Published in final edited form as:

Free Radic Biol Med. 2014 February ; 67: 255–264. doi:10.1016/j.freeradbiomed.2013.10.814.

Endothelin-1 stimulates catalase activity through the PKC δ mediated phosphorylation of Serine 167

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Abstract

Our previous studies have shown that endothelin-1 (ET-1) stimulates catalase activity in endothelial cells and lambs with acute increases in pulmonary blood flow (PBF), without altering gene expression. The purpose of this study was to investigate the molecular mechanism by which this occurs. Exposing pulmonary arterial endothelial cells (PAEC) to ET-1 increased catalase activity and decreased cellular hydrogen peroxide (H₂O₂) levels. These changes correlated with an increase in serine phosphorylated catalase. Using the inhibitory peptide δ V1.1, this phosphorylation was shown to be PKC δ dependent. Mass spectrometry identified serine167 as the phosphorylation site. Site-directed mutagenesis was used to generate a phospho-mimic (S167D) catalase. Activity assays using recombinant protein purified from *E.coli* or transiently transfected COS-7 cells, demonstrated that S167D-catalase had an increased ability to degrade H₂O₂ compared to the wildtype enzyme. Using a phospho-specific antibody, we were able to verify that pS167 catalase levels are modulated in lambs with acute increases in PBF in the presence and absence of the ET receptor antagonist, tezosentan. S167 is being located on the dimeric interface suggesting it could be involved in regulating the formation of catalase tetramers. To evaluate this possibility we utilized analytical gel-filtration to examine the multimeric structure of recombinant wildtype- and S167D-catalase. We found that recombinant wildtype catalase was present as a mixture of monomers and dimers while S167D catalase was primarily tetrameric. Further, the incubation of wildtype catalase with PKC δ was sufficient to convert wildtype catalase into a tetrameric structure. In conclusion, this is the first report indicating that the phosphorylation of catalase regulates its multimeric structure and activity.

Keywords

post-translational regulation; phosphorylation; catalase

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INTRODUCTION

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 [1]. 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 [2]. In this model, the acute increase in PBF was followed by compensatory pulmonary vascular constriction that was, in part, mediated by an increase in endothelin-1 (ET-1) [2]. In our recent studies we have shown that shear stress and ET-1 have opposing effects on NO signaling and that this is mediated through the modulation of hydrogen peroxide (H₂O₂) and the phosphorylation of eNOS at S1177 [3]. In pulmonary arterial endothelial cells (PAEC), ET-1 decreases-, while acute shear stress enhances-, the levels of H₂O₂ and pS1177-eNOS [3]. Further, the alterations in H₂O₂ and pS1177-eNOS correlate with changes in both catalase serine phosphorylation and activity rather than changes in catalase expression [3, 4]. However, little is known regarding the post-translational regulation of catalase and the available data suggest that the identified modifications result in the attenuation of catalase activity. For example, catalase has been shown to be redox sensitive, with oxidative modifications of cysteine C377 being attributed to the inhibition of catalase in patients with asthma [5]. In addition, it has been demonstrated that free thiols such as cysteine or glutathione can directly attenuate catalase activity [6, 7] although the key cysteine residues involved in catalase thionylation have not been identified. Catalase has also been shown to be sensitive to inhibition by NO released from cigarette smoke [8] and by peroxynitrite released during inflammation [9]. However, our recent study suggests that catalase can also be stimulated via the phosphorylation of serine residues [3]. However, the specific phosphorylation sites and the mechanism underlying the modulation of catalase activity are unresolved and was the focus of our study.

Our data indicate that the PKC δ -dependent phosphorylation site of catalase is located at S167. Further, using a phospho-specific antibody raised against pS167 we demonstrated that this site is enhanced in PAEC exposed to ET-1 and attenuated in lambs with acute increases in PBF exposed to the ET receptor antagonist, tezosentan. Importantly, the S167D phosphomimic catalase mutant was found to exhibit greater catalytic activity than wildtype catalase. Finally, we found that the phosphorylation of S167 leads to structural changes within catalase that promote the formation of the tetrameric structure required for maximal activity.

