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Hepatocytes Express IL-2 In vitro in The Presence of Both HBx Stimulation and Mild Oxidative Stress*

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Abstract Hepatocellular carcinoma (HCC) has an extremely poor prognosis which is caused mainly by high frequency of metastatic recurrence. We previously reported that high interleukin (IL)-2 levels in peritumoral tissue were closely associated with a decreased incidence of intrahepatic tumor recurrence in patients with hepatitis B virus (HBV)-associated HCC, and the immune staining of IL-2 primarily was found in tumor-surrounding hepatocytes. However, whether hepatocytes can express IL-2 *in vitro* and underlying mechanism remain to be investigated. Here, we compared IL-2 expression levels in peritumoral tissue from HCC patients between HBV-positive group and HBV-negative group. We then overexpressed HBV x protein (HBx) in immortalized human liver cell line THLE-2, and mimic microenvironmental oxidative stress with hydrogen peroxide (H_2O_2) treatment *in vitro*. We demonstrated that hepatocytes can express IL-2 *in vitro* through MAP3K7/NF-κB pathway in the presence of both HBx stimulation and mild oxidative stress. Targeting microenvironmental oxidative stress offers a promise for prevention and therapy of HBV-associated HCC metastatic recurrence.

Key words hepatocellular carcinoma, interleukin-2, HBx
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Hepatocellular carcinoma (HCC) is one of the most common and aggressive human malignancies worldwide [1–2]. Its high frequency of metastatic recurrence results in an extremely poor prognosis[3–4]. It remains to be challenges to understand the mechanism underlying invasion and metastases of HCC. Our previous study revealed that high interleukin (IL)-2 levels in tumor-surrounding hepatic tissues were significantly associated with a decreased incidence of intrahepatic tumor recurrence and a prolonged overall survival in patients with hepatitis B virus (HBV)-associated HCC, and the immune staining of these IL-2 primarily was found in tumor-surrounding hepatocytes [5]. But whether hepatocytes can express IL-2 under certain conditions *in vitro* and what signaling pathways are involved in this process remain to be investigated.

The incidence and deaths of HCC in China has been more than half of the world, mainly attributed to the high incidence of HBV infection and subsequent liver cirrhosis[1, 6]. Among the total HCC cases used in our previous study, >90% had a history of HBV infection and a majority were chronic HBV carriers[5]. Therefore, we suppose that HBV-associated chronic inflammatory response or accompanying microenvironmental oxidative stress is associated with

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IL-2 expression.

In the present study, in order to verify if IL-2 expression in tumor-surrounding hepatocytes are associated with HBV infection, we first compared IL-2 expression levels in tumor-surrounding hepatic tissue from HCC patients between HBV-positive group and HBV-negative group. Accumulating evidences indicate that HBV x protein (HBx) is required for HBV replication and involved in HBV-related carcinogenesis, so we decide to use HBx to conduct the next experiments. We overexpressed HBx in immortalized human adult liver cell line THLE-2, and mimic oxidative stress with hydrogen peroxide (H_2O_2) treatment *in vitro* [7–8]. We explored whether HBx and/or H_2O_2 stimulation induce THLE-2 to express IL-2, and further analyzed what signaling pathway contributed to IL-2 expression of THLE-2 under these inducements.

1 Materials and methods

1.1 Samples from patients

A total of 38 HCC patients who had undergone curative resection in Zhongshan Hospital (Shanghai, China) from February, 2006 to September, 2009 were enrolled in this study. The diagnosis of HCC was confirmed by two pathologists. Peritumoral hepatic tissues were obtained 2–3 cm away from the tumor. All patients were divided into two groups. One group (n =20) consisted of HCC patients with histories of HBV infection and they were chronic HBV carriers, in contrast, another group (n =18) consisted of HCC patients without histories of HBV infection. These samples were used for the immunohistochemical analysis. This study was approved by the Zhongshan Hospital Research Ethics Committee. Informed consent was obtained according to the committee's regulations and the Declaration of Helsinki.

