



## STUDY OF PI3K/Akt SIGNAL PATHWAY IN HBV X PROTEIN INDUCING RAT GLOMERULAR MESANGIAL CELL PROLIFERATION

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### ABSTRACT

**【Objective】** To study the role of PI3K/Akt signal pathway in the HBV X Protein inducing on glomerular mesangial cell (GMC) proliferation. **【Methods】** The HBV X gene was amplified by PCR, the target gene was inserted into eukaryotic expression vector pCI-neo to constitute pCI-neo-X, which then transfect it into cultured rats GMCs. The PI3K/Akt signal pathway was inhibited by LY294002. The level of HBx, Akt, p-Akt and  $\beta$ -actin protein in glomerular mesangial cells were assayed by Western blotting. Mesangial cell proliferation in different groups were assayed by MTT colorimetry. **【Results】** The GMC transfected pCI-neo-X plasmid expressed HBx protein steadily after 36h, compared with that of GMC+pCI-neo group cells were significantly proliferated at 36h and 48h ( $p < 0.05$ ). The group of GMC+pCI-neo-X cells proliferation was inhibited after LY294002 being added, compared with that of empty vector group p-Akt was slightly expressed ( $p < 0.05$ ). **【Conclusion】** HBV X protein can induce GMC proliferation and PI3K/Akt signal is one of the most crucial transduction pathway inducing it.

**Keywords:** hepatitis B virus X protein ; glomerular mesangial cell ; PI3K/Akt ; cell proliferation

Hepatitis B antigenemia has been associated with glomerulonephritis for more than 40 years[1]. It is a well-known complication of chronic hepatitis B virus (HBV) infection[2,3]. Most patients present with predominantly renal manifestations of either proteinuria or the nephrotic syndrome. Almost all cases of hepatitis B virus-associated glomerulonephritis (HBV-GN) are of membrane nephropathy, however, mesangial proliferation and sclerosis also have been commonly reported[4-7]. A few cases, membrane proliferative glomerulonephritis is reported with significant mesangial cell interposition, glomerular basement membrane (GBM) reduplication, and subendothelial glomerular deposits[6,8]. Strikingly, HBV DNA can be detected in various proliferative glomerulonephritis, including type I and type III membrane proliferative glomerulonephritis (MPGN) as well as crescentic glomerulonephritis[8,9], suggesting that HBV infection may contribute to the development of glomerulonephritis. However, the related studies and the underlying mechanisms remain largely unclear.

GMC proliferation and excessive deposition of extracellular matrix proteins has been identified contributing to the progression of chronic kidney disease, including HBV-GN. The HBV X gene encoded protein (HBx) plays an important role in promoting cell proliferation and blocking apoptosis in human liver normal cells and tumor cells[10-12]. Our previous study showed that HBx can induce proliferation of cultured mesangial cell line *in vitro*, and that TNF- $\alpha$  high expression is closely related to the GMCs proliferation[13]. Phosphatidylinositol-3-kinase/Akt signaling pathway is important in cell proliferation[14], we hypothesized that HBx might contribute to the development of glomerulonephritis.

Therefore, in the present study, we investigated the effects of HBx in cultured MGCs to determine the underlying mechanisms. We observed the effects of compound that selectively inhibits PI3K/Akt signal pathway. This kinase plays different roles in different cells, promoting cell proliferation or apoptosis.

## 1.MATERIAL AND METHODS

### 1.1 Construction of HBV X gene-containing vector:

The pCI-neo-X plasmid containing full-length X gene of HBV was constructed. The sense primer is 5'-CCG CTC GAG GTA TAC ATC ATT TCC ATG GC-3', and the antisense primer is 5'-CCG GAA TTC GAG ATG ATT AGG CAG AGG TG-3'. The full-length X gene of HBV fragment was from the wild type HBV and transferred into the *EcoR I/XhoI* site of pCI-neo vector.

