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Recombinant HBsAg inhibits LPS-induced COX-2 expression and IL-18 production by interfering with the NFκB pathway in a human monocytic cell line, THP-1

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***Background/Aims*: Hepatitis B virus suppresses the human immune-system and HBsAg inhibits the induction of cytokines by LPS in human macrophages, but the mechanisms involved remain unclear. COX-2 and its product, PGE2, play a role in hepatits B and IL-18 has also been shown to inhibit HBV infection in vivo. We investigated whether rHBsAg affects induction of COX-2 and IL-18 by LPS and, if so, which signal pathways are involved in a human monocytic cell line, THP-1.**

***Methods*: Cell culture, Western blotting for COX-2, ERK and IKB-α, immunofluorescence for HBsAg and NFκB protein and ELISA for PGE2, IL-18 and IL-12 were performed.**

***Results*: rHBsAg inhibits LPS-induced COX-2 expression in a time- and dose-dependent manner by blocking the ERK and NFκB pathways. LPS-induced IL-18 production was also down-regulated by rHBsAg by interfering mainly with the NFκB pathway. PGE2 reversed the inhibition of LPS-induced IL-18 production by rHBsAg. rHBsAg was also found to inhibit the induction of IL-12 by LPS in THP-1 cells.**

***Conclusions*: These results showed a novel anti-inflammatory property of rHBsAg which involves inhibition of COX-2 and suggested that hepatits B virus may regulate IFN-γ production by inhibiting IL-18 and IL-12 production.**

***Keywords*: rHBsAg; Cyclooxygenase 2; Interleukin 18; NFκB pathway; Human monocytic cell line**

1. Introduction

Hepatitis B is one of the major liver diseases with 400 million people estimated to be chronic HBV carriers worldwide. Analysis of the interaction between HBV and immunocytes holds great promise for the understanding of the mechanism of persistent infection of hepatitis B virus [1]. It has been shown that hepatitis B virus suppresses human immune-system and HBsAg inhibits the release of LPS-induced cytokines by human monocytes [2,3]. Studies with HBV transgenic mice have led to the generally accepted idea that the tolerance at the T cell level is responsible for the establishment of the persistent state [4]. These reports suggest that HBV infection or virus products interfere with the function of monocytes, contributing to the development of HBV persistence. However, the mechanism and signal pathways by which HBsAg affects monocyte function remain unknown.

Cyclooxygenase 2 (COX-2) is a rate-limiting enzyme involved in the production of prostaglandins (PGs), prostacyclin and thromboxanes. COX-2 is induced in response to a variety of proinflammatory agents and cytokines [5,6]. Over-expression of COX-2 has been demonstrated in various tumors and chronic inflammatory

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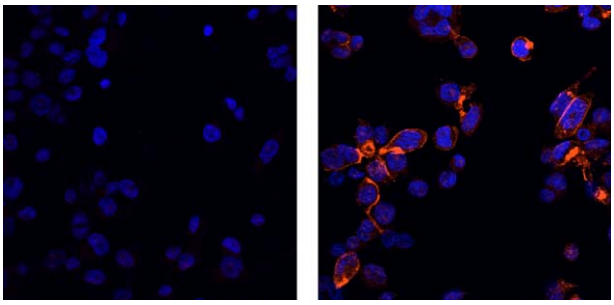

Fig. 1. Attachment of rHBsAg to cell membrane. Immunofluorescence staining for HBsAg in THP-1 cells after rHBsAg treatment. Cells on chamber slides were treated with PMA for 24 h, with rHBsAg (100 μg/ml) for 4 h, and incubated with goat anti-HBsAg antibody and TRITC-labeling anti-goat IgG. Immunofluorescence signals developed were detected by Laser Scanning Microscope LSM510. Strong red fluorescence signals were observed mainly on the cell membrane.

diseases, such as rheumatoid arthritis and ulcerative colitis [7,8]. Recent studies have shown that COX-2 and its important product, PGE2, are associated with liver pathogenesis, including hepatitis, fibrosis and cancer [9–12].

