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Inflammation Research

Neutrophil elastase inhibitor (sivelestat) reduces the Levels of inflammatory mediators by inhibiting NF-κB

S. Hagiwara, H. Iwasaka, S. Hidaka, A. Hasegawa and T. Noguchi

Department of Anesthesiology and Intensive Care Medicine, Oita University Faculty of Medicine, 1-1 Idaigaoka-Hasamamachi, Yufu City, Oita 879-5593, Japan, Fax +81-97-586-5949, e-mail: saku@med.oita-u.ac.jp

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Abstract. *Objective:* Sivelestat sodium hydrate (sivelestat) is a specific synthetic inhibitor of neutrophil elastase (NE). Various studies suggest that sivelestat treatment reduces inflammation. In this study, we tested the hypothesis that sivelestat acts as an inhibitor of inflammatory mediators and prevents nuclear factor-κB (NF-κB) activation.
Methods: In the presence and absence of sivelestat, the mouse macrophage cell line RAW 264.7 was stimulated with lipopolysaccharide (LPS) and the levels of inflammatory mediators (TNF-α, IL-6 and high mobility group box 1 (HMGB1)) and nitrite in the cell supernatant were measured, along with inducible nitric oxide synthase (iNOS) expression.
Results: While LPS administration increased the secretion of inflammatory mediators and nitric oxide (NO), sivelestat decreased the secretion of these mediators. Cell signaling studies demonstrated that sivelestat decreased NF-κB activation by inhibiting IκB phosphorylation.
Conclusion: Sivelestat may inhibit the various inflammatory mediators through NF-κB inhibition.

Key words: Lipopolysaccharide – Sivelestat sodium hydrate – HMGB1 – Neutrophil elastase

Introduction

Acute respiratory distress syndrome (ARDS) commonly occurs in cases of severe sepsis and yields a mortality rate of 25–31 and few effective therapeutic strategies are currently available [1]. Despite recent improvements in our understanding of the molecular underpinnings of ARDS, acute lung injury (ALI) remains refractory to treatment.

A specific neutrophil elastase (NE) inhibitor, sivelestat, has been developed in Japan [2]. This agent is characterized as having no effects on proteases other than NE [2]. Previous studies reported that sivelestat reduced lung injury associated with systemic inflammatory response syndrome (SIRS) in humans [3] and decreased endotoxin-induced lung injury in animal models [4]. Indeed, plasma NE levels were significantly elevated in patients with ALI and ARDS compared to healthy volunteers [5]. In addition, clinical studies demonstrated that sivelestat improved the arterial oxygen tension-to-inspired oxygen fraction (PaO2/FiO2) and decreased the length of stay in the intensive care unit and days on a ventilator for patients with ALI and ARDS [6]. Moreover, treatment of ARDS with sivelestat reduced serum cytokine levels [7]. Sivelestat is also effective in animal models of ARDS/ALI. These results suggest that sivelestat might be useful for the treatment of SIRS and ARDS/ALI.

While the efficacy of sivelestat for ALI patients remains controversial, a Japanese phase III study reported promising results [8]. In that study, sivelestat administration reduced the artificial ventilation period and duration of stay in the intensive care unit. Additionally, we recently demonstrated the effectiveness of sivelestat in animal models of ARDS/ALI [9]. However, the mechanism(s) by which sivelestat confers protection is not well understood.

During severe inflammation, excessive oxidative stress is observed and nitric oxide (NO) has been postulated to be a key regulator. Various inflammatory stimuli, including lipopolysaccharide (LPS), stimulate endogenous NO production by activating inducible nitric oxide synthase (iNOS) [10]. Overproduction of NO subsequently initiates a cascade of inflammatory responses that lead to tissue injury and, eventually, multiple organ dysfunction [11].

