A Selective Thromboxane A₂ (TXA₂) Synthase Inhibitor, Ozagrel, Attenuates Lung Injury and Decreases Monocyte Chemoattractant Protein-1 and Interleukin-8 mRNA Expression in Oleic Acid–Induced Lung Injury in Guinea Pigs

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Abstract. This study examined the effect of ozagrel, a thromboxane A₂ synthase inhibitor, on the accumulation of leucocytes and chemokine mRNA expression in lungs experimentally injured using oleic acid (OA). OA injection into guinea pigs rapidly increased thromboxane A₂ generation and subsequently increased total protein concentration and the numbers of macrophages and neutrophils in bronchoalveolar lavage fluid and increased monocyte chemoattractant protein-1 and interleukin-8 mRNA expression in the whole lung. Administration of ozagrel prevented these changes associated with OA injection. Ozagrel is a promising drug candidate for preventing acute lung injury.

Keywords: ozagrel, oleic acid, lung injury

Acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) are among the most severe forms of lung injury and carry a very high fatality rate (1, 2). In ARDS/ALI, non-cardiogenic pulmonary edema develops as a result of vascular hyper-permeability induced by inflammatory reactions in which there is an activation of alveolar macrophages, neutrophil infiltration, and production of inflammatory mediators such as eicosanoids (2).

Therapeutic strategies against ARDS/ALI based on the regulation of eicosanoids, such as inhibition of phospholipase A₂ and cyclooxygenase-2 and blocking and activation of eicosanoid receptors, have been advocated (3). Inhibition of thromboxane A₂ (TXA₂) synthase may be one such strategy. TXA₂ is known as a potent vasoconstrictive eicosanoid that can modulate pulmonary arterial pressure and hypoxic pulmonary vasoconstriction. Excessive production of TXA₂ in the lungs of patients with ARDS/ALI and ARDS/ALI animal models has been observed; it appears to be involved in pulmonary gas exchange dysfunction (3). In our previous study, we demonstrated that TXA₂ is involved in the early phases of oleic acid (OA)–induced lung injury, a representative model of ARDS/ALI, and that the rapidly acting TXA₂ synthase inhibitor ozagrel can prevent hypoxemia associated with OA-induced lung injury (4). Although TXA₂ seems to involve the development of the lung injury via modulation of pulmonary circulation, the precise roles of TXA₂ in lung injury and the usefulness of TXA₂ synthase inhibition against lung injury, particularly in terms of inflammatory reactions such as chemokine expression and subsequent leucocyte migration, remain uncertain.

High concentration of chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), and recruitment of leucocytes were found in bronchoalveolar lavage fluid (BALF) in ARDS/ALI, suggesting they play an important role in the develop-
ment of these diseases, and their inhibition seems to be an important therapeutic target (2, 5, 6). In vitro studies using a cell culture system indicated that TXA₂ has the potential to evoke chemokine expression (7, 8). These findings lead to the hypothesis that TXA₂ might be involved in the development of lung injury via chemokine expression and the accumulation of leucocytes; furthermore, TXA₂ synthase inhibition by ozagrel may impede such an immunological response. Building upon the previous suggestion, we designed in vivo experiments to demonstrate the effect of ozagrel on inflammatory cell accumulation and chemokine mRNA expression in lungs experimentally injured using OA in guinea pigs.

Lung injury in male Hartley guinea pigs (420 – 500 g) was induced as described previously (4, 9). Briefly, guinea pigs were anesthetized with pentobarbital sodium (Nembutal®; Dainabot Co., Osaka), and catheters (1.1-mm outer diameter) were inserted into subclavian veins for injection of reagents. Lung injury was induced by injection of 15 μL/kg OA. Total protein concentration, number of leucocytes, and concentration of thromboxane B₂ (TXB₂) in BALF were used as lung injury parameters. Chemokine mRNA expression in whole lungs was determined by the reverse transcriptase-polymerase chain reaction (RT-PCR). The following specific primers were designed to assess gene expressions: MCP-1 (210 bp) sense: 5’CTTCTGTGCCTGCTGGTCTAT3’, antisense: 5’GGGTCAGCACAGACTCCTT3’; IL-8 (193 bp) sense: 5’TGGTCGTGACAAAGTTGGTC3’, antisense: 5’CTGCACCCACTTCTTG3’; etoxin (290 bp) sense: 5’ATGAAAGTCTCCAAGCGTTC3’, antisense: 5’TTACGCGTTTGTAGTTTTGGA5’; and regulated-upon-activation, normally T-cell–expressed and –secreted chemokine (RANTES) (190 bp) sense: 5’TACATGAAAGGTCGACAGCTG3’, antisense: 5’CTAGCTCATCTCCAAGAGTTG3’; and β-actin (279 bp) sense: 5’CCAACCTGGACGACATGAG3’, antisense: 5’CATACCCCTCAGGTAG3’. This study was approved by the Animal Care and Use Committee of Kumamoto University and is in accordance with National Institutes of Health guidelines for the care and handling of animals.

