs in human HCC cell line HepG2 and MHCC97H cells in comparison with that in human normal hepatic cell line HL-7702 cells (L-02), and the influence of isoproterenol (ISO) on the growth of these HCC cells using blocking agents in correlation with ß2-AR and its downstream signaling pathways. We found that α1-AR was down-regulated and ß2-AR was up-regulated in HepG2 and MHCC97H cells. ISO dose-dependently promoted the growth of both HepG2 and MHCC97H cells. ISO-induced growth and survival of HCC cells were effectively attenuated by ICI 118551, U0126 and PD153035, but not by H-89 or LY294002. ISO transiently activated MAPK/ERK1/2 in tumor cells which could be blocked either by ICI 118551 or U0126, but not by H-89, LY294002, or PD153035. These findings indicate that ISO mimicking a mitogen promoted the growth of HepG2 and MHCC97H cells via ß2-AR-mediated activation of both MAPK/ERK1/2 dependent and independent signaling pathways, and ISO activated MAPK/ERK1/2 by an EGFR-independent mechanism.

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Abbreviations: α1-AR, alpha 1-adrenergic receptor; AKT/PKB, AKT/protein kinase B; ß2-AR, beta2-adrenergic receptor; EGFR, epithelial growth factor receptor; ERK1/2, extracellular signal-regulated kinase 1/2; HCC, hepatocellular carcinoma; ISO, isoproterenol; MAPK, mitogen activated protein kinase; PKA, cAMP-dependent protein kinase; PI3K, phosphatidylinositol-3-kinase

Key words: stress hormone, isoproterenol, adrenergic receptor, extracellular signal-regulated kinase 1/2, hepatocellular carcinoma

Introduction

Clinical, epidemiologic and experimental studies indicate that psychological stress influences the incidence and progression of cancers. These effects of psychological stress on cancer cells are mediated by the key stress hormones and their corresponding receptors (1). The adrenergic receptors (ARs) belong to the superfamily of G-protein-coupled receptor that can activate several known intracellular signaling pathways for the growth and invasion of cancer cells (2). However, the mechanisms that account for mediating the effect of stress hormones on cancer cells are not fully understood. The majority of the data show that stress hormones inhibit the growth of some cancer cells via α1-AR (3,4), while stimulate the growth of other cancer cells via ß2-AR (5-7). The influence of stress hormones on cancer cells depends on the type of ARs expressed in tumor cells.

Hepatocellular carcinoma (HCC) is a highly aggressive and the most common malignancy in livers with poor prognosis (8). The mechanisms that regulate the growth and invasion of HCC cells are poorly understood. Recent studies demonstrated that psychological stress affected the severity of chronic viral hepatitis and cirrhosis (9-11). Moreover, the expression pattern of α1-AR and ß2-AR in human HCC was aberrant from that in normal liver tissues (12). Experimental studies demonstrated that the loss of α1-AR conferred the growth-promoting effect of stress hormones on human HCC cells (3). However, in addition to the dysregulation of α1-AR, ß2-AR was up-regulated in human HCC tissues (12). The key stress hormones epinephrine and norepinephrine promoted hepatocarcinogenesis via ß2-AR in animals (13-16). There is presently no study investigating the effect of stress hormones in correlation with ß2-AR on human HCC cells. The implication of the elevated ß2-AR in human HCC remains unknown.

Table I. Primers used for reverse transcription-PCR.

Name	Forward	Reverse	Size (bp)
α1A-AR	5'-GAAGGGCAACACAAGGACAT-3'	5'-CCTAGACTTCCTCCCCGTTC-3'	310
α1B-AR	5'-TGGGGAGAGTTGAAAAATGC-3'	5'-AATGAAGTAGTTGGTGGGCG-3'	204
ß1-AR	5'-CGCCTCTTCGTCTTCTTCAACTG-3'	5'-ACATCGTCGTCGTCGTCGTC-3'	236
ß2-AR	5'-TGCCAATGAGACCTGCTGTGAC-3'	5'-TGTGTTGCCGTTGCTGGAGTAG-3'	526
ß3-AR	5'-GTGTGACCGCCAGCATCG-3'	5'-AGAAGAGGAAGGTAGAAGGAGACG-3'	295
ß-actin	5'-ATCGTGCGTGACATTAAGGAGAAG-3'	5'-AGGAAAGGAAGGCTGGAAGAGTG-3'	179

The aim of this study was to examine the expression of α1- and ß-ARs in human HCC cell lines and the influence of the ß-AR agonist isoproterenol (ISO) on the growth of these HCC cells.