Release of various mediators (including cytokines and the high mobility group box 1 (HMGB1) protein) correlates with severity of inflammation, as observed in SIRS and ALI [12, 13, 14]. The cytokines TNF-α and IL-6 play a central role in the pathogenesis of the acute inflammatory response and some studies report that high levels of TNF-α and IL-6 correlate with the severity of disease [15, 16]. Additionally, HMGB1

Correspondence to: S. Hagiwara

enhances the inflammatory response during septic shock [17, 18], and a later release and action may be generally observed compared to that for cytokines such as TNF-α and IL-1β [13]. Therefore, inhibitors of cytokines and HMGB1 might prove beneficial in the treatment of SIRS and ALI because they affect both the early and late inflammatory responses.

The transcription factor NF-κB regulates the expression of many inflammatory genes including TNF-α and IL-6 [19]. NF-κB activation involves the regulation of p65 and p50 subunit transactivation [20]. Aberrant activation of NF-κB is associated with systemic inflammation, as observed in cases of septic shock [21].

Sivelestat may inhibit the secretion of HMGB1 from LPS-stimulated murine macrophages. To test these hypotheses, we investigated the effect of sivelestat treatment on the secretion of various mediators by RAW264.7 cells. We further examined NF-κB activity and IκB phosphorylation to elucidate the mechanism which controlled these effects.

Materials and Methods

Materials

LPS (O127:B8) was obtained from Sigma-Aldrich (St Louis, MO). Sivelestat was donated by Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Antibodies to iNOS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphorylated IκB α (p-IκB α) and IκB α (Cell Signaling Technology, Beverly, MA), and β-actin (Abcam, Cambridge, UK) were purchased and stored at 4°C or -20°C.

Cell Culture

The RAW 264.7 macrophage-like cell line obtained from the American Type Culture Collection was maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 5% heat-inactivated fetal bovine calf serum, penicillin (50 units/ml, Gibco BRL, Grand Island, NY), and streptomycin (50 µg/ml, Gibco BRL, Grand Island, NY) at 37 °C in 5% CO2. The medium was removed and replaced with RPMI 1640 containing 5% fetal bovine serum (FBS) (for most experiments) or Opti-MEM (Sigma-Aldrich, St. Louis, MO) (for experiments measuring HMGB1 levels in conditioned media). RAW264.7 cells were simultaneously treated with sivelestat and stimulated with LPS. Samples of the culture supernatant were obtained at various time points, as described in the next section. Cells were lysed using the Mammalian Protein Extraction Reagent Kit (PIERCE Biotechnology, Rockford, IL) or Nuclear and Cytoplasmic Extraction Reagent Kit (PIERCE Biotechnology, Rockford, IL). Homogenates were boiled (5 min) prior to the addition of dithiothreitol. Cytoplasmic and nuclear protein extracts were analyzed by western blot as described below.

Measurement of cytokine and HMGB1 secretion

Samples of culture supernatant were taken at 0, 1.5, 3, 4.5, 6, 9, 12, 18 and 24 h time points. "0 h" refers to the time point immediately prior to LPS administration. HMGB1, IL-6, and TNF-α levels were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (HMGB1, Shino-Test Corporation, Tokyo, Japan; IL-6 and TNF-α, R&D Systems Inc., Minneapolis, MN).

Determination of nitrite concentration

Samples of culture supernatant were taken at 0, 1.5, 3, 4.5, 6, 9, 12, 18 and 24 h time points. "0 h" refers to the time point immediately prior to LPS administration. NO is rapidly oxidized to nitrite in biological fluids. Therefore, nitrite content in samples was measured as a proxy for NO. Nitrite concentrations were determined using a commercial kit according to the manufacturer's protocol (R&D Systems Inc., Minneapolis, MN). This kit measures nitrite concentration using a modification of the Griess assay, which is a colorimetric assay that measures absorbance at 540 nm. Nitrite concentration was calculated using a standard curve and expressed as micromoles per liter.

