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Attenuation of LPS-induced iNOS expression by 1,5-anhydro-D-fructose

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ABSTRACT

1,5-anhydro-D-fructose (1,5-AF), a monosaccharide formed from starch and glycogen, exhibits antioxidant and antibacterial activity, and inhibits cytokine release by attenuating NF-κB activation in LPS-stimulated mice. The present study examined whether 1,5-AF inhibits lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) *in vitro* and *in vivo*. We found that 1,5-AF significantly blocked the production of NO, and protein and mRNA expression of iNOS, and up-regulated IL-10 production *in vitro*. We also investigated the effects of 1,5-AF on acute lung inflammation in C57BL/6J mice. We found that protein and mRNA expression of iNOS in lung tissues were inhibited by 1,5-AF pretreatment. In addition, the serum level of IL-10 was upregulated by 1,5-AF. Collectively, the iNOS transcriptional and translational inhibitory effects of 1,5-AF seem to be prolonged and enhanced by the production of IL-10. These results suggest that 1,5-AF could be a useful adjunct in the treatment of acute lung inflammation.

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Introduction

Inflammation is a central feature of many pathophysiological conditions in response to tissue injury and host defense against invading pathogens [1]. Macrophages are the main proinflammatory cells involved in the responses to invading pathogens and release many proinflammatory molecules, including nitric oxide (NO). Excessive NO production has been implicated in the pathogenesis of inflammatory tissue injury and in several disease states [2,3]. In activated macrophages, the transcriptionally expressed inducible nitric oxide synthase (iNOS) is responsible for the prolonged enhanced production of NO. Thus, pharmacological inhibition of NO production offers promising targets for therapeutic intervention in inflammatory disorders.

1,5-anhydro-D-fructose (1,5-AF) is a newly identified monosaccharide that is formed directly from starch or glycogen through an α-1,4-glucan lyase reaction (EC 4.2.2.13). During its formation, the carbonyl group does not undergo hemiacetal bonding, but it is instead fully hydrated in aqueous solution so that it may play a metabolically active role [4]. 1,5-AF has been found in fungi [5], red algae [6], *Escherichia coli* [7] and rat liver tissue [8]. 1,5-AF is likely to act as an antioxidant for scavenging reactive oxygen species (ROS) induced by phorbol myristate acetate (PMA) in THP-1 cells, copper-mediated LDL oxidation [9,10], or as antimicrobial agents [10], and can attenuate NF-κB activation [11]. ROS and NO, a reactive nitrogen species (RNS), are believed to be important mediators that lead to lung injury [12] .

Interleukin (IL)-10 is a cytokine that has important anti-inflammatory and antiproliferative properties, and attenuates the severity of various disease states. Furthermore, IL-10 suppresses cellular production of NO, a molecular signal in the inflammatory process, and down-regulates the expression of iNOS, which is regulated as a transcription factor of NF-κB activation in macrophages during acute lung injury [13]. Thus, increased IL-10 levels are required for attenuation of inflammation.

In this study, we investigated whether 1,5-AF affects NO production via its anti-inflammatory activity. We conducted this study to explore the anti-inflammatory effects of 1,5-AF on iNOS expression in lung tissues from C57BL/6J mice and in the murine macrophage cell line RAW264.7, which can be stimulated with LPS to mimic a state of infection and inflammation [14].

Material and methods

Cell culture and treatment. The murine macrophage-like RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone Logan, UT). The cells were pretreated with

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1,5-AF (0–500 μg/ml) for 2 h and stimulated with LPS (500 ng/ml; O111:B4, Alexis Biochemical, San Diego, CA) in serum-free Opti-MEM-I medium (Invitrogen, Carlsbad, CA) for various durations. Cells were extracted for iNOS experiments, and the supernatant was collected for NOx measurement.

Measurement of nitrite. The measurement of NO*x* (NO^{2-} + NO^{3-}) in the supernatant was performed according to the method of Misko et al. [15] with minor modifications. In brief, 2,3-diaminonaphthalene (DAN) was dissolved in 0.62 N HCl at a concentration of 0.05 mg/ml. NO^{3-} in culture medium was reduced to NO^{2-} with nitrate reductase (14 mU) and NADPH (40 μM) at room temperature (RT) for 5 min. The media were then collected and aliquots of each sample (100 μl) were placed into 96-well plates. DAN (10 μl) was then added to each well at RT. After 10 min, 5 μl of 2.8 N NaOH was added to each well, and the plate was read on an Appliskan luminescence spectrometer (excitation 360 nm, emission 440 nm) (Thermo Fisher Scientific, Waltham, MA). Standard curves were made with concentrations of sodium nitrite ranging from 0.04 to 10 μM in phenol red-free DMEM.

