Pulmonary Pharmacology & Therapeutics 23 (2010) 308–315

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

Inhibition by ethyl pyruvate of the nuclear translocation of nuclear factor-κB in cultured lung epithelial cells

Ayako Mizutani [a,c], Noriko Maeda [a], Seikichi Toku [a], Yoichiro Isohama [b], Kazuhiro Sugahara [c], Hideyuki Yamamoto [a,*]

[a] Department of Biochemistry, School of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan
[b] Department of Chemico-Pharmacological Sciences, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan
[c] Department of Anesthesiology, School of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan

ARTICLE INFO

Article history:
Received 28 October 2009
Received in revised form
15 February 2010
Accepted 4 March 2010

Keywords:
Alveolar epithelial cells
A549 cells
Ethyl pyruvate
Inflammation
Nuclear factor-κB
Tumor necrosis factor α

ABSTRACT

Tumor necrosis factor α (TNFα) is a cytokine inducing inflammatory responses. It has been reported that ethyl pyruvate has anti-inflammatory actions through inhibition of the transcription mediated by nuclear factor-kappa B (NF-κB). By reporter gene assay, we first confirmed that TNFα activated the NF-κB pathway in cultured alveolar epithelial cells, A549 cells. This activation was strongly inhibited by ethyl pyruvate in a concentration-dependent manner. Treatment of the cells with TNFα-induced phosphorylation and degradation of IκBα within 15 min. The level of IκBα protein was increased from 30 min, suggesting an increase in the NF-κB-mediated transcription of IκBα. Ethyl pyruvate did not affect the changes in IκBα within 15 min, but strongly inhibited the increase in the IκBα protein level from 30 min. An immunoblot analysis revealed that ethyl pyruvate inhibited the nuclear translocation of RelA from 5 min of the treatment with TNFα. These results strongly suggested that ethyl pyruvate inhibited the NF-κB pathway through inhibition of the nuclear translocation of RelA. Ethyl pyruvate may be a good therapeutic drug for inflammation in which activation of the NF-κB pathway is involved.

1. Introduction

Nuclear factor-kappa B (NF-κB) is a central mediator of human immune responses. In mammals, there are at least five NF-κB proteins, RelA, p50, RelB, p52 and RelC, and they form a variety of homodimers and heterodimers (for review, see [17]). The dimers exist in the cytoplasm in inactive forms due to the binding of an inhibitory protein called IκB (for review, see [17]). Many inflammatory conditions, including bacterial and viral infections, rapidly activate the NF-κB signaling pathway. Tumor necrosis factor α (TNFα), a cytokine especially important in inducing inflammatory responses, also activates this pathway. Activation of NF-κB is induced by the phosphorylation, ubiquitination and degradation of IκB. Then, NF-κB dimers translocate to the nucleus where they stimulate the transcription of hundreds of genes that participate in inflammatory responses. Therefore, pharmacological inhibition of NF-κB should substantially attenuate inflammatory responses.

Ethyl pyruvate, an aliphatic ester derived from pyruvate, has been shown to be an effective anti-inflammatory agent in a variety of *in vitro* and *in vivo* model systems [5]. For example, ethyl pyruvate has been reported to ameliorate injuries following endotoxemia [21], alcoholic hepatitis [24] and acute pancreatitis [25]. In many of these models, it has been reported that ethyl pyruvate represses the expression of various pro-inflammatory genes, including inducible nitric oxide synthase, TNFα, cyclooxygenase-2, and interleukin-6 [18,21,26]. Some of these anti-inflammatory effects of ethyl pyruvate may be due to inhibition of the NF-κB signaling pathway [7,8]. However, the molecular mechanisms by which ethyl pyruvate inhibits the NF-κB signaling pathway are not fully understood. In spite of all the data about the effects of ethyl pyruvate on inflammatory processes in various tissues, its effects on lung injury have not been carefully examined.

