

Tezosentan Increases Nitric Oxide Signaling Via Enhanced Hydrogen Peroxide Generation in Lambs With Surgically Induced Acute Increases in Pulmonary Blood Flow

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ABSTRACT

We have previously shown that acute increases in pulmonary blood flow (PBF) are limited by a compensatory increase in pulmonary vascular resistance (PVR) via an endothelin-1 (ET-1) dependent decrease in nitric oxide synthase (NOS) activity. The mechanisms underlying the reduction in NO signaling are unresolved. Thus, the purpose of this study was to elucidate mechanisms of this ET-1–NO interaction. Pulmonary arterial endothelial cells were acutely exposed to shear stress in the presence or absence of tezosentan, a combined ET_A/ET_B receptor antagonist. Shear increased NO_x, eNOS phospho-Ser1177, and H₂O₂ and decreased catalase activity; tezosentan enhanced, while ET-1 attenuated all of these changes. In addition, ET-1 increased eNOS phospho-Thr495 levels. In lambs, 4 h of increased PBF decreased H₂O₂, eNOS phospho-Ser1177, and NO_x levels, and increased eNOS phospho-Thr495, phospho-catalase, and catalase activity. These changes were reversed by tezosentan. PEG-catalase reversed the positive effects of tezosentan on NO signaling. In all groups, opening the shunt resulted in a rapid increase in PBF by 30 min. In vehicle- and tezosentan/PEG-catalase lambs, PBF did not change further over the 4 h study period. PVR fell by 30 min in vehicle- and tezosentan-treated lambs, and by 60 min in tezosentan/PEG-catalase-treated lambs. In vehicle- and tezosentan/PEG-catalase lambs, PVR did not change further over the 4 h study period. In tezosentan-treated lambs, PBF continued to increase and LPVR to decrease over the 4 h study period. We conclude that acute increases in PBF are limited by an ET-1 dependent decrease in NO production via alterations in catalase activity, H₂O₂ levels, and eNOS phosphorylation. *J. Cell. Biochem.* 114: 435–447, 2013. © 2012 Wiley Periodicals, Inc.

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Elevated shear stress within the pulmonary vasculature is a factor in the pathophysiology of several pulmonary hypertensive disorders [Morales and Loscalzo, 1997]. Acute increases in shear stress accompany surgically induced increases in pulmonary blood flow (PBF), such as those associated with the repair of several common congenital cardiac defects [Tworetzky et al., 2000]. We have previously described a novel model of a

surgically induced acute increase in PBF, created by the placement of a large (8 mm) vascular graft between the aorta and pulmonary artery in an intact juvenile lamb [Oishi et al., 2006]. In this model, the acute increase in PBF was followed by compensatory pulmonary vascular constriction that was, in part, mediated by an endothelin-1 (ET-1) dependent decrease in endothelial nitric oxide synthase (eNOS) activity. However, the mechanisms

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linking increased PBF, ET receptor activation, and a decrease in NOS activity remain unclear.

A large body of evidence demonstrates that eNOS is dynamically regulated at the transcriptional [Searles, 2006], post-transcriptional [Searles, 2006], and post-translational levels [Fulton et al., 2001; Rafikov et al., 2011]. In addition, factors such as intracellular location [Shaul, 2002], protein-protein interactions [Rafikov et al., 2011], and substrate and cofactor availability [Sharma et al., 2012] can all regulate eNOS activity. Furthermore, mechanical forces can impact eNOS. We have also shown that acute increases in fluid shear stress can increase eNOS activity *in vitro*, independent of changes in eNOS expression [Kumar et al., 2010]. The shear-mediated increase in eNOS activity appears to be regulated, in part, by the phosphorylation status of eNOS [Kumar et al., 2010]. A key phosphorylation site on eNOS is located at serine 1177 (Ser1177) [Fulton et al., 1999]. Recently, we demonstrated that in pulmonary artery endothelial cells (PAEC) acute increases in shear stress were accompanied by: decreased catalase activity, increased hydrogen peroxide (H₂O₂) production, increased eNOS phosphorylation at Ser1177, and increased NO generation [Kumar et al., 2010]. These findings, however, contrast the ET-1 mediated decrease in NOS activity in our lamb model of increased PBF [Oishi et al., 2006]. Therefore, the purpose of this study was to explore the potential role of H₂O₂ signaling under conditions of acutely increased PBF *in vivo*, using our lamb model. We hypothesized that acute increases in PBF are associated with ET receptor-mediated alterations in H₂O₂ signaling that result in decreased NOS activity and bioavailable NO. The juvenile lamb is ideally suited to mimic the cardiopulmonary physiologic changes noted in infants undergoing surgical-induced acute changes in PBF. Many of the fetal and neonatal PBF patterns have been investigated in the lamb and have been found to mimic the physiology of humans. In addition, its size facilitates the ability to safely perform the surgical interventions needed, monitor vascular pressures and flow, and perform intermittent peripheral lung biopsies.

Our findings indicate that acute increases in PBF in lambs, results in an ET receptor mediated increase in catalase activity that decreases H₂O₂ levels, eNOS phosphorylation at Ser1177, and bioavailable NO. These effects can be modulated by both ET receptor antagonism and augmentation of catalase activity.

MATERIALS AND METHODS

CELL CULTURE

Primary cultures of ovine PAEC were isolated as described previously [Wedgwood et al., 2001]. Cells were maintained in DMEM containing phenol red supplemented with 10% fetal calf serum (Hyclone, Logan, UT), antibiotics, and antimycotics (MediaTech, Herndon, VA) at 37°C in a humidified atmosphere with 5% CO₂-95% air. Cells were utilized between passages 3 and 10.

SHEAR STRESS AND ET-1 TREATMENT

Laminar shear stress, in the presence and absence of tezosentan (5 μM), was applied using a cone-plate viscometer that accepts six-well tissue culture plates, as described previously [Kumar et al., 2010]. This method achieves laminar flow rates that represent

physiological levels of laminar shear stress in the major human arteries, which is in the range of 5–20 dyn cm⁻² [Laurindo et al., 1994]. Confluent serum-starved PAEC (16 h) were also treated with ET-1 (100 nM; Sigma, St Louis, MO) and exposed or not to laminar shear stress. In certain experiments, PAEC were also exposed to the ET_A receptor antagonist, BQ-123 (10 μM; Sigma) or the ET_B receptor antagonist, Res 701-3 (10 μM; American Peptide, Sunnyvale, CA). The media and cells were collected and used for further experiments. PAEC were also treated with ET_B receptor agonist 4-ala ET-1 (100 nM; Phoenix Pharmaceutical, Burlingame, CA) for 4 h.

WESTERN BLOTTING

Serum-starved PAEC (16 h) were sheared with or without tezosentan (5 μM) for 4 h and solubilized with a lysis buffer containing 1% Triton X-100, 20 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Pierce). Insoluble proteins were precipitated by centrifugation at 14,000 rpm for 10 min at 4°C, and the supernatants were then subjected to SDS-PAGE on 4–20% polyacrylamide gels and transferred to a Polyvinylidene fluoride (PVDF) membrane (BioRad). The membranes were blocked with 5% non-fat dry milk or 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween (TBST). The primary antibodies used for immunoblotting were anti-catalase, anti-phospho-serine (1:500; Calbiochem), anti-phospho-Ser1177 eNOS, anti-eNOS (1:1,000; BD Biosciences), ET_A receptor (Santa Cruz Biotechnology), and ET_B receptor (Santa Cruz Biotechnology). For the studies to determine the ratios of pThr-495 eNOS or p-Ser1177 eNOS to total eNOS two separate gels were run. Membranes were then washed with TBST three times for 10 min, incubated with the appropriate secondary antibody coupled to horseradish peroxidase, washed again with TBST as described above, and the protein bands visualized with ECL reagent (Pierce) using a Kodak 440CF image station. Loading was normalized by re-probing the membranes with an anti-β-actin antibody (1:2,500; Sigma).

DETERMINATION OF CATALASE ACTIVITY

Catalase activity was measured as described [Kumar et al., 2010]. This method is based on the rate of degradation of H₂O₂ to form water and oxygen over time. In both cell and ovine lung studies, total protein extracts (40 μg) were diluted to 1 ml in 50 mM phosphate buffer (pH 7.0). One milliliter of 10 mM H₂O₂ solution was added, and the decomposition of the substrate was recorded by the decrease in absorbance at 240 nm over a 60 s period. Catalase activity was expressed as degradation of H₂O₂ · mg protein⁻¹ · min⁻¹.