IL-18 was originally identified as an IFN-γ-inducing factor in mice exposed to *P. acnes* and lipopolysaccharide (LPS). Macrophages were first identified as IL-18-secreting cells following activation with the appropriate stimuli, such as LPS, phagocytic substances and microbial infections [13,14]. IL-18 induces IFN-γ production directly, an important anti-viral factor, in NK cells [15] and also acts in synergy with IL-12 to induce IFN-γ in $CD4^+$ T cells. IL-18 enhances cytotoxicity of T cells and NK cells involved in liver pathogenesis [16]. IL-18 has also been shown to suppress HBV infection in vivo [17]. On the other hand, it is not clear whether HBV regulates IL-18 production. It is also unclear whether COX-2 regulates HBsAg functions alone or in combination with IL-18 in monocytes.

In this study, we investigated whether HBsAg (subtype, adr) is involved in LPS-induced COX-2 expression and production of IL-18 and IL-12 in a monocytic cell line, THP-1. Our finding showed that rHBsAg inhibits LPS-induced COX-2 expression and IL-18 production by interfering with the NFκB pathway. The ERK pathway was also involved in production of PGE2, an important product of COX-2, and IL-18. PGE2 reversed the inhibitory effect of rHBsAg on LPS-induced IL-18 production. These results showed that HBV may affect the function of monocytes via the NFκB and ERK pathways.

2. Materials and Methods

2.1. Reagents

Recombinant HBsAg (rHBsAg, subtype adr) which did not contain a pre-S2 region was purchased from Cortex Biochem (Sanleandro, USA). Goat polyclonal anti-phospho-ERK, rabbit polyclonal anti-ERK, goat polyclonal anti-COX-2, monoclonal anti-NFκB p65 and anti-IκB-α antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The NFκB inhibitor, AAVALLPAVLLAL-LAPVQRKRQKLMP, was purchased from Santa Cruz Biotechnology, PD98059 from Calbiochem (La Jolla, CA), and LPS and PGE2 from Sigma (Sigma, St Louis, MO, USA).

2.2. Cell culture

A human monocytic cell line, THP-1, was obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 with 10% foetal bovine serum (FBS) at 37 °C under 5% CO_2/95% air. To analyze the effect of rHBsAg on THP-1 cells, cells (1×10^6 cells) were pre-treated for differentiation with 10 nM phorbol 12-myrisate 13-acetate (PMA) for 24 h, and then treated with rHBsAg (25–100 μg/ml) for different lengths of time. As a confirmation, human peripheral blood monocyte cell (PBMC) was drawn from a healthy volunteer. Human PBMC was separated by Ficoll-paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. PBMC (1×10^6 cells) was treated with rHBsAg for different concentration before LPS-induction. As a negative control, denatured rHBsAg (0.1% SDS, 100 °C for, 10 min) was added at same concentration in medium of THP-1.

2.3. Western blotting

Cells (5×10^5) were sonicated in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulforyl fluoride, 45 μg/ml aprotinin, 100 mM sodium orthovanadate) and measured for the total protein using a BCA Protein Assay Kit (Pierce, IL, USA). Samples containing the same amount of total protein were electrophoresed in SDS gels, and transfered to polyvinylidene difluoride membranes. The membranes were incubated with different primary antibodies and then with biotinylated anti-goat, -rabbit or -mouse IgG (Vector, Laboratories, Burlingame, USA). Signals were developed with an ABC kit (Vector, Burlingame, USA) and diaminobenzine.

2.4. Immunofluorescence for HBsAg and NFκB p65 proteins

Cells (1×10^4) were cultured in chamber slides (Asahi Techno, Tokyo, Japan) and pre-treated with PMA for 24 h. Cells were then incubated with rHBsAg (100 μg/ml), fixed with 10% formalin neutral buffer solution for 10 min, treated with methanol for 10 min, and microwaved for 5 min to repair the antigen in 10 mmol/l citrate buffer (pH 6.0) and increase permeability. Cells were incubated with goat anti-HBsAg polyclonal or mouse anti-NFκB p65 monoclonal antibodies at 4 °C overnight, and then with TRITC-labeled anti-goat IgG (1:100) or FITC-labeled anti-mouse IgG (1:100) (Vector, Laboratories, Burlingame, CA, USA), respectively, for 60 min at room temperature. Immunofluorescence developed was analyzed under Laser Scanning Microscope LSM510 (Carl Zeiss, Oberkochen, Germany).