Western blot analysis. As described previously [16], RAW264.7 cells were washed in ice-cold PBS, lysed with lysis buffer (0.5 M Tris–HCl, 10% SDS, 10% 2-mercaptoethanol, and 20% glycerol). Next, 30 μg/ml of protein was subjected to SDS–PAGE and then transferred to nitrocellulose membranes (Whatman, Cassel, Germany). The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.04% Tween 20 (TBST) and incubated with iNOS antibodies (Ab) (Upstate Inc., Lake Placid, NY) or anti-β-actin Ab (Santa Cruz Biotechnology, Santa Cruz, CA) in TBST supplemented with 1% non-fat dried milk. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary Abs (MP Biomedicals, LLC, Santa Ana, CA) diluted to 1:3000 in TBST supplemented with 2.5% non-fat dried milk. Immunoreactive proteins were detected with an enhanced chemiluminescence detection system (Amersham Biosciences).

Flow cytometric assessment of cell viability. RAW264.7 cells were collected and fixed with 70% ethanol at −20°C for 20 min. After washed with phosphate-buffered saline (PBS), the cells were centrifuged and stained with propidium iodine (PI) solution (PI 20 μg/ml and RNase 625 μg/ml in PBS) for 20 min in the dark. The PI fluorescence was measured with an Epics XL flow cytometer (Beckman Coulter, High Wycombe, Bucks, UK).

Animal studies and treatment protocol. As described previously [17], 7-week-old, male C57BL/6J mice were obtained from Kyudou (Kumamoto, Japan). Animal protocols were approved by the Frontier Science Research Center, Kagoshima University and were conducted according to National Institutes of Health (NIH) guidelines. The mice were housed in a pathogen-free environment under controlled light and humidity conditions, and were provided food and water *ad libitum*. Mice were divided into four groups and treated with: (1) saline solution, (2) 1,5-AF, (3) LPS, and (4) 1,5-AF and LPS ($n = 6$ per group). Mice were given an intraperitoneal (i.p.) injection of LPS (2 mg/kg, Sigma, O55:B5, 1×10^6 EU/mg) or saline, immediately after i.p. injection of 1,5-AF (38.5 mg/kg body weight) or saline. Four hours after the injection, blood was drawn by intracardiac penetration and collected in capillary blood collection tubes (Terumo, Tokyo, Japan). Serum was collected and stored at −80 °C. Lung tissue was obtained immediately after the mice were killed and fixed in 10% neutral-buffered formalin (Nacalai Tesque, Inc, Kyoto, Japan).

RT-PCR. As described previously [17], total RNA was extracted from RAW264.7 cells or lung tissues of mice using an RNAqueous kit (Ambion, Inc., Texas). RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). The relative mRNA expression levels were determined using an Applied Biosystems 7300 Real-Time PCR System with a TaqMan Universal PCR Master Mix (Applied Biosystems) and NOS2 primers (Mn00440485_ml). The expression levels were calculated as the ratio of the mRNA level for a given gene relative to the mRNA level for glyceraldehyde-3-phosphate dehydrogenase (Mm99999915_ml) in the same cDNA sample.

Immunohistochemistry. Paraffin-embedded 5-μm-thick lung sections were deparaffinized and dehydrated. Antigen retrieval was performed using antigen-unmasking solution (Vector Laboratories Inc., Burlingame, CA). Slides were blocked using Block ACE™ (Dainippon Sumitomo Pharma Co., Osaka, Japan) and incubated with rat monoclonal anti-macrophage Ab (1:100 dilution; Abcam, Tokyo, Japan) or rabbit anti-iNOS polyclonal Ab (1:100 dilution; Santa Cruz Biotechnology) at 4 °C in PBS containing 1% bovine serum albumin. Slides were washed with TBST and incubated with Histofine Simple Stain Mouse MAX-PO (Nichirei, Tokyo, Japan). The slides were washed and stained with 3,3′-diaminobenzidine (DAB; Dako Envision Kit, Glostrup, Denmark). Counterstaining was performed with hematoxylin.

Statistical analysis. Data are expressed as means ± SE. Differences between means were evaluated using unpaired two-sided Student's *t*-test ($P < 0.05$ was considered significant).