1.2 Cell culture and treatments

As described in previous study[9–10], THLE-2 cells (ATCC, Manassas, VA) were cultured in BEGM medium (Lonza) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL) in plates coated with bovine serum albumin/collagen/fibronectin at 37℃ in a humidified incubator containing 5% CO_2. THLE-2 cell line does not has background of HBV infection. In order to mimic oxidative stress, cells were stimulated with 0.05 mmol/L or 1 mmol/L H_2O_2 for 1 h. To inhibit NF-κB activity, cells were treated with 1.2 μmol/L Caffeic Acid Phenethyl Ester (CAPE, Selleckchem), a selective NF-κB inhibitor for 48 h. LYTAK1, a MAP3K7 (TAK1) kinase-selective inhibitor, is a gift from Paul J Chiao's Laboratory in the University of Texas M. D. Anderson Cancer Center. To inhibit MAP3K7, cells were treated with 10 nmol/L LYTAK1 for 48 h.

1.3 Reagents

The following antibodies and reagents were used in this study: control β-actin antibody was purchased from Sigma-Aldrich Corporation (St. Louis, MO); antibodies to P65, p-P65, TAK1 and p-MAPK3 were purchased from Cell Signaling Technology Inc (Beverly, MA); anti-hepatitis B virus X antigen antibody, anti-IL1A antibody, anti-GAPDH antibody and anti-IL2 antibody were obtained from ABCam (Cambridge, UK); secondary antibody (Alexa Fluor 488 conjugate) was purchased from Thermo Fisher Scientific (Rockford, USA).

1.4 Plasmid construction and establishment of stable cell lines

The full length of the coding sequence of HBx was cloned into the lentiviral vector pCDHCMV-MCS-EF1-puro (SBI, USA) to generate the lentiviral expressing constructs. Lentiviruses were generated by transfecting lentiviral-expressing vectors HBx/pLKO.1, together with packaging vector psPAX2 and envelope plasmid pMD2.G into 293T cells. At 48 to 72 h after transfection, viral culture supernatants from 293T cells were collected, filtered through a 0.45 μm filter, and used to infect the exponentially growing target cells THLE-2 with 50% confluence in the presence of 8 mg/L polybrene (Sigma-Aldrich Corporation).

1.5 Western blotting

Western blotting was conducted as described previously[11]. Briefly, cells were subjected to lysis with RIPA buffer, and cell lysates were separated by electrophoresis and transferred to PVDF membrane. After blocking, membranes were incubated with primary antibodies. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibody and signal was detected by exposure to X-ray film.

1.6 Enzyme-linked immunosorbent assays(ELISAs)

Cells were collected through centrifugation. For protein preparation, 250 μl lysis buffer (1% CHAPS, 50 mmol/L DTT, 10 mmol/L EDTA, 1 mmol/L PMSF, 1 mg/L pepstatin A, and 1 mg/L leupeptin in pH 7.2

PBS) were added to 0.1 g of cells then mixed vigorously. Insoluble substances were removed by centrifugation, and the supernatants harvested. The IL-2 expression levels were detected by ELISA (R&D, USA) following the manufacturer's instructions as described previously [5]. Analyses were performed in duplicate.

1.7 Immunohistochemistry

Immunohistochemistry was carried out as described previously [5]. IL-2 immunostaining was assessed using the semiquantitative histological score (H-score) approach, which combines the intensity and area of cells positive for IL-2 expression. Ten random 400× microscopic fields per slide were evaluated. The mean percentage of positively stained cells was scored as follows: 0%–25% (1); 26%–50% (2); 51%–75% (3); and 76%–100% (4). Staining intensity was categorized as follows: –(0); +(1); ++(2) and +++(3). The evaluation of immunohistochemical variables was performed without knowledge of the clinicopathologic data by two independent investigators.