MATERIALS AND METHODS

Cell Culture

Primary cultures of ovine pulmonary arterial endothelial cells (PAEC) were isolated as described previously [10]. Cells were maintained in DMEM containing 1g/L glucose and 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. COS-7 cells were cultured and maintained in the same constituent medium but with a higher glucose concentration (4.5g/L glucose).

Western Blotting

Serum-starved PAEC (16 h) were treated with ET-1 (100 nM) for 0–4 h as previously described [3]. After treatment cells were 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 (Thermo Fisher Scientific Inc., Rockford, IL). 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 (Bio-Rad, Hercules, CA). 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-phospho-PKC δ (Tyr311, Cell Signaling, Beverly, MA), anti-total PKC δ (Cell Signaling, Beverly, MA), anti-catalase (Research Diagnostic, Inc, Flanders NJ), or anti-phosphoserine antibody (EMD Millipore Corporation, Billerica, MA). Membranes were 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 were visualized with ECL reagent (Thermo Fisher Scientific Inc., Rockford, IL) using a Kodak 440CF image station. Loading was normalized by re-probing the membranes with an anti-(β -actin antibody (1:2,500; Sigma, St. Louis, MO).

Immunoprecipitation analysis to detect phospho-catalase

PAEC were serum-starved for 16h then treated with ET-1 (100nM) for 4 h. The cells were then lysed in ice-cold lysis buffer. For each immunoprecipitation, cell lysates were incubated with anti-catalase antibody (Research Diagnostic, Inc, Flanders NJ) for 2 h at 4°C and then with protein G Plus/Protein A agarose suspension (EMD Millipore Corporation, Billerica, MA) 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 subjected to Western blot analysis using either an anti-phosphoserine antibody or PKC δ as described above. The same blot was re-probed with the anti-catalase antibody to normalize for the levels of catalase immunoprecipitated in each sample.

Immunocytochemistry

PAEC cells were split into four 4-chamber slides. The cells were allowed to become confluent and transferred to serum free growth media overnight at 37°C. On the day of the experiment, the cells were exposed or not to ET1 (100 nM) for 4 hours at 37 °C. Four percent paraformaldehyde (500 μ l, Fisher Scientific) was added to each well for 15 minutes then aspirated and replaced with PBS (1ml). The slides were then incubated at 4 °C overnight. The PBS was then replaced with 0.25% triton 100X (500 μ l) for 30 minutes at room temperature. The wells were washed 3 times with PBS for 2 minutes each and 2% BSA (500 μ l) was added for 30 minutes at room temperature as a blocking agent. Subsequently, primary antibodies for catalase (raised in rabbit, 1:200 dilution) and PMP70 (raised in mouse, 1:200 dilution, Sigma Aldrich) were added and incubated for 1h at room temperature. The primary antibodies were aspirated and the wells washed 3 times with PBS for 2 minutes each. Secondary antibodies, Alexa fluor 594 anti-rabbit IgG (Invitrogen, 1:500 dilution) and Alexa Fluor 488 goat anti mouse IgG (Invitrogen, 1:500 dilution) were then added to each well and incubated in the dark for 1h. Cells were then washed and mounting media-Gold antifade reagent with DAPI (Invitrogen) added. The cells were then imaged using a computer-based confocal DeltaVision imaging system (Applied Precision Inc.) utilizing appropriate excitation and emission wavelengths for red and green fluorescence.

Generation of a phospho-S167-catalase specific antibody

A catalase phospho pS167 specific antibody was raised using a synthetic phosphopeptide antigen CFPSFIH(pS)QKRNPQ, where pS represent phosphoserine. The peptide was used to immunize rabbits. Phosphopeptide-reactive rabbit antiserum was first purified by affinity chromatography. Further purification was carried out using immunodepletion by non-phosphopeptide CFPSFIHSQKRNPQ resin chromatography, after which the resulting eluate was tested for antibody specificity by ELISA and immunoblotting.