DETECTION OF NO_x

NO generated by PAEC in response to shear was measured in the media using an NO-sensitive electrode with a 2-mm diameter tip (ISO-NOP sensor, WPI) connected to an NO meter (ISO-NO Mark II, WPI) as described previously [Kumar et al., 2010]. NO levels in the lung lysate were determined by indirect method of measuring the levels of nitrite as we have previously described [McMullan et al., 2000]. Briefly, samples were deproteinized by adding cold ethanol to the sample (1:4 v:v) and then concentrated using a speed-vac. Potassium iodide/acetic acid reagent was prepared fresh daily. This

reagent was added to a septum sealed purge vessel and bubbled with nitrogen gas. The gas stream was connected via a trap containing 1N NaOH, to a Sievers 280i Nitric Oxide Analyzer (GE). Deproteinized samples were injected with a syringe through a silicone/Teflon septum. Results were analyzed by measuring the area under curve of the chemiluminescence signal using the Liquid software (GE).

MEASUREMENT OF H₂O₂ LEVELS

A modified H₂DCFDA oxidation method was used to detect H₂O₂ levels [Wedgwood and Black, 2005]. For the cell culture studies, H₂O₂ levels were detected in the media. For the lambs studies, snap-frozen peripheral lung tissue samples stored at -80°C were powdered. Powdered tissue samples (20 mg) were homogenized in PBS (100 µl) and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was then used for detection of H₂O₂ levels. Fifty microliters of culture media or lung lysate were incubated with H₂DCFDA (25 µM; Calbiochem) for 30 min in the dark with or without 100 U ml⁻¹ catalase (Sigma). Purified catalase was used to confirm that the oxidation of H₂DCFDA was H₂O₂ dependent. The samples were measured with excitation at 485 nm and emission at 530 nm in Fluoroskan Ascent FL (Thermo Electron, Waltham, MA). Oxidation of H₂DCFDA was converted into micromolars of H₂O₂ by using a standard curve prepared from the oxidation of known concentration of H₂O₂ as we published previously [Kumar et al., 2010].

IMMUNOPRECIPITATION TO DETECT PHOSPHO-CATALASE

For each immunoprecipitation, 1,000 µg of lung lysate was incubated with an anti-catalase antibody overnight at 4°C followed by incubation with a protein G Plus/Protein A agarose suspension (Calbiochem) for 1 h at 4°C. The immune complexes were washed three times with lysis buffer and boiled in SDS-PAGE sample buffer for 5 min. Agarose beads were pelleted by centrifugation, and the protein supernatants were loaded and run on 4-20% polyacrylamide gels, followed by transfer of the proteins to nitrocellulose membranes. The membranes were blocked with 2% BSA in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 2 h at room temperature, incubated with anti-phosphoserine antibody (Calbiochem) overnight at 4°C, washed three times with TBST (room temperature, 10 min), and then incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce). The reactive bands were visualized with the SuperSignal West Femto maximum sensitivity substrate kit (Pierce) using a Kodak 440CF image station. The same blot was reprobated with anti-catalase antibody to normalize for the levels of catalase immunoprecipitated in each sample.

LAMB MODEL OF SURGICALLY INDUCED ACUTE INCREASES IN PBF

Fifteen juvenile lambs (4-6 weeks of age) were fasted for 24 h, with free access to water. The lambs were then anesthetized with ketamine hydrochloride (15 mg kg⁻¹ IM). Under additional local anesthesia with 2% lidocaine hydrochloride, polyurethane catheters were placed in an artery and vein of each hind leg. These catheters were advanced to the descending aorta and the inferior vena cava, respectively. The lambs were then anesthetized with ketamine hydrochloride (~0.3 mg kg⁻¹ min⁻¹), diazepam (0.002 mg kg⁻¹ min⁻¹), and fentanyl citrate (1.0 µg kg⁻¹ h⁻¹),

incubated with a 7.0 mm OD cuffed endotracheal tube, and mechanically ventilated with a V.I.P Bird[®] Sterling (Palm Springs, CA) time-cycled, pressure-limited ventilator. Pancuronium bromide (0.1 mg kg⁻¹ dose⁻¹) was given intermittently for muscle relaxation. Utilizing strict aseptic technique, a midsternotomy incision was then performed, and the pericardium was incised. With the use of side biting vascular clamps, an 8.0 mm Gore-tex[®] vascular graft (~2 mm length) (W.L. Gore and Associates, Milpitas, CA), which was occluded by vascular clips, was anastomosed between the ascending aorta and main pulmonary artery with 7.0 proline (Ethicon Inc., Somerville, NJ), using a continuous suture technique. Utilizing a purse-string suture technique, polyurethane catheters were placed directly into the right and left atrium, and main pulmonary artery. An ultrasonic flow probe (Transonics Systems, Ithaca, NY) was placed around the left pulmonary artery to measure PBF. The midsternotomy incision was then temporarily closed with towel clamps. An intravenous infusion of Lactated Ringers and 5% dextrose (75 ml h⁻¹) was begun and continued throughout the study period. Cefazolin (500 mg, IV) and gentamicin (3 mg kg⁻¹, IV) were administered before the first surgical incision. The lambs were maintained normothermic (39°C) with a heating blanket.

EXPERIMENTAL PROTOCOL

After a 30 min recovery, baseline measurements of the hemodynamic variables (pulmonary and systemic arterial pressure, heart rate, left PBF, left and right atrial pressures), and systemic arterial blood gases and pH were measured. A peripheral lung wedge biopsy was obtained for determinations of: H₂O₂ levels, catalase expression and activity, phospho-catalase levels, eNOS phosphorylation at Thr495 and Ser1177, and NO_x levels. A side-biting vascular clamp was utilized to isolate peripheral lung tissue from a randomly selected lobe, and the incision was cauterized. Approximately 300 mg of peripheral lung were obtained for each biopsy.

In control lambs (N = 5), an infusion of normal saline (vehicle) was initiated and continued throughout the study period. In a second group (N = 5), an infusion of tezosentan (a combined ET_A and ET_B receptor antagonist) was initiated at 0.5 mg kg⁻¹ h⁻¹ and continued throughout the study period. The dose of tezosentan was determined from previous work that demonstrates physiologically significant blockade of ET-1-induced vascular alterations [Fitzgerald et al., 2004]. In a third group (N = 5), an intravenous dose of polyethylene glycol (PEG)-catalase (15,000 units kg⁻¹) was administered before the initiation of the tezosentan infusion. Tezosentan (0.5 mg kg⁻¹ h⁻¹) was then administered throughout the study period. The dose of PEG-catalase (15,000 units kg⁻¹) was based on studies that demonstrated significant (nine-fold) sustained increases in plasma catalase activity [Carpenter et al., 2001]. In our initial study describing this model, we showed that a sham procedure (which was identical to the procedure above except that the surgical clips were not removed from the vascular graft) did not produce any appreciable hemodynamic or biochemical alterations [Oishi et al., 2006].

One hour following the initiation of the infusion (vehicle, tezosentan, or tezosentan plus PEG-catalase), the vascular clips were removed from the aortopulmonary graft, opening the shunt. Hemodynamic variables were monitored continuously for 4 h. Four hours following shunt opening, a lung biopsy was obtained for

determinations of: H₂O₂ levels, catalase expression and activity, phospho-catalase levels, eNOS phosphorylation at Thr495 and Ser1177, and NO_x levels.

At the end of the protocol, all lambs were killed with a lethal injection of sodium pentobarbital followed by bilateral thoracotomy as described in the NIH Guidelines for the Care and Use of Laboratory Animals. All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco and Georgia Health Sciences University.

DRUG PREPARATION

Tezosentan (molecular weight 649.6, Actelion Pharmaceuticals Ltd, Allschwil, Switzerland) was diluted in sterile 0.9% saline to a final concentration of 1 mg ml⁻¹ and used immediately. Polyethylene glycol-conjugated catalase (2,000–5,000 units mg⁻¹ protein; Sigma) was diluted in 5 ml sterile 0.9% saline and used immediately.