1.8 Immunofluorescence

Cells were fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Then cells were incubated with 5% BSA at RT for 1 h before incubation with IL-2 antibody at 4℃ overnight. Alexa Fluor 488 goat anti-rabbit IgG was then added to samples and incubated at RT for 30 min. Samples were stained with DAPI (Molecular Probes) at RT for 10 min. Finally, slide covers were mounted on glass slides with ProLong Gold antifade reagent (Invitrogen). Images were taken by Carl Zeiss LSM710 (Germany) confocal microscope and analyzed by Carl Zeiss LSM710 software.

1.9 Quantitative PCR

Total RNA was extracted from cells using TRIZOL. First-strand DNA was synthesized with an iScript cDNA Synthesis Kit (Bio-Rad). The real-time PCR reactive reagent was Brilliant SYBR Green QPCR master mix (Stratagene). The PCR reaction was run on the IQ5 PCR system (Bio-Rad). GAPDH mRNA expression was used as an internal control. Quantitative PCR data for mRNA expression was normalized by their own internal control. Three independent experiments were conducted for each experimental condition. Primers used to amplify the IL-2 gene were 5′ CAAACCTCTGGAGGAAGTGC 3′ and 5′ GGTTGCTGTCTCATCAGCAT 3′.

Quantitative real-time RT-PCR was also performed using a custom Taqman arrays (Applied Biosystems, California, USA) that included 27 genes associated with inflammatory response and cytokine pathways. They are FOS (Hs00170630_m1), JAK1 (Hs01026983_m1), JAK3 (Hs00169663_m1), AKT1 (Hs00178289_m1), JUN (Hs99999141_s1), MAP2K1 (Hs00605615_mH), MAPK1 (Hs01046830_m1), MAPK3 (Hs00385075_m1), PIK3C3 (Hs00176908_m1), SOS1 (Hs00362308_m1), NFAT5 (Hs00232437_m1), MAP3K7 (Hs00177373_m1), MAP2K3 (Hs00177127_m1), MYD88 (Hs00182082_m1), NFKB1 (Hs00765730_m1), PIK3C3 (Hs00176908_m1), RELA (Hs00153294_m1), RELB (Hs00232399_m1), TRAF2 (Hs00184192_m1), IL2RA (Hs00166229_m1), IL2RB (Hs01081697_m1), STAT3 (Hs00374280_m1), STAT6 (Hs00598625_m1), PIK3R1 (Hs00381459_m1), TLR4 (Hs00152939_m1), IL1A (Hs00174092_m1) and NOS2 (Hs00167248_m1). Analyses were conducted in accordance with the manufacturer's protocol. Samples were run in duplicates.

1.10 Statistical analysis

Statistical analysis was performed with SPSS version 15.0 (SPSS Inc., Chicago, IL). Comparison of two groups was conducted using the Student *t* test. Multiple comparisons were analyzed by one-way analysis of variance. Two-tailed *P*-values less than 0.05 were considered significant.

2 Results

2.1 IL-2 expression in tumor-surrounding hepatocytes was associated with HBV infection

We detected protein express levels of IL-2 in tumor-surrounding hepatic tissues from HCC patients with or without HBV infection history (n=20 and n=18 respectively) using immunohistochemistry method. Our results showed that IL-2 expression levels in tumor-surrounding hepatic tissues from HCC patients with HBV infection history were significantly higher than that of those patients without HBV infection history (Figure 1a, 1b). IL-2 was stained mainly in hepatocytes in the noncancerous hepatic tissues. These data suggest that IL-2 expression in tumor-surrounding hepatocytes correlates with HBV infection, and chronic inflammatory response against HBV might be a contributing factor for IL-2 expression of hepatocytes.