2.5. Measurement of IL-18, IL-12 and PGE_2 by ELISA

IL-18, IL-12 and PGE_2 in the medium were measured by using a Human IL-18 ELISA Kit (MBL, Nagoya, Japan), a Human IL-12 ELISA Kit (Biosource, Camarillo, USA) and a PGE_2 High Sensitivity ELISA Kit (R&D Systems, Minneapolis, USA), respectively.

2.6. Statistical analysis

Statistical significance was determined using *t*-test. The significance level was set at $P<0.05$.

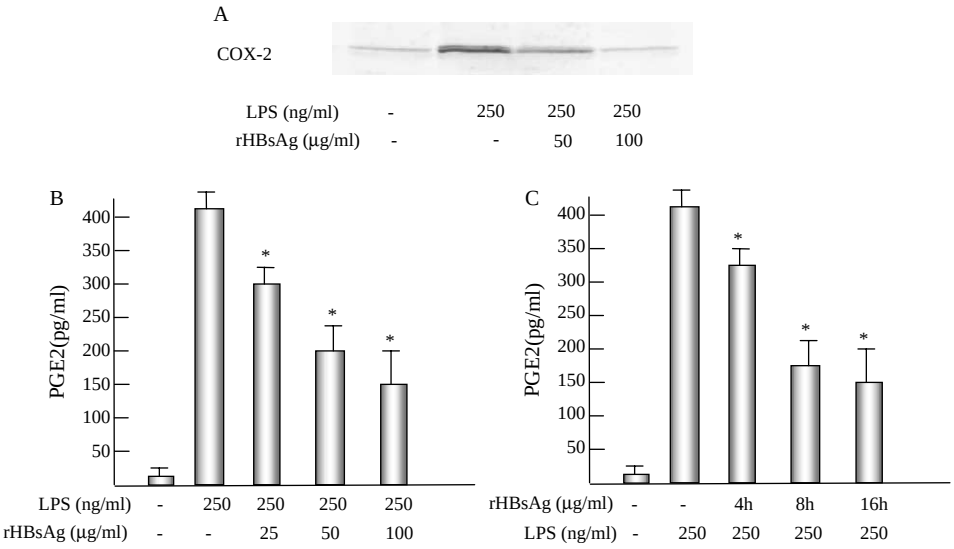

Fig. 2. Inhibition of LPS-induced COX-2 expression and PGE2 production by rHBsAg. Cells in a 12-well dish were treated by 10 nM PMA for 24 h, with 25, 50 and 100 μg/ml rHBsAg for 16 h, stimulated by LPS (250 ng/ml) for 8 h, and analyzed for COX-2 and PGE2. (A) Analysis of COX-2 in cells by Western blotting. The cells pre-treated by rHBsAg showed weaker expression of COX-2 than the cells without HBsAg pre-treatment in response to LPS stimulation. (B) Dose-dependent inhibition of LPS-induced PGE2 by rHBsAg. Analysis of PGE2 in medium by ELISA. (C) Time-course analysis of inhibition of LPS-induced PGE2 by rHBsAg. Data from three independent experiments are shown as the mean ± SD. *$P < 0.05$.

3. Results

3.1. rHBAsAg adhered to the cell membrane

To obtain the physical evidence for the interaction of rHBsAg with THP-1 cells, cells were cultured in chamber slides, pre-treated with PMA for 24 h, treated with rHBsAg (100 μg/ml) for 4 h, and then analyzed for HBsAg by immunofluorescence staining. Strong red fluorescence was observed on the membrane of HBsAg-treated cells, indicating that rHBAs adhered to the cell membrane (Fig. 1).