### 1.2 Cell culture and plasmid transfection:

GMCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) purchased from Sigma Aldrich. (New York, USA), containing 100U/ml penicillin, 0.1mg/ml streptomycin, and 10% heated-inactivated fetal

bovine serum (FBS) (Invitrogen) in a humidified incubator with 5%CO<sub>2</sub> at 37°C. When the cells were grown to 80% confluence, pCI-neo or pCI-neo-X plasmid was transfected into GMCs by lipofection technique<sup>[16]</sup>. The transfected cells were called GMC-pCI-neo and GMC-pCI-neo-X cells, respectively. A mixture solution containing 2 ml serum-free opti-MEM (Invitrogen) (pre-warmed to 37°C), 4µg plasmid DNA, 10µl lipofectamine 2000 was added into each well. After incubation for 6 hours, 2 ml DMEM-0.5%FBS medium was added into the wells, while untransfected GMCs in serum-free opti-MEM were used as controls.

### 1.3 Western blot analysis:

Proteins (HBx, Akt, p-Akt and β-actin) were extracted from GMCs and GMC-pCI-neo-X and were subjected to Western blot analysis using specific antibodies for β-actin (Boster, Wuhan, P. R. China), and HBx. In brief, the harvested cells (~2×10<sup>8</sup> cells) were resuspended in 200µl of PBS and sonicated 5 times. Cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were electrotransferred onto nitrocellulose membrane. The membrane was then blocked with milk casein (5% by weight in PBS) for 24 hours, and then hybridized with each diluted primary antibody for 24 hours at 4°C. The membrane was then washed 4 times and incubated for additional 15 min with TBST (10mM Tris-Cl, pH 7.4, 150 mMNaCl, 0.1% Tween 20). Finally, the membrane was hybridized with horse radish-peroxidase (HRP)-conjugated secondary antibody. Specific immunoblotting bands were visualized by ECM-associated fluorography.

### 1.4 MTT assay:

An MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] kit (Sigma Co.) was used to measure the metabolic activities of viable cells and the degree of cell proliferation. Subconfluent monolayer GMCs were detached from the cultured dishes by trypsin and centrifuged at 200 g for 5 min. The cell pellets were re-suspended in the fresh media (2.5×10<sup>4</sup> cells/ml), and then 200 µl the suspension was transferred into each well of a flat-bottomed 96-well plate. The cells were incubated at 37°C with 5%CO<sub>2</sub> for different time (6, 24, 36 or 48 hours). At the end of the incubation, the cultured medium was replaced by 200 µl fresh medium. MTT (2.5 mg dissolved in 50 µl of dimethylsulfoxide) was then added to each well and incubated for 4 hours at 37°C. To remove formed MTT-formazan crystals, 200 µl DMSO was added into each well, 25 µl glycine buffer (0.1 M, NaCl, pH 10.5) was then added to each well. Viable cells were detected by ELISA analyzer at 570 nm.

### 1.5 Statistical data analysis:

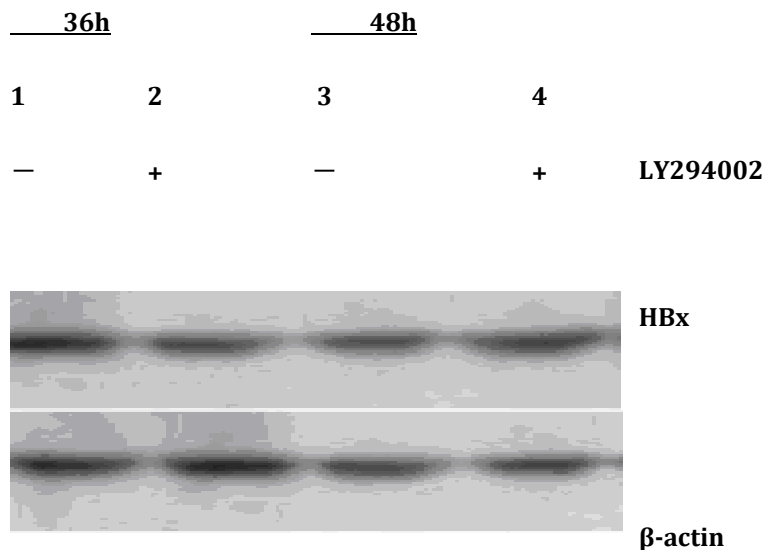
All data were expressed as mean ± SE. One-way ANOVA (with SPSS13.0 software) was used to assess the significance for the differences between different experimental groups. p<0.05 was considered statistically

significant.

## 2. RESULTS

### 2.1 Effects of HBV X gene transfection on expression of HBx in pCI-neo-X plasmid transfected GMCs *in vitro*:

Figure 1 shows the time course of transfection-induced changes (relative to  $\beta$ -actin) in HBx, demonstrating that HBx expression was not affected by LY294002 at 36 and 48 hrs after the pCI-neo-X plasmid transfection.