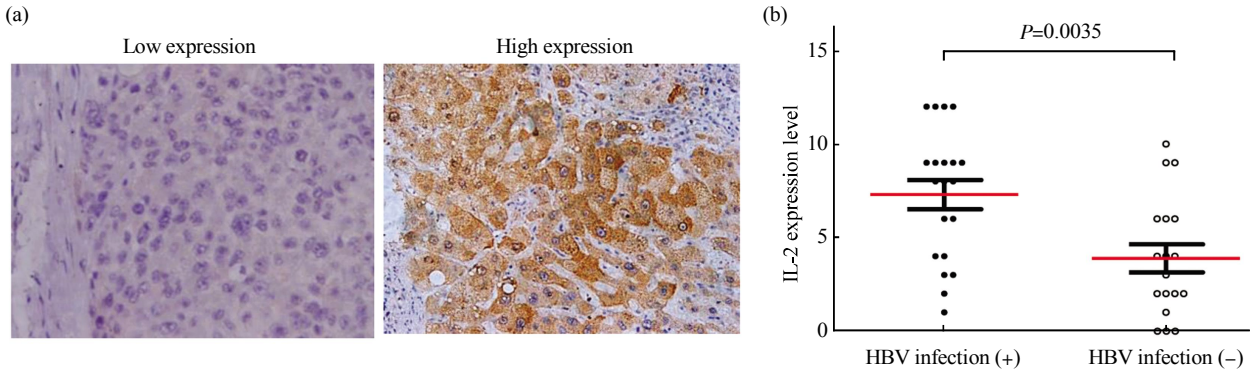

Fig. 1 IL-2 expression in tumor-surrounding hepatic tissues is associated with HBV infection

(a) Expression patterns of IL-2 immunohistochemistry of representative tumor-surrounding hepatic tissues with high or low expression levels are shown (×200). (b) IL-2 expression levels in tumor-surrounding hepatic tissues were compared between HCC patients with HBV infection histories and those without HBV infection histories. ●: HBV infection (+); ○: HBV infection (–).

2.2 HBx and mild oxidative stress induced hepatocytes to express IL-2 in vitro

HBx protein, a 17-ku protein encoded by the "X" open reading frame of HBV genome, is functionally associated with cellular activities such as apoptosis, signaling, and cell growth[12–14]. THLE-2 cell line is an immortalized human liver cell line. To determine whether HBx promotes IL-2 expression by hepatocytes, we established the THLE-2 cell line stably expressing HBx (THLE-2/HBx) using a lentivirus-mediated overexpression system (Figure 2a). We observed that THLE-2/HBx didn't express IL-2 compared to control (Figure 2b, 2c). In addition, we treated THLE-2 cells with different concentration of H_2O_2 [8]. We found that neither high concentration (1 mmol/L) nor low concentration (0.05 mmol/L) of H_2O_2 made THLE-2 to elevate significantly IL-2 expression comparing with controls (Figure 2d, 2e). However, we found that after treated with the low concentration of H_2O_2, THLE-2/HBx cells increased significantly both mRNA and protein expression levels of IL-2 compared to THLE-2/HBx cells treated with vehicle(Figure 2f, 2g). Interestingly, after treated with the high concentration of H_2O_2, THLE-2/HBx cells didn't augment expression levels of IL-2 compared to THLE-2/HBx cells treated with vehicle (Figure 2f, 2g). In contrast, THLE-2/HBx cells treated with the high concentration of H_2O_2 tend to reduce IL-2 expression comparing with THLE-2/HBx cells treated with the low concentration of H_2O_2 (Figure 2f, 2g).

To further determine subcellular location of IL-2 expressed by THLE-2/HBx, THLE-2/HBx cells were subjected to immunofluorescence staining of IL-2. It was observed that IL-2 was distributed to the cytoplasm of THLE-2/HBx after the treatment of the low concentration of H_2O_2, while few IL-2 was stained in THLE-2/HBx cells after the treatment of vehicle (Figure 2h, 2i). These results suggest that HBV infection and mild oxidative stress, rather than strong oxidative stress, induce hepatocytes to express IL-2.