MEASUREMENTS

Pulmonary and systemic arterial, and right and left atrial pressures were measured using Sorenson Neonatal Transducers (Abbott Critical Care Systems, Chicago, IL). Mean pressures were obtained by electrical integration. Heart rate was measured by a cardiometer triggered from the phasic systemic arterial pressure pulse wave. Left PBF was measured on an ultrasonic flow meter (Transonic Systems, Ithaca, NY). All hemodynamic variables were measured continuously utilizing the Gould Ponemah Physiology Platform (Version 4.2) and Acquisition Interface (Model ACG-16; Gould Inc., Cleveland, OH), and recorded with a Dell Inspiron 5160 computer (Dell Inc., Round Rock, TX). Blood gases and pH were measured on a Radiometer ABL5 pH/blood gas analyzer (Radiometer, Copenhagen, Denmark). Hemoglobin concentration and oxygen saturation were measured by a co-oximeter (model 682, Instrumentation Laboratory, Lexington, MA). Pulmonary vascular resistance (PVR) was calculated using standard formulas. Body temperature was monitored continuously with a rectal temperature probe.

STATISTICAL ANALYSIS

Statistical calculations were performed using the GraphPad Prism V. 4.01 software. The means ± SE was calculated for all samples, and significance was determined by either the unpaired or paired *t*-test or ANOVA. For ANOVA, Newman-Keuls post hoc testing was also utilized. A value of *P* < 0.05 was considered significant.

RESULTS

ET RECEPTOR ANTAGONISM ENHANCES SHEAR-MEDIATED INCREASES IN NO GENERATION IN PAEC

Our previous *in vivo* study indicated that increased PBF combined with tezosentan treatment resulted in an increase in NOS activity [Oishi et al., 2006]. Therefore, our first step was to determine NO_x concentrations in the media of PAEC exposed to acute shear stress (20 dyn cm⁻², 4 h) in the presence and absence of tezosentan. As expected, NO_x levels were significantly increased in response to shear stress (Fig. 1A). Tezosentan potentiated the shear-mediated increase in NO_x (Fig. 1A).

ET RECEPTOR ANTAGONISM INCREASES SHEAR-MEDIATED PHOSPHORYLATION OF eNOS AT SER1177 IN PAEC

In order to investigate potential mechanisms for the ET receptor-mediated increase in NO generation with acute shear stress in PAEC, we performed Western blot analysis to measure phosphorylation of eNOS at Ser1177, a site known to increase eNOS activity. Again PAEC were exposed to acute shear stress (20 dyn cm⁻², 4 h) in the presence and absence of tezosentan. Consistent with our previous studies [Kumar et al., 2010], we found that acute shear stress significantly increased pSer1177 eNOS levels (Fig. 1B). While, in the presence of tezosentan, the increase in pSer1177 eNOS induced by shear stress was potentiated (Fig. 1B).

ET RECEPTOR ANTAGONISM ENHANCES SHEAR-MEDIATED INCREASES IN H₂O₂ GENERATION IN PAEC

Our previous studies have shown that shear stress increases Ser1177 eNOS in PAEC via Akt activation secondary to increased H₂O₂



Fig. 1. ET receptor antagonism enhances shear-mediated increases in NO generation in pulmonary arterial endothelial cells. PAEC were acutely exposed to shear stress (20 dyn cm⁻², 4 h) in the presence or absence of the combined ET receptor antagonist, tezosentan (5 μM). Shear-mediated increase in NO generation was potentiated in the presence of tezosentan (A). The increase in NO correlated with an increase in the ratio of pSer1177 eNOS versus total eNOS (B) in both shear and shear with tezosentan. The increases in NO generation are attenuated by PEG-catalase (100 U ml⁻¹; A). Data are mean ± SEM; n = 4–6. **P* < 0.05 vs. no shear, †*P* < 0.05 vs. shear alone, ‡*P* < 0.05 vs. shear and tezosentan.

generation [Kumar et al., 2010]. Therefore, we next measured cellular H_2O_2 levels, as estimated by H_2DCFDA oxidation, in PAEC exposed to acute shear stress (20 dyn cm^{-2} , 4 h) in the presence and absence of tezosentan. Consistent with our previous studies, our results indicated that cellular H_2O_2 levels were significantly increased in response to shear (Fig. 2A). While in the presence of tezosentan, the cellular H_2O_2 levels induced by shear stress was potentiated (Fig. 2A). The addition of catalase decreased the signal under both conditions, indicating specificity of the assay for H_2O_2 (Fig. 2A). Confirming the important role of H_2O_2 , the addition of catalase attenuated the shear and tezosentan mediated increases in NO_x (Fig. 1A).

ET RECEPTOR ANTAGONISM POTENTIATES A SHEAR-MEDIATED DECREASE IN CATALASE ACTIVITY IN PAEC

We have previously shown that shear stress decreases catalase activity in PAEC [Kumar et al., 2010]. Therefore, we determined catalase expression and activity in PAEC exposed to acute shear stress (20 dyn cm^{-2} , 4 h) in the presence and absence of tezosentan. Consistent with our previous studies, our results indicated that catalase protein levels did not change in response to shear (Fig. 2B), but catalase activity was significantly decreased (Fig. 2C). In the presence of tezosentan, there was a further significant reduction in catalase activity in PAEC exposed to shear stress (Fig. 2C). Tezosentan had no affect on catalase protein levels (Fig. 2B).

ET-1 DECREASES NO SIGNALING IN PAEC BY INCREASING CATALASE ACTIVITY

As we have previously shown that ET-1 decreases in NOS activity in our lamb model of acutely increased PBF [Oishi et al., 2006] we next determined if this was due to modulation of pSer1177 or pThr495 eNOS levels and the role of ET_B receptor signaling. Our data indicate that ET-1 significantly decreased H_2O_2 levels in PAEC (Fig. 3A). This decrease in H_2O_2 correlated with a significant increase in catalase activity (Fig. 3B). The decrease in H_2O_2 correlated with a significant decrease in p-Ser1177 eNOS (Fig. 3C) and a significant increase in pThr495 eNOS (Fig. 3D). Overall these changes resulted in a significant decrease in NO_x levels (Fig. 3E). The addition of the ET_B -receptor antagonist, Res701-3 blocked all these changes (Fig. 3A–D). To further confirm the role of ET_B receptor activation in the reduction in NO signaling we exposed PAEC to the ET_B receptor-specific agonist, 4-ala ET-1. We found that 4-ala ET-1 significantly decreased both H_2O_2 levels (Fig. 3F) and catalase activity (untreated, Fig. 3G) without altering catalase protein levels (Fig. 3H). 4-ala ET-1 also significantly decreased NO_x levels (Fig. 3I).

ET-1 ATTENUATES THE SHEAR-MEDIATED INCREASE IN H_2O_2 AND NO GENERATION IN PAEC BY INCREASING CATALASE ACTIVITY

In our lamb model of acute increases in PBF, there are both increased shear stress and ET-1 levels [Oishi et al., 2006]. Thus, we next determined the affect on H_2O_2 and NO signaling if we exposed PAEC to shear stress in the presence of ET-1. Our data indicate that ET-1

