Acute lung injury is an inflammatory syndrome that increases the permeability of the blood-gas barrier, resulting in high morbidity and mortality. Despite intensive research, treatment options remain limited. We investigated the protective efficacy of tezosentan, a novel, dual endothelin receptor antagonist, in an experimental model of alpha-naphthylthiourea (ANTU)-induced acute lung injury in rats. ANTU was intraperitoneally (i.p.) injected into rats at a dose of 10 mg/kg. Tezosentan was injected 30 minutes before ANTU was subcutaneously (s.c.) injected at doses of 2, 10, or 30 mg/kg, 60 minutes before ANTU was injected at doses of 2, 10, or 30 mg/kg (i.p.), and 90 minutes before ANTU at a dose of 10 mg/kg (i.p.). Four hours later, the lung weight/body weight (LW/BW) ratio and pleural effusion (PE) were measured. When injected 30 minutes before ANTU at doses of 2, 10, or 30 mg/kg (s.c.), tezosentan had no effect on lung pathology. When injected 30 minutes before ANTU at doses of 2, 10, or 30 mg/kg (i.p.) or 90 minutes before ANTU at a dose of 10 mg/kg (i.p.), tezosentan significantly decreased the PE/BW ratio and had a prophylactic effect on PE formation at all doses. Therefore, tezosentan may attenuate lung injury. Furthermore, its acute and inhibitory effects on fluid accumulation were more effective in the pleural cavity than in the interstitial compartment in this experimental model.

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Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are syndromes of acute respiratory failure that are secondary to increased permeability and noncardiogenic pulmonary edema [1–4]. ALI/ARDS is a major cause of morbidity, death, and cost in
intensive care units. The American-European consensus conference on ARDS defined ALI/ARDS as a “syndrome of inflammation and increased permeability.” Hence, it is now widely accepted that the pathophysiology of ALI/ARDS is driven by aggressive inflammatory reactions that damage the alveolar-capillary units [3–5]. Inflammatory mediators directly amplify endothelial injuries or recruit inflammatory cells into the vascular, interstitial, and alveolar spaces [5,6]. However, the mechanisms that result in lung injury, the time course of these syndromes, the involved inflammatory pathways, and associated cell repair processes are not well understood [7].

The endothelins (ETs) are a family of 21 amino acid peptides with powerful vasoconstrictive properties [8]. At least three isoforms of ET have been identified—ET-1, ET-2, and ET-3. ET-1 is the most common. Two main ET receptors have also been identified: ETA and ETB [9]. Initially described as strong vasoconstrictors, ETs are now also believed to have potent proinflammatory effects [2]. For example, transgenic mice that overexpress ET-1 release increased amounts of tumor necrosis factor (TNF)-α, interferon-γ, interleukin (IL)-1, and IL-6 [10]. ETs are abundant in the lungs and are synthesized in both the airways and pulmonary vasculature. Because ET-1 may act as an immune modulator, an increase in ET-1 may contribute to lung injuries by inducing the expression of cytokines, including TNF, IL-6, and IL-8 [9]. All these findings suggest that ET-1 has a role in respiratory physiology and pathophysiology. The ET system is activated in clinical settings and, following various types of experimental lung injuries, ET receptor antagonists have been reported to affect different levels of pathophysiology [10–13].

Alpha-naphthylthiourea (ANTU), a widely used rodenticide, causes ALI in a dose-dependent manner by changing the permeability of the lung microvasculature [14]. Because the effects of ANTU are specifically directed at the lungs, the use of this agent has become a popular means of investigating the physiological changes that result from acute lung injury [15–17]. Morphological studies indicate that the capillary endothelial cells of the lung are the primary cellular target of ANTU toxicity. Injury to the endothelium appears as blebbing and scalloping of the cell surface, eventually resulting in the loss of the endothelial barrier. This loss of endothelial barrier integrity results in increased capillary permeability and the production of interstitial and alveolar edema [18,19]. There are no reports on the effects of tezosentan on ANTU-induced ALI. Therefore, this study investigated the effect of the novel, dual ET receptor antagonist, tezosentan, on an ANTU-induced model of ALI in rats.