Analysis of cells treated with rHBsAg for 72 h by typan blue and DAPI staining showed no significant increases in cell death by the rHBsAg treatment (data not shown).

3.2. rHBsAg (adr) inhibits the induction of COX-2 and PGE2 by LPS

The effect of HBsAg on the induction of COX-2 by LPS by incubating THP-1 cells, pre-treated with PMA, with 100 μg/ml rHBsAg for 16 h, stimulating with 250 ng/ml LPS for 4 h and then performing COX-2 analysis by Western blotting was studied. The results showed that rHBsAg inhibits LPS-induced COX-2 expression (Fig. 2A).

For the effect of HBsAg on the induction of PGE2, cells were treated with PMA and rHBsAg followed by LPS as described above for COX-2 and then analyzed for PGE2 by ELISA. The results showed that rHBsAg inhibited LPS-induced PGE2 expression in a dose-dependent (Fig. 2B) and time-dependent (Fig. 2C) manner.

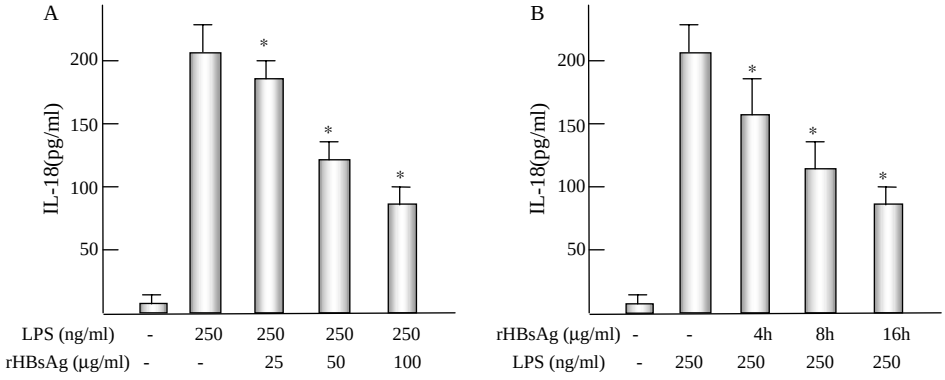

Fig. 3. Effect of rHBsAg on LPS-induced IL-18 production. Cells were treated with rHBsAg at 25, 50 and 100 μg/ml for 16 h and then with 100 μg/ml rHBsAg for 4, 8 and 16 h. LPS (250 ng/ml) was added to the medium and incubation continued for 8 h. IL-18 in medium was analyzed by ELISA. (A) Dose-dependent inhibition of LPS-induced IL-18 by rHBsAg. (B) Time-dependent inhibition of LPS-induced IL-18 by rHBsAg. Data from three independent experiments are shown as the mean ± SD. *$P < 0.05$.