Materials and methods

Stimulants, inhibitors and antibodies. (±)-Isoproterenol hydrochloride (ISO) and the ß2-AR antagonist ICI 118551 were obtained from Sigma-Aldrich Corporation (St Louis, MO, USA). Rabbit antibodies against α1AB-AR, ß2-AR, EGFR and phospho-EGFR (Tyr1086) were obtained from Upstate Biotech (Lake Placid, NY, USA). Rabbit antibodies against p44/42-MAPK (ERK1/2) and phospho-ERK1/2 (T202/Y204) were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). The specific secondary antibody, the specific PAK inhibitor H-89, PI3K inhibitor LY294002, EGFR tyrosine kinase inhibitor PD153035 and MEK1/2 inhibitor U0126 were obtained from Calbiochem-Novabiochem Corporation (San Diego, CA, USA).

Cell culture and treatment. Human normal liver cell line HL-7702 (L-02) and hepatoma cell line HepG2 were obtained from ATCC (Rockville, MD, USA), and the high invasive human HCC cell line MHCC97H was obtained from Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China). L-02 and MHCC97H cells were maintained and propagated in DMEM with high glucose supplemented with 15% fetal bovine serum (HyClone, Logan, UT, USA). HepG2 cells were maintained and propagated in RPMI-1640 supplemented with 10% fetal bovine serum. All cultured cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2 and experiments were done using 70-80% confluent cultures.

Reverse transcription-PCR. Total cellular RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The RNA concentration was measured by GeneQuant II (Pharmacia, Uppsala, Sweden) at 260 nm. Reverse transcription reaction and cDNA synthesis was performed in accordance with the manufacturer's instructions (Invitrogen).The specific primers used for the evaluation are listed in Table I. ß-actin was used as internal control and MMLV free reactions served as negative controls. The amplification step includes 35 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec. The PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The correct molecular weight was confirmed by a molecular weight marker (Boehringer Mannheim Biochemicals).

Western blot analysis. Cells were washed twice in phosphate buffered saline (PBS), lysed in ice-cold radioimmune precipitation (RIPA) buffer, and then centrifuged for 10 min at 4˚C. Supernatant was collected and protein concentrations were determined and adjusted to 2 mg/ml using the Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates were mixed with 3X Laemmli buffer and heated for 5 min at 95˚C. They were then resolved by SDS-PAGE (8% polyacrylamide gels), transferred to polyvinylidene difluoride membrane (Immobilon™; Millipore Corp.) by electroblotting. The membrane was blotted with 10% non-fat milk, washed in TBS Tween and incubated with primary rabbit polyclonal antibodies overnight at 4˚C. After washing with TBS Tween, they were incubated with secondary antibody solution (horseradish peroxidase conjugated IgG) for 60 min at room temperature. Membranes were washed again with TBS Tween before detection using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The following primary antibodies were used at dilutions: anti-α1-AR, 1:400; anti-ß2-AR, 1:400; anti-EGFR, 1:800; anti-phospho-EGFR, 1:800; anti-ERK1/2, 1:800; anti-phospho-ERK1/2, 1:800; anti-ß-actin, 1:400. Secondary antibody was used at dilutions of 1:10,000.