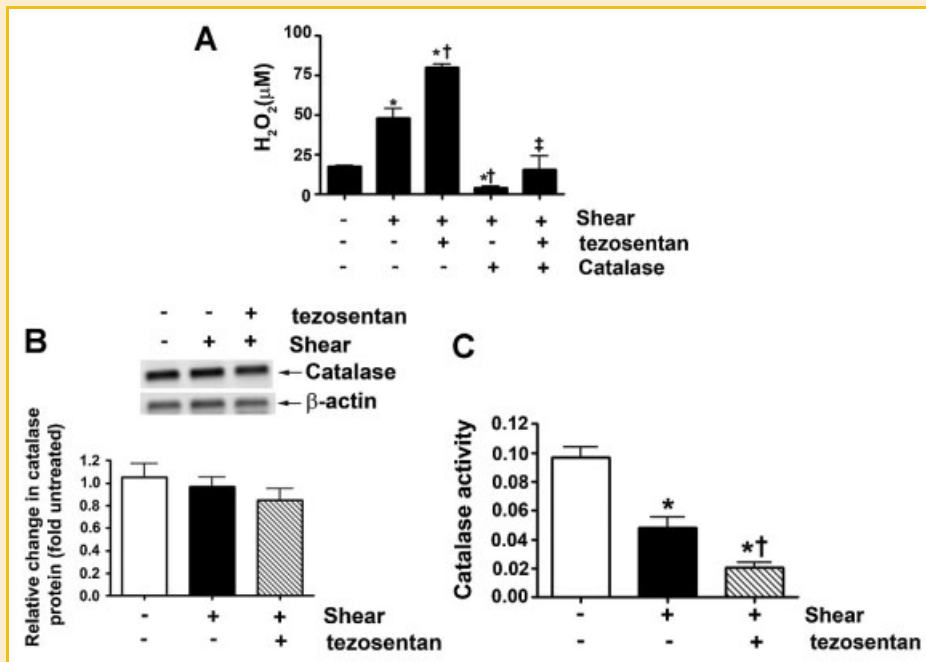


Fig. 2. ET receptor antagonism potentiates shear-mediated increase in H_2O_2 via a decrease in catalase activity. PAEC were acutely exposed to shear stress (20 dyn cm^{-2} , 4 h) in the presence or absence of the combined ET receptor antagonist, tezosentan ($5 \mu\text{M}$). The levels of H_2O_2 were determined in the media by H_2DCFDA oxidation reaction and whole cell extracts ($20 \mu\text{g}$) were subjected to Western blot analysis in order to determine changes in catalase protein levels. Catalase activity (H_2O_2 degraded/min/mg protein) was also determined. The shear-mediated increase in H_2O_2 was enhanced by tezosentan (A) and attenuated by PEG-catalase (100 U ml^{-1} , A). Neither shear nor tezosentan changed catalase protein levels (B). However, acute shear stress decreased catalase activity, which was further attenuated by tezosentan (C). Data are mean \pm SEM; $n = 3-6$. * $P < 0.05$ vs. no shear, $^\dagger P < 0.05$ vs. shear alone, $^\ddagger P < 0.05$ vs. shear and tezosentan.

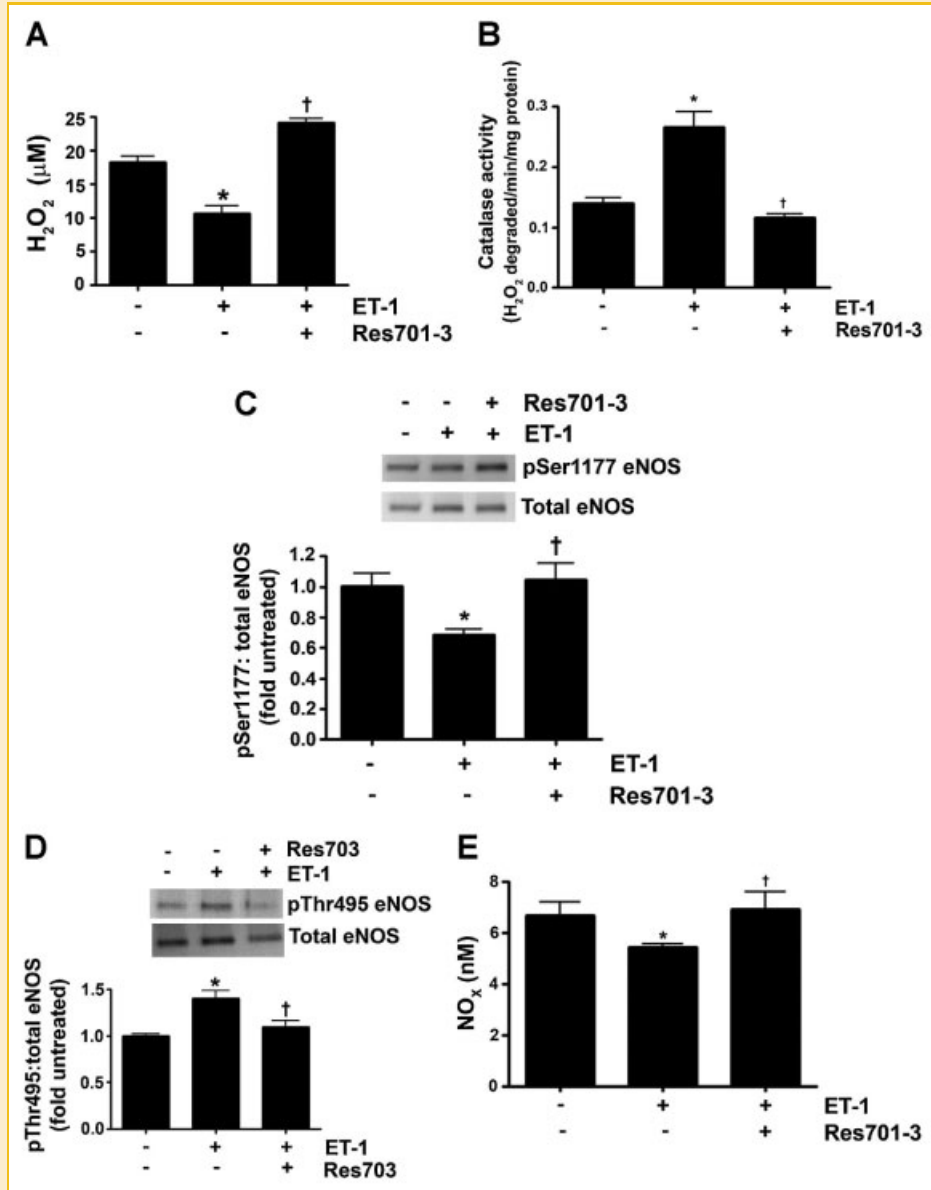


Fig. 3. ET-1 treatment decreases H₂O₂ levels and NO generation via ET_B receptor signaling. PAEC were exposed to ET-1 (100 nM, 4 h) in the presence or absence of the ET_B receptor antagonist Res701-3 (10 μM). The media was used for the measurement of H₂O₂ and NO levels. The cell lysate was then used for determination of the pSer1177- and pThr495-eNOS, total eNOS, catalase activity and catalase protein levels. ET-1 decreased H₂O₂ levels (A), and increased catalase activity (B, H₂O₂ degraded/min/mg protein). ET-1 also decreased pSer1177 eNOS levels (C) but increased the level of pThr495 eNOS (D) as determined by Western blot analysis. NO generation was also decreased with ET-1 treatment (E). The ET_B receptor antagonist, Res701-3 blocked all of these effects (A–E). PAEC were also treated with the ET_B receptor agonist, 4-ala ET-1 (100 nM, 4 h). H₂O₂ levels decreased with 4-ala ET-1 treatment (F) while catalase activity (H₂O₂ degraded/min/mg protein) increased (G). Catalase protein levels did not change (H). NO levels were also decreased by 4-ala ET-1 (I). Data are mean ± SEM; n = 4–6. **P* < 0.05 vs. untreated, †*P* < 0.05 vs. ET-1 alone.

significantly reduced both the shear-mediated increases in NO_x (Fig. 4A) and H₂O₂ (Fig. 4B) indicating that ET-1 is dominant over shear stress with respect to NO and H₂O₂ generation.

IN LAMBS, EXPOSURE TO ACUTE INCREASES IN PBF DECREASES LUNG TISSUE H₂O₂ LEVELS, WHICH IS REVERSED BY ET RECEPTOR ANTAGONISM

H₂O₂ levels, estimated by H₂DCFDA oxidation, were determined in peripheral lung biopsies taken before (pre) and 4 h after (post) shunt opening in lambs in the absence (vehicle-treated) and presence of

tezosentan. In vehicle-treated lambs, lung tissue H₂O₂ levels were significantly decreased after 4 h of increased PBF (Fig. 5A). However, in tezosentan-treated lambs, lung tissue H₂O₂ levels were significantly increased (Fig. 5A).

IN LAMBS, EXPOSURE TO ACUTE ELEVATIONS IN PBF INCREASES LUNG TISSUE CATALASE ACTIVITY, INDEPENDENT OF PROTEIN EXPRESSION, WHICH IS REVERSED BY ET RECEPTOR ANTAGONISM

In order to further explore potential mechanisms for these in vivo differences in H₂O₂ levels, we measured catalase

