Effects of a dual endothelin-1 receptor antagonist on airway obstruction and acute lung injury in sheep following smoke inhalation and burn injury

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ABSTRACT

Studies have suggested that ET-1 (endothelin-1) is associated with lung injury, airway inflammation and increased vascular permeability. In the present study we have tested the hypothesis that treatment with a dual ET-1 receptor antagonist will decrease airway obstruction and improve pulmonary function in sheep with combined S+B (smoke inhalation and burn) injury. Twelve sheep received S+B injury using the following protocol: six sheep were treated with tezosentan, an ETA and ETB receptor antagonist, and six sheep received an equivalent volume of vehicle. Physiological and morphological variables were assessed during the 48 h study period and at the end of the study. There was no statistically significant difference in the \( P_aO_2/F_iO_2 \) (partial pressure of O\(_2\) in arterial blood/fraction of O\(_2\) in the inspired gas) ratio of the tezosentan-treated animals compared with controls; however, lung lymph flow was significantly higher \((P < 0.05)\) in the treated animals. PVRI (pulmonary vascular resistance index) was significantly reduced \((P < 0.05)\) in the tezosentan-treated animals. Assessment of NOx (nitric oxide metabolite) levels in plasma and lymph showed significantly elevated \((P < 0.05)\) levels in the tezosentan-treated animals compared with levels in untreated sheep. The degree of bronchial obstruction was similar in both treated and control sheep; however, bronchiolar obstruction was reduced in sheep treated with tezosentan. Histopathologically, no difference in the degree of parenchymal injury was detected. In conclusion, administration of a dual ET-1 receptor antagonist prevented an increase in PVRI after injury and reduced the degree of bronchiolar obstruction in sheep with S+B; however, treated sheep showed higher levels of NOx and increased lung lymph flow. Tezosentan treatment was ineffective in protecting against acute lung injury in this model.

INTRODUCTION

In the last decade, care of burn victims has greatly improved with the use of broad spectrum antibiotics, effective resuscitation and early removal of the necrotic tissue. However, morbidity and mortality of burn victims with inhalation injury has not been reduced [1]. Understanding the pathogenesis of ALI (acute lung

Key words: acute lung injury, airway obstruction, endothelin-1, inflammation, mucous secretion, nitric oxide, tezosentan.

Abbreviations: ALI, acute lung injury; ET-1, endothelin-1; \( F_iO_2 \), fraction of O\(_2\) in the inspired gas; MPO, myeloperoxidase; NO, nitric oxide; NOx, NO metabolite; iNOS, inducible NO synthase; \( P_aO_2 \), partial pressure of O\(_2\) in arterial blood; PVRI, pulmonary vascular resistance index; S+B, smoke inhalation and burn.

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injury) following smoke inhalation injury is important for the development of treatment modalities to care for these victims better.

Previous studies have demonstrated in an ovine model that a preparation of cooled cotton S+B (smoke inhalation and burn) injury models the pathophysiology of these injuries in humans [2,3]. In sheep, initial injury from smoke inhalation is limited to the trachea and upper bronchial airways and is characterized by epithelial exfoliation, mucous secretion, increased vascular permeability and acute inflammatory cell infiltration [2]. Additional studies have shown that the development of ALI following smoke inhalation injury is neutrophil dependent and, in part, mediated by NO (nitric oxide) ([4–6]; for review, see [7]). In a recent study [8], smoke inhalation injury caused the airways to become extensively obstructed, with the degree of obstruction correlating with the severity of ALI. Major components of airway obstructive material are mucus, fibrin and inflammatory cells.

In the last decade, numerous studies have shown an association of ET-1 (endothelin-1) with airway disease [9] and ALI [10]. ET-1 has also been shown to have profibrinolytic properties [11–15] and may also be a mediator of glandular secretion [16–18] and promote bronchial oedema [15]. These studies, in addition to a recent study reporting that smoke inhalation injury enhances the expression of ET-1 in airways of sheep after injury [19], suggest that this peptide may play an important role in airway obstruction and ALI in a sheep model of S+B injury.