Cell proliferation assay. Cell proliferation was investigated through MTT methods. Briefly, $1x10^4$ cells per well were plated into flat-bottomed 96-well plates (Costar, Corning, NY, USA). After 24 h, cells were serum-starved overnight and incubated with different concentrations of stimulants with or without inhibitors for 6, 12, 24, 48 and 96 h. Control wells were treated with 0.1% DMSO alone. Subsequently, 20 μl MTT (5 g/l) was added to each well and incubated for an additional 4 h. Plates were centrifuged for 5 min at 1,000 rpm, and the medium was carefully discarded. The formed formazan

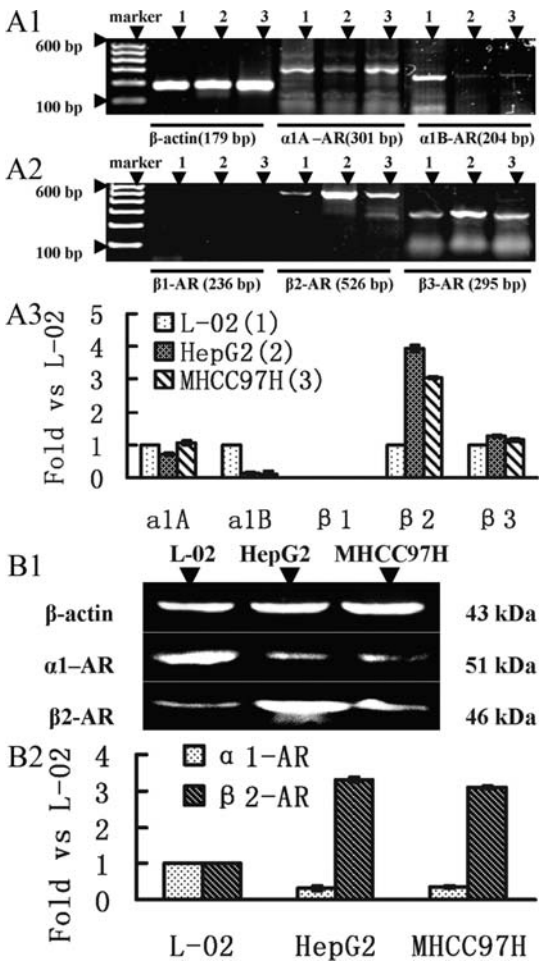

Figure 1. The expression of α1- and ß2-ARs in L-02, HepG2 and MHCC97H cells. The expression of α1- and ß2-ARs was detected using reverse transcription-PCR and Western blotting. Result was presented as fold change vs. control (L-02). (A1 and 3) The expression of α1A- and α1B-AR mRNA. (A2 and 3) The expression of ß1-, ß2- and ß3-AR mRNA. (B1 and 2). The expression of α1-AR and ß2-AR protein. α1-AR was detected using a specific antibody against both α1A- and α1B-ARs.

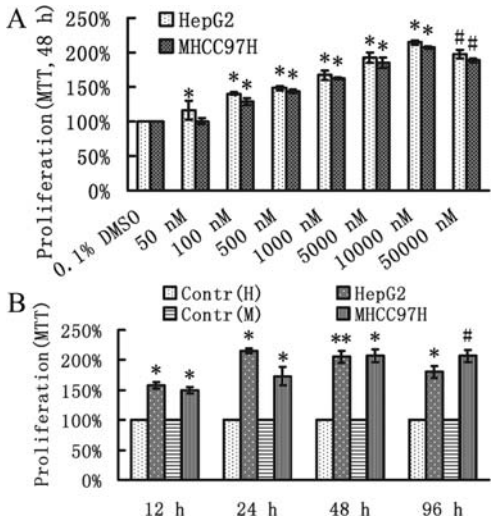

Figure 2. The growth-promoting effect of isoproterenol (ISO) on HepG2 and MHCC97H cells. Cell proliferation was assessed by MTT assay. Data were expressed as a percentage of the control (0.1% DMSO) OD value. (A) Cells were serum-starved overnight and then incubated with increasing doses of ISO for 48 h (h). *p<0.05 vs. control. $^{\#}$p<0.05 vs. control and p>0.05 vs. 5000 nM of ISO. (B) Cells were incubated with 10 μM of ISO for 12, 24, 48 and 96 h. *p<0.05 vs. control. **p<0.05 vs. control and p>0.05 vs. 24 h. $^{\#}$p<0.05 vs. control and p>0.05 vs. 48 h.

crystals were dissolved in 100 μl of DMSO, and absorbance was read at 570 nm using a spectrophotometer.