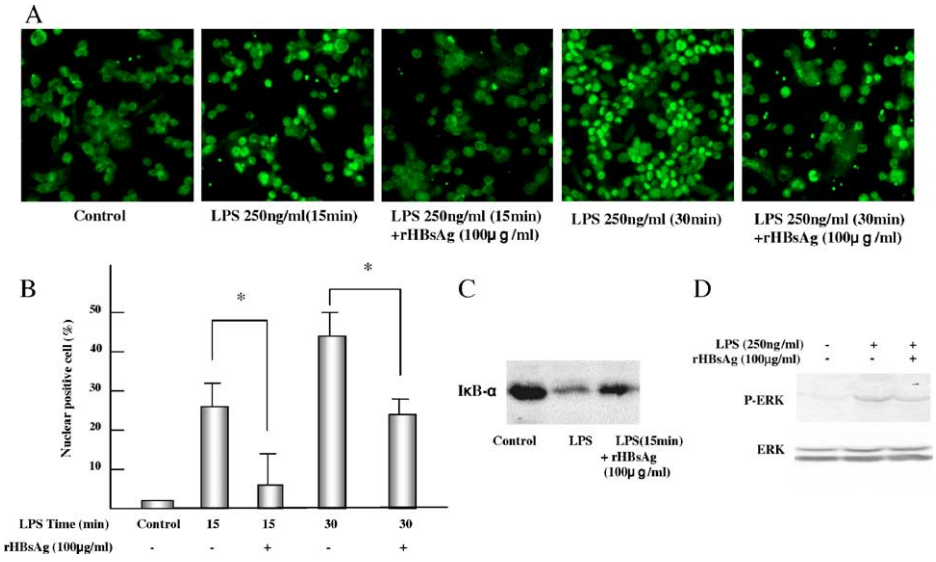

Fig. 4. Effects of rHBsAg on LPS-induced NFκB pathway and ERK activation. (A) Analysis of NFκB p65 protein by immunofluorscence staining. Cells, in chamber slide, were treated with rHBsAg at 100 μg/ml for 16 h, and with LPS (250 ng/ml) for 15 or 30 min. NFκB p65 protein was analyzed by immunofluorescence staining using mouse anti-NFκB p65 antibody. (B) Percentage of nuclear positive cells. More than 1000 cells/slide were counted. LPS-induced entrance of NFκB p65 protein from the cytoplasm to the nucleus was delayed by rHBsAg treatment. (C) The LPS-induced degradation of IκB-α was down-regulated by rHBsAg treatment. (D) Effect of rHBsAg on the ERK pathway. THP-1 cells were treated with rHBsAg (100 μg/ml) for 16 h, with LPS (250 ng/ml) for 30 min, and analyzed for phosphorylated ERK by Western blotting. LPS-induced phosphorylation of ERK was inhibited by rHBsAg treatment. Data from three independent experiments are shown as the mean ± SD. *$P < 0.05$.

3.3. rHBsAg inhibits LPS-induced IL-18 production

To analyze the effect of rHBsAg on IL-18 production induced by LPS, PMA-pre-treated cells (1×10^6 cells) were incubated with 25, 50 and 100 μg/ml rHBsAg for 16 h, with LPS (250 ng/ml) for 8 h, and analyzed for IL-18 released into medium by ELISA. As shown in Fig.3A, rHBsAg inhibited LPS-induced IL-18 production in a dose-dependent manner. Time-course analysis of the effect of rHBsAg (100 μg/ml) showed that rHBsAg inhibited LPS-induced IL-18 production in a time-dependent manner (Fig. 3B).

To confirm the effect on another system, human PBMC from a healthy volunteer was stimulated by LPS (250 ng/ml) after100 μg/ml rHBsAg treatment for 16 h. rHBsAg inhibited LPS-induced IL-18 and PGE2 production in human PBMC at 48.4 and 42%. As a negative control, a denatured treatment of rHBsAg eliminated the inhibitory effect on IL-18 and PGE2 in THP-1 cell.

3.4. rHBsAg inhibits LPS-induced activation of NFκB and ERK pathways

To investigate the mechanism by which rHBsAg inhibited LPS-induced COX-2 expression, the NFκB pathway, an important pathway for COX-2 expression and IL-18 production was analyzed by determining NFκB p65 protein