Materials and methods

Animal model/assessment of ALI

This study was carried out using Wistar albino male rats weighing 200–240 g (animal laboratory of Zonguldak Karaelmas University, Zonguldak, Turkey). The rats were housed under standard laboratory conditions with a 12-h light/dark cycle and were allowed free access to food and water. The procedures and protocols of this study were in accordance with our institutional guidelines, which parallel the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health, 1985). Approval for this study was obtained from the Animal Experiments Local Ethics Committee of Zonguldak Karaelmas University.

During the experiment, the animals were placed in separate cages and kept at room temperature (22 °C). ANTU (suspended in olive oil at a concentration of 4 mg/mL) was injected intraperitoneally (i.p.) at a dose of 10 mg/kg. When injected into the rats, ANTU produces pulmonary edema, as indicated by increases in the lung weight/body weight ratio (LW/BW) and pleural effusion (PE), reaching its maximum effect within 4 hours. The control group received the same volume of just olive oil. Four hours later, the animals were anesthetized with thiopental sodium (50 mg/kg i.p.) and exsanguinated by cutting the abdominal aorta.

The thorax was then opened, and any PE was carefully collected by suction and the volume was measured. Care was also taken to prevent contamination of the PE with blood. The lungs were removed, and all surrounding tissues were dissected and weighed using an analytical balance. The volume of the PE (mL) and the LW/BW and pleural effusion/body weight (PE/BW) ratios were calculated and as indices of ALI.

Experimental protocol

The animals were divided into 10 groups of 10 animals per group. The groups are described in Table 1. Tezosentan was dissolved into a solution of sterile saline. All of the drugs were prepared daily. The drug solution was kept in dark containers in order to protect it from light-induced decomposition.

Histological examination

For the histopathological examinations, the lungs were immersed in 10% formalin and allowed to fix for 2–3 days. Then, 10-μm cross sections were processed for standard

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<sup>a</sup> Tezosentan was administered 30 minutes before ANTU injection.
<sup>b</sup> Tezosentan was administered 60 minutes before ANTU injection.
<sup>c</sup> Tezosentan was administered 90 minutes before ANTU injection.
hematoxylin and eosin (H-E) staining. All of the lobes of each lung were subject to histological examination. Then, the sections were examined by light microscopy and photographed.

Chemicals

The following chemicals were used in this study: α-naphthylthiourea (Interchim, Montluçon, France) was suspended in olive oil (4 mg/ml) and was a gift from Dr. E. Schillinger, Schering AG, Berlin, Germany. Olive oil was purchased from Sigma (St. Louis, MO, USA). Tezosentan was purchased from Actelion Pharmaceuticals LTD, 4123 Allschwil, Switzerland.

Statistical analysis of results

The results are expressed as means ± SEM. The groups were compared using one-way analysis of variance (ANOVA), followed by the Tukey test when significance was indicated. A p-value of <0.05 was considered significant. Statistical analyses were performed using SPSS for Windows 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Effects of α-naphthylthiourea on the pulmonary vasculature

Upon histopathological examination, ANTU triggered severe pulmonary injury, as indicated by perivascular, peribronchial, and alveolar septal edema, erythrocyte extravasation, and inflammatory cell infiltration (Fig. 1B, C); no changes were observed in the control or olive oil-treated rats (Fig. 1A). Therefore, the olive oil group was used as a control group.

Significant lung edema developed 4 hours after the i.p. injection of 10 mg/kg ANTU, as indicated by an increase in the LW/BW ratio and PE when compared with the control rats. The LW/BW ratio was 67.8 ± 3.2 × 10^4 for ANTU-treated rats and 44.4 ± 1.8 × 10^4 for the control rats (p < 0.05) (Fig. 2A). A mean volume of 4.1 ± 0.4 mL PE was extracted from the ANTU-treated rats; none was extracted from the control rats (p < 0.05) (Fig. 2B, C).

The effects of tezosentan on ANTU-induced pulmonary edema

When injected 30 minutes before ANTU at doses of 2, 10, or 30 mg/kg s.c., tezosentan produced no prophylactic effects on ANTU-induced pulmonary edema (LW/BW ratio) (Fig. 2A) or PE formation (Fig. 2B, C).

When injected 60 min before ANTU at doses of 2, 10, or 30 mg/kg i.p., tezosentan led to a significant drop in the PE/BW ratio (p < 0.05) and had a prophylactic effect on ANTU-induced PE formation at every dose tested (Fig. 2B, C). Although the 30-mg/kg dose of tezosentan did not significantly affect the LW/BW ratio, it did cause a reduction in lung injury, as shown by the results of the histopathological examination of this group (Fig. 3).