Apoptosis analysis. Cells were plated in six-well plates at $3x10^5$ per well. After incubated with stimulants in absence or presence of inhibitors for the indicated times, cells were harvested by trypsinization and washed with PBS. Cells were fixed in ice-cold 80% ethanol, washed, and resuspended in 1 ml PBS; treated with 10 μl RNase A (21 mg/ml); and stained with 5 μl propidium iodide at 1 mg/ml for 30 min at room temperature in the dark. The stained cells were analyzed by flow cytometry (BD LSRII System, BD Biosciences, San Jose, CA, USA). To discriminate apoptotic cells from live cells and necrotic cells, the treated cells were washed in PBS, resuspended in 100 ml binding buffer containing FITC-conjugated annexin V (Becton-Dickinson, San Jose, CA, USA) and propidium iodide (PI) annexin V(+)/P(-) and annexin V(+)/PI(+) represent the cells in early apoptosis and late apoptosis/necrosis, respectively.

Statistical analysis. Immunoblot signals were first quantitated using densitometry and Image J software (version 1.34s; by Wayne Rasband, NIH, Bethesda, MD, USA). Results were expressed as the mean ± SEM of three separate experiments and analyzed by the computer program SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA), using one-way analysis of variance (ANOVA) followed by the Tukey's t-test. P<0.05 were considered statistically significant.

Results

ß2-AR was increased and α1-AR was decreased in HepG2 and MHCC97H cells. Both α1A- and α1B-AR mRNA were expressed in the human normal hepatic cell line and HCC cell lines. However, α1B-AR mRNA was decreased in the tumor cells (0.13-fold in HepG2 and 0.1-fold in MHCC97H vs. L-02) (Fig. 1A1 and A3). Meanwhile, much higher level of ß2-AR mRNA was detected in tumor cells (3.9-fold in HepG2 and 3.1-fold in MHCC97H vs. L-02), with no detectable ß1-AR mRNA and similar level of ß3-AR mRNA in both normal and malignant cells (Fig. 1A2 and A3). The level of ß2-AR protein was increased by 3.3-fold in HepG2 and 3.1-fold in MHCC97H vs. L-02, while the level of total α1-AR protein was markedly decreased in the two tumor cell lines (0.3-fold in HepG2 and 0.34-fold in MHCC97H vs. L-02) (Fig. 1B1 and B2).

Growth-promoting effects of ISO on HepG2 and MHCC97H cells. Because ß2-AR was greatly increased in both HepG2 and MHCC97H cells, we used the ß-AR agonist ISO as a stimulant to observe whether the growth of HepG2 and MHCC97H cells could be influenced. ISO dose-dependently stimulated the proliferation of HepG2 and MHCC97H cells.