Initially, we examined the change in time course parameters in the lung injury system. An intravenous injection of 15 μL/kg OA caused an increase in total protein concentration in BALF, a marker of pulmonary vascular hyper-permeability, in a time-dependent manner (Fig. 1A). The maximum increase, approximately 2.5-fold of that at 0 h, was observed 3 h after OA injection. This result is in good agreement with previous results evaluated by Evans blue extravasation (4). OA increased the number of leucocytes in BALF. Total leucocytes were quantified using a hemocytometer and a differential leucocyte count was determined by light microscopy with two blinded observers and a pathologist. C: Changes in TXB₂ production in BALF. Concentration of TXB₂ was analyzed by an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Each bar represents a mean ± S.E.M. (n = 5 – 6). Statistical analyses were made using repeated measured ANOVA followed by Tukey’s post-hoc tests. *P<0.05 and **P<0.01, compared with the values at 0 h.

Fig. 1. Time-course parameters of lung injury induced with OA. Bronchoalveolar lavage was performed 0, 0.75, 1.5, 3, and 6 h after OA injection with 10 mL ice-cold saline containing 0.7 mM ethylene-diaminetetraacetic acid disodium and 10 μg/mL indomethacin (Sigma, St. Louis, MO, USA). BALF was filtered by immediate centrifugation (250 × g. 4°C, 10 min). The supernatant was analyzed for total protein concentration and TXB₂ concentration, and the cell pellet was resuspended in 2 ml of RPMI medium containing 10% fetal bovine serum for the determination of leucocytes. A: Changes in total protein concentration in the BALF after OA injection. Total protein concentration in the BALF was measured using a bio-analyzer (Hitachi 7600; Hitachi, Tokyo). B: Changes in the number of cells in BALF after OA injection. Cells recovered from BALF were stained with Turk's stain solution (Nacalai Tesque, Kyoto) and counted using a Bürker-Türk hemocytometer. A cytospin was prepared using CytoSpin (Universal 32R; Hettich Zentrifugen, Tuttingen, Germany) and stained with May-Grünwald Giemsa stain (Merck, Darmstadt, Germany), and a differential leucocyte count was determined by light microscopy with two blinded observers and a pathologist. C: Changes in TXB₂ production in BALF. Concentration of TXB₂ was analyzed by an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA). Each bar represents a mean ± S.E.M. (n = 5 – 6). Statistical analyses were made using repeated measured ANOVA followed by Tukey’s post-hoc tests. *P<0.05 and **P<0.01, compared with the values at 0 h.
Reverse transcription was performed with 1 primer and myeloblastosis virus RT were used for cDNA synthesis. PCR kit (AMV) Ver. 2.1 (Takara Bio, Inc., Otsu). Oligo dT-adapter protocol. RT-PCR analysis was performed using the Takara RNA Carlsbad, CA, USA) (8 mL). Total RNA from the lungs was extracted in TRIzol (Invitrogen, Inc., Carlsbad, CA, USA) (8 mL/1 g tissue) according to manufacturer’s protocol. RT-PCR analysis was performed using the Takara RNA PCR kit (AMV) Ver. 2.1 (Takara Bio, Inc., Otsu). Oligo dT-adapter primer and myeloblastosis virus RT were used for cDNA synthesis. Reverse transcription was performed with 1 μg of RNA at 42°C for 60 min, 99°C for 5 min, and 5°C for 5 min. Each experiment included samples containing no RT in the following RT-PCR, to exclude amplification from contaminating genomic DNA. Equal loading was shown with β-actin bands.

were significantly increased 3 and 6 h after OA injection compared with that at 0 h. The number of macrophages and neutrophils was significantly increased with OA injection, while the number of eosinophils and lymphocytes was unaffected (Fig. 1B). OA injection also caused an increase in the level of TXB₂ in BALF. A significant increase in TXB₂ was observed 0.75 and 1.5 h after OA injection (Fig. 1C). Although the time course for the increase in total protein seemed to coincide with an increase in leucocytes, the increase in TXB₂ occurred earlier. As shown in Fig. 2, MCP-1 and IL-8 mRNA was prominently increased with OA injection, both peaking 3 h after OA injection; significant changes in eotaxin and RANTES mRNA were not observed by OA injection. These results suggest that inflammatory reactions, characterized by MCP-1 and IL-8 mRNA expression and infiltration of macrophages and neutrophils, are observed for OA-induced lung injury as well as for clinical ARDS/ALI and other models of lung injury (5, 6, 10). In addition, these results indicate that TXA₂ production precedes chemokine expression and leucocyte migration.