2.3 RELA and MAP3K7 were involved in IL-2 expression of THLE-2/HBx under mild oxidative stress

We further want to know what molecules relevant to inflammatory response and cytokine pathways were changed when THLE-2/HBx expressed IL-2 under mild oxidative stress. We analyzed 27 genes associated with inflammatory response and cytokine pathways using custom Taqman arrays by real-time RT-PCR method. It was found that mRNA levels of IL-1A, MAPK3, RELA and MAP3K7 were significantly increased when THLE-2/HBx was treated with the low concentration of H_2O_2 comparing with the THLE-2/HBx cells treated with vehicle (Figure 3a). We further verified the changes of these genes at protein levels by Western blotting. Since nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB)/RELA (P65) activation involves phosphorylation of P65 proteins[15–16], phosphorylated P65 (p-P65) also were analyzed. It is demonstrated that protein levels of P65, p-P65 and MAP3K7 were also significantly elevated when THLE-2/HBx cells were stimulated with the low

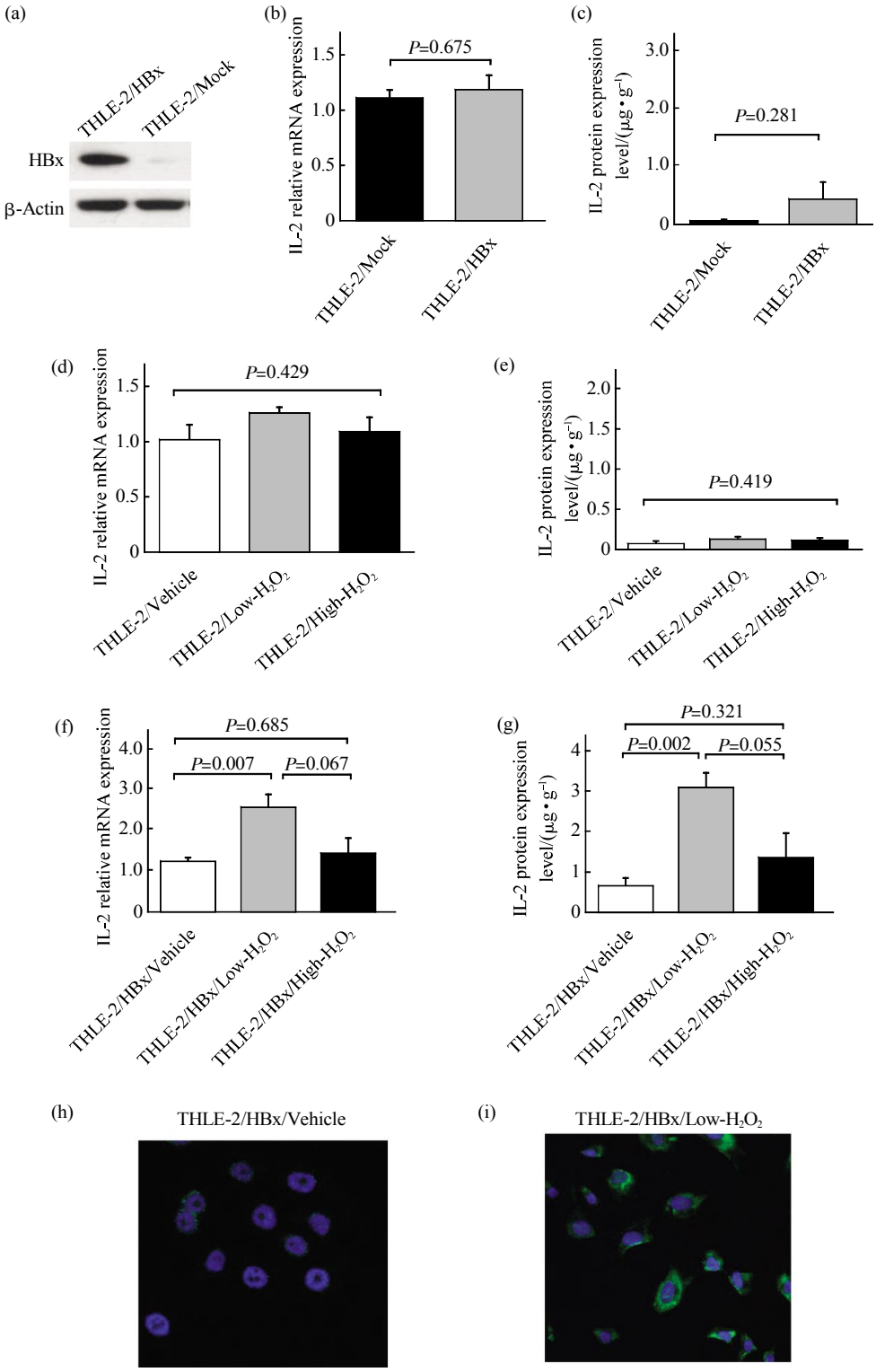

Fig. 2 HBx and mild oxidative stress promote hepatocytes to express IL-2 in vitro