Western blot

Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were washed with phosphate buffered saline (PBS) containing 0.1% Tween 20 and 5% skim milk (PBS-T). The membranes were incubated with primary antibody (1:1000 dilution). After secondary antibody incubation, blots were developed using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) and exposed on Hyperfilm ECL (Amersham, Buckinghamshire, UK). The film was then scanned and the band concentration was calculated by the quantification of the integrated optical density of the appropriate band using the Image J 1.37v software program (National Institute of Health, Bethesda, MD).

NF-κB Binding Assay

Samples of the cells were taken at 0, 1 and 2 h time points. "0 h" refers to the time point immediately prior to LPS administration. The DNA binding activity of NF-κB (p50/p65) was determined using an ELISA-based nonradioactive NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA).

Statistical analysis

For descriptive purposes, all continuous data were presented as the mean ± SD. The data were compared using nonparametric tests and analyzed by the Mann–Whitney U-test for comparison between two independent groups. The Kruskal-Wallis test was used for comparison between all groups. P-values less than 0.05 were considered statistically significant.

Results

Effect of sivelestat on HMGB1 secretion

The secretion of HMGB1 into the culture supernatant was measured 24 h following LPS administration. HMGB1 levels in the culture supernatant increased after the LPS administration, but the secretion was significantly inhibited by the administration of 100 µg/ml sivelestat (Figure 1). The most effective dose of sivelestat was 100 µg/ml, and this dose was used for subsequent experiments.

Effect of sivelestat on cytokine secretion

TNF-α levels in the culture supernatant increased 3 h after LPS administration (Figure 2A). Sivelestat significantly inhibited the secretion of TNF-α in response to LPS administration. Similarly, IL-6 levels in the culture supernatant also increased following LPS administration (Figure 2B), and its increase was inhibited by sivelestat (Figures 2 A, B).

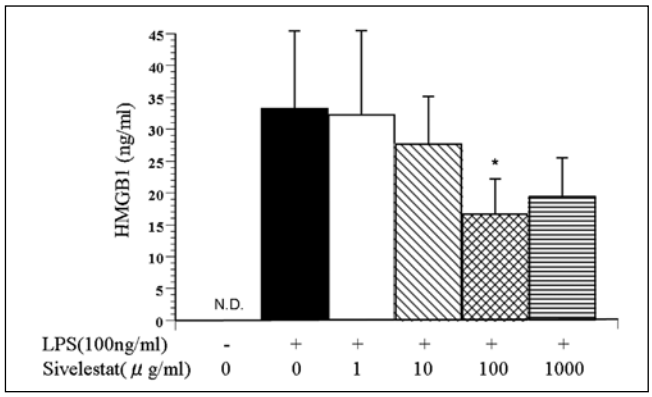

Fig. 1. Effect of sivelestat on HMGB1 secretion by LPS-stimulated murine macrophages.
Murine macrophages treated with or without sivelestat (1, 10, 100, and 1000 μg/ml) were stimulated with LPS (100 ng/ml) for 20 h. Supernatants were prepared and HMGB1 levels were examined by ELISA. Results are expressed as the mean ± SD. *denotes a significant difference compared to LPS-only treated cells ($p < 0.05$). N.D.: not detected.

