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Phosphatidylinositol 3-kinase modulates IL-18-induced nuclear factor-κB activation

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KEY WORDS interleukin 18；1-phosphatidylinositol 3-kinase；antisense oligonucleotides；NF-kappa B；enzyme-linked immunosorbant assay；reverse transcriptase polymerase chain reaction

ABSTRACT

AIM：To investigate whether phosphatidylinositol (PI) 3-kinase is involved in interleukin-18 (IL-18)-induced nuclear factor-κ B (NF-κB) activation. **METHODS**：Antisense PI 3-kinase oligonucleotide (ODN) was delivered by lipofectin encapsulation into cultured human hepatocellular carcinoma HepG2 cells. PI 3-kinase mRNA expression was assayed by semiquantitative reverse transcription-PCR. The levels of NF-κB were measured by sandwich ELISA. **RESULTS**：(1) Antisense PI 3-kinase ODN blocked PI 3-kinase mRNA expression. (2) IL-18 activated NF-κB from basal level of 0.153 ± 0.008 to 1.942 ± 0.017. (3) Antisense PI 3-kinase ODN inhibited IL-18-induced NF-κB activation in a concentration (1 – 8 mg/L)- and time (5 – 24 h)-dependent fashion. When the cells were treated with antisense PI 3-kinase ODN 2 mg/L for 8 h，a maximum inhibitory rate was 35.2 % from 1.942 ± 0.017 control to 1.259 ± 0.018. **CONCLUSION**：PI 3-kinase is necessary for IL-18-stimulated NF-κB activation.

INTRODUCTION

Interleukin-18 (IL-18)，a novel proinflammatory cytokine，is involved in the progression of pathogenicity in chronic inflammatory diseases including endotoxin-shock，hepatitis，and autoimmune-diabetes[1]. Many of the proinflammatory effects of IL-18 are regulated largely by nuclear factor-κB (NF-κB)[1]. In resting cells，NF-κB is present in the cytoplasm，where it is bound to an inhibitor protein IκB. In response to IL-18，IκB is degraded，and NF-κB is transported to the nucleus to switch on the expression of certain genes[2]. Elucidation of the IL-18 signal transduction pathway leading to NF-κB activation should provide valuable insight into potential mechanisms to alleviate inflammation.

Recently，phosphatidylinositol (PI) 3-kinase has been found to be involved in IL-1 signaling cascade that results in translocation of NF-κB[3]. Since IL-18 is related to the IL-1 family in terms of primary structure，3-dimensional structure，receptor family，signal transduction pathways，and biological effects[1]，we postulated that PI 3-kinase was required for IL-18-induced NF-κB activation. To test this hypothesis，we evaluated the effects of PI 3-kinase on IL-18-stimulated NF-κB activation.

MATERIALS AND METHODS

Cell lines and reagents Human hepatocellular carcinoma HepG2 cells were obtained from American Type Culture Collection and cultured to confluency at 37 ℃ in a humidified atmosphere of 5 % CO_2 + 95 % air in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum. Lipofectin was purchased from Gibco，BRL. IL-18 was purchased from R&D systems. Goat polyclonal antibody against the carboxy terminus of NF-κB p65 and rabbit polyclonal antibody against the amino terminus of NF-κB p65 were obtained from Santa Cruz Biotechnology，Inc，USA. Goat anti-rabbit IgG-HRP was purchased from Sino-American Biotechnology Co. Spin or vacuum (SV) total RNA isolation system and reverse transcription-PCR (RT-PCR) kit were supplied by Promega Corporation.

Synthesis of oligonucleotides (ODN) and selection of sequence targets The sequences of ODN against PI 3-kinase were：antisense，5′-CTC AGC ACT CAT GTT TGC-3′；sense，5′-GCA AAC ATG AGT GCT GAG-3′. Sense ODN was used as control. ODN were synthesized by Sangon Co Ltd and modified with

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phosphorothioate.