The aim of the present study was to test the hypothesis that ET-1 contributes to the pathophysiology of ALI and airway obstruction in sheep with S+B injury. To test the hypothesis, sheep with S+B injury were given the dual ETA and ETB receptor antagonist tezosentan. Statistical analysis of physiological and histological variables in the treated and control animals was used to test the hypothesis.

**MATERIALS AND METHODS**

The present study was approved by the Animal Care and Use Committee of the University of Texas Medical Branch and conducted in compliance with the guidelines for the care and use of laboratory animals of the National Institutes of Health and the American Physiology Society. The animals used in the present study were female range-bred adult sheep (approx. 40 kg in weight). Preparation for injury and chronic study has been described previously [5]. Briefly, surgical preparation included endotracheal intubation, cannulation of the right femoral artery and vein and catheterization of the pulmonary artery and the left atrium. To measure and collect lung lymph, a thoracotomy in the sixth intercostal space was performed and the efferent vessel of the caudal mediastinal lymph node was cannulated with Silastic medical-grade tubing (0.635 mm internal diameter; 1.194 mm outer diameter; Dow Corning, Midland, MI, U.S.A.). Following surgical preparation, the animals were allowed to recover for 5–7 days and given free access to food and water.

**Injury protocols**

Prior to injury, the animals were deeply anaesthetized with 3% halothane. Previous studies have described in detail the protocols for S+B injury [3,20]. Briefly, smoke inhalation injury was induced by burning 40 g of cotton towelling in a modified bee smoker attached to a tracheostomy tube. The temperature of the smoke was monitored continuously to ensure that it did not exceed 40 °C. Four series of 12 breaths (total 48 breaths) were delivered. Carboxyhaemoglobin concentration in the arterial blood was monitored (CO-Oximeter 482; Instrumentation Laboratory, Lexington, MA, U.S.A.) after each 12 breaths to ensure that each animal had received an equivalent dose.

Cutaneous burn injury (40%, third-degree injury) was inflicted with a Bunsen burner, with each flank of the animal receiving a 20% total body surface area burn. The degree of injury is a full-thickness burn, including both the epidermis and dermis, in which the nerve endings are heat-destroyed, so the sheep does not feel pain after the procedure.

**Resuscitation**

Following injury, animals were given free access to food. Water intake was restricted and the animals were resuscitated with Ringer’s lactate solution (4 ml/percentage body surface area burned per kg of body weight per 24 h) [21]. During the study period, the animals were monitored in a critical care facility. Blood gases were monitored to maintain normal pulmonary arterial oxygen tension. Ventilation (Servo Ventilator 900C; Siemens, Elema, Sweden) was performed with a PEEP (positive end-expiratory pressure) of 5 cm H2O and a tidal volume of 15 ml/kg of body weight.

During the first 3 h following injury, the inspiratory O2 concentration was maintained at 100% and the respiratory rate was kept at 30/min to induce rapid clearance of carboxyhaemoglobin after smoke inhalation. After three hours, ventilation rate and FiO2 (fraction of O2 in the inspired gas) were adjusted according to blood gas analysis to maintain the Paco2 (partial pressure of CO2 in arterial blood) between 25 and 30 mmHg and the arterial O2 saturation above 90%.

To test our hypothesis that ET-1, through interaction with both ETA and ETB receptors, contributes to airway obstruction and pulmonary dysfunction, we administered the dual ETA and ETB receptor antagonist tezosentan [5-isopropyl-pyridine-2-sulphonic acid 6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2-(2-1H-tetrazol-5-yl-pridin-4-yl)-pyridin-4-ylamide] [22], 1 h
prior to injury and continuously during the 48 h study period. Five animals received a bolus of tezosentan prior to injury (3 mg/kg of body weight in 5% dextrose), with an infusion dose of 1 mg·kg⁻¹·h⁻¹ of body weight·h⁻¹ during the 48 h study period. One animal received a 2-fold increase in the bolus dose (6 mg/kg of body weight) and an increase in the infusion dose from 1 to 3 mg·kg⁻¹·h⁻¹ of body weight·h⁻¹). Six animals served as controls, receiving S+B injury, but also the same volume of 5% dextrose solution as the treated animals over the 48 h study period. The dose of tezosentan given was suggested by the manufacturer and similar to the dose used in a recent study showing tezosentan reduced systemic and pulmonary vascular resistance in a model of endotoxin-induced lung injury in sheep [23].