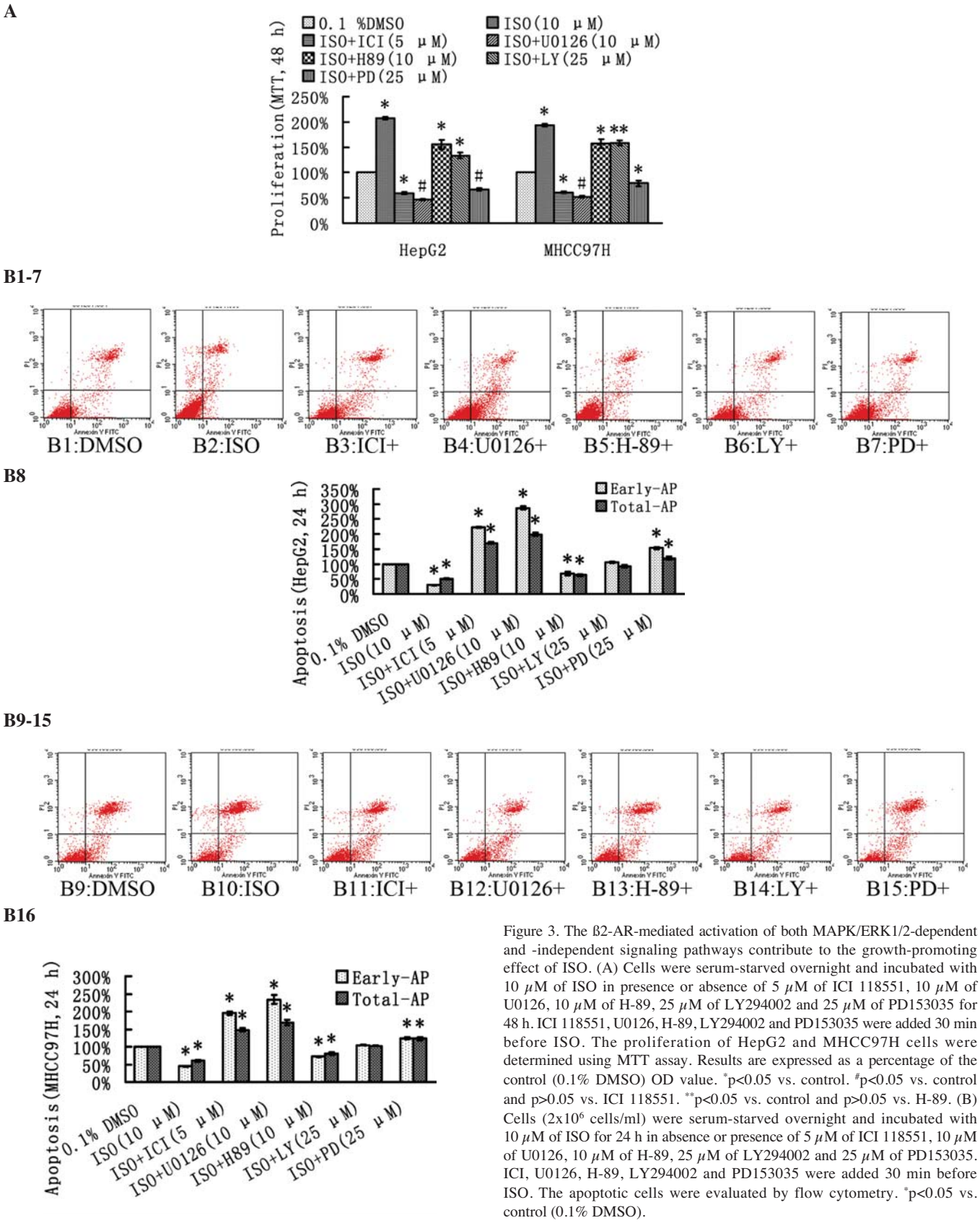

Figure 3. The ß2-AR-mediated activation of both MAPK/ERK1/2-dependent and -independent signaling pathways contribute to the growth-promoting effect of ISO. (A) Cells were serum-starved overnight and incubated with 10 μM of ISO in presence or absence of 5 μM of ICI 118551, 10 μM of U0126, 10 μM of H-89, 25 μM of LY294002 and 25 μM of PD153035 for 48 h. ICI 118551, U0126, H-89, LY294002 and PD153035 were added 30 min before ISO. The proliferation of HepG2 and MHCC97H cells were determined using MTT assay. Results are expressed as a percentage of the control (0.1% DMSO) OD value. *p<0.05 vs. control. $^{\#}$p<0.05 vs. control and p>0.05 vs. ICI 118551. **p<0.05 vs. control and p>0.05 vs. H-89. (B) Cells (2x10^6 cells/ml) were serum-starved overnight and incubated with 10 μM of ISO for 24 h in absence or presence of 5 μM of ICI 118551, 10 μM of U0126, 10 μM of H-89, 25 μM of LY294002 and 25 μM of PD153035. ICI, U0126, H-89, LY294002 and PD153035 were added 30 min before ISO. The apoptotic cells were evaluated by flow cytometry. *p<0.05 vs. control (0.1% DMSO).