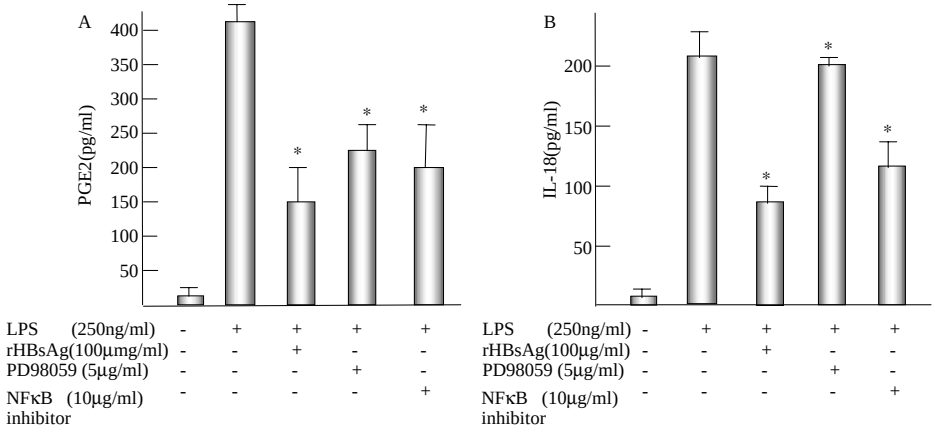

Fig. 5. Effects of inhibitors of the ERK and NFκB signal pathways on LPS-induced expression of COX-2, PGE2 and IL-18. (A) PD98059 (ERK inhibitor) (5 μg/ml) and AAVALLPAVLLALLAPVQRKRQKLMP (NFκB inhibitor) (10 μg/ml) blocked significantly LPS-induced PGE2 production. (B) The NFκB inhibitor, but not the ERK inhibitor (PD98059) inhibited LPS-induced IL-18 production. Data from three independent experiments are shown as the mean ± SD. *$P < 0.05$.

by immunofluorsescence staining (Fig. 4A). The number of cells positive for nuclear NFκB p65 was significantly lower in the rHBsAg-treated cells than in the control cells (Fig. 4B) and LPS-induced IκB-α degradation was inhibited (Fig. 4C), indicating that rHBsAg inhibited LPS-induced activation of the NFκB signal pathway.

In order to confirm the role of the NFκB pathway in our system, cells were treated with 10 μg/ml AAVALLPAVLLALLAPVQRKRQKLMP, an NFκB pathway inhibitor, for 2 h before LPS treatment, and analyzed for production of PGE2 and IL-18. As shown in Fig. 5 (A and B), the NFκB pathway inhibitor suppressed LPS-induced PGE2 and IL-18 production. These results suggest that the NFκB pathway plays an important role in PGE2 and IL-18 production.

We next examined the effect of rHBsAg on the ERK pathway. Cells (1×10^6) were treated with 100 μg/ml rHBsAg for 12 h, with 250 ng/ml LPS for 30 min, and then analyzed for phosphorylated ERK by Western blotting. This result showed that LPS-induced phosphorylation of ERK was inhibited by rHBsAg (Fig. 4D).

In order to understand the significance of ERK in LPS-inducing COX-2 and IL-18 production, THP-1 cells were treated with a selective inhibitor of phosphorylation of ERK, PD98059 (5 μg/ml), for 2 h before LPS treatment. PD98059 inhibited the production of PGE2 about 50% (Fig. 5A), but had no effect on IL-18 production (Fig. 5B). These results suggest that the ERK pathway was involved in LPS-induced COX-2 expression and PGE2 production. However, our results provided no evidence that the ERK pathway was involved in LPS-induced IL-18 production in THP-1 cells. The reason why PD98059 inhibited the expression of COX-2 and PGE2 production, but not IL-18 production is not clear at present. It is possible that the NFκB pathway may compensate for the loss of the ERK pathway caused by PD98059.

3.5. PGE2 reverses the inhibition of IL-18 production by rHBsAg

To investigate the relationship between the COX-2/PGE2 pathway and IL-18 production, we studied the effect of PGE2 on IL-18 production in THP-1 cells. PGE2 did not induce the IL-18 production significantly, but it enhanced LPS-induced IL-18 production (Fig. 6). In addition, PGE2 reversed the inhibition of LPS-induced IL-18 production by rHBsAg in a dose-dependent manner (Fig. 6). These results showed that PGE2 promoted LPS-induced IL-18 production.

3.6. rHBsAg inhibits LPS-induced IL-12 production in a dose-dependent manner

To analyze the effect of rHBsAg on the induction of IL-12 by LPS, THP-1 cells (1×10^6 cells), pre-treated by 10 nM PMA for 24 h, were treated with rHBsAg at 25, 50 and 100 μg/ml for 16 h and with LPS (250 ng/ml) for 8 h, and analyzed for IL-12 in the medium by ELISA. As shown in Fig. 7, LPS-induced IL-12 production was inhibited by rHBsAg in a dose-dependent manner.