**Figure 1:** Representative Western blot data, showing that expression of HBx was not affected by LY294002

\* “—”inhibitor free, “+”added inhibitor

### 2.2 GMCs proliferation in different time:

**GMCs proliferation were detected** by MTT, at 36h and 48h, in GMC+pCI-neo-X group, cell proliferation was markedly, compared with GMC group and GMC+pCI-neo group, separately. ( $p < 0.05$ ), which indicated that HBx can promoting GMC proliferative. See table 1.

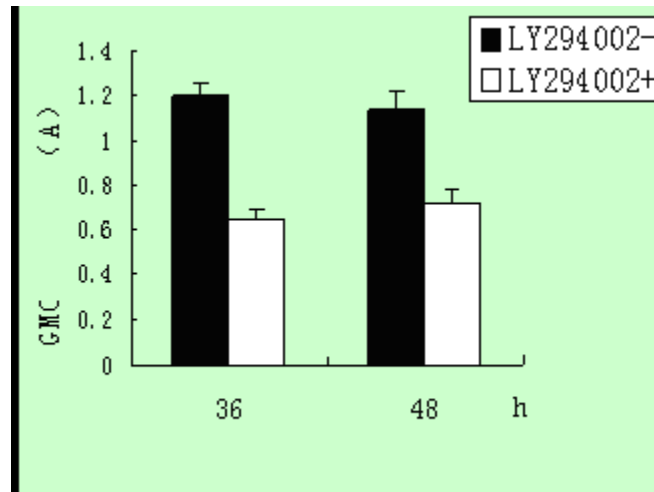
|               | 0h        | 6h        | 24h       | 36h        | 48h        |
|---------------|-----------|-----------|-----------|------------|------------|
| GMC           | 0.26±0.06 | 0.34±0.06 | 0.37±0.05 | 0.69±0.09  | 0.64±0.16  |
| GMC+PCI-neo   | 0.31±0.06 | 0.28±0.07 | 0.47±0.08 | 0.67±0.15  | 0.72±0.17  |
| GMC+PCI-neo-X | 0.33±0.07 | 0.33±0.08 | 0.65±0.13 | 1.46±0.30* | 1.74±0.30* |

**Table 1** : GMC proliferation in deferent time ( $\bar{x} \pm s$ , n=3)

\*GMC group vsGMC+pCI-neo group,  $p < 0.05$

### 2.3 PI3K inhibitor on GMCs proliferation:

Figure 2 showed that incubation of the HBV X gene-transfected GMCs with LY294002, detecting GMCs absorbance at 36h、48h, LY294002- group vs LY294002+ group ( $p < 0.05$ ) .It was indicated that HBx can promote GMC proliferation through PI3K/Aktpathway. See table 2.