The minimal dose of ISO with a significant growth-promoting effect on HepG2 cells was 50 nM (116.3±4.1% of control, p<0.05) and on MHCC97H cells was 100 nM (128.8±5.3% of control, p<0.05) (Fig. 2A). The maximal dose of ISO with the most efficient growth-promoting effect on HepG2 cells was 10000 nM (214.5±3.5% of control, p<0.05) and on MHCC97H cells was 10000 nM (207±1.9% of control, p<0.05) (Fig. 2A). The growth-enhancement was trigged at

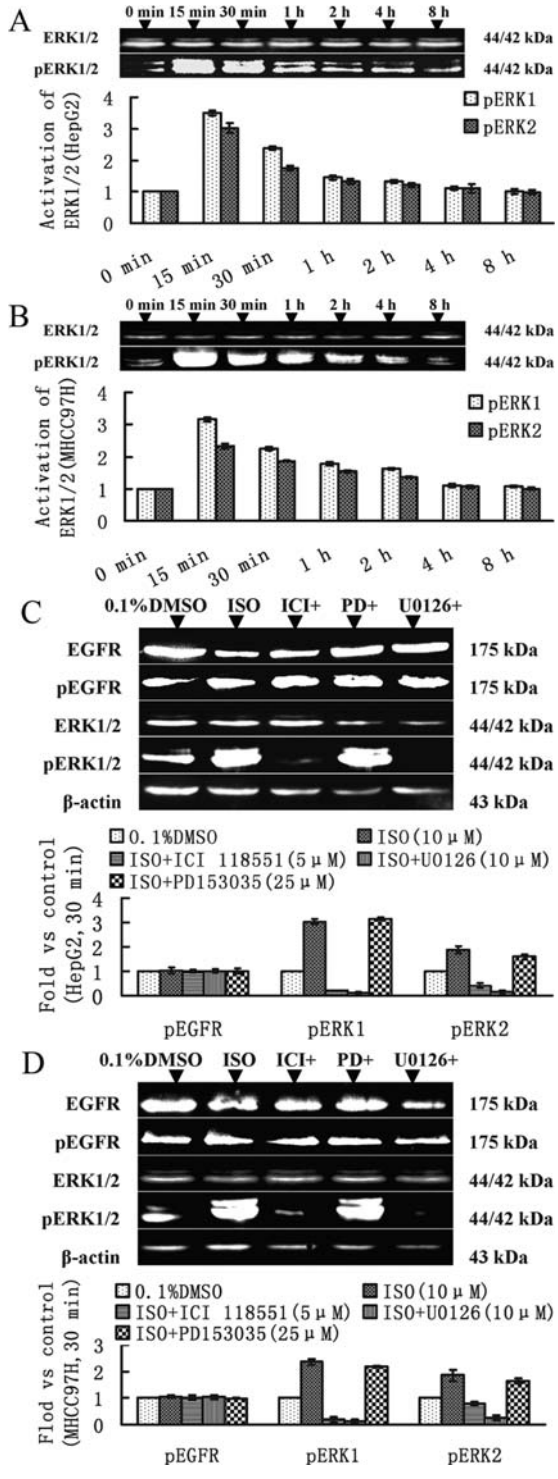

Figure 4. ISO activated MAPK/ERK1/2 by a ß2-AR-mediated and EGFR-independent mechanism. (A and B) Cells were serum-starved overnight and incubated with 10 μM of ISO for 15, 30 min, 1, 2, 4 and 8 h. The result is presented as fold change vs. control (0.1% DMSO). ISO transitionally activated ERK1/2 in HepG2 and MHCC97H cells. (C and D) Cells were incubated with 10 μM of ISO for 30 min in absence or presence of 5 μM of ICI 118551, 10 μM of U0126, and 25 μM of PD153035. ICI 118551, U0126 and PD153035 were added 30 min before ISO. The phosphorylated-EGFR (pEGFR) and pERK1/2 were determined by Western blotting. The result is presented as fold change vs. control (0.1% DMSO).