Determination of Catalase Activity and Michaelis-Menten binding constant

Catalase activity was measured as described previously [4]. This method is based on the rate of degradation of H_2O_2 to form water and oxygen over time. Total protein extracts (40 μg) were diluted to 1 ml in 50 mM phosphate buffer (pH 7.0). One milliliter of 10 mM H_2O_2 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 $\text{H}_2\text{O}_2 \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$. To calculate the Michaelis-Menten binding constant, catalase activity was assessed at different concentration of H_2O_2 . All the concentrations were run in duplicate. Using a thermo-controlled spectrophotometer (Hitachi US-2900), a measure of time scan was set at 1200 seconds and the change in absorbance at 240nm was monitored. A cuvette containing phosphate buffer was used as a blank. H_2O_2 solution in PBS (800 μl) was pipetted into a cuvette and 8 μl of catalase solution, containing NADPH (10 μM), was added and mixed well by inversion. The reaction rate was calculated, and the K_m value for wild type- and the S167D mutant-catalase was determined.

Expression and purification of recombinant human catalase—To purify human recombinant catalase, 50ml of terrific broth was premixed with kanamycin (100mg/ml) and chlorophenicol (50mg/ml), and inoculated with *E. coli* BL21 cells transformed with the pET28b plasmid containing either a complete human catalase cDNA sequence [11] or a phospho-mimic mutant, S167D-catalase. Bacteria were grown overnight at 37°C (260 rpm) then used to inoculate 2.8L Fernabach flasks (6 $\times$ 1.5L) containing terrific broth (52g/L) as the culture medium and supplemented with kanamycin (100mg/ml) and riboflavin (15mg). Flasks were placed on an orbital shaker and were allowed to grow at 37°C (200 rpm). The OD₆₀₀ was checked periodically during the growth period until it reached 0.8–1.0 (4–5h) then adenosine-5'-triphosphate (ATP, 200 μM final concentration) and isopropyl-beta-D-thiogalactopyranoside, dioxane free (IPTG, 1mM final concentration, to induce the T7 promoter) was added and the cells incubated for 18–20 hours at 25°C (200 rpm). Bacteria were then harvested by centrifugation using a FiberLite F6 6 $\times$ 1000 rotor at 4°C (3500 rpm/2700g) for 20 min. The pellet was immediately transferred into lysis buffer (40mM Tris-HCl, 5% glycerol, 1mg/ml lysozyme) containing a protease inhibitor cocktail for use with histidine-tagged proteins (Sigma, St. Louis, MO), ribonuclease A from bovine pancreas (Sigma, St. Louis, MO), and deoxyribonuclease I from bovine pancreas (10⁶ units, Sigma, St. Louis, MO) were then added. The pellet was gently rocked for 30 min at 4°C, sonicated on ice, and then subjected to ultracentrifugation at 4°C (60,000 rpm/37,1000g) for 1 hour and 45 min. The supernatant was loaded onto a Hisprep FF 16/10 column (charged with 0.1M NiSO_4) using binding buffer (40mM Tris-HCl, 100mM NaCl, 5% glycerol, 30mM imidazole) at 0.1ml/min flow. The column was washed with washing buffer (40mM Tris-HCl, 300mM NaCl, 5% glycerol, 30 mM imadizole) using a flow rate of 1.5ml/min, and a base line was obtained resulting in the washing out of non-histidine-tagged proteins. Elution of histidine-tagged protein was accomplished using elution buffer (40mM Tris-HCl, 300mM NaCl, 5% glycerol, 400mM imidazole) at 1.0ml/min flow. Collected fractions were loaded for size-exclusion gel filtration on a HiLoad 26/60 Superdex 200 prep grade column using catalase gel filtration buffer (60mM Tris-HCl, 100mM NaCl, 5% glycerol) at 0.2ml/min flow. Fractions were collected in 5ml amounts for analysis by Coomassie blue staining and mass spectrometry. Desalting was then performed for fractions containing catalase using a HiPrep 26/10 desalting column and catalase gel filtration buffer at flow rate of 0.5ml/min. All purification steps were performed at 4°C. Protein homogeneity was confirmed using Coomassie blue staining and Western blot analysis using an anti-catalase antibody (Research Diagnostics Inc., Flanders, NJ). The final protein concentration was then determined in each fraction then stored at –80°C until used.