Results

1,5-AF inhibits LPS-induced NO production in RAW264.7 cells

Murine macrophage-like RAW264.7 cells are commonly used to investigate anti-inflammatory responses [14]. To investigate

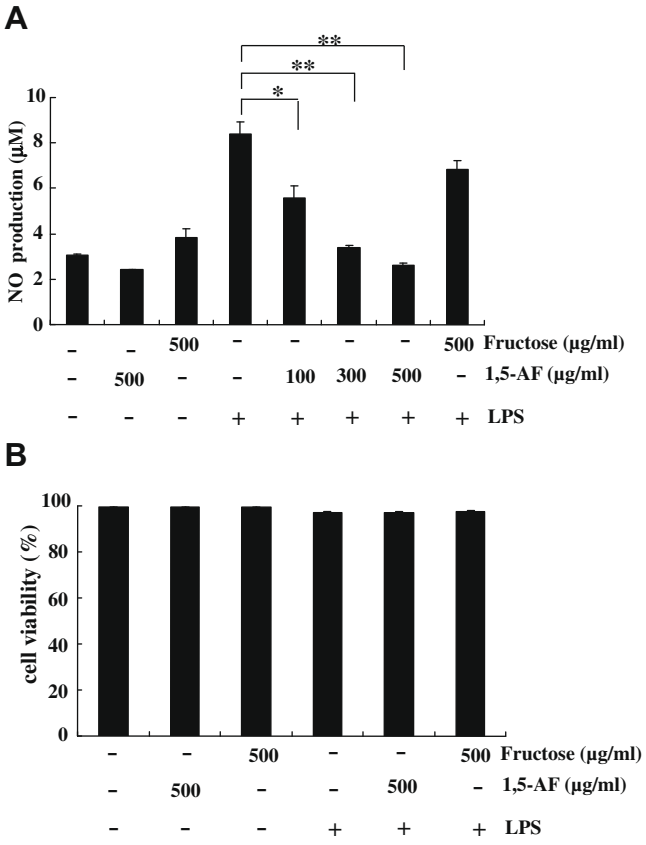

Fig. 1. 1,5-AF inhibits LPS-induced NO production in RAW264.7 cells. Cells were pretreated for 2 h with the indicated concentration of 1,5-AF and were then stimulated with LPS (500 ng/ml) for 18 h. (A) The culture media were collected and assayed for nitrite production. (B) Cells were collected and assayed for cell viability by flow cytometry. The values are expressed as means ± SE of triplicate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences versus the control group.

whether 1,5-AF suppresses NO production in LPS-treated RAW264.7 cells, RAW264.7 cells were preincubated with 1,5-AF (0–500 μg/ml) or fructose (500 μg/ml) as an analog of 1,5-AF, and were then stimulated with LPS for 18 h. As shown in Fig. 1A ,1,5-AF significantly suppressed NO production compared with that in cells stimulated with LPS alone ($P < 0.01$, $P < 0.05$, Fig. 1A) in a dose-dependent manner; however, fructose had no effect on LPS-induced NO production (Fig. 1A).

To exclude the potential for cytotoxic activity of 1,5-AF, we examined whether 1,5-AF affects cell viability with flow cytometry. As shown in Fig. 1B, the cell viability was not affected by 500 μg/ml 1,5-AF, a concentration that significantly inhibited LPS-stimulated production of NO (Fig. 1A).

1,5-AF inhibits LPS-induced iNOS protein and gene expression in RAW264.7 cells

To investigate whether the inhibition of NO production is caused by reduced iNOS protein expression, Western blot analysis was performed on the LPS-treated lysates with or without 1,5-AF pretreatment. As shown in Fig. 2 A, 1,5-AF pretreatment also inhibited iNOS protein expression in a dose-dependent manner. Expression of β-actin was also determined in the same blot as the loading control and was noted as a consistent band. Furthermore, we performed RT-PCR analysis for iNOS mRNA. As shown in Fig. 2B, 1,5-AF treatment significantly inhibited the expression of iNOS mRNA in LPS-stimulated RAW264.7 cells compared with LPS alone in a dose-dependent manner (LPS alone: 1.06 ± 0.029; 300 μg/ml 1,5-AF: 0.810 ± 0.08, $P < 0.05$; 500 μg/ml 1,5-AF: 0.681 ± 0.08, $P < 0.01$). However, the use of fructose as an analog had no effect on the expression of iNOS mRNA (0.880 ± 0.067).