12 h and peaked at 24 h for HepG2, and trigged at 12 h and peaked at 48 h for MHCC97H after the treatment of ISO (10000 nM/10 μM) (Fig. 2B).

ß2-AR-mediated activation of both MAPK/ERK1/2-dependent and -independent signaling pathways contribute to the growth-promoting effect of ISO on tumor cells. It has been demonstrated that the most frequently associated signaling pathways that downstream ß2-AR (G-protein-coupled receptor) are the adenyl cyclase/cyclic adenosine monophospate-dependent protein kinase (cAMP/PKA), mitogen activated protein kinase/extracellular signal-regulated kinase 1/2 (MAPK/ERK1/2) and phosphatidylinositol-3-kinase/ AKT or protein kinase B (PI3K/AKT) signaling pathways (2,17-19). We used the selective ß2-AR antagonist ICI 118551 to confirm the ß2-AR signaling, and the PKA inhibitor H-89, the MEK-specific inhibitor U0126, the PI3K inhibitor LY294002 and the specific EGFR tyrosine kinase inhibitor PD153035 to rule out the possible involvement of these signaling pathways in mediating the effect of ISO. The treatment of ISO (10 μM) resulted in a significant increase in proliferation (HepG2: 207.6±2.2% of control, $p<0.05$; MHCC97H: 193.4±2.4% of control, $p<0.05$) and decrease in the percentage of apoptotic cells (HepG2: 50.9±2.2% of control, $p<0.05$; MHCC97H: 60.6±3.1% of control, $p<0.05$). The ISO-induced proliferation and survival of tumor cells were effectively attenuated by ICI 118551 (decreased proliferation of HepG2: 58.9±2.5% of control, $p<0.05$; decreased proliferation of MHCC97H: 60.6±2% of control, $p<0.05$; increased apoptosis of HepG2: 170.2±3.5% of control, $p<0.05$; increased apoptosis of MHCC97H: 147.9±4.9% of control, $p<0.05$), U0126 (decreased proliferation of HepG2: 46.4±1% of control, $p<0.05$; decreased proliferation of MHCC97H: 51.8±2% of control, $p<0.05$; increased apoptosis of HepG2: 199±5.6% of control, $p<0.05$; increased apoptosis of MHCC97H: 168.7±7.3% of control, $p<0.05$) or PD153035 (decreased proliferation of HepG2: 66.3±2.4% of control, $p<0.05$; decreased proliferation of MHCC97H: 78.8±5.9% of control, $p<0.05$; increased apoptosis of HepG2: 119.4± 5.5% of control, $p<0.05$; increased apoptosis of MHCC97H: 122.9±4.3% of control, $p<0.05$), but not by H-89 and LY294002 (Fig. 3A and B1-16).

ISO activates MAPK/ERK1/2 by a ß2-AR-mediated and EGFR-independent mechanism. The ISO-induced activation of MAPK/ERK1/2 in HepG2 and MHCC97H cells was further demonstrated by Western blotting. The addition of ISO resulted in an immediate activation of ERK1/2 in both HepG2 and MHCC97H cells. As early as 15 min after the addition of ISO (10 μM), the level of phosphorylated-ERK1/2 (pERK1/2) was increased by an average of 3.49/3.02-fold in HepG2 cells (Fig. 4A) and 3.15/2.33-fold in MHCC97H cells vs. controls (Fig. 4B). The activation of ERK1/2 was transitional, with no detectable elevation of pERK1/2 at 8 h after ISO stimulation (Fig. 4A and B). The ß2-AR signaling was confirmed using ICI 118551, the specificity of the phosphorylation of ERK1/2 was confirmed using U0126, and the possible involvement of EGFR was ruled out using PD153035 (17-19). ISO-induced phosphorylation of ERK1/2 was effectively inhibited either by ICI 118551 (5 μM) or U0126 (10 μM), but not by PD153035 (25 μM). No detectable changes of pEGFR were observed in presence of ISO (Fig. 4C and D).