Haemodynamic and oxygenation variables
During the 48 h study period, vascular pressures were measured using transducers (model PX-1800; Baxter, Irvine, CA, U.S.A.), which were adapted with a continuous flushing device. The transducers were connected to a haemodynamic monitor (model 78304A; Hewlett Packard, Santa Clara, CA, U.S.A.). All haemodynamic measurements were made with the animals in a standing position. Zero calibrations were taken at the level of the olecranon joints of the front leg. Cardiac output was measured by the thermodilution technique using a cardiac output computer (Baxter). Ice-cold 5% dextrose solution was used as the indicator. The cardiac index was calculated using standard equations. Blood gases and acid/base balance were measured using a blood gas analyser (Instrumentation Laboratory, Lexington, MA, U.S.A.). Arterial and mixed venous blood gas results were corrected for body temperature. Oxyhaemoglobin saturation and carboxyhaemoglobin concentrations were analysed with a CO-oximeter (Instrumentation Laboratory). Blood gases and cardiopulmonary variables were measured at 3, 6, 12, 18, 24, 30, 36, 42 and 48 h after injury. Baseline measurements were taken approx. 1 h before sedation and injury. The PaO₂ (partial pressure of O₂ in arterial blood)/FiO₂ ratio was calculated at each time interval.

Lung lymph and NOx (NO metabolite) measurements
Lung lymph flow (ml/h) was measured with a graduated test tube and stopwatch. NO and NOx (nitrate and nitrite) levels were measured in both plasma and lymph by a chemiluminescence assay using an NO analyser (Antec Instruments, Houston, TX, U.S.A.) [24]. Samples were acquired for analysis at 3, 6, 12, 18, 24, 36, 42 and 48 h after injury. Baseline measurements were obtained approx. 1 h before sedation and injury. Changes in NOx levels were analysed relative to baseline.

Histological analysis
At the end of the study, all of the animals were killed with an overdose of ketamine, followed by an intravenous injection of saturated KCl solution. Following verification of death, the trachea and lungs were removed and tissue for histology was collected in a systematic way. A transverse slice approx. 1 cm thick was taken through the middle of the lower lobe of the right lung, injected with 10% buffered formalin and immersed in the same fixative for 3–5 days. Following fixation, the tissue slice was sampled into four blocks for light microscopic analysis. Based on planimetric measurements, the sampling protocol allowed for an average of 5.7 ± 10.5% of the tissue slice to be examined histologically. A technician unaware of the sample status sampled the lung slice for histological processing. Samples were processed into paraffin, sectioned at 4 µm and stained with haematoxylin and eosin.

Semi-quantitative scoring for the degree of the histopathological change was accomplished using a standardized protocol. Using masked slides from the first three blocks from each animal, 24 × 10 objective fields of view were scored systematically for the degree of congestion, oedema, inflammation and haemorrhage. For each field, a score of 0–4 was noted for each parameter, with 0 = normal, 1 = light, 2 = moderate, 3 = strong and 4 = intense. Following scoring of the 24 fields, a mean score for the individual parameter was calculated for each animal. Additionally, a total histopathological score was obtained by calculating the mean of the four separate scores. This method has been used to characterize the degree of parenchymal injury in a model of smoke inhalation injury and sepsis [25]. This method has also shown decreases in parenchymal pathology scores in studies in which pharmacological agents have attenuated the development of ALI in sheep after S+B injury [26,27].