(a) HBx expression in THLE-2 cell lines with HBx over-expression (THLE-2/HBx) was validated by Western blotting. THLE-2/Mock is control cells. (b, c) IL-2 mRNA and protein expression levels in THLE-2/HBx and THLE-2/Mock cells were analyzed respectively by real-time PCR and ELISA. (d, e) IL-2 mRNA and protein expression levels in THLE-2 cells with indicated treatments were analyzed respectively by real-time PCR and ELISA. (f, g) IL-2 mRNA and protein expression levels in THLE-2/HBx cells with indicated treatments were analyzed respectively by real-time PCR and ELISA. Error bars represent ±*SD* from three independent experiments. (h, i) Confocal microscopy analyses showed IL-2 staining in the indicated cells and treatments. THLE-2/Vehicle, THLE-2/Low-H_2O_2 and THLE-2/High-H_2O_2 denotes THLE-2 cells treated with PBS, the low concentration of H_2O_2 and the high concentration of H_2O_2 respectively. THLE-2/HBx/vehicle, THLE-2/HBx/Low-H_2O_2 and THLE-2/HBx/High-H_2O_2 denotes THLE-2/HBx cells treated with PBS, the low concentration of H_2O_2 and the high concentration of H_2O_2 respectively.

concentration of H_2O_2 (Figure 3b). However, IL-1A and MAPK3 in THLE-2/HBx cells stimulated with the low concentration of H_2O_2 were not significantly higher than that in the THLE-2/HBx cells stimulated with vehicle (Figure 3a). These results imply that NF-κB/RELA and MAP3K7 might contribute to IL-2 expression of THLE-2/HBx in the condition of mild oxidative stress.

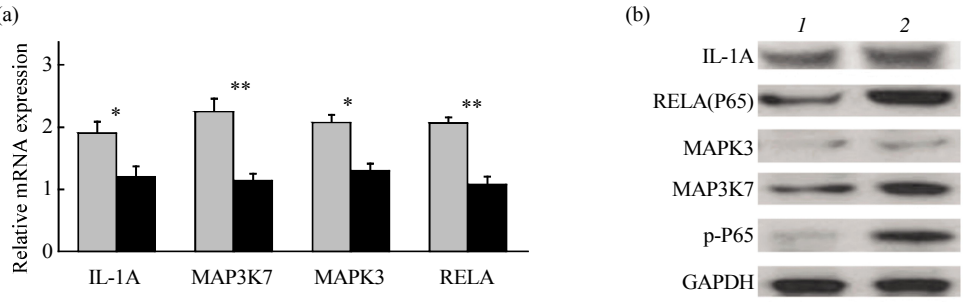

Fig. 3 The inflammation-associated molecules were involved in IL-2 expression of THLE-2/HBx under the low concentration of H_2O_2

(a) The mRNA of indicated genes were significantly differentially expressed between THLE-2/HBx/Low-H_2O_2 and THLE-2/HBx/Vehicle based on real-time PCR analyses. □: THLE-2/HBx/Low-H_2O_2; ■: THLE-2/HBx/Vehicle. (b) The protein levels of differentially expressed genes were further analyzed by Western blotting. Error bars represent ±*SD* from three independent experiments. THLE-2/HBx/Vehicle and THLE-2/HBx/Low-H_2O_2 denotes THLE-2/HBx cells treated with vehicle and the low concentration of H_2O_2 respectively. **P*<0.05; ***P*<0.01. *1*: THLE-2/HBx/Vehicle; *2*: THLE-2/HBx/ Low-H_2O_2.