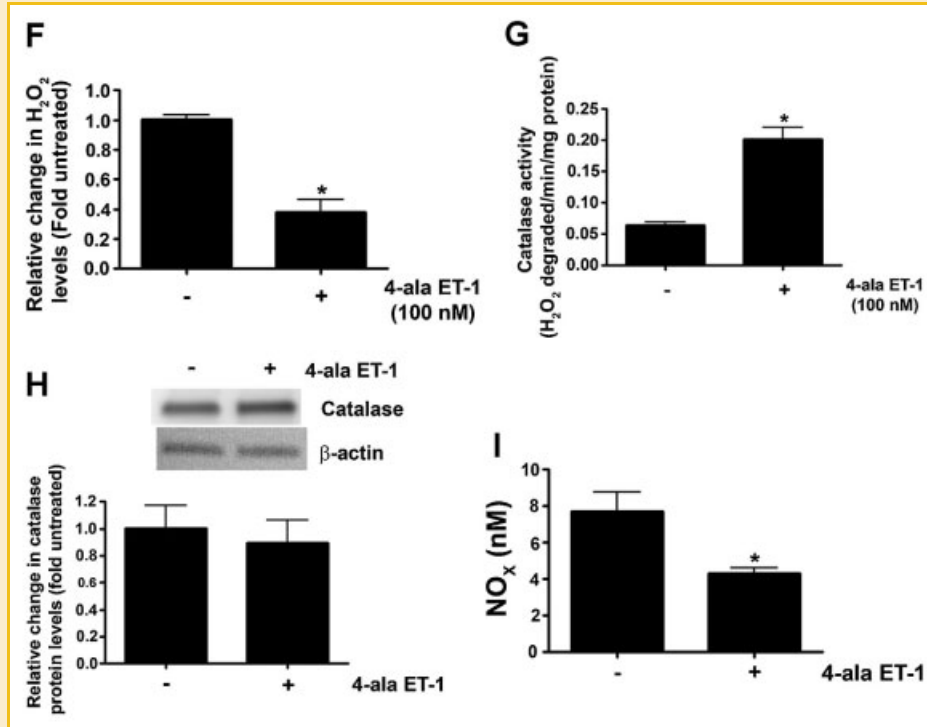


Fig. 3. (Continued)

protein levels and activity in peripheral lung biopsies taken before and 4 h after shunt opening in lambs treated or not with tezosentan. We did not find differences in catalase protein levels post shunt opening in either group (Fig. 5B). However, in vehicle-treated lambs, lung catalase activity was significantly increased 4 h after shunt opening (Fig. 5C). In contrast, in tezosentan-treated lambs, lung tissue catalase activity was significantly decreased 4 h after shunt opening (Fig. 5C). These data are consistent with altered catalase activity contributing to the in vivo differences in H₂O₂ levels with and without tezosentan.

IN LAMBS, EXPOSURE TO ACUTE ELEVATIONS IN PBF INCREASES LUNG TISSUE p-Ser CATALASE SERINE LEVELS

Our previous in vitro studies indicated that shear stress induced decreases in catalase activity were associated with a decrease in catalase serine phosphorylation [Kumar et al., 2010]. Therefore, in order to determine whether a similar mechanism occurs in vivo, we measured pSer-catalase levels in peripheral lung biopsies taken before and 4 h after shunt opening. We found that lung tissue pSer-catalase levels were significantly increased 4 h after shunt opening (Fig. 5D) while in the presence of tezosentan, pSer-catalase levels were significantly decreased (Fig. 5D).

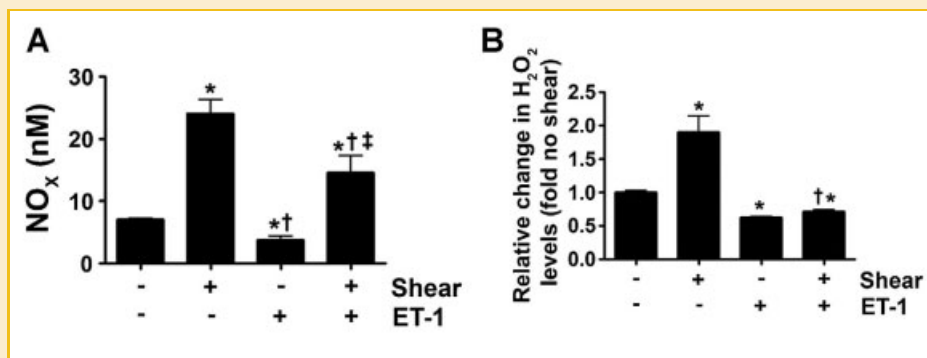


Fig. 4. ET-1 attenuates shear-mediated increase in NO generation and H₂O₂ levels in pulmonary arterial endothelial cells (PAEC). PAEC were acutely exposed to shear stress (20 dyn cm⁻², 4 h) in the presence or absence of ET-1 (100 nM, 4 h) and the media used for the detection of H₂O₂ and NO_x. The shear-mediated increase in NO_x (A) and H₂O₂ (B) was attenuated in the presence of ET-1. Data are mean ± SEM; n = 6–12. *P < 0.05 vs. no shear, †P < 0.05 vs. shear alone, ‡P < 0.05 vs. ET-1 alone.

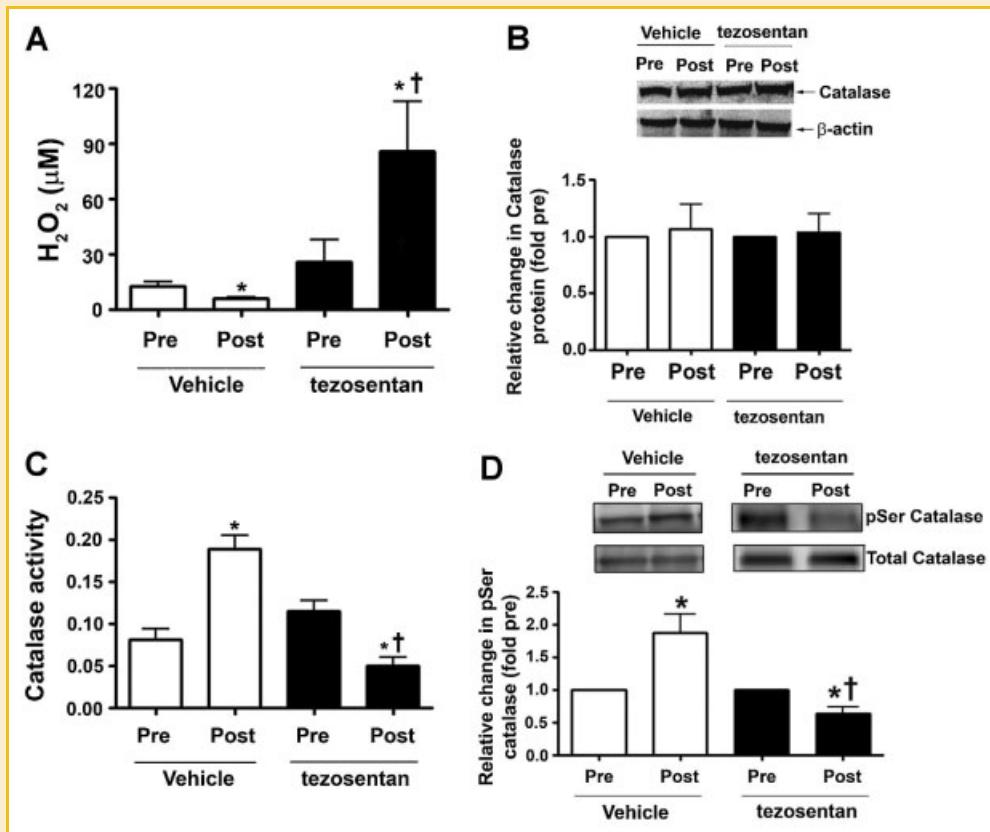


Fig. 5. ET receptor antagonism increases lung tissue H_2O_2 levels, in association with attenuated catalase activity, in lambs acutely exposed to increased PBF. Lung biopsies were taken before (Pre) and 4 h after (Post) the increase in PBF. In vehicle-treated lambs, H_2O_2 levels, as estimated by H_2DCFDA oxidation, decreased after 4 h of increased PBF; whereas, in tezoesentan-treated lambs, H_2O_2 levels increased (A). Western blot analysis performed on lysates (20 μ g) prepared from Pre and Post lung biopsies, revealed no change in catalase protein after 4 h of increased PBF, in either vehicle- or tezoesentan-treated lambs (B). However, in vehicle-treated lambs, lung tissue catalase activity was enhanced after 4 h of PBF; whereas, in tezoesentan-treated lambs catalase activity decreased (C). Lung lysate (1 mg) were also subjected to immunoprecipitation (IP), using an antibody specific to catalase followed by Western blot (IB) analysis using a specific antiserum raised against phosphorylated-serine residues. In vehicle-treated lambs, the increase in catalase activity was associated with an increase in phospho-serine catalase (pSer-catalase), whereas, in tezoesentan-treated lambs pSer-catalase levels were decreased (D). Data are mean \pm SEM; $n = 5-7$. * $P < 0.05$ vs. pre shunt opening, $^{\dagger}P < 0.05$ vs. vehicle-treated post.