When injected 90 min before ANTU at a dose of 10 mg/kg i.p., tezosentan caused a significant decrease in PE formation (p < 0.05) (Fig. 2B, C). The most significant protective effect on PE formation was seen in this group.

Figure 1.
(A) Normal histological appearance of olive oil-injected lungs. Representative hematoxylin-eosin (H-E) staining (40× magnification). (B) Prominent perivascular, peribronchial, and alveolar septal edema, erythrocyte extravasation, and inflammatory cell infiltration after ANTU treatment (H-E, 10×). (C) Higher magnification of pathology due to ANTU (H-E, 40×).
Tezosentan significantly increased the LW/BW ratio of this group (Fig. 2A).

**Discussion**

This study is the first to show that tezosentan, a dual endothelin ETA/ETB receptor antagonist, causes significant inhibition of PE (PE/BW ratio) formation when injected i.p. 60 and 90 min before ANTU administration and has a prophylactic effect on ANTU-induced PE formation. Conversely, it had no significant preventive effect on pulmonary edema (LW/BW ratio). The preventive effect of tezosentan on PE was seen only following i.p. injection.

Furthermore, tezosentan caused an increase in the LW/BW ratio when injected (i.p.) 90 min before ANTU. Therefore, tezosentan has complex effects on the pathology of this ANTI-induced ALI model that depend on the dose, application time, and route, similar to that noted in the literature [12].

When injected i.p., tezosentan significantly decreased the PE/BW ratio and had a prophylactic effect on ANTU-induced PE formation at all doses. When injected s.c. at the same doses, it had no prophylactic effect on ANTU-induced PE formation. These results suggest that the i.p. administration of tezosentan was more effective in our experiment. Whether administered via i.p. or s.c. injection, tezosentan had no prophylactic effect on ANTU-induced changes to the LW/BW ratio.

Our results show that a single i.p. injection of tezosentan significantly decreases PE formation. Plasma leakage into the pleural cavity and fluid accumulation in the interstitial compartment are important pathophysiological changes in ANTU-induced ALI. Many edemagenic agents have been reported to cause pulmonary endothelial damage, which allows excessive fluid to invade into the interstitium. There are three possible routes for the subsequent escape of excessive fluid: clearance by the lymphatic system, passage into the alveolar airways, and passage into the pleural cavity. The majority of edemagenic agents enhance the passage of fluid into the alveolar airways, presumably by damaging the alveolar epithelium lining, resulting in alveolar edema. However, ANTU causes severe PE, possibly because of the sensitivity of the mesothelial cell lining layer and because it targets capillary endothelial cells [14]. The exact mechanism responsible for the formation of PE is unclear, but we postulate that the acute inhibitory effects of tezosentan on fluid accumulation have a greater effect on the pleural cavity than the interstitial compartment in this model of ALI. The mechanisms responsible for the effects of tezosentan on PE pathophysiology are an important and poorly understood subject. However, we have insufficient data that can be used to reach a conclusion about the augmentative effects
of tezosentan on pulmonary edema (LW/BW ratio) at a dose of 10 mg/kg when injected 90 minutes before ANTU. This is still under investigation in our laboratories.

Alpha-naphthylthiourea produces relatively selective pulmonary toxicity, as manifested by nonhemorrhagic edema and extensive PE. The exact mechanisms that are responsible for ANTU in pulmonary tissues are not clear. It has been postulated that vasoactive substances that originate from the pulmonary vasculature and Airways contribute to ANTU-induced pulmonary edema. The mechanisms of ANTU-induced lung damage have been studied extensively and result in prominent damage to the vascular endothelium. This damage is partially mediated by arachidonic acid metabolites [20]. Following the intravenous (i.v.) injection of radiolabeled ANTU, covalent binding to macromolecules has been observed in the lungs, and desulfurization of this toxic compound produces reactive molecules [21]. ANTU is also partially metabolized by cytochrome P450 monooxygenase in both the liver and lung microsomes into an intermediate, which is also capable of covalent binding [22]. We have shown for the first time the roles of oxidized low-density lipoprotein [23] and inducible nitric oxide synthase (iNOS) enzyme [24, 25] in ANTU-induced ALI.