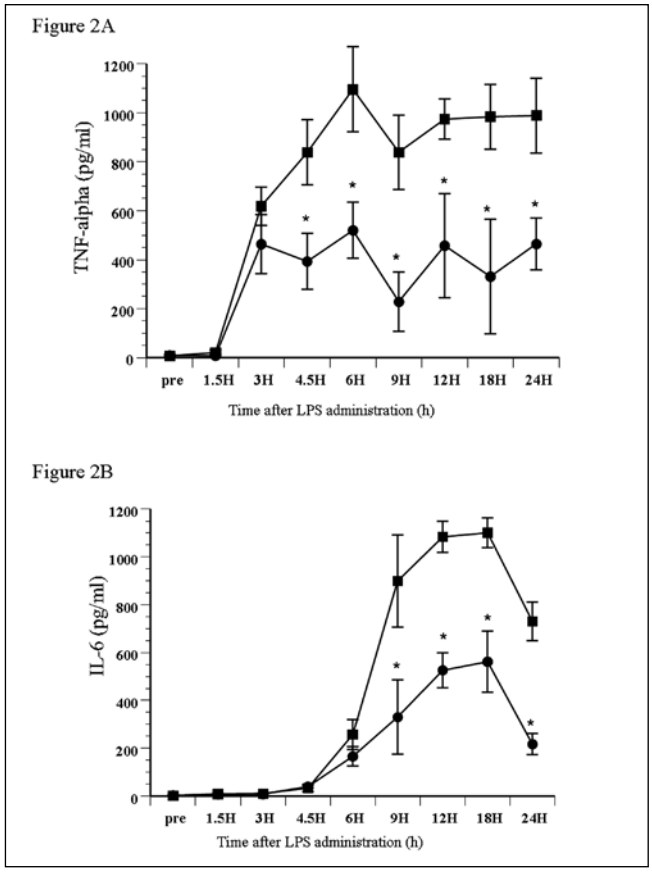

Fig. 2. Effect of sivelestat on TNF-α and IL-6 production by LPS-stimulated murine macrophages.
Murine macrophages treated with or without sivelestat (100 μg/ml) were stimulated with LPS (100 ng/ml) for the indicated durations. Squares represent LPS treatment without sivelestat and circles represent LPS treatment with sivelestat. Supernatants were collected and TNF-α(**A**) and IL-6 (**B**) levels were determined by ELISA. Results are expressed as the mean ± SD. * denotes a significant difference compared to cells treated with only LPS ($p < 0.05$).

Effect of sivelestat on serum nitrite levels and iNOS expression

Nitrite is a metabolic product of NO that is often used as a marker to indicate NO production. Nitrite concentrations were measured in culture supernatant at the indicated times after LPS administration. We observed a LPS-induced increase in serum nitrite levels that was significantly inhibited by sivelestat treatment (Figure 3). The observed increase in iNOS expression in murine macrophage RAW264.7 cells by LPS administration was also diminished by sivelestat treatment (Figure 4).

Sivelestat inhibits the IKK pathway and modulates NF-κB

Since the NF-κB pathway plays a crucial role in the secretion of cytokines and NO, we measured the activation of the NF-κB subunits p50 and p65 in the nucleus. Treatment of cells with LPS led to a robust activation of p50 and p65. This activation was partially blocked by sivelestat (Figure 5). The IκB kinase (IKK) pathway was examined as another potential regulator of NF-κB. Sivelestat inhibited IκB α degradation resulting from LPS stimulation (Figure 6). In addition, IκB α phosphorylation (p-IκB) in RAW264.7 cells increased after LPS administration, and this phosphorylation was also inhibited by sivelestat (Figure 6).

Discussion

In this study, we demonstrate that sivelestat inhibited HMGB1 secretion and production of cytokines and NO in LPS-stimulated murine macrophages by inhibiting NF-κB activation. This lack of NF-κB activation may be due to inhibition of IκB phosphorylation by sivelestat.

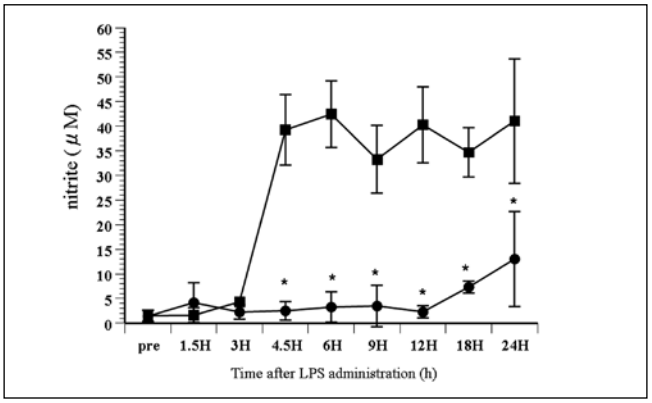

Fig. 3. Effect of sivelestat on serum nitrite levels after LPS stimulation of murine macrophages.
Murine macrophages treated with or without sivelestat (100 μg/ml) were stimulated with LPS (100 ng/ml) for the indicated durations. Squares represent LPS treatment without sivelestat and circles represent LPS treatment with sivelestat. Supernatants were collected and nitrite concentrations were determined by a modification of the Griess assay. Results are expressed as the mean ± SD. *denotes a significant difference compared with cells treated only with LPS ($p < 0.05$).