IN LAMBS, EXPOSURE TO ACUTE ELEVATIONS IN PBF MODULATES eNOS PHOSPHORYLATION AND DECREASES BIOAVAILABLE NO

We measured eNOS phosphorylation at Ser1177 and Thr495 in peripheral lung biopsies taken before and 4 h after shunt opening in lambs treated or not with tezoesentan. In vehicle-treated lambs, pSer1177 eNOS was significantly decreased (Fig. 6A) while pThr495 eNOS levels significantly increased (Fig. 6B) 4 h after shunt opening. However, in tezoesentan-treated lambs, pSer1177 eNOS was significantly increased (Fig. 6A) while pThr495 eNOS significantly decreased (Fig. 6B) 4 h after shunt opening. Further, NO_x levels significantly decreased 4 h after shunt opening in vehicle-treated lambs but significantly increased in tezoesentan-treated lambs (Fig. 6C). The changes were not due to changes in ET receptor density, as ET_A - or ET_B -receptor protein levels were not altered (Fig. 6D-G).

PEG-CATALASE ATTENUATES THE EFFECTS OF ET RECEPTOR ANTAGONISM IN LAMBS EXPOSED TO INCREASED PBF

Our results indicated that alterations in H_2O_2 were central to the augmentation of bioavailable NO in tezoesentan-treated lambs

exposed to increased PBF. In order to further examine this association, PEG-catalase was administered to an additional group of tezoesentan-treated lambs exposed to increased PBF. In contrast to lambs treated with tezoesentan-alone, we found no differences in lung tissue H_2O_2 levels, as estimated by H_2DCFDA oxidation (Fig. 7A), pSer1177 eNOS (Fig. 7B), or NO_x levels (Fig. 7D) in lambs treated with PEG-catalase in combination with tezoesentan during 4 h of increased PBF. However, pThr495 eNOS levels were significantly increased (Fig. 7C).

THE CO-ADMINISTRATION OF PEG-CATALASE AND TEZOESANTAN RESTORES COMPENSATORY PULMONARY VASCULAR CONSTRICTION

In order to investigate the physiologic relevance of these biochemical alterations in vivo, we studied the hemodynamic effects of the co-administration of PEG-catalase and tezoesentan, compared with tezoesentan alone or the vehicle control in our lamb model of surgically induced acutely increased PBF [Oishi et al., 2006]. As expected, and consistent with our previous study [Oishi et al., 2006], in vehicle-treated lambs, opening of the aortopul-

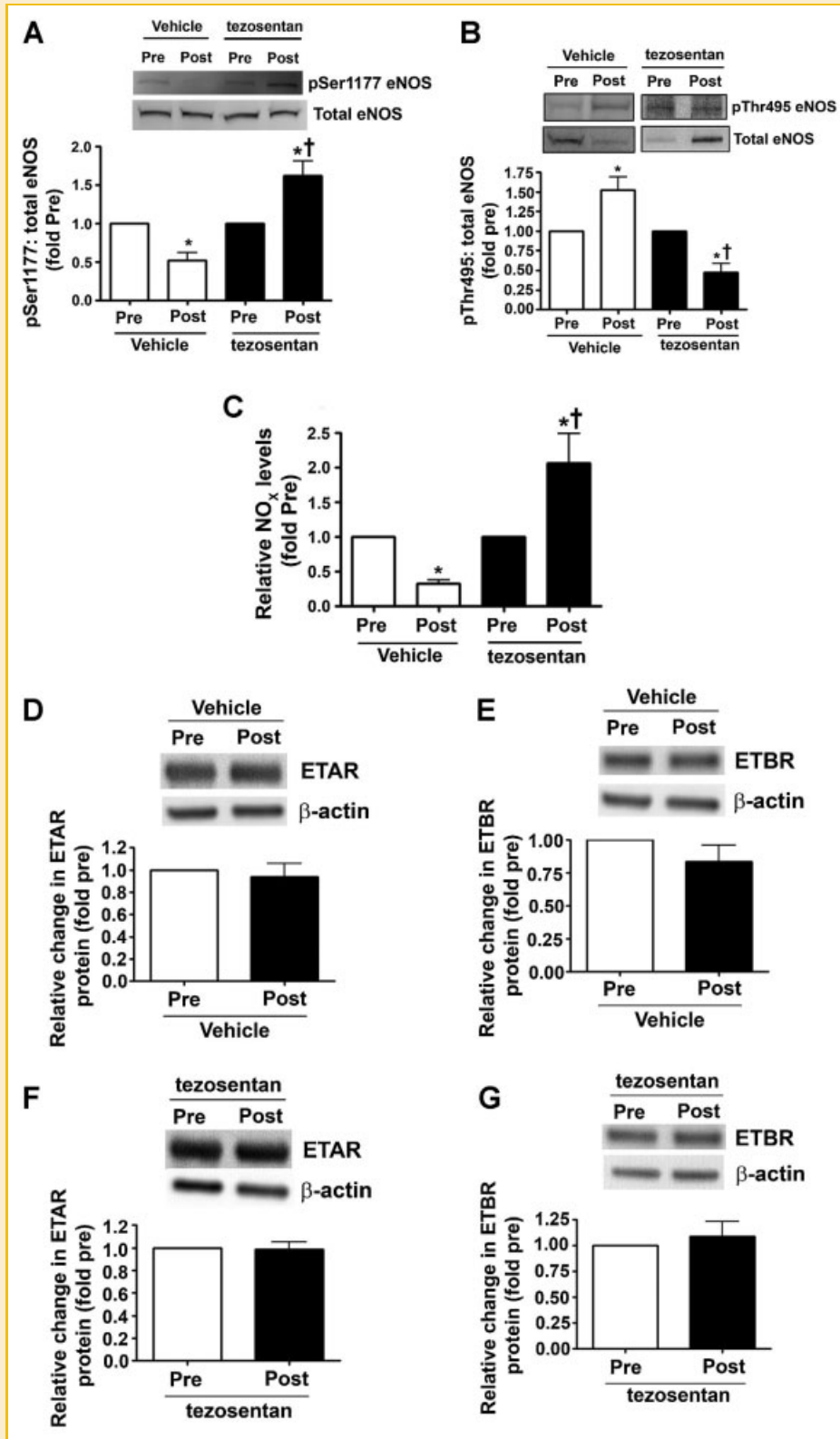


Fig. 6. ET receptor antagonism increases p-Ser1177 eNOS levels and NO in lambs acutely exposed to increased PBF. PBF was acutely increased in 4-week-old lambs in the absence (vehicle-treated) or presence of the ET receptor antagonist tezosentan ($0.5 \text{ mg kg}^{-1} \text{ h}^{-1}$). Lung biopsies were taken before (Pre) and 4 h after (Post), the increase in PBF. Lysates ($40 \mu\text{g}$) prepared from these samples were subjected to Western blot analysis and probed with anti-pSer1177- or anti-pThr495-eNOS antibodies. For normalization, duplicate samples were run and blotted, then probed with an antibody for total eNOS. In vehicle-treated lambs, pSer1177 eNOS levels decreased after 4 h of increased PBF; whereas, in tezosentan-treated lambs pSer1177 eNOS levels increased (A). In vehicle-treated lambs, pThr495 eNOS levels increased after 4 h of increased PBF; whereas, in tezosentan-treated lambs pThr495 eNOS levels decreased (B). The changes in phosphorylation corresponded with a reduction in NO_x levels after 4 h of increased PBF in vehicle-treated lambs, but an increase in NO_x levels in tezosentan-treated lambs (C). No changes in either ET_A or ET_B receptor protein levels were observed (D–G). Data are mean \pm SEM; $n = 4$ –5. * $P < 0.05$ vs. pre shunt opening, † $P < 0.05$ vs. vehicle-treated post.