1,5-AF suppresses LPS-induced pulmonary iNOS expression and mRNA production in C57BL/6J mice

iNOS is a important molecule in lung tissue injury and is primarily expressed by activated macrophages [18]. Thus, to confirm the above results, we examined whether 1,5-AF suppressed iNOS expression in the LPS-challenged C57BL/6J mice. As shown in Fig. 3B and C and analyzed in Supplementary Fig. 1A, 1,5-AF pretreatment for 2 h significantly suppressed iNOS expression in the pulmonary parenchyma in LPS-challenged C57BL/6J mice compared with LPS alone (58 ± 1% vs. 23 ± 0.2%, $P < 0.01$). Few iNOS-positive parenchymal cells were found in the control mice or in the mice treated with 1,5-AF alone (Fig. 3A and D). iNOS expression was greater in specimens obtained from mice exposed to LPS alone than in those obtained from 1,5-AF- and LPS-treated mice. Similar results were obtained from real-time PCR analysis of iNOS mRNA in lung tissue. iNOS mRNA was up-regulated in mice exposed to LPS, whereas pretreatment with 1,5-AF decreased LPS-induced iNOS mRNA expression (Supplementary Fig. 1B; $P < 0.05$ compared with LPS-challenged mice).

1,5-AF increases IL-10 levels in RAW264.7 cell and in C57BL/6J mice

IL-10 also exerts an anti-inflammatory role in acute lung inflammation [13]. iNOS expression in macrophages is down-regulated by IL-10 during acute lung injury [13]. Therefore, we next investigated whether 1,5-AF alters the production of IL-10. RAW264.7 cells were preincubated with 1,5-AF (0–500 μg/ml) for 1 h and then challenged with LPS. There was a significant dose-dependent increase in IL-10 production (Fig. 4A). Furthermore, in LPS-challenged C57BL/6J mice (Fig. 4B), the IL-10 levels were in-

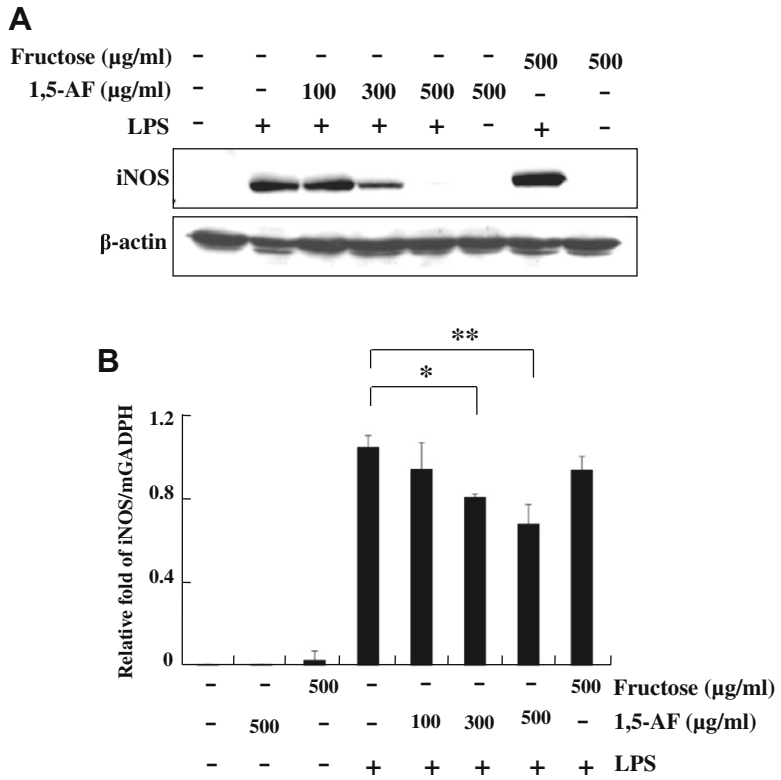

Fig. 2. 1,5-AF inhibits LPS-induced iNOS protein levels and mRNA expression in RAW264.7 cells. Cells were pretreated for 2 h with the indicated concentration of 1,5-AF and were then stimulated with LPS (500 ng/ml). (A) Whole-cell lysates were prepared after 12 h of stimulation, and were analyzed for the presence of iNOS using Western blotting with anti-iNOS Ab. The blot was stripped of the bound Abs and reprobed with β-actin to confirm equal loading. (B) Total RNA was extracted after stimulation for 6 h. iNOS and GAPDH mRNAs were measured by real-time RT-PCR. iNOS mRNA levels were normalized against GAPDH. The values are expressed as means ± SE of triplicate experiments, with four samples per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences versus the control group.