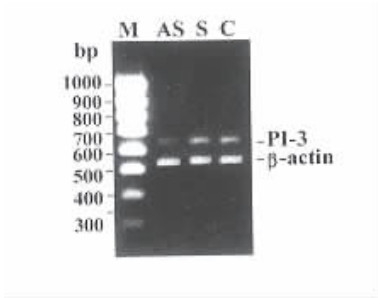
RT-PCR for detecting PI 3-kinase mRNA expression Cells grown to confluency were exposed to IL-18 100 kU·L^{-1} for 30 min. The cultures were washed with DMEM to remove IL-18. Cells were then treated with lipofectin-encapsulated sense PI 3-kinase ODN 2 mg/L, or lipofectin-encapsulated antisense PI 3-kinase ODN 2 mg/L for 8 h. The cells in control group were incubated in DMEM without any treatment. Total RNA of cells was extracted, reverse transcribed, and amplified by PCR. Amplified DNA was visualized by ethidium bromide stain of 1 % agarose gel. Results from three independent experiments were quantified by BIO-PROFIL/BIO-CAPT/BIO-1D$^{(++)}$ analysis software (VILBER LOURMAT). Since β-actin was housekeeping gene, amplification signals for PI 3-kinase mRNA were normalized with the amplification signals of β-actin mRNA. The PI 3-kinase 5′ primer (nucleotides 56 to 77 of human sequence) was 5′-GGT ACC AGT ACA GAG CGC TGT A-3′; the 3′ primer (nucleotides 685 – 706) was 5′-CGG AGC TTT GTA CTT CTG GAG C-3′. The primers complementary to the human β-actin gene were: the 5′ primer, 5′-GTG GGG CGC CCC AGG CAC CA-3′; the 3′ primer, 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ (synthesized by Sangon Ltd).

Nuclear protein extraction and ELISA for detecting NF-κB Cells grown to confluency were exposed to IL-18 100 kU·L^{-1} for 30 min. The cultures were washed with DMEM to remove IL-18. Cells were then treated with lipofectin-capsulated antisense PI 3-kinase ODN. Subsequently, the cultures were washed and exposed to IL-18 100 kU·L^{-1} for 1 h. The cells in basal group were incubated in DMEM without any treatment. Nuclear proteins of cells were extracted by the method described previously[4] and NF-κB was detected using sandwich ELISA. Ninety six-well microtiter plates (Sigma) were coated with goat polyclonal antibody against the carboxy terminus of NF-κB p65 (0.003 g·L^{-1}, 100 μL/well) overnight at 4 ℃. Then the plates were blocked with 1 % BSA overnight at 4 ℃. The plates were washed. Nuclear proteins diluted at 1∶500 100 μL were added to the plates at 37 ℃ for 2 h. The plates were washed, and rabbit polyclonal antibody against the amino terminus of NF-κB p65 was added and incubated at 37 ℃ for 2 h. The plates were washed and incubated with goat anti-rabbit IgG-HRP diluted at 1∶1000 (100 μL/well) at 37 ℃ for 2 h. The plates were washed, and *o*-phenylenedeiamine (OPD) 2 mmol·L^{-1} with 0.006 % H_2O_2 in citric acid-phosphate buffer (100 μL/well) was added. Then the plates were incubated at 37 ℃ for 1 h. The reaction was stopped by adding citric acid 2 mol·L^{-1} (50 μL/well). The absorbance at 450 nm was measured with a Model 450 microplate reader (BIORAD).

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared with t test.

RESULTS

Effects of antisense PI 3-kinase ODN on PI 3-kinase mRNA expression The results from RT-PCR showed that antisense PI 3-kinase ODN inhibited PI 3-kinase expression ($P < 0.01$), while the sense ODN had no effect (Fig 1).



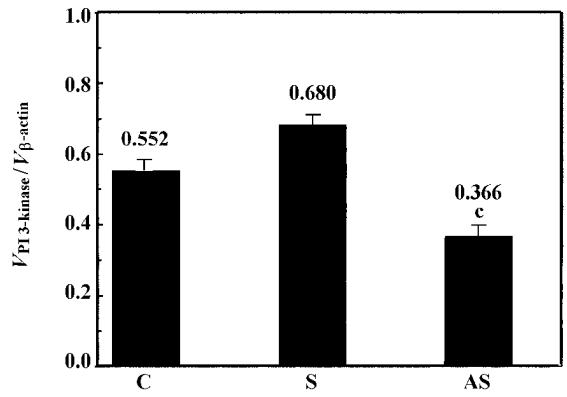


Fig 1. Antisense PI 3-kinase ODN inhibited PI 3-kinase mRNA expression. n = 3 independent experiments. $\bar{x} \pm s$. M: DNA size marker; S: sense PI 3-kinase ODN; AS: antisense PI 3-kinase ODN; C: control; V: volume of peak.