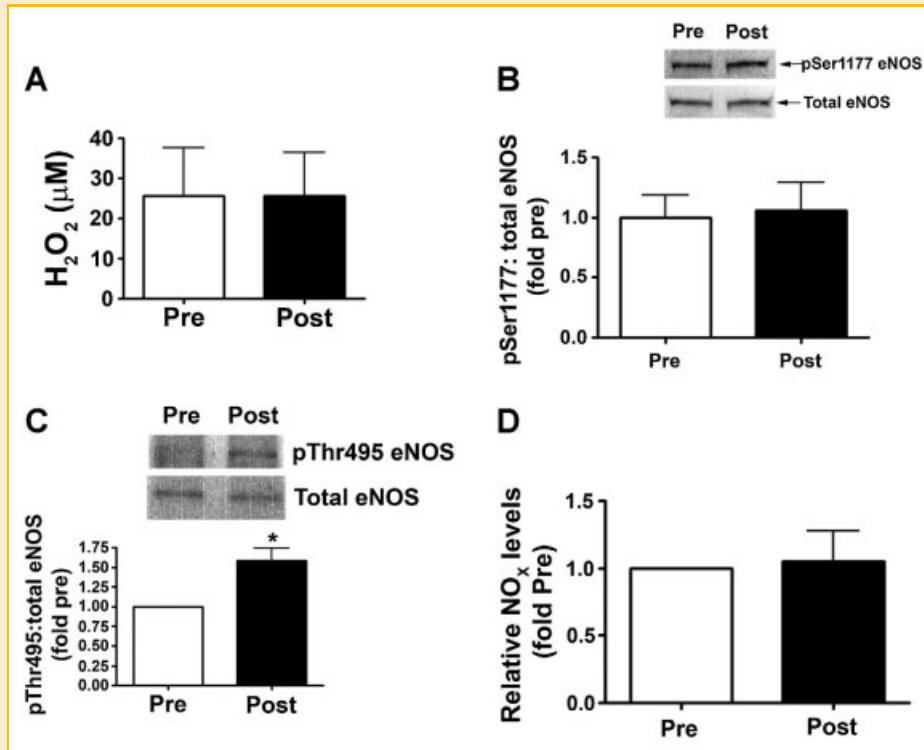


Fig. 7. PEG-conjugated catalase attenuates the beneficial effect of ET receptor antagonism on NO signaling in lambs acutely exposed to increased PBF. PBF was acutely increased in 4-week-old lambs. Lambs were treated with the ET receptor antagonist tezosentan ($0.5 \text{ mg kg}^{-1} \text{ h}^{-1}$) in combination with PEG-catalase ($15,000 \text{ units kg}^{-1}$). Lung biopsies were taken before (Pre) and 4 h after (Post), the increase in PBF. In contrast to the findings in tezosentan-treated lambs, lambs treated with tezosentan in combination with PEG-catalase, did not demonstrate increases in lung tissue H_2O_2 levels, as estimated by H_2DCFDA oxidation, after 4 h of increased PBF (A). Lysates ($40 \mu\text{g}$) prepared from Pre and Post lung biopsies were subjected to Western blot analysis and probed with anti-pSer1177- or anti-pThr495-eNOS antibodies. For normalization, duplicate samples were run and blotted, then probed with an antibody for total eNOS. In contrast to the findings in tezosentan-treated lambs, lambs treated with tezosentan in combination with PEG-catalase, did not demonstrate increases in pSer1177 eNOS levels after 4 h of increased PBF (B) while pThr495 levels were increased (C). Lung tissue NO_x levels did not increase in lambs treated with tezosentan in combination with PEG-catalase (D). Data are mean \pm SEM; $n = 4$. * $P < 0.05$ vs. pre shunt opening.

monary vascular graft resulted in a rapid and significant increase in PBF (from 38.6 ± 15.4 to $84.7 \pm 20.0 \text{ ml min}^{-1} \text{ kg}^{-1}$ at 30 min) and a significant decrease in left PVR (LPVR; from 0.29 ± 0.16 to $0.15 \pm 0.05 \text{ mmHg ml}^{-1} \text{ min}^{-1} \text{ kg}^{-1}$ at 30 min), which did not change further over the subsequent 4-h study period (Fig. 8A and B). Also consistent with our previous study, in tezosentan-treated lambs, opening of the vascular graft resulted in a rapid and significant increase in PBF (from 41.33 ± 14.24 to $82.72.4 \pm 25.41 \text{ ml min}^{-1} \text{ kg}^{-1}$ at 30 min) and a significant decrease in LPVR (from 0.31 ± 0.15 to $0.18 \pm 0.08 \text{ mmHg ml}^{-1} \text{ min}^{-1} \text{ kg}$ at 30 min). However, unlike vehicle-treated lambs, PBF and LPVR did not reach a plateau but rather continued to change from 30 min to 4 h after shunt opening (Fig. 8A and B). Furthermore, by 150 min PBF was significantly higher than in vehicle-treated lambs; likewise, by 180 min LPVR was significantly lower than in vehicle-treated lambs (Fig. 8A and B). Thus, our results indicated that acute increases in PBF were followed by compensatory pulmonary vascular constriction that limited progressive increases in PBF and decreases in PVR in vehicle-treated lambs and that were attenuated by ET receptor antagonism.

In lambs treated with PEG-catalase in combination with tezosentan ($0.5 \text{ mg kg}^{-1} \text{ h}^{-1}$), opening of the aortopulmonary vascular graft also resulted in a rapid and significant increase in

PBF (from 49.5 ± 17.7 to $80.6 \pm 27.6 \text{ ml min}^{-1} \text{ kg}^{-1}$ at 30 min). Interestingly, however, LPVR did not decrease immediately (LPVR; 0.26 ± 0.10 to $0.27 \pm 0.17 \text{ mmHg ml}^{-1} \text{ min}^{-1} \text{ kg}$ at 30 min), but did significantly decrease by 60 min after shunt opening (LPVR; $0.19 \pm 0.04 \text{ mmHg ml}^{-1} \text{ min}^{-1} \text{ kg}$). Like vehicle-treated, and in contrast to tezosentan alone, PBF and LPVR did not change further over the 4-h study period in lambs treated with PEG-catalase in combination with tezosentan (Fig. 8A and B).

DISCUSSION

The results of this study reveal novel interactions between ET receptor activation, H_2O_2 signaling, and NO production under conditions of increased PBF. In our original study establishing the model of acute surgically induced increased PBF, we found that PBF was limited by compensatory pulmonary vascular constriction mediated, in part, by increased ET-1 levels and ET receptor activation that decreased NOS activity [Oishi et al., 2006]. This study elucidates the mechanisms for these interactions. Specifically, our data indicate that increased PBF, via increased ET receptor activation, increases catalase activity, due to increased catalase phosphorylation, which in turn decreases H_2O_2 levels.

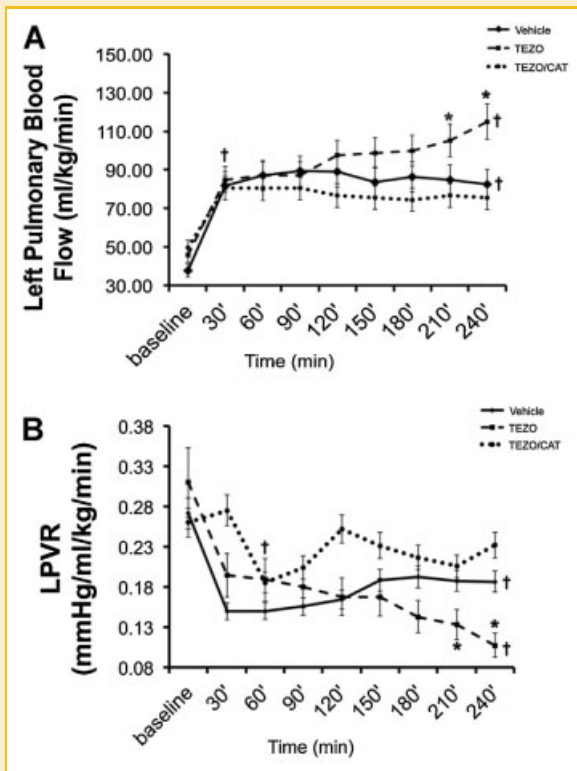


Fig. 8. The co-administration of PEG-catalase and tezosentan restores compensatory pulmonary vascular constriction that is impaired by tezosentan alone, in lambs with acutely increased PBF. Left PBF and left PVR (LPVR) following aortopulmonary shunt opening in vehicle, tezosentan ($0.5 \text{ mg kg}^{-1} \text{ h}^{-1}$, TEZO), and tezosentan ($0.5 \text{ mg kg}^{-1} \text{ h}^{-1}$) combined with PEG-catalase-treated lambs ($15,000 \text{ units kg}^{-1}$; TEZO/CAT). (A) PBF increases sharply by 30 min following aortopulmonary shunt opening in all groups. PBF continues to increase in the TEZO group (dashed line), but not in the vehicle (solid line) or TEZO/CAT (dotted line) groups over the 4-h study period; PBF is higher in the TEZO group than the other groups at 210 and 240 min. PBF remains above baseline in all groups at 240 min. B: LPVR decreases sharply by 30 min following aortopulmonary shunt opening in the vehicle (solid line) and TEZO (dashed line) groups. In the vehicle group, LPVR does not change further over the 4-h study period. In the TEZO/CAT (dotted line) group, LPVR decreases to levels similar to the other groups by 60 min. Furthermore, in the TEZO/CAT group LPVR returns to levels similar to baseline by 120 min without further changes over the 4-h study period. In the TEZO group, LPVR continues to decrease following aortopulmonary shunt opening over the 4-h study period, such that LPVR is lower than in the vehicle and TEZO/CAT groups at 210 and 240 min. Data are mean \pm SD. Vehicle $N = 8$, TEZO $N = 6$, TEZO/CAT $N = 5$, $^*P < 0.05$ vs. vehicle and TEZO/CAT, $^{\dagger}P < 0.05$ vs. baseline.