Acute lung injury is characterized by alveolar damage with a disruption of the alveolar epithelial barrier, inflammatory cells and protein rich edema fluid influx into the alveolus and surfactant abnormalities [6,19]. TNFα has been reported to down-regulate surfactant production by alveolar epithelial type II cells [2]. A549 cells are immortalized alveolar epithelial cells, which retain many

Abbreviations: NF-κB, Nuclear factor-κB; TNFα, Tumor necrosis factor α; DMEM, Dulbecco's modified Eagle's medium.

* Corresponding author. Department of Biochemistry, School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan. Tel.: +81 98 895 1112; fax: +81 98 895 1404
E-mail address: hideyuki@med.u-ryukyu.ac.jp (H. Yamamoto).

1094-5539/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.pupt.2010.03.002

of the characteristics of alveolar epithelial type II cells. This cell line has been used as a model to study the responses of alveolar epithelial type II cells to several treatments [4,9,10,13].

In the present study, we found that ethyl pyruvate inhibited the TNFα-induced activation of NF-κB in A549 cells; therefore, we closely examined the molecular mechanisms responsible for this inhibition.

2. Materials and methods

2.1. Materials

The following chemicals and reagents were obtained from the indicated sources: [γ-^{32}P]ATP, Perkin Elmer, Inc. (Boston, MA); Dulbecco's modified Eagle's medium (DMEM), ethyl pyruvate, 1M HEPES, pH 7.0–7.6, phosphate-buffered saline (PBS), and reduced glutathione (GSH) ethyl ester, Sigma Chemical Co. (St Louis, MO); fetal calf serum (FCS), HyClone (Logan, UT); phorbol 12-myristate 13-acetate (PMA), Promega Corp., Madison, WI; protease inhibitor cocktail and phosphatase inhibitor cocktail (EDTA free), Nacalai Tesque (Kyoto, Japan); SDS-PAGE molecular weight standards, Bio-Rad (Richmond, CA); anti-IκBα, anti-phospho-IκBα, anti-RelA, anti-p50, anti-RelB, anti-p52, and anti-RelC antibodies, Cell Signaling (Beverly, MA); anti-β-actin antibody, Sigma Chemical Co. (St Louis, MO); anti-RelA monoclonal antibody, Santa Cruz (Santa Cruz, CA); and TNFα, Wako (Osaka, Japan). Other chemicals were of analytical grade.

2.2. Culture of A549 cells, transfection of luciferase reporter gene and dual-luciferase reporter gene assay

A549 cells were grown on Petri dishes (Nunc, Roskilde, Denmark) in a culture medium consisting of 500 ml of DMEM containing 0.584 g/L of L-glutamate and 4.5 g/L of glucose and 10% (vol/vol) heat-inactivated FCS as described previously [9,13]. Primary Human Venous Endothelial Cells (HUVEC) and the culture medium for the cells were obtained from DS Pharma Biomedical Co. (Osaka, Japan). The NF-κB luciferase reporter gene (pNF-κB-Luc) was obtained from Stratagene Co. (La Jolla, CA). The firefly-luciferase reporter gene, which has five-tandem repeats of the κB-motif (TGGGGACTTTCCGC) upstream of the TATA-box, was transfected into A549 cells. To adjust the variation in transfection efficacy among culture dishes, the cells were co-transfected with phRL-TK (Promega, Co. WI), which contains the herpes simplex virus thymidine kinase promoter to provide the constitutive expression of *Renilla* luciferase. The cells were co-transfected with pNF-κB-Luc (2.0 μg of plasmid DNA) and phRL-TK (0.2 μg of plasmid DNA) using 4 μl of FuGENE HD transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) in 2 ml of standard medium. After 36 h, the medium was exchanged to the complete growth medium, and the cells were further cultured for 24 h. In some experiments, A549 cells were pretreated with 5 or 10 mM ethyl pyruvate for 60 min. The cells were treated for 4 h with 100 ng/ml of TNFα in the presence or absence of ethyl pyruvate. When we examined the effects of GSH ethyl ester, the cells were pretreated with 6 mM GSH ethyl ester for 15 min before treatment with TNFα [18]. The activities of firefly luciferase and *Renilla* luciferase were measured by the Dual-Luciferase Reporter Assay System (Promega, Co. WI) with a luminometer (TD-20/20, Turner designs, Sunnyvale, CA) according to the manufacturer's protocol. The ratio of luminescence from the reaction mediated by firefly luciferase to that from the reaction mediated by *Renilla* luciferase was determined.