Discussion

Increasing data indicate that stress hormones and their corresponding receptors also influence the carcinogenesis and progression of HCC (3,9-16). However, there is presently no study investigating the influence of stress hormones in correlation with the ß2-AR on human HCC cells. This report presents the primary findings from our study on the expression of α1- and ß2-AR in human HCC cell lines and the influence of ISO on the growth of these tumor cells.

The expression patterns of α1- and ß-ARs in HepG2 and MHCC97H cells was determined in comparison with that in human normal hepatic cell line L-02. α1A- and α1B-AR mRNA were expressed in both benign and malignant cells. However, the level of α1B-AR mRNA was markedly decreased in cancer cells (Fig. 1A1 amd A3). Much higher level of ß2-AR mRNA was detected in tumor cells, with no detectable ß1-AR mRNA and a similar level of ß3-AR mRNA in both benign and malignant cell lines (Fig. 1A2 and A3). The changes in expression of α1- and ß2-ARs were further demonstrated by Western blotting (Fig. 1B1 and B2) and the results confirmed the previous findings in human HCC tissues that ß2-AR was overexpressed in human HCC (12).

We demonstrated that the activation of ß-AR was sufficient for initiating some pro-proliferative signaling pathways in both HepG2 and MHCC97H cells. The ß-AR agonist ISO dose-dependently stimulated the growth of both HepG2 and MHCC97H cells. Exposure of HepG2 and MHCC97H cells to ISO resulted in an increased cell proliferation (Fig. 2A and B, 3A), enhanced cell survival (Fig. 3B2, B8, B10 and B16).

As demonstrated in several studies, the most common signaling pathways downstream ß2-AR in tumor cells are the cAMP/PKA, the MAPK/ERK1/2 and the PI3K/AKT signaling pathways (2,17-19). Clinical studies demonstrate that the high expression and activity of ERK1/2 and PI3K/AKT in HCC cells is associated with rapid tumor progression (20,21), whereas, the majority of studies on cAMP/PKA show that it is associated with the growth inhibition of HCC cells (22). It is not known whether they are also the mitogenic effectors downstream ß2-AR in HCC cells. This was determined by pre-treating HCC cells with the ß2-AR antagonist ICI 118551, the PKA inhibitor H89, the MEK inhibitor U0126, the PI3K inhibitor LY294002 and the specific EGFR tyrosine kinase inhibitor PD153035. We found that the ß2-AR-mediated activation of both MAPK/ERK1/2-dependent and -independent signaling pathways contributed to the growth-promoting effect of ISO on HepG2 and MHCC97H cells, because the ISO-induced proliferation and survival of tumor cells were effectively attenuated by ICI 118551, U0126 and PD153035, but not by H89 or LY294002 (Fig. 3A, B3, B4, B7, B8, B11, B12, B15 and B16). However, the results also indicated that the effect of ISO may also be mediated by transactivation of EGFR (Fig. 3A, B7, B8, B15 and B16). This was further determined by Western blotting. ISO transitionally activated MAPK/ERK1/2 in both HepG2 and MHCC97H cells (Fig. 4A and B). The ISO-induced activation of MAPK/ERK1/2 was blocked either by ICI 118551 or U0126, but not by PD153035 (Fig. 4C and D). The activity of EGFR was not markedly affected by the treatment of ISO either. These findings indicated that ISO activated MAPK/ERK1/2 in HepG2 and MHCC97H cells by a ß2-AR-mediated and EGFR-independent mechanism.

In conclusion, the results of this study confirmed that α1-AR was down-regulated and ß2-AR was up-regulated in human HCC cells (12). The ß-AR agonist ISO mimicking a mitogen significantly promoted the growth of HepG2 and MHCC97H cells via ß2-AR-mediated activation of both MAPK/ERK1/2-dependent and -independent signaling pathways, and ISO activated MAPK/ERK1/2 by a ß2-AR-mediated and EGFR-independent mechanism.

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