The second histopathological study was assessment of airway obstruction using a method described previously [8]. Briefly, all histological slides were combined, randomly sorted and masked prior to observation. For each slide, the tissue was scanned with a ×4 objective. For each cross-sectioned airway, the percentage of airway lumen obstructed (0–100%) was estimated. Each airway was also classified as either a bronchus or a bronchiole. Bronchi were defined as airways with supporting cartilage and/or mucous glands. Bronchioles lacked cartilage and mucous glands. The main bronchus was excluded from the analysis, because cast material in this large airway is usually displaced during gross tissue collection. After all slides had been examined, mean airway obstruction scores were determined for each animal. Individual animal data were then placed into the control and treated groups for statistical analysis.
Quantification of MPO (myeloperoxidase)-stained neutrophils
This procedure was conducted to provide a measure of neutrophil infiltration in the parenchyma of the study animals. Histological sections were obtained from block #2 for each animal. Tissue was deparaffinized and pretreated with bacterial protease (Type XXIV; 6 μg in 200 ml of Tris/HCl buffer, pH 7.6; Sigma, St. Louis, MO, U.S.A.) for 30 min at room temperature and 0.6 % H2O2 (Sigma) for 30 min to quench endogenous peroxidase. Immunolabelling was accomplished with the use of a Vectastain® Elite ABC peroxidase kit (Vector Laboratories, Burlingame, CA, U.S.A.). Briefly, tissues were incubated for 1 h at room temperature in 10 % normal goat serum to block non-specific staining. Tissue was then incubated overnight at 4 °C with a rabbit polyclonal antibody to human MPO (Calbiochem, La Jolla, CA, U.S.A.) diluted 1:1000 in normal goat serum. Tissue was washed with buffer and incubated for 1 h with biotinylated goat anti-rabbit IgG, washed with buffer and incubated for 1 h with the ABC reagent. Visualization of the peroxidase-labelled antibody complex was accomplished by incubating the tissues for 5 min with a chromogen solution (Vector Laboratories). Tissue was then counterstained with eosin. This immunostaining protocol produces a black precipitate for antigen localization with non-immunostained tissue staining red.

To measure the degree of MPO immunolabelling, three-phase colour analysis was performed with AnalySiS imaging software (Soft Imaging Systems, Lakeview, CO, U.S.A.). Briefly, a digital image of parenchyma was obtained with a × 40 objective lens. Using the image analysis software, the three phases (red = eosin-stained tissue; black = immunostained cells; and white = alveolar space) were defined by selecting a minimum of 20 points within each phase area. Using this procedure, approx. 99.9 % of the pixels in each digital image were assigned to a particular phase. The phase protocol was saved and used on all images analysed. To measure the degree of parenchymal immunostaining for MPO in the tezosentan-treated animals compared with controls, all slides were masked prior to analysis. Similarly, fixed and sampled tissue samples from five surgically prepared animals without injury (sham) were also included in the analysis. Five × 40 fields of parenchyma were captured/animal. Images were from non-overlapping regions of parenchyma and were acquired by viewing one slide/animal from left to right. For each image, the imaging software determined the ratio of the number of pixels stained black to the number of pixels stained red. The ratio of black (MPO; μm²) relative to the eosin-stained septal area (μm²) was recorded for each image. A mean ratio was obtained from the five measurements from each animal and was recorded for the appropriate treatment group.

Statistical analysis
Summary statistics of data are expressed as means ± S.E.M. Statistical analysis of the physiological data was accomplished by ANOVA for a two-factor experiment with repeated measurements over time. Unpaired Student’s t tests were used to detect time points of statistical significance (post-hoc analysis). Statistical analysis of the comparison between groups of the degree of airway obstruction, histopathological score and measurement of neutrophil sequestration in the parenchyma was accomplished with Mann–Whitney rank sum tests. All statistical tests were conducted with SigmaStat software (SPSS, Chicago, IL, U.S.A.).

RESULTS
PVRI (pulmonary vascular resistance index) was decreased significantly (P < 0.05) in tezosentan-treated animals compared with controls (Figure 1), and the significant decreases were detected 6–42 h after injury (P < 0.05; Figure 1).

Assessment of pulmonary function showed that tezosentan was ineffective in attenuating the decrease in PaO2/FiO2 in injured animals. Both study groups exhibited ALI within 24 h after injury. No statistically significant difference in mean PaO2/FiO2 values between treated and untreated groups was detected at any time point. Two animals from the tezosentan-treated group were killed early, one at 24 and the other at 36 h after injury, due to a PaO2/FiO2 ratio below 100. All animals in the control group survived the study period of 48 h. The two tezosentan-treated animals killed early had each been given the bolus dose of 3 mg/kg of body weight, followed by 1 mg·kg⁻¹·body weight·h⁻¹ after injury.