To evaluate efficacy, 80 mg/kg ozagrel, identified in our previous study (4) as the dosage capable of fully inhibiting TXA₂ production, was administered intravenously 30 min prior to OA injection; lung injury parameters were measured 3 h after OA injection. To ascertain the effect on TXA₂ production, we measured the levels of TXB₂ in BALF 1.5 h after OA injection. Administration of ozagrel prevented an increase in expression of MCP-1 and IL-8 mRNA in whole lungs 3 h after OA injection (Fig. 3: A and B). There was a significant decrease in the MCP-1 / β-actin and IL-8 / β-actin ratios in the OA + ozagrel group compared with the OA + saline group, as measured by optical densitometry. In addition, increases in the number of macrophages and neutrophils and in total protein in the BALF 3 h after the OA injection were markedly attenuated by ozagrel (Fig. 3: C and D). Ozagrel significantly inhibited the increase in TXB₂ levels in BALF 1.5 h after OA injection (Fig. 3E). Moreover, plasma TXB₂ levels were significantly increased by OA injection (41.7 ± 19.6 pg/mL at 0 min vs. 129.6 ± 19.9 pg/mL at 6 min after OA injection, P<0.05, n = 5), whereas no significant increases were observed in the ozagrel-treated group (21.2 ± 3.8 pg/mL at 0 min vs. 27.1 ± 4.7 pg/mL 6 min after OA injection, n = 5). These results indicated that the selective TXA₂ synthase inhibitor ozagrel reduces pulmonary vascular hyper-permeability, the inflammation that is manifested by infiltration of macrophages and neutrophils, and the over-expression of MCP-1 and IL-8 mRNA in lungs induced by OA injection.

Since pathophysiological changes induced by OA injection into animal models are similar to those in patients with ARDS/ALI, this lung injury has been widely used as an experimental model for ARDS/ALI (10). Although some inflammatory mechanisms seem to be involved in OA-induced lung injury (4, 9, 10), the precise mechanisms remain unclear. Results of this study suggest that the intervention of TXA₂ in leucocyte activation may be via chemokine mRNA expression, which supports the in vitro evidence reported by Ishizuka et al. (7) that TXA₂ has the potential to regulate chemokine mRNA expression induced by tumor necrosis factor-α or platelet-activating factor. In addition, in vivo and in vitro reports indicated that TXA₂ also plays a role in the expression of adhesion molecules, such as intercellular adhesion molecule-1 (11, 12). Taken together, TXA₂ is a pro-inflammatory mediator that facilitates, at least partly, chemokine expression and leucocyte activation in OA-induced lung injury. To substantiate this suggestion, further studies that examine the effects of TXA₂-receptor antagonist or TXA₂-receptor knockout on chemokine expression and leucocyte activation in OA-induced lung injury and that
determine other chemokine expressions in lung injury are required.

Previous reports on the usefulness of the antifungal agent ketoconazole, known to inhibit generation of TXA\(_2\), as a prophylactic drug against ARDS/ALI (13, 14) demonstrated that the drug significantly decreases TXA\(_2\) generation, the incidence of ARDS/ALI, and mortality in patients with risk factors of ARDS/ALI, such as sepsis. However, ketoconazole does not reduce TXA\(_2\) generation and death in patients whose ARDS/ALI had already progressed (15). In a previous study, we showed that ozagrel, administered simultaneously with or 30 min prior to OA injection, prevents hypoxemia, but ozagrel administered after OA injection has little effect. In this study, we further confirmed that ozagrel administered 30 min before OA injection prevents lung injury manifested by pulmonary vascular hyper-permeability, chemokine expression, and leucocyte accumulation in the lungs with injury induced by OA. Taking these findings together, we suggest that prophylactic and/or therapeutic use of rapidly acting TXA\(_2\) synthase inhibitors is a promising strategy for the prevention of ARDS/ALI. Further basic and clinical experiments are needed to establish the efficacy of TXA\(_2\) synthase inhibitors for patients with critical illnesses or injuries (i.e., sepsis, trauma, and fat emboli).

In summary, our results indicate that TXA\(_2\) synthase inhibition by ozagrel prevents lung injury and decreases IL-8 and MCP-1 mRNA expression in OA-injured lungs, as well as supports the possibility that ozagrel is a promising candidate against ARDS/ALI.
References