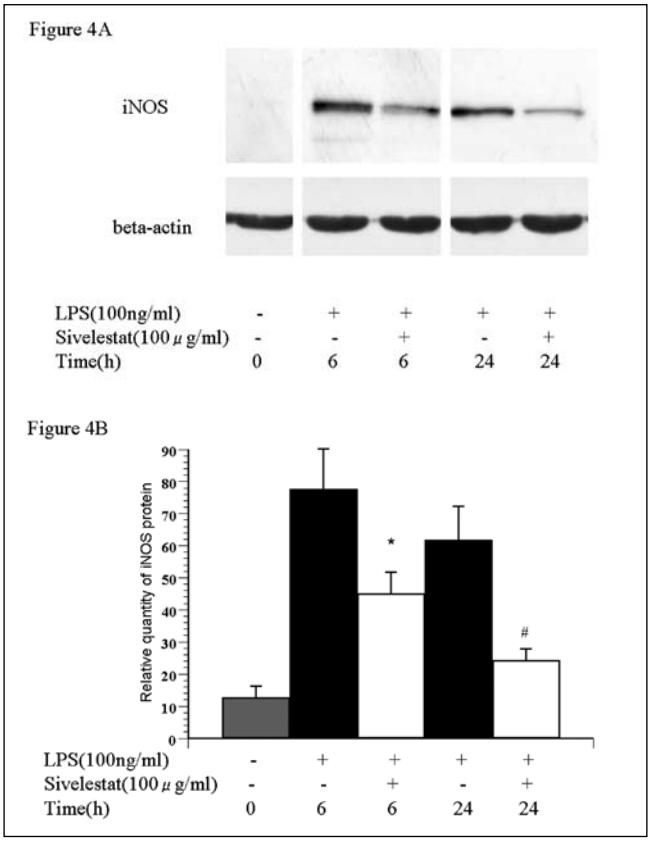

Fig. 4. Changes in iNOS protein expression after LPS stimulation of murine macrophages.
(**A**) Murine macrophages treated with or without sivelestat (100 μg/ml) were stimulated with LPS (100 ng/ml) for the indicated durations. The expression of iNOS after the treatment of cells with LPS, with or without sivelestat, was detected by western blot. The blots were also probed with an antibody against β-actin as a loading control. (**B**) The signal intensities for the density based on an immunoblot analysis of iNOS protein. iNOS was quantified using an image analyzer. The density of the signal intensities was lower for the cells treated with both LPS and sivelestat compared to cells treated only with LPS. Results are expressed as the mean ± SD. * denotes a significant difference compared with the LPS-only group at 6 h ($p<0.05$). # denotes a significant difference compared with the LPS-only group at 24 h ($p<0.05$).

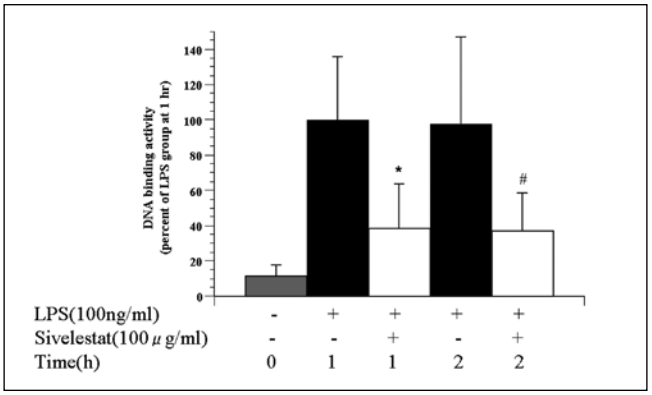

Fig. 5. Effect of sivelestat on the LPS-induced increase of p50/p65 DNA binding activity.
The DNA binding activity assay showed a marked decrease in p50/p65 DNA binding activity in nuclear fractions from RAW264.7 cells treated with LPS and sivelestat compared to cells treated only with LPS. Results are expressed as the mean ± SD. * denotes a significant difference compared with the LPS-only group at 1 h ($p<0.05$). # denotes a significant difference compared with the LPS-only group at 2 h ($p<0.05$).