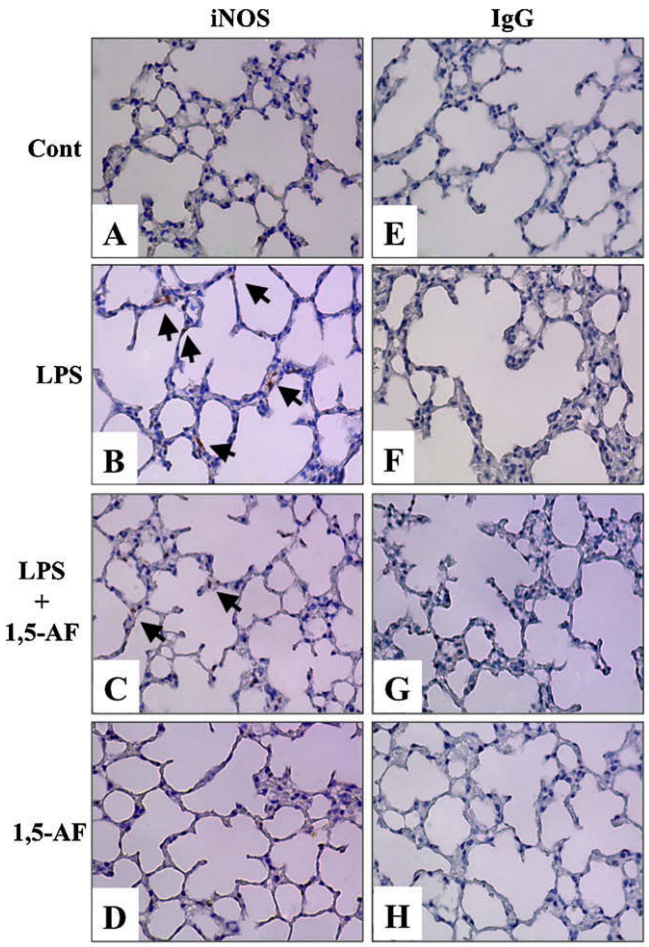

Fig. 3. 1,5-AF inhibits LPS-induced iNOS expression in the lung tissue of C57BL/6J mice. Seven-week-old male mice were challenged for 4 h with either saline alone (A) or LPS (B) immediately after 1,5-AF (38.5 mg/kg) (C) or saline treatment and 1,5-AF alone (D). The lung tissues were stained with iNOS Ab (A–D) and isotype IgG (E–H). The brown regions indicate the DAB-positive area (arrows). Original magnification: 400×.

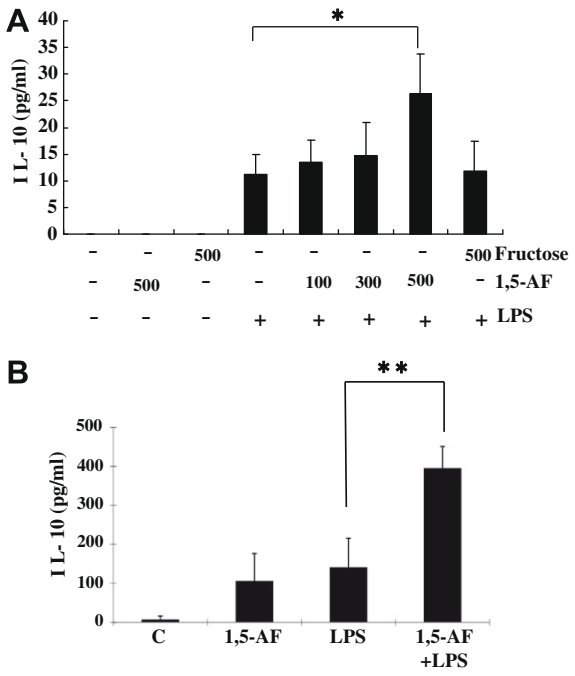

Fig. 4. 1,5-AF upregulates IL-10 production in LPS-stimulated RAW264.7 cells and mice serum. (A) Cells were pretreated for 2 h with the indicated concentration of 1,5-AF and were then stimulated with LPS (500 ng/ml) for 6 h. The concentration of IL-10 released into the supernatant was measured by ELISA. The values are expressed as means ± SE of triplicate experiments. (B) Mice were treated as described in Fig. 3. The serum IL-10 concentrations were measured by ELISA. The values are expressed as means ± SE with six mice per group. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences versus the control group.

creased after treatment with 1,5-AF plus LPS compared with LPS treatment alone (Fig. 4B; $P < 0.01$).