In-gel catalase activity—In gel catalase activity was determined using the inhibition of exogenous horseradish peroxidase/H₂O₂-mediated diaminobenzidine (DAB) oxidation after semi-native gel electrophoresis was used to separate the various catalase forms (monomer, dimer, tetramer). After electrophoresis the gels were soaked with DAB (0.7mg/ml) and HRP (1µg/ml) in PBS for 1h then washed twice with deionized water and developed by applying H₂O₂ solution (3mM). In this reaction catalase activity is determined through the appearance of a colorless band against a dark background.

Gel filtration chromatography—To examine the oligomeric composition of the catalase we utilized analytical gel filtration. One hundred µl of each sample, at a concentration 1 mg/ml, was injected into a Tosoh TSKgel G3000SWxl gel filtration column. Using a flow rate of 0.5ml/min, monomer, dimer, trimer and tetramer fractions were eluted in 100mM phosphate buffer (pH=7.0) using an HPLC system (GE) and analyzed by measuring the absorption at 260nm.

Detection of H₂O₂ levels

The Amplex Red Reagent (Life Technologies, Grand Island, NY) was used to detect H₂O₂ levels in the phenol red free media as previously described [12]. Briefly, an equal amount (50µl) of media was incubated at 37°C for 30min in master mix solution containing Amplex Red reagent, horseradish peroxidase, and a buffer solution. After the end of the incubation period, the fluorescence was read using a fluorescent plate reader (Fluoroskan Ascent, Thermo Fisher Scientific Inc., Rockford, IL) using an excitation wavelength of 530nm and an emission wavelength of 590nm. Fluorescent units were then converted into concentrations of H₂O₂ through extrapolation from a standard curve using known amounts of H₂O₂.

Lamb model of surgically induced acute increases in PBF—This was carried out as recently described [2]. Briefly, juvenile lambs (4–6 weeks of age) underwent a midsternotomy incision and the pericardium was incised. Using side biting vascular clamps, an 8.0mm Gore-tex® vascular graft (~2mm 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. The midsternotomy incision was then temporarily closed with towel clamps. An intravenous infusion of Lactated Ringers and 5% Dextrose (75ml/h) was begun and continued throughout the study period. Cefazolin (500mg, IV) and gentamicin (3mg/kg, IV) were administered before the first surgical incision. The lambs were maintained normothermic (39°C) with a heating blanket. In control lambs an infusion of normal saline (vehicle) was initiated and continued throughout the study period. In a second group an infusion of tezosentan (a combined ET_a and ET_b receptor antagonist) was initiated at 0.5mg/kg/hour 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 [13]. At the end of the 4h 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 Regents University.

MALDI-TOF-TOF mass spectrometry—Peptide calibration standards and matrix CHCA were obtained from Applied Biosystems (Carlsbad, CA). All spectra were taken on an ABSciex 5800 MALDI-TOF-TOF mass spectrometer in positive reflector mode (10 kV) with a matrix of CHCA. At least 1000 laser shots were averaged to obtain each spectrum. Masses were calibrated to known peptide standards. Five microliter aliquots of the catalase