Previous studies demonstrated that treatment of ALI/ARDS with sivelestat led to reduced cytokine production and improved lung function [6, 7]. One of the proposed mechanisms was NE inhibition by sivelestat [2]. However, other potential mechanisms were not elaborated upon. We demonstrate that sivelestat reduced production of various inflammation mediators through NF-κB inhibition. Therefore, sivelestat inhibits NE production and may also have anti-inflammatory properties.

Cytokines are important mediators of inflammation in various disease states [22]. Previous studies demonstrated the mutual relationship between HMGB1 and cytokines [13] and the proinflammatory properties of HMGB1 [23]. Therefore, cytokines and HMGB1 are thought to play a crucial role in regulating inflammation. We observed that the cytokine and HMGB1 levels were significantly decreased in response to sivelestat treatment (Figures 1, 2). These results indicate that in addition to inhibiting cytokine production, sivelestat inhibits HMGB1 during LPS stimulation of murine macrophages. Therefore, sivelestat may be useful for regulating the inflammatory response.

Inducible nitric oxide synthase (iNOS) and nitrite levels were significantly inhibited by sivelestat treatment (Figure 3, 4). iNOS is typically activated under systemic inflammatory conditions including septic shock [24] and its increased expression promotes nitric oxide (NO) release, leading to organ damage. NO plays a key role in inflammation and has been implicated in a wide variety of disease processes [24]. Furthermore, TNF-α stimulates iNOS activity [25]. These results suggest that the anti-inflammatory actions of sivelestat in response to LPS administration may occur by inhibiting cytokines and iNOS. iNOS inhibition may be related to TNF-α inhibition.

NF-κB coordinates the induction of several genes leading to the production and secretion of pro-inflammatory cytokines when NF-κB is freed from an inhibitory action of IκB [26]. Moreover, iNOS activity in macrophages is first regulated and modulated by cellular receptor molecules via NF-κB pathway activation [27]. In addition, NF-κB activation contributes to HMGB1 secretion after LPS administration [28]. We demonstrate that sivelestat inhibited LPS-induced NF-κB activation (Figure 5). This may, in turn, inhibit the activation of macrophages and the amounts of various inflammatory mediators secreted.

NF-κB associates with inhibitory IκB proteins which sequester NF-κB in the cytoplasm. Phosphorylation of IκB by IκB kinases (IKKs) is required to release NF-κB from this inhibition [29]. Indeed, LPS stimulation of murine macrophages activates several intracellular signaling pathways, including the IκB kinase (IKK)/NF-κB pathway [30]. Our results suggest that sivelestat impinges on the IKK pathway to dampen the inflammatory response by repressing IκB phosphorylation and subsequent NF-κB activation (Figure 5). Accordingly, the inhibition of IκB phosphorylation by sivelestat during LPS-induced inflammation may limit NF-κB activation.