4. Discussion

The results of this study are summarized in Fig. 8. The NFκB and ERK pathways were involved in the inhibition of

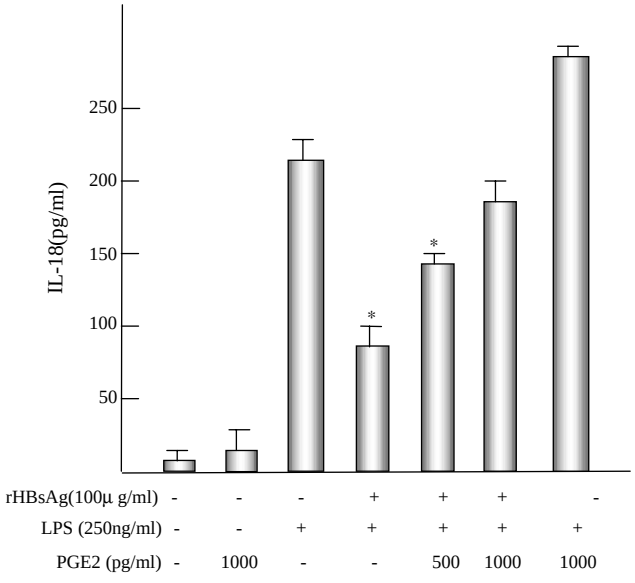

Fig. 6. Suppression of the inhibitory effect of rHBsAg on LPS-induced IL-18 production by PGE2. Cells were treated with rHBsAg at 100 μg/ml for 16 h, with PGE2 (500 or 1000 pg/ml) for 2 h, and then with LPS (250 ng/ml) for 8 h. IL-18 in medium was analyzed by ELISA. Results are shown as the mean±SD of three separate experiments.

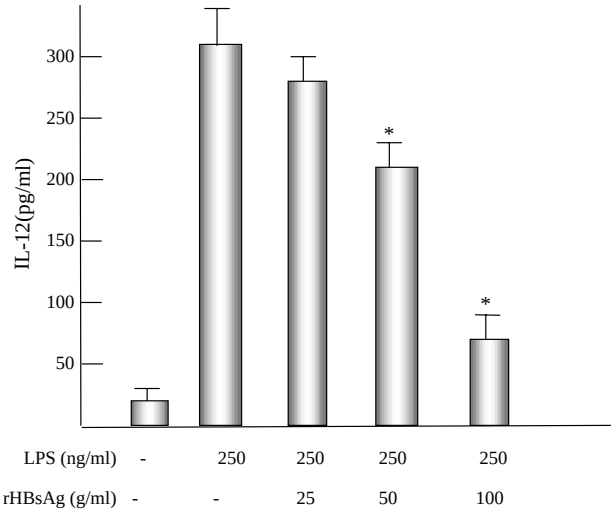

Fig. 7. A dose-dependent inhibition of LPS-induced IL-12 production by rHBsAg. Cells were treated with rHBsAg at 25, 50 and 100 μg/ml for 16 h, with LPS (250 ng/ml) for 8 h, and analyzed for IL-12 in the medium by ELISA. Results are shown as the mean±SD of three separate experiments. *: $P<0.05$.

LPS-induced COX-2 expression and the production of PGE2 by rHBsAg in THP-1 cells. rHBsAg inhibited LPS-induced COX-2 expression and PGE2 production by inhibiting both NFκB and ERK pathways. LPS-induced production of IL-18 was also down-regulated by rHBsAg through inhibition of the NFκB pathway. This inhibitory effect of rHBsAg on IL-18 production was reversed by the addition of exogenous PGE2. Our findings show that rHBsAg inhibits COX-2, PGE2 and IL-18 production by suppressing THP-1 cell responses to LPS stimulation, suggesting a novel immunoregulatory role for rHBsAg and a novel relationship between IL-18 and PGE2 in rHBsAg-treated monocytes. The further study about how HBsAg regulates the level of IFN-gamma in vivo will be significant to understand HBV persistence.