(band from catalase immunoprecipitation above 60 kDa) tryptic digest (18h incubation with 10ng MS grade trypsin at 28C in 25mM ammonium bicarbonate) were cleaned on a C18 ZipTip (Millipore, Bedford MA) as per manufacturer's instructions. Bound peptides were desalted with two 5µl washes of 0.1% TFA and then eluted with 2.5µl of aqueous, acidic acetonitrile (75% CH₃CN, 0.1% TFA). The eluant was mixed with 2.5µl of freshly prepared CHCA stock solution (20 mg/ml CHCA in aqueous acetonitrile as above), and 1.5µl portions of this mixture were spotted onto a MALDI sample plate for air drying. 1.5µl of crude peptides were additionally mixed with 1.5µl of CHCA and spotted. MS and MS/MS spectra (1609.4 m/z corresponded to DPILFPSFIHS¹⁶⁷(PO₄)QK sequence from catalase) were analyzed in Protein Pilot 3.0 and Mascot Distiller software packages.

Molecular modeling—We used the available structure of catalase (PDB ID – 1QQW). To model this structure we utilized Yasara software [14]. The geometry of human catalase was automatically optimized using the steepest descent energy minimization algorithm in the solvent implicit model.

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-1 treatment enhances catalase activity in pulmonary arterial endothelial cells through PKCδ dependent phosphorylation

Our initial studies confirmed that ET-1 exposure (100nM) produced a significant increase in catalase activity after 4h (Fig. 1 A) without altering catalase protein levels (Fig 1 B). The increase in catalase protein levels correlated with a reduction in H₂O₂ levels in the culture medium (Fig. 1 C) and an increase in phospho-serine catalase levels (Fig 1 D). Immunocytochemistry to evaluate the colocalization of catalase (red) with the peroxisomal marker, PMP70 (green) suggests that ET-1 does not alter the distribution of catalase to the peroxisome (Fig. 1 E & F). Significant catalase staining is also observed outside the peroxisome although it is possible that this represents catalase trafficking to the peroxisome. Calculation of the Pearson coefficient indicates that ~85% of catalase co-localizes with peroxisomes (Fig 1 F). Phosphorylation of PKCδ at Tyr311 is considered to reflect its increased activity and ET-1 treatment increased phospho-Tyr311 PKCδ which was attenuated by the PKCδ inhibitory peptide, δV1.1 (Fig 2 A). δV1.1 exposure also attenuated the ET-1 mediated increase in catalase activity (Fig 2 B) and prevented the decrease in H₂O₂ levels (Fig 2 C). Together these data reflect the importance of PKCδ as a downstream signaling mediator of ET-1.

Identification of the catalase phosphorylation site as serine (S) 167

Purified catalase was incubated with PKCδ then subjected to 1D gel electrophoresis followed by in-gel tryptic digestion and mass spectrometry. The MS spectra of the catalase tryptic digests were then analyzed to identify mass shifts of 80Da as an indication of a phosphorylation event. The identified peptide fragments were then analyzed by MS/MS to confirm the position of the phosphorylation site. An MS/MS spectrum containing a 1609 m/z fragment was obtained in positive reflector mode using air as the collision gas (Fig. 3 A). The MS/MS fragmentation pattern produced was then fitted to the known catalase sequence and identified the phosphorylation modification as S167 within the peptide sequence DPILFPSFIHS*(PO₄)QK (Fig. 3 A). The following collision fragments were identified: y4, y5-H₂O, y7-H₂O, indicated as solid blue lines; b2, b3, b6-H₂O, b8-H₂O, b12 indicated as