Assessment of the rate of PaO2/FiO2 decline in the tezosentan-treated group and the injured control group.
is shown in Figure 2. Comparison of the degree of pulmonary dysfunction and histopathology in the tezosentan-treated animal given the 6 mg/kg of body weight bolus and 3 mg·kg⁻¹ of body weight·h⁻¹ dose compared with the treated animals that received the lower dose (3 mg/kg of body weight bolus and 1 mg·kg⁻¹ of body weight·h⁻¹) showed no trend towards decreased obstruction or improvement in pulmonary function, suggesting that administration of a higher dose did not change the study outcome.

Comparison of the degree of increase in lung lymph flow relative to baseline showed a statistically significant increase in animals that received tezosentan at all time points after injury, except at 18 and 24 h (Figure 3). At the end of the study, the degree of lymph flow was approx. 4-fold higher than the flow observed in the injured but untreated group (Figure 3).

Analysis of NOx levels in the plasma and lung lymph of tezosentan-treated animals showed an approx. 2-fold increase compared with controls at baseline. In plasma, the NOx levels were significantly greater (P < 0.05) at 6 h after injury (Figure 4A). No significant difference in the NOx levels in lung lymph fluid were observed (Figure 4B).

In the histological analysis of airway obstruction, 63 and 102 bronchi were scored in the injured control and tezosentan-treated animals respectively. The range of scores in the injured untreated group and the tezosentan group was 10.8–68.4 and 8.4–55.0 respectively. Mean degrees of obstruction were 26.8 ± 17.2 in the tezosentan-treated group and 27.1 ± 12.4 in the untreated group. No significant difference in the mean degree of obstruction was evident (P = 0.59). In the assessment of bronchiolar obstruction, 600 airways were examined from the six animals in the tezosentan group compared with 806 airways in the control group. Analysis of the degree of airway obstruction in bronchioles showed a significant decrease (P = 0.04) in the degree of obstruction in the tezosentan-treated group (14.3 ± 3.6) compared with...
DISCUSSION

In the present study, a comparison of PVRI between the control animals and animals treated with tezosentan showed that tezosentan prevented an increase in PVRI after S+B injury, suggesting strongly that the dose of tezosentan used in our study was pharmacologically effective. However, treatment with tezosentan was unsuccessful in attenuating the development of ALI in sheep after S+B injury.

The physiological parameter that defines ALI, $P_{aO_2}/F_iO_2 \leq 300$, was similar in both the tezosentan-treated and injured control animals. An additional feature of ALI from smoke inhalation injury in sheep is an increasing degree of lung lymph flow. Comparison of the rate of lymph flow between the control and tezosentan-treated animals showed a significant ($P < 0.05$) increase in the degree of lymph flow in treated animals at nearly all time points after injury.

Measurement of airway obstruction in the two study groups showed a trend towards decreased bronchial obstruction in the tezosentan-treated animals compared with controls and a significant decrease in the degree of bronchiolar obstruction in the tezosentan-treated animals. These results suggest that treatment may have reduced the degree of mucous secretion or airway inflammation, both of which contribute to bronchiolar obstruction [8]. In our assessment of airway obstruction, the number of bronchi and bronchioles included in the analysis was different in the two groups. As presented in the Materials and methods section, approx. $51.7 \pm 10.5 \%$ of the midsection slice was sampled for histology. The sampling protocol followed is not selective for bronchi visible in the midsection slice, but generally yields an ample sample size. We believe the smaller number of bronchi and bronchioles observed in the tezosentan group was a consequence of the variability in sampling of the midsection slice, and is not likely to have biased the results. Semi-quantitative histopathological analysis showed no significant difference in the degree of congestion, oedema, inflammation or haemorrhage in the parenchyma of treated animals compared with controls.