COX-2 is produced by a highly inducible gene expressed in response to cell activation processes, such as growth, differentiation, and inflammation [1,18]. It is a crucial enzyme in the process of PGs' synthesis. Prostaglandins have been recognized as important mediators of hepatic, renal, cardiovascular and pulmonary functions and their synthesis alters during the courses of several physiopathological situations [19,20]. PGE2 plays an important role in liver pathogenesis. For example, PGE2 delays collagen formation and up-regulates tissue inhibitor of metalloproteinases-2 leading to matrix accumulation in rat liver [21]. It has been shown that plasma levels of PGE2 increase with the advancement of liver cirrhosis [22]. COX-2 expression has not been described previously in relation with HBV protein. In this study, we demonstrated a novel relationship between the HBV antigen and COX-2 expression,which is an important pro-inflammation reaction in monocytes.

IL-18 together with IL-12 strongly induces IFN-γ production in T and NK cells, augments their activity, enhances the expression of the Fas ligand, and induces the production of GM-CSF [23–26]. IFN-γ has a central role in host defenses in both innate and acquired immunity. IFN-γ is secreted from T cells and NK cells upon stimulation with antigens or mitogens, but a detailed regulatory mechanism for IFN-γ production has not yet been fully elucidated in relation to hepatitis B virus infection. IL-18 has the potential to control HBV replication during self-limited infection in vivo through production of IFN-γ [17]. IL-12 exhibits various physiological activities, one of which is the induction of IFN-γ [27]. In this study, we showed that rHBsAg have an important role, previously unknown by affecting IL-18 and IL-12 production, which may be important for persistent HBV infection in chronic HBV carrier.

Recent reports have shown that different relationships exist between IL-18 and PGE2 in different cells. It has been reported that PGE2 simulates the production of IL-18 in microglial cells [28]. PGE2 inhibits IL-18-induced ICAM-1 and B7.2 expression in mononuclear cells [29]. A relatively strong correlation has been reported between IL-18 and PGE2 levels in synovial fluid of osteoarthritis [30]. PGE2

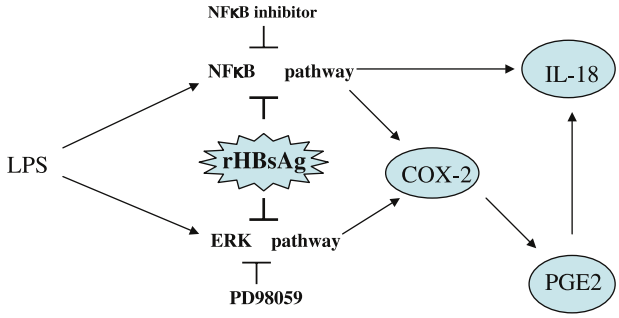

Fig. 8. The relationship between LPS-induced production of COX-2, PGE2 and IL-18 in THP-1 cells and sites of inhibition by rHBsAg. LPS induces COX-2/PGE2 and IL-18 by ERK and NFκB pathways. rHBsAg inhibits both the ERK and NFκB pathways, resulting in inhibition of LPS-induced the expression of COX-2 and the production of PGE2 and IL-18. The NFκB inhibitor inhibits the LPS-induced production of PGE2 and IL-18 while the ERK inhibitor (PD98059) inhibits LPS-induced COX-2/PGE2 expression only. Exogenous PGE2 reverses the inhibition of LPS-induced IL-18 production by rHBsAg.

was shown to suppress IFN-γ synthesis by NK cell in a recent report [31]. In this study, we reported that PGE2 reversed the inhibitory effect of rHBsAg on LPS-induced IL-18 production in THP-1 cells through a pathway other than ERK and NFκB pathways. Four PGE2 receptor subtypes, EP1, EP2, EP3 and EP4, are known to mediate PGE2 functions, singly or in combination [32]. It is of interest to identify the receptor(s) involved in the reversion of rHBsAg-induced inhibition of IL-18 expression by PGE2 in THP-1 cells. It is also of interest to investigate how PGE2 affects the final IFN-γ level by different effects to monocyte and NK cell.

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