solid green lines and immonium ions Q, FP, S(PO₄)Q, FSP, HS(PO₄)Q, SFIH, HIS(PO₄)Q, PILFP, SFIHS(PO₄) and FIHS(PO₄)Q indicated as solid black lines. MS analysis of PAEC cells treated with ET-1 identified the same 1609 m/z peptide fragment in tryptic digests of immunoprecipitated catalase (Fig. 3 B), however the amount of the fragment was insufficient to allow further MS/MS analysis. On the basis of our MS data, we then generated a polyclonal antibody specific for pS167 on catalase. Using Western blot analysis we confirmed that pS167 catalase levels were elevated in ET-1 exposed PAEC and this increase was attenuated in the presence of δ V1.1 (Fig. 3 C). To begin to elucidate if the changes we observed *in vitro* had physiologic importance we utilized our lamb model with acutely increased PBF. We have recently shown that these lambs have increases in catalase serine phosphorylation and that this correlates with increased catalase activity [2]. Both these events are attenuated in the presence of the ET-receptor antagonist, tezosentan [2]. Western blot analysis of lung extracts revealed a decrease in both phospho-PKC δ (Fig. 3 D) and pS167 catalase (Fig. 3 E) in tezosentan treated lambs suggesting that the PKC δ -catalase interaction has a physiologic relevance.

To further explore the role of pS167 in catalase we generated phospho-mimic mutant S167 catalase in which the serine residue at 167 was replaced by aspartic acid (D) to mimic the negative charge introduced by phosphorylation. The S167D-catalase mutant was then purified using a bacterial expression system. After his-tag affinity purification the protein was further purified using size exclusion chromatography using a G-75 gel filtration column. The resulting recombinant protein was then subjected to SDS-PAGE and Coomassie blue staining and identified a single monomeric band (Fig 4 A) and confirmed as catalase by MS (data not shown). Michaelis-Menten kinetic analysis was then used to determine the K_m of both wildtype- and the S167D mutant-catalase for H₂O₂. The Michaelis-Menten constant, K_m was decreased in the S167D mutant ($K_m=52.4$) compared to wild-type catalase ($K_m=82.3$) (Fig 4 B). The activity of S167D catalase was then compared to the wildtype enzyme by measuring their rates of H₂O₂ decomposition. The ability of S167D catalase to decompose H₂O₂ was found to be two-fold higher than wildtype catalase under the same conditions (Fig 4 C) correlating well with the decreased K_m (Fig. 4 B). Similarly, when COS-7 cells were transiently transfected with wildtype- and S167D-catalase (Fig. 4 D) catalase activity was ~2-fold higher in the S167D-catalase transfected cells compared to wildtype expressing cells (Fig. 4 E), although over-expression levels were similar (Fig. 4 D).

PKC δ dependent phosphorylation enhances catalase activity by stimulating tetramer assembly

Using semi-native gel electrophoresis we found that recombinant catalase purified from *E.coli* was a mixture of monomers, dimers, and tetramers (Fig 5 A right panel). Importantly, zymography indicated that only the dimeric and tetrameric fractions exhibited activity while the monomer fraction was completely inactive (Fig 5 A left panel). An analysis of the catalase crystal structure (PDB ID 1QQW) revealed that Serine 167 is buried within the tetrameric structure suggesting it would not be accessible for phosphorylation (Fig 5 B). However, further analysis revealed that S167 could be water accessible when catalase was in a dimeric form (Fig 5 C). This observation led us to hypothesize that phosphorylation of S167 is the driving force for the assembly of the catalase dimer into the fully active, tetrameric structure. To test this potential mechanism, we utilized recombinant wildtype catalase and phospho-mimic S167D mutant protein in combination with analytical gel filtration to allow us to monitor the oligomeric structure of catalase. Our data indicate that recombinant catalase purified from *E. coli* is predominantly present in a dimeric form (Fig. 5 D upper panel). Importantly, the incubation of catalase with recombinant PKC δ is sufficient to trigger the assembly of the catalase dimer into a tetrameric structure (Fig. 5 D, middle

panel). Similar to wildtype catalase exposed to PKC δ , S167D catalase is predominantly tetrameric (Fig. 5 D, lower panel). A summary of the gel filtration data is shown in Table 1.