Decreased H_2O_2 signaling is associated with a decrease in eNOS activation and decreased NO production. A central role for ET receptor activation is demonstrated by the reversal of this sequence with ET receptor antagonism. Furthermore, a role for H_2O_2 signaling is demonstrated by the attenuation of the effects of ET receptor antagonism with catalase supplementation.

It is well established that laminar shear stress is an important stimulus for NO production by endothelial cells. However, the controlling mechanisms are complex and incompletely understood. A number of studies have shown that eNOS activity is regulated in a reciprocal fashion by phosphorylation at Thr495 and Ser1177, with

phosphorylation of Thr495 inhibiting [Fleming et al., 2001] and Ser1177 stimulating [Dimmeler et al., 1999] the enzyme. Thus, in a situation where ET-1 levels are high, pThr495 will predominate and NO generation in the lung will be attenuated, vasoconstriction will occur and this will result in an increase in PVR. Conversely, when pSer1177 levels are high NO signaling will be enhanced, which will result in pulmonary vasodilation and a reduction in PVR. Acutely, the overall balance between pThr495 and pSer1177 will determine how much NO is generated in the lung and thus whether the pulmonary vessels are maintained in a relaxed or constricted state. Further, if this phosphorylation imbalance is maintained, and as NO is an inhibitor of SMC proliferation [Mooradian et al., 1995], this would lead to the development of endothelial dysfunction, vascular remodeling and ultimately, the development of pulmonary hypertension. Indeed in our previous studies in the lamb, in which there has been chronic attenuation of NO signaling over several days to weeks, we have observed both endothelial dysfunction and vascular remodeling [Fratz et al., 2011]. The signaling pathways leading to the phosphorylation of eNOS at Th495 and S117 have been partially elucidated, indicating that phosphoinositide-3-kinase [Thomas et al., 2002], protein kinase C (PKC) [Michell et al., 2001], protein kinase B (Akt) [Fulton et al., 1999], and H_2O_2 [Kumar et al., 2010] play important roles. Here, we show here that the addition of tezosentan, a combined ET_A/ET_B receptor antagonist, enhanced NO signaling through increases in H_2O_2 and phosphorylation of eNOS at Ser1177. The increases in H_2O_2 were mediated by decreases in catalase activity. Conversely, in PAEC exposed to ET-1 alone or in combination with shear stress, catalase activity was increased, H_2O_2 levels were decreased and pSer1177 eNOS and NO_x levels were attenuated while pThr495 eNOS levels were enhanced. Thus, it appears that in PAEC, ET-1 signaling is dominant and tempers shear-induced NO production. This is particularly interesting given our earlier findings in which lambs with increased PBF had increased levels of ET-1 and decreased eNOS activity, and our more recent findings in PAEC showing that over expression of preproET-1 cDNA decreased eNOS expression, at least in part, due to increased PKC δ signaling [Sud and Black, 2009]. We have also shown that over expression of PKC δ increases catalase activity and attenuates the shear-mediated increases in H_2O_2 and pSer1177 eNOS levels [Kumar et al., 2010]. The present in vivo findings are in agreement with these in vitro data. We found that after 4 h of increased PBF in lambs, catalase activity increased, in association with catalase phosphorylation, and H_2O_2 levels decreased. However, increased PBF in combination with ET receptor antagonism had the opposite effect on catalase activity and H_2O_2 levels.

At the same time, it is important to recognize differences between the in vitro and in vivo situations. In PAEC, we demonstrated shear-mediated increases in NO_x production that were enhanced by ET receptor antagonism; whereas, in lambs increased PBF decreased NO_x production, which was reversed by ET receptor antagonism. Although PAEC produce ET-1, a 21 amino acid polypeptide, the effects of ET-1 are mediated by at least two receptor populations, ET_A and ET_B . The ET_A receptors are located on smooth muscle cells and mediate vasoconstriction, whereas the ET_B receptors are located on endothelial cells and mediate vasodilation [Arai et al., 1990; Sakurai et al., 1990; Wong et al., 1995]. In

addition, a second subpopulation of ET_B receptors is located on smooth muscle cells and mediates vasoconstriction [Shetty et al., 1993]. Therefore, in PAEC, the effects of tezosentan are likely attributable exclusively to ET_B receptor antagonism, whereas in lambs the effects of both ET_A and ET_B receptor antagonism are likely manifest. This is an important point as there are differential effects of ET-1 on H₂O₂ production in PAEC and pulmonary arterial smooth muscle cells (PASMC) [Wedgwood and Black, 2005]. ET-1 increases H₂O₂ production, in an ET_A receptor dependent manner, in PASMC monocultures, and fetal PAEC/PASMC co-cultures. However, in PAEC monoculture, ET-1 decreases H₂O₂ levels, in an ET_B receptor dependent manner [Wedgwood and Black, 2005]. In this study, we extended these findings to show that ET-1 decreases stimulatory eNOS phosphorylation at pSer1177 and increases the phosphorylation of the inhibitory site at Thr495. Together these changes in phosphorylation lead to a decrease in NO_x levels in PAEC. These changes correlate with an increase in catalase activity. Thus, the findings of this study add to a growing body of literature that indicates that reactive oxygen species in general, and H₂O₂ in particular, are important in the regulation of signaling within the pulmonary vasculature. The results of the present study indicate that ET-dependent alterations in catalase activity play an important role in modulating H₂O₂ levels in lambs exposed to increased PBF, presumably due to alterations in its clearance. However, it is worth noting that we did not determine if there were alterations in H₂O₂ production in our models. The dismutation of superoxide, by superoxide dismutase, is the primary source of H₂O₂ within the vasculature. Therefore, a decrease in superoxide production might also lead to decreased H₂O₂ levels under conditions of increased PBF. However, our previous studies are not consistent with this mechanism, as we have shown increased superoxide production secondary to acutely increased PBF in a fetal lamb model of constriction of the ductus arteriosus [Hsu et al., 2010] as well as increases in superoxide in lambs with chronically increased PBF [Oishi et al., 2008].

Opening of the aortopulmonary shunt resulted in a significant increase in PBF, more than doubling in each group. This increase in PBF must be associated with an increase in shear stress, but it is important to recognize that shear stress was not measured directly in the lambs and that levels of shear stress likely differ with location within the pulmonary vascular bed. Nevertheless, our model is powerful in that, unlike physiologic increases in PBF such as those that occur with exercise, we created a pathologic situation in which the pulmonary to systemic blood flow ratio is greater than one. The palliation of several congenital cardiac defects that is often complicated by dynamic elevations in PVR is associated with a similar physiology. In addition, the pathophysiology of various disease processes that can lead to pulmonary vascular dysfunction likely involves pathologic increases in PBF. For example, congenital diaphragmatic hernia, a known etiology of persistent pulmonary hypertension of the newborn, is often associated with ipsilateral pulmonary hypoplasia, which results in abnormally increased PBF to the contra-lateral lung [Moreno-Alvarez et al., 2008]. Pneumectomy and chronic thromboembolic pulmonary hypertension with asymmetric lesions would also increase PBF to portions of the pulmonary circulation [Mercier et al., 2009]. Furthermore, in

patients with all types of pulmonary arterial hypertension, disease progression often results in obstructive pulmonary arteriopathy, which can lead to regional increases in PBF and shear stress in other less affected portions of the lung [Galie et al., 2008]. Therefore, the novel signaling interactions demonstrated in the present study may have implications for a number of disease processes.

In summary, we found that the compensatory pulmonary vascular constriction that limits PBF in lambs after the placement of an aortopulmonary shunt is mediated, at least in part, by ET receptor activation, increased catalase phosphorylation and activity, decreased H₂O₂ levels, modulation of eNOS phosphorylation, and reduced NO production. ET receptor antagonism during increased PBF reverses this signaling, preserves NO generation, and prevents the compensatory increase in PVR.

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