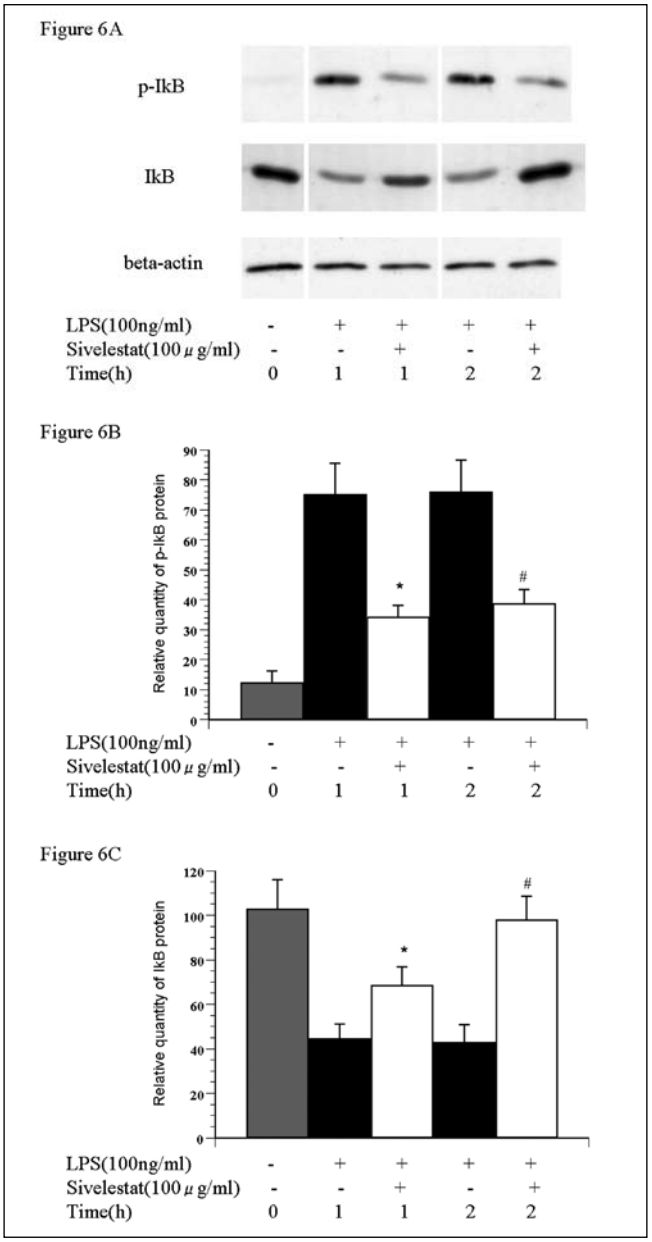

Fig. 6. Effect of sivelestat on LPS-induced IκB phosphorylation.
(**A**) Murine macrophages treated with or without sivelestat (100 μg/ml) were stimulated with LPS (100 ng/ml) for the indicated durations. The cytoplasmic levels of phosphorylated IκB were determined by western blot analysis using antibodies specific for 1) phosphorylated IκB α (p-IκB, upper); 2) total IκB α (IκB, middle); and 3) β-actin as a loading control (lower). (**B**) The signal intensities for the density were based on an immunoblot analysis of p-IκB protein. p-IκB was quantified using an image analyzer. The density of the signal intensities was lower for cells treated with both LPS and sivelestat, compared to cells treated only with LPS. Results are expressed as the mean ± SD. * denotes a significant difference compared with the LPS-only group at 1 h ($p<0.05$). # denotes a significant difference compared with the LPS-only group at 2 h ($p<0.05$). (**C**) The signal intensities for the density were based on an immunoblot analysis of IκB protein. IκB was quantified using an image analyzer. The density of the signal intensities was higher for the cells treated with both LPS and sivelestat compared to cells treated only with LPS. Results are expressed as the mean ± SD. * denotes a significant difference compared with the LPS-only group at 1 h ($p<0.05$). # denotes a significant difference compared with the LPS-only group at 2 h ($p<0.05$).

In conclusion, our results suggest that sivelestat may exert an anti-inflammatory effect due to its ability to inhibit the production and secretion of cytokines and HMGB1, as well as NO secretion. These might be related to the inhibition of NF-κB in sivelestat-treated cells. One important caveat of this study is the high concentration of sivelestat used. Such high concentrations may non-specifically affect intracellular proteases (such as the proteasome) and thereby inhibit NF-κB activation. Further studies will be required to elucidate the mechanisms by which sivelestat regulates inflammation.

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