Discussion

The present study revealed that 1,5-AF inhibits LPS-induced NO production in the murine macrophage-like cell line RAW264.7, and protects mice from LPS-induced lung injury by down-regulating the expression of iNOS and up-regulating the production of IL-10.

ROS and NO, an RNS, are produced by phagocytes, such as macrophages, in response to LPS stimulation [19]. The excessive synthesis of NO by iNOS acts as a major macrophage-derived inflammatory mediator and is also involved in the development of inflammatory disease [20]. Our previous study in THP-1 cells showed that 1,5-AF inhibits the formation of ROS because of the presence of enediol forms [9]. Furthermore, other studies have shown that compounds with antioxidant activity such as curcumin [17] and resveratrol [21] also inhibit the production of NO and expression of iNOS. In our present study, 1,5-AF inhibited LPS-induced production of NO and dose-dependently decreased the amount of iNOS protein and its mRNA production in RAW264.7 cells. Thus, these findings suggest that pretreatment with 1,5-AF has an antioxidant effect that may inhibit iNOS expression at the transcriptional and translational levels.

In response to LPS stimulation, the inflammatory cellular infiltrates in the lung predominantly consist of neutrophils and macrophages [22]. In turn, these activated macrophages generate ROS [23] and release many inflammatory mediators, including iNOS [24] and proinflammatory cytokines [25]. This perpetuates a vicious cycle to continue the production of cytotoxic mediators, ultimately leading to profound injury, such as acute lung injury [22]. Moreover, iNOS inhibitors prevent LPS-induced acute respiratory distress syndrome (ARDS) [26]. Our results, which agree with those of previous studies, show that treatment with 1,5-AF significantly suppresses LPS-induced iNOS expression in C57BL/6J mice. This implies that 1,5-AF has an important anti-inflammatory effect on acute lung inflammation.

Increasing the production of the anti-inflammatory cytokine IL-10 could also inhibit proinflammatory mediators such as IL-6 [27] and iNOS [28]. The increased level of IL-10 in the lung of patients with ARDS is associated with improved survival [29] and IL-10-knockout mice show increased iNOS expression and NO production in lung tissue [13]. In the present study, pretreatment with 1,5-AF enhanced the LPS-induced production of the counter-regulatory cytokine IL-10 compared with that with LPS stimulation alone both *in vitro* and *in vivo* and may thus play an inhibitory role in LPS-induced iNOS transcription and translation.

A previous report has revealed that proinflammatory mediators are regulated by the transcription factor NF-κB in LPS-induced lung inflammation [30]. In our study, we found that 1,5-AF slightly suppressed iNOS expression at 3 h (data not shown). However, it did markedly suppress iNOS mRNA and protein expression at 6 h and 12 h, respectively. 1,5-AF is shown to inhibit the translocalization of NF-κB p65 independently of IκBα degradation, and decreased the levels of proinflammatory cytokines such as IL-6,

TNF-α and MCP-1 at 4 h [11]. Furthermore, iNOS expression was dependent on NF-κB activation, thus, suggesting that 1,5-AF may directly inhibit iNOS expression by attenuating NF-κB activation as well as by its antioxidant effects.

The present study indicated that 1,5-AF may inhibit iNOS expression by up-regulation of the anti-inflammatory cytokine IL-10. Since IL-10 has been shown to inhibit the translocalization of NF-κB p65, which was dependent on the degradation of IκBα [31]. The inhibition of iNOS expression (6 h) by 1,5-AF occurred at the same time and consequently increased the expression of IL-10 (6 h). Therefore, these findings suggest that 1,5-AF may directly inhibit iNOS expression and NO production via NF-κB inactivation in the early phase and indirectly via increased IL-10 levels, which may sustain the anti-inflammatory effects of 1,5-AF. Thus, these results raise the possibility that the NF-kB inactivation and increased IL-10 level caused by the action of 1,5-AF may attenuate iNOS expression.

Collectively, our results suggest that 1,5-AF acts as a selective inflammatory inhibitor and this anti-inflammatory effect was augmented by the production of IL-10. In turn, IL-10 inhibited LPS-induced iNOS over-expression in RAW264.7 cells and in the lung tissue of mice. Based on these results, we have clarified the mechanism of 1,5-AF activity, which may be used in the treatment of inflammatory diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.108.

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