DISCUSSION

ET-1 is a 21 amino acid polypeptide secreted by endothelial cells. It is one of the most potent vasoconstrictors known [15]. ET-1 acts in an autocrine/paracrine manner and binds to the G-protein-coupled receptors, endothelin type A (ETA) and type B (ETB), to produce its physiological effects [16]. Patients with pulmonary hypertension have increased expression of preproendothelin-1 mRNA and increased levels of ET-1 in both the lung and plasma [17, 18]. This increase in ET-1 production results in increased right atrial pressure, increased pulmonary vascular resistance, decreased pulmonary artery oxygen saturation and increased mortality in patients with pulmonary hypertension. ET-1 also promotes vascular cell proliferation due to its mitogenic potential [19]. Our previous work, has also shown an increase in ET-1 levels in a lamb model of surgically induced acute increases in PBF [2]. Further, we recently reported, in the same model, that inhibition of ET-1 signaling using the combined receptor antagonist, tezosentan decreased pulmonary vascular resistance (PVR) and increased PBF while subjecting these animals to tezosentan along with PEG-catalase increased PVR [3]. Further we have shown that ET-1 attenuates the NO generation required for vasodilation by stimulating catalase activity [3]. The data presented in this study reveal that ET-1 increases catalase activity, without altering protein levels or peroxisome/cytosol redistribution. The increase in catalase activity, we observed, correlates with a significant decrease in K_m value from 82.3mM in wildtype to $K_m=52.4$ mM in phospho-mimic S167D mutant. Interestingly, the previously reported K_m values for catalase vary greatly, ranging from 3mM [20] to 1.1M [21]. This variability has not been resolved but it has been suggested that both the catalase kinetic is complex and the K_m are time dependent [21]. Further, Chance and colleagues have shown that at high H_2O_2 concentrations, catalase forms Compound II, thought to be involved in self-inhibition [22, 23]. Compound II formation will contribute significantly to the kinetics in the initial stages of the reaction, when H_2O_2 concentrations are higher, and become less important at the end of reaction, when H_2O_2 becomes depleted. Thus, it is possible that the published differences in the K_m for purified catalase occur due to the use of different experimental conditions making comparisons between studies difficult. However, it is important to note that we determined the K_m of purified wildtype- and S167D-catalase under the same experimental conditions. Thus, we can state that the S167D mutant protein does exhibit a reduction in K_m .

Another important finding in our studies is the possible role of a PKC δ -dependent post-translational phosphorylation event, at S617, in stimulating catalase activity. That PKC δ is stimulated by ET-1 is supported by prior studies. For example, the sustained exposure of PAEC to ET-1, mediated via its over-expression, decreases eNOS promoter activity, protein levels, and NO generation. The decrease in transcription correlated with increased activity of PKC δ and STAT3 [24]. The increase in STAT3 activity and decrease in eNOS promoter activity were inhibited by the over-expression of dominant negative mutants of PKC δ or STAT3 [24]. Further, we have previously shown that that PKC δ inhibition potentiates the shear-mediated increases in eNOS expression and activity, while PKC δ activation inhibits these events [25]. Interestingly we have also shown that shear stress decreases PKC δ activity and enhances NO signaling through increases in H_2O_2 and S1177 phosphorylation [3, 4]. However, when PAEC are exposed to both shear and ET-1, the ET-1 effect is dominant and NO signaling is attenuated [3]. Further, these effects on NO signaling correlate with changes in serine phosphorylation of catalase, catalase activity and H_2O_2 levels such that increased NO signaling is associated with decreases in catalase phosphorylation, catalase activity and increases in H_2O_2 while the opposite is true when NO signaling is attenuated. It should also be noted that ET-1 appears to have cell specific effects as we have previously shown that