2.4 THLE-2/HBx cells expressed IL-2 partly through MAP3K7/NF-κB pathway under mild oxidative stress

To investigate if IL-2 expression of THLE-2/HBx under mild oxidative stress is dependent to MAP3K7/ NF-κB pathway, we used MAP3K7 and NF-κB/P65 inhibitor to attenuate MAP3K7 and NF-κB activity respectively, and then observed changes of IL-2 expression. We used these inhibitors referring to previous studies[17–18]. The results showed that THLE-2/ HBx cells significantly decreased both mRNA and protein levels of IL-2 when stimulated with the low concentration of H_2O_2 in the presence of MAP3K7 inhibitor (LYTAK1) comparing with these cells stimulated with the low concentration of H_2O_2 in the absence of LYTAK1 (Figure 4a, 4b and 4c). Likewise, we found that THLE-2/HBx cells also significantly reduced both mRNA and protein levels of IL-2 when stimulated with the low concentration of H_2O_2 in the presence of P65 inhibitor (CAPE) comparing with these cells stimulated with the low concentration of H_2O_2 in the absence of CAPE (Figure 4a, 4b and 4c). All the data indicated that IL-2 expression of THLE-2/HBx in the condition of mild oxidative stress was partly dependent to MAP3K7/ NF-κB pathway.

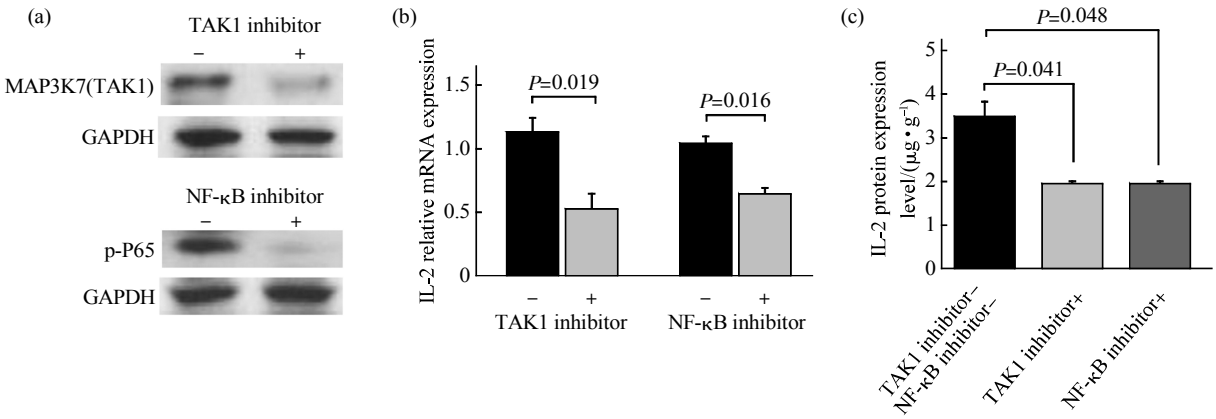

Fig. 4 THLE-2/HBx/Low-H_2O_2 expresses IL-2 through MAP3K7/NF-κB pathway

(a) Western blotting analyses validated that the protein expression levels of MAP3K7 (TAK1) and p-P65 in THLE-2/HBx/Low-H_2O_2 were reduced in the presence of relevant inhibitor. (b, c) IL-2 expression levels in THLE-2/HBx/Low-H_2O_2 with indicated treatments were analyzed respectively by real-time PCR and ELISA. THLE-2/HBx/Low-H_2O_2 denotes THLE-2/HBx cells stimulated with the low concentration of H_2O_2.