2.3. Preparation of cell extracts

A549 cells were washed once in PBS and lysed on ice in a homogenization buffer containing 20 mM Tris–HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 0.1% (vol/vol) Triton X-100, a protease inhibitor cocktail, and a protein phosphatase inhibitor cocktail [14]. We diluted the protease inhibitor cocktail and the protein phosphatase inhibitor cocktail as directed by the manufacturer. The homogenate was centrifuged at 13,000 × *g* at 4 °C for 10 min to obtain the supernatant (cell extract). The cell extract thus obtained was heat-treated in the presence of SDS-PAGE sample buffer [11] at 100 °C for 2 min.

2.4. SDS-PAGE and immunoblot analysis

SDS-PAGE was performed by the method of Laemmli [11], followed by an immunoblot analysis [20]. Immunoreactive proteins were detected using the enhanced chemiluminescence detection kit (GE Healthcare UK Ltd., Little Chalfont, England) as directed by the manufacturer. For reprobing, the membrane was incubated with stripping buffer [62.5 mM Tris–HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% (wt/vol) SDS] at 50 °C for 30 min [23]. The membrane was then washed with a blocking solution containing 2.5% (wt/vol) BSA or 5% (wt/vol) skim milk, 100 mM Tris–HCl, pH 7.5, 0.9% (wt/vol) NaCl, and 0.1% (vol/vol) Tween-20, and Tris-buffered saline with Tween-20 (TTBS) containing 100 mM Tris–HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween-20 at room temperature and subjected to an immunoblot analysis.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of A549 cells was extracted using an RNeasy mini kit (QIAGEN, MD, USA) according to the manufacturer's directions, and the amount of total RNA was determined by measuring the absorbance at 260 nm. First-strand cDNA was synthesized from 2 μg of total RNA in a 20 μl reaction volume using a TAKARA RNA PCR kit (AMV) Ver.3.0 (Takara Bio Inc., Otsu, Japan) with an Oligo dT-Adaptor primer. Preliminary PCR experiments were performed to identify the linear amplification conditions for each product. Amplification of human IκBα and GAPDH was performed for 30 cycles (30 s at 95 °C, 30 s at 62 °C, 2 min at 72 °C) and 21 cycles (30 s at 95 °C, 30 s at 60 °C, 2 min at 72 °C), respectively. For the amplification of IκBα, we used as a sense primer, GACGAGGAGTACGAGCAGATGGTCAAG, and as an antisense primer, GACACGTGTGGCCATTGTAGTTGGTAG. For the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we used as a sense primer, ACCACAGTCCATGCCATCAC, and as an antisense primer, TCCACCACCCTGTTGCTGTA. The PCR products were separated by electrophoresis on a 1% agarose gel, visualized by ethidium bromide staining, and quantified using a LAS4000 mini (Fuji Film, Tokyo, Japan).

2.6. Preparation of nuclear extracts of A549 cells and HUVEC

Nuclear extracts of A549 cells and HUVEC were prepared according to the methods of Yamamoto et al. [22], with some modifications. Briefly, the cells cultured in a 60-mm plastic dish were harvested by scraping in 400 μl of Buffer A (10 mM HEPES-KOH, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 0.1 mM sodium orthovanadate, protease inhibitor cocktail, and protein phosphatase inhibitor cocktail) for 5 min on ice. After centrifugation at 3000 × *g* for 2 min, the nuclear pellet was resuspended in Buffer B (50 mM HEPES-KOH, pH 7.8, 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium orthovanadate, protease inhibitor cocktail, and protein phosphatase inhibitor cocktail) and incubated for 30 min on ice. The supernatant (nuclear extracts) was collected after centrifugation at 14,000 × *g* for 15 min at 4 °C. The nuclear extracts were stored at −80 °C prior to use.