Effects of antisense PI 3-kinase ODN on activation of NF-κB induced by IL-18 IL-18 stimulated NF-κB activation from basal level of absorbance (A) 0.153 ± 0.008 to 1.942 ± 0.017 and antisense PI 3-kinase ODN blocked IL-18-stimulated NF-κB activation in a

concentration- and time-dependent fashion. When the cells were treated with antisense PI 3-kinase ODN 2 mg/L for 8 h, NF-κB levels were reduced by 35.2 % from 1.942 ± 0.017 control to 1.259 ± 0.018 ($P < 0.01$) (Tab 1).

Tab 1. Antisense PI 3-kinase ODN inhibited IL-18-induced NF-κB activation. $n = 3$ wells. $\bar{x} \pm s$. $^{c}P < 0.01$, $^{f}P < 0.01$, $^{i}P < 0.01$ *vs* respective control.

Antisense PI 3-kinase ODN/mg/L	NF-κB/A_{450} 5 h	8 h	24 h
0	1.954 ± 0.037	1.942 ± 0.017	1.937 ± 0.040
1	1.630 ± 0.022^{c}	1.512 ± 0.009^{f}	1.590 ± 0.012^{i}
2	1.375 ± 0.017^{c}	1.259 ± 0.018^{f}	1.303 ± 0.008^{i}
4	1.422 ± 0.022^{c}	1.389 ± 0.011^{f}	1.413 ± 0.011^{i}
8	1.808 ± 0.017^{c}	1.702 ± 0.013^{f}	1.738 ± 0.035^{i}

DISCUSSION

Antisense ODN have recently generated considerable interest as a tool for research and also as potential therapeutic agents for human diseases predominantly due to their specific potential. The specificity of antisense PI 3-kinase ODN was supported by our observation that antisense PI 3-kinase ODN inhibited expression of PI 3-kinase mRNA, while they did not attenuate housekeeping gene β-actin expression (Fig 1). The specific inhibition of PI 3-kinase mRNA expression by antisense PI 3-kinase ODN led to the concentration- and time-dependent inhibition of NF-κB activation. A maximum inhibition of NF-κB activation occurred when the cells were treated with antisense PI 3-kinase ODN 2 mg/L for 8 h. The reason why higher concentration (> 2 mg/L) of antisense PI 3-kinase ODN produced a weaker effect has yet to be investigated.

As mentioned above, antisense PI 3-kinase ODN blocked IL-18-induced NF-κB activation via inhibition of PI 3-kinase mRNA expression. It suggests that PI 3-kinase modulates IL-18-induced NF-κB activation. The mechanisms by which PI 3-kinase modulates IL-18-induced NF-κB activation remain to be clarified. However, several signaling molecules have been shown to affect directly or indirectly the pathway resulting in the activation of NF-κB. First, protein kinase C (PKC) ζ has been implicated as a regulator of NF-κB activation. Since the phosphorylated lipid products of PI 3-kinase activate PKC ζ, PKC ζ acts downstream of PI 3-kinase[3]. Second, ceramide is critical in initiating the events leading to NF-κB activation via degradation of IκB[2]. Ceramide enhances PI 3-kinase activity[5], so it may lie upstream of PI 3-kinase. Finally, mitogen-activated protein kinase (MAPK) pathway is involved in NF-κB activation. Raf-1 kinase[2], Jun N-terminal kinase (JNK)[6], and p38 MAPK[6] have been shown to modulate NF-κB activation. Their activity can be mediated by PI 3-kinase[7].

Taken together, in this study, PI 3-kinase has been identified for the first time as a signal molecular in IL-18 signaling pathway to activate NF-κB. It provides a therapeutic target for IL-18-induced inflammation.

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磷脂酰肌醇 3-激酶调控白介素-18 诱导核因子-κB 活化

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关键词　白介素 18；1-磷脂酰肌醇 3-激酶；反义寡核苷酸类；核因子-κB；酶联免疫吸附测定；逆转录聚合酶链反应

目的：研究磷脂酰肌醇（PI）3-激酶在白介素 18（IL-18）诱导核因子-κB（NF-κB）活化中的作用．方法：Lipofectin 介导反义 PI 3-激酶寡核苷酸转染 HepG2 细胞．用逆转录 PCR 法检测 PI 3-激酶 mRNA 表达水平，以 Sandwich ELISA 法检测 NF-κB 的活化．结果：（1）反义 PI 3-激酶寡核苷酸抑制 PI 3-激酶 mRNA 表达．（2）IL-18 诱导 NF-κB 活化．（3）反义 PI 3-激酶寡核苷酸呈时间（5 – 24 h）和浓度（1 – 8 mg/L）依赖性地抑制 IL-18 诱导的 NF-κB 活化．结论：PI 3-激酶调控白介素-18 诱导的 NF-κB 活化．

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