**Figure2:** PI3K inhibitor on GMC proliferation( $\bar{x} \pm s$ , n=3)

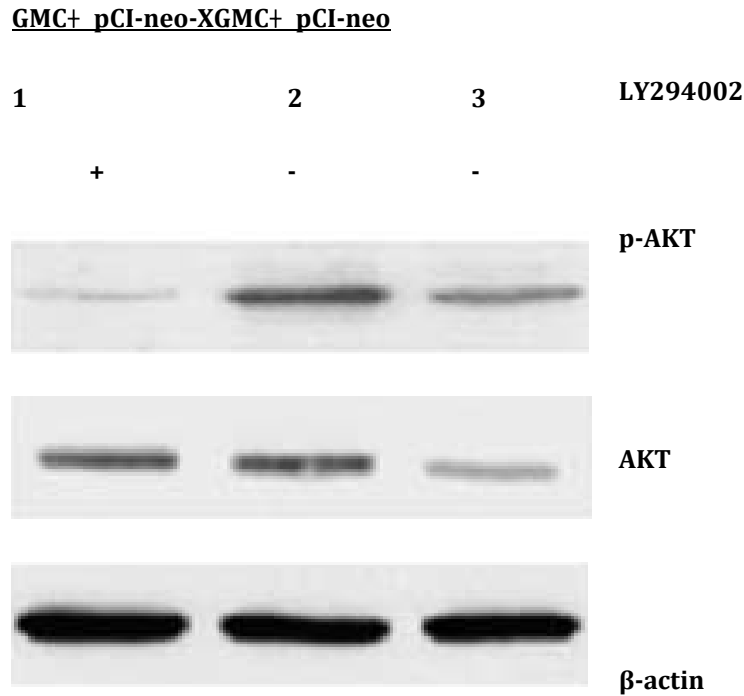
A : absorbance

LY294002- group vs Y294002+ group,  $p < 0.05$

### 2.4 LY294002 on p-Akt:

When GMC+PCI-neo-X proliferation reach 80% infusion, add 50μmol/L LY294002, p-Akt expression is higher in GMC+PCI-neo-X (-) groupthan that in GMC+PCI-neo-X (+) group , but p-Aktis lower in GMC+PCI-

neo-X (-)group .See Fig.3.



**Figure 3:** PI3K inhibitor decreased p-AKT expression

1 : GMC+PCI-neo-X (-) group, 2 : GMC+PCI-neo-X (+) group, 3 : GMC ; “—”inhibitor free, “+”added inhibitor

### 3. DISCUSSION

The major findings of the present study are that, first, HBV X gene infection (or transfection) significantly increased HBx expression in the transfected GMCs. Since the changes in HBx expression displayed similar time courses, Second, blockade of PI3K/Akt signal pathway by LY294002 significantly attenuated HBx-induced GMCs proliferation. The GMCs proliferation can be seen in some HBV-GN. The mechanisms underlying GMC proliferation remains unclear. HBV DNA was detected in the glomerular of HBV-GN. The present study demonstrated that HBV X gene can be transfected into rat GMCs and functionally express HBx in these cells at 36 to 48 hours after transfection. In addition, transfection of HBV X gene also promoted GMC proliferations. These data suggest that HBx can promote GMC proliferation. This is consistent with our previous study[13].

HBx can activate many cell signal transduction pathways, including PI3K/Akt , ERKs[15,16], p38[17], and NF-kappa B[18,19]. ERKs, p38 and NF-kappa B pathway have been done in our previous study, and showed that ERKs and NF-kappa B pathways were involved. However, PI3K/Akt pathway have not studied. For this purpose, we used the selective PI3K/Akt inhibitor to block its signal pathway. We found that **LY294002** significantly attenuated HBx-induced TNF-a expression and also GMC proliferation (data not shown), suggesting that PI3K/Akt pathways may underlie the action of HBx on the GMC proliferation.

HBx activates Ras and rapidly induces a cytoplasmic signaling cascade linking Ras, Raf, and mitogen-activated protein kinase (MAP kinase), leading to transcriptional transactivation. HBx strongly elevates levels of GTP-bound Ras, activated and phosphorylated Raf, and tyrosine-phosphorylated and activated MAP kinase. Transactivation of transcription factor AP-1 by HBx is blocked by inhibition of Ras or Raf activities but not by inhibition of Ca(2+)- and diacylglycerol-dependent protein kinase C. HBx was also found to stimulate DNA synthesis in serum-starved cells. HBx protein therefore stimulates Ras-GTP complex formation and promotes downstream signaling through Raf and MAP kinases, and may influence cell proliferation[20].

The serine/threonine kinase Akt is a major signaling molecule in the regulation of ECM protein synthesis and cell proliferation [21,22]. It has been reported that the PI3K-AKT signaling pathways was activated in glomeruli of diabetic rats as well as in mesangial cells cultured under high-glucose conditions[22].

We found that treatment with PI3K inhibitors, LY294002, inhibited the activation of Akt in mesangial cells, indicating that phosphorylation of Akt depended on the activities of PI3K. we concluded that HBx-induced mesangial cell proliferation were dependent on the activation of PI3K/Akt signaling pathways. The relationship between the activation of PI3K/Akt and mesangial cell proliferation have not determined, but HBx-induced proliferation maybe related to up-regulated cyclin D1 expression[23]. These results demonstrated that HBx has the ability to promote mesangial cell proliferation in vitro, and its stimulatory effects on cell proliferation and PI3K/Akt signaling pathways in cultured mesangial cells.

From our study, we can supposed that GMCs proliferation was contributed to HBx. Activation of PI3K/Akt signaling pathways induced by HBx may play a partial role in GMCs proliferation. However, cell proliferation is related to many cytokines expression and cell cycle. The exact mechanism remains unknown. Further investigation should be indicated.

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