Previous studies in this ovine model have demonstrated that the development of ALI was neutrophil-dependent. In the present study, we used light microscopic phase analysis to measure the ratio of MPO staining to the septal area to determine the degree of neutrophil sequestration in the parenchyma. Mean ratios were similar in the injured groups and were approx. 2-fold greater than in tissue without injury; however, the difference was not statistically significant ($P > 0.05$).

Additional variables examined in the present study included NOx levels in both plasma and lymph. Results showed a trend of higher NOx levels in both plasma and lymph of the treated animals compared with controls. The increase was significantly greater ($P < 0.05$) early after injury. Recent studies have demonstrated that NO is important in the pathophysiology of ALI in sheep after S + B injury. Inhibition of iNOS (inducible NO synthase) has been shown to attenuate the increased vascular permeability after S + B injury [6] and, additionally, to attenuate the degree of ALI after inhalation injury [5]. Recent studies have shown decreased levels of airway obstruction in sheep treated with an iNOS inhibitor after S + B injury [26–28].

Numerous studies have examined the possible mechanisms by which NO may be involved in the pathophysiology of disease processes. NO has been shown to react with superoxide to yield the reactive nitrogen species peroxynitrite ($ONOO^-$) [29–31]. Peroxynitrite has...
cytotoxic potential through reactions with protein and non-protein sulphhydrils, oxidation of lipids, nitrosation of tyrosine and induction of single-strand breaks in DNA [32,33]. However, studies have also shown that NO may promote the chemotactic potential of neutrophils [34–37] and stimulate the production of proinflammatory agents [36].

In vascular biology, ET-1 and NO are clearly established as mediators of vascular tone [31,38]. ET-1 binding to the ET₁ receptor is coupled with constitutive endothelial cell NO synthase (eNOS) production of NO [39]. Additionally, ET-1, through interaction with both ETₐ and ETₐ receptors, is a mediator of airway tone. ET-1 binding to the ET₁ receptor promotes bronchial smooth muscle cell contraction, whereas interaction with the ETₐ receptor promotes NO-induced relaxation [40]. Although ET-1 has been shown to modulate the expression of NO, Markewitz et al. [41], using an vitro assay, showed that ET-1, through the ET₁ receptor pathway, inhibited the activity (approx. 30 %) and mRNA (approx. 50 %) expression of iNOS. A study by Beck et al. [42] showed a similar effect of ET-1 binding to ET₁ receptors for inhibition of iNOS transcription in glomerular mesangial cells stimulated with cytokines. These studies and the higher NOx levels found in the plasma and lymph in tezosentan-treated animals in the present study suggest that tezosentan treatment may have promoted increased activity of iNOS, thereby promoting increased injury in our study animals. This hypothesis would explain the observed increased NOx levels and the increased lymph flow observed in the present study. Clearly, further studies assessing the effects of ET-1 receptor antagonists on iNOS expression in this injury model are warranted.

Although ET-1 and NO are clearly related in the maintenance of vascular and airway tone, each of these mediators has been linked to submucosal gland secretion. Two studies have shown that ET-1 can inhibit [43] or promote [44] mucous secretion from isolated bronchial mucous glands. However, in neither of these studies was the receptor-mediated pathway of activation identified. Furthermore, NO has been shown to induce submucosal gland secretion [45], although a relationship between ET-1 and NO interaction in the process of glandular secretion has not been studied. Future studies to assess the ability of single ET-1 receptor-mediated pathways to inhibit glandular secretion and their effects on iNOS expression are needed.

In conclusion, administration of a dual ET-1 receptor antagonist in sheep with S+B injury produced both positive and adverse effects. Positive effects included preventing an increase in PRV and, in support of our study hypothesis, tezosentan reduced the degree of bronchial obstruction. However, the degree of improvement in obstruction was not sufficient to attenuate the degree of ALI. Adverse effects observed with tezosentan treatment included increased lung lymph flow and higher NOx levels in the plasma and lymph of the treated animals. These data, together with the extensive literature defining a relationship of ET-1 and NO, suggest that further studies using ET-1 and NO suggest that further studies using ET-1 receptor antagonists and examination of NOS isoform expression and activity in this large animal injury model are needed.

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272 R. A. Cox and others


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