Additionally, ET-1 has potent constrictor effects on the arteries in vitro and produces a long-lasting pressor response. ET-1 mediates the changes seen in pulmonary hypertension. Endothelin receptor antagonists have been approved for the treatment of pulmonary arterial hypertension [26]. These drugs may offer new opportunities for managing patients with lung disease, especially ALI/ARDS, because no pharmacological agent has been convincingly shown to improve the clinical prognosis of these patients [2]. It has been suggested that ETs are involved in the pathogenesis of ALI [2, 8–13, 23]. Many types of experimental lung injury result from increased circulating ET-1, bronchoalveolar lavage (BAL) ET-1, and lung tissue ET-1 [8, 27]. The ET-1 levels in humans are also increased in sepsis, burns, ALI, and ARDS [28, 29]. In patients who have succumbed to ARDS, there was also a marked increase in tissue ET-1 immunostaining in the vascular endothelium, alveolar macrophages, smooth muscle, and airway epithelium compared with the lungs of patients who died without ARDS [30].

The mechanisms by which ET-1 contributes to pulmonary edema remain uncertain but may include altering the vascular reactivity, resulting in increased capillary pressure and fluid filtration [31]; however, the effect of ET-1 on vascular permeability is controversial. Although several studies have suggested that ET-1 increases microvascular permeability [32, 33], others have found no effect [34]. However, it has also been suggested that ET-1 decreases transvascular fluid flux by stabilizing the integrity of the endothelial barrier [35, 36]. Similarly, ET-1 has been shown to inhibit edema formation that is induced by various chemical stimuli in rat and rabbit skin [37, 38]. In a previous study [39], we found that i.v. injection of ET-1 into rats 15 minutes before ANTU significantly reduced the LW/BW ratio at a dose of 3 nmol/kg, but not at doses of 0.3 or 1 nmol/kg. Furthermore, decreases in the PE/BW ratio were significant at 1 and 3 nmol/kg ET-1. Phosphoramidon, endothelin converting enzyme inhibitor, did not alter the edema-producing effect of ANTU indicated the lack of participation of the ET-peptide cascade in this pathological event. We suggest that the long-lasting vasoconstrictive nature of ET-1 may factor into this preventive effect because no resolution regarding these parameters was observed with angiotensin II (AT II), a relatively short-acting vasoconstrictor peptide. Endothelin receptor antagonists have been evaluated in different animal models of ALI/ARDS. While studies have shown that they have protective effects in some models of ALI/ARDS [12, 13, 40–42], their efficacy may vary in different experimental models of lung injury or following different modes of administration. This was reflected in a study by Hubloue et al. [43] on oleic acid-induced lung injury in dogs where pretreatment with bosentan, another dual ET receptor antagonist, reduced pulmonary hypertension, whereas the same treatment started 90 minutes after the insult had no effect. Similarly, Cox et al. [44] found that tezosentan does not provide clear protection against ALI that was induced by smoke inhalation or burns in sheep. This suggests that the timing of intervention is important in dynamic progressive processes such as ALI. Their study supports our results, namely that tezosentan causes a significant increase in pulmonary edema formation, unlike the other groups, when injected i.p. 90 minutes before ANTU. Similarly, Ruetten et al. [45] found that pretreatment with a nonselective ET receptor antagonist in endotoxin-challenged rats markedly augmented hemodynamic deterioration and reduced the 6-hour survival rate. Conversely, Rossi et al. [12] found that tezosentan efficiently counteracts pulmonary edema in sepsis-related ALI in pigs at a dose of 1 mg/kg, whereas a 10-fold greater dose of tezosentan resulted in adverse effects, most notably hemodynamic deterioration and early death. These results indicate that tezosentan can have opposite results depending on the dose and route of administration.

Our results indicate that ET peptides play a role in ANTU-induced ALI. Tezosentan has a significant protective effect on massive PE formation, but it has no preventive effect on pulmonary edema and no significant histopathological recovery was observed. These results suggest that the nonselective ET receptor antagonist, tezosentan, when administered i.p., has complex effects on inflammation and tissue injury, as indicated in this and other models of ALI. Our results corroborate earlier findings regarding the complex effects of tezosentan on ALI pathology. We conclude that the ET system is involved in the pathophysiology of lung disease, including ALI, but further studies are warranted to determine the specific mechanisms responsible for the favorable effects of ET receptors and ET receptor antagonism.

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References

Tezosentan and acute lung injury