3 Discussion

It is well known that IL-2 is produced mainly by Th1 cells and it can activate T lymphocytes and stimulate proliferation of them. The epithelial cells are also reported to be able to express endogenous IL-2[19]. Moreover, the augmented intrahepatic IL-2 expression in patients with chronic hepatitis C has been noted[20-21]. Kasprzak *et al*.[22] reported that IL-2 expression was elevated in hepatocytes of patients with chronic hepatitis C virus (HCV) infection. Our previous study reveals that IL-2 levels in tumor-surrounding hepatocytes are associated with good prognosis of patients with HCC. But what facilitates hepatocytes to express IL-2 and how hepatocytes express IL-2 have been not clear till now. In this study, we first demonstrated that HBV infection combined with microenvironmental mild oxidative stress promote hepatocytes to express IL-2. Intrahepatic Th1-like cytokines facilitate doubtlessly elimination of pathogens. Our results suggest that hepatocytes might contribute to removal of hepatitis virus at early stage of the disease.

IL-2's transcription is mediated by multiple transcription factors, including nuclear factor of activated T cells (NFAT) family proteins[23], NF-κB, activator protein-1 (AP-1) and so on[24]. In the present study, we demonstrated that hepatocytes express IL-2 through TAK1/NF-κB signaling pathway. Since the cytokine signaling pathways tested in our study are limited, other pathways contributing to IL-2 expression in hepatocytes can't be ruled out.

In this study, we also observed that HBx overexpression combined the low concentration of H_2O_2 rather than the high concentration of H_2O_2 induced hepatocytes to product IL-2. The oxidative stress refers to the physiological disturbance between the reactive oxygen species (ROS) such as H_2O_2 or O_2- and the ability of the body to eliminate ROS[25]. It was reported that HBV replication could produce oxidative stress, and increased oxidative stress were observed in HBV-associated HCC [26-28]. Imbalance between production of ROS and their removal by antioxidant mechanism leads to damage of important biomolecules and cells[29]. Our study also showed that hepatocytes didn't express IL-2 under condition of strong oxidative stress. Budhu *et al*. reported that increasing intrahepatic Th1 cytokines might inhibit metastases of HCC. Previously our study also reveals that intrahepatic IL-2 is a protective cytokine for patients against intrahepatic metastasis of HCC. Therefore, regulating the balance between production and removal of ROS may be important for IL-2 expression by hepatocytes, and hence favors to inhibition of HCC metastasis. In addition, high or low concentration of H_2O_2 has already reported to have different roles on immune cells and HCC cells. Higher concentrations of H_2O_2 inhibit the production of T cell growth factor (TCGF) by immune cells and promote HCC cell apoptosis, conversely, lower concentrations of H_2O_2 augment TCGF production by immune cells and inhibit HCC cell apoptosis. This might explain that long-lasting high oxidative stress from chronic inflammation might play a vital pathogenic role in the progression leading to cancer[30-32]. We suppose that a low dose of H_2O_2 may be helpful for blocking the development cancer and treating cancer.

In conclusion, hepatocytes can express IL-2 *in vitro* through MAP3K7/NF-κB pathway in the presence of both HBV stimulation and mild oxidative stress. Targeting microenvironmental oxidative stress offers a promise for prevention and therapy of HCC metastasis.

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肝细胞在 HBx 刺激和低氧压力时表达 IL-2*

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摘要　肝细胞肝癌有极端坏的预后，主要归因于它的高转移性复发．我们曾经报道过对于乙型肝炎相关性肝癌患者，癌周组织的白介素 2 (IL-2)水平越高就倾向于有更低的肝内转移复发率，而且 IL-2 的免疫组化显示主要着色于癌周的肝细胞．然而，是否肝细胞能在体外表达 IL-2 以及其内在机制仍未被揭示．本研究里，比较了有和没有肝炎病史的肝癌患者的癌周组织 IL-2 表达水平，然后在永生化的成人肝细胞 THLE-2 里过表达了乙型肝炎病毒 x 蛋白(HBx)，并用过氧化氢处理模拟氧压力微环境．实验证实肝细胞在 HBx 刺激和低氧压力同时存在下能通过 MAP3K7/NF-κB 通路表达 IL-2．研究结果提示靶向调节微环境氧化压力为预防和治疗乙肝相关性肝癌的转移复发提供了新的方向．

关键词　肝细胞肝癌，白介素 2，乙型肝炎病毒 x 蛋白

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