ET-1 increases both superoxide [26] and H₂O₂ levels in monocultures of pulmonary arterial smooth muscle cells (PASMC) mediated via ETA receptor signaling while H₂O₂ are decreased in monocultures of PAEC via ETB receptor signaling [27, 28]. However, in co-cultures the increase in H₂O₂ in PASMC appears to overcome the increased catalytic activity of catalase in the PAEC and H₂O₂ levels [27]. Thus, the effect of ET-1 on H₂O₂ levels during the development of pulmonary hypertension appears to be complex, being determined by both increased generation and enhanced degradation. Thus, the net effect of ET-1 on H₂O₂ levels will then be determined by the relative expression of ET subtypes in the vascular wall. The endothelial levels of the ETB subtype receptor are decreased during the development of PH while the SMC levels of the ETA receptor subtype increase [29, 30]. Thus, it is likely that the eventual outcome will be an overall increase in H₂O₂ as the generation of H₂O₂ in the SMC layer becomes dominant with the loss of the ETB receptors ability to increase catalase activity and maintain the level of H₂O₂ in a physiologic range to sustain NO signaling. It should also be noted that the role of H₂O₂ on NO signaling is complex. We, and others, have shown that H₂O₂ can both stimulate [31] and inhibit [27, 28] eNOS expression and stimulate [32–34] and inhibit [35] eNOS activity. The effect of H₂O₂ on eNOS activity appears to be concentration and time-dependent with lower levels stimulating activity [33, 34] and higher levels inhibiting the enzyme [35]. Indeed cell culture data indicate that in the same EC, concentrations of H₂O₂ up to 60 μM stimulate eNOS activity [4] while concentrations of >100 μM attenuates both eNOS activity [36, 37] and gene expression [27, 37, 38]. Further, lambs with sustained increases in PBF have increased levels of H₂O₂ [39] but this correlates with a reduction in NO signaling [40]. Thus, it is important to recognize that H₂O₂ has a physiologic component that can enhance NO signaling [31, 32] and a pathologic component that decrease NO signaling [4, 36, 41]. Thus, the data we present here, both *in vitro* and *in vivo*, add to this complexity by demonstrating that changes in PKCδ dependent catalase phosphorylation, by modulating cellular H₂O₂, can regulate NO signaling. This likely occurs through alterations in the pp60^{Src}-PI3 kinase-Akt axis and S1177 phosphorylation of eNOS [3, 4, 42]. We summarize our view on the ROS mediated regulation of the ET-1 induced signaling in PAEC in Figure 6. Briefly, we speculate that, as ET-1 mediated calcium influx has been reported to stimulate NOX activity [43], NOX stimulation results in increased ROS production, which in turn can stimulate eNOS through pp60^{Src} activation [44] and could directly activate PKCδ through oxidation of important Zn-S bonds [45]. However, the overstimulation of PKCδ, as reported here, leads to increased catalase activity, decreasing ROS levels and thereby inhibiting NO production from eNOS, i.e., acts as a negative feedback loop.

PKC is a family of serine/threonine-related protein kinase that plays a key role in many cellular functions and affects many signal transduction pathways [46]. PKCδ has been shown to be involved in regulating various cellular functions in endothelial cells [4, 47, 48]. It is well established that activation of PKCδ induces its translocation from the cytosol to membrane and ET-1 has been shown to stimulate the translocation of PKCδ to the membrane in both the human saphenous vein endothelial cells [49] and the porcine coronary artery [50]. The activation of PKCδ also involves the phosphorylation of tyrosine residues [51] although the importance of this phosphorylation is controversial, as studies have reported this phosphorylation can increase [52–54] or decrease PKCδ activity [55]. However, phorbol 12-myristate 13-acetate (PMA), a well-established activator of PKC family members, increases the phosphorylation of PKCδ at Tyr311 and is used as a marker for PKCδ activation [56, 57]. It should be noted that ET-1 also enhances intracellular calcium. Thus, it is possible that other PKC isoforms may also be able to phosphorylate and stimulate catalase activity. Alternatively, it is possible that other signals, besides ET-1, that stimulate PKC activity may also be able to regulate NO signaling through catalase phosphorylation and decreases in H₂O₂ levels. However, further studies, perhaps using isoform specific PKC knockout mice, will be required to test these possibilities. It is also

important to note that the regulation of catalase by phosphorylation is not a well-explored area despite six decades of research into the biochemistry of catalase. Proteomic analysis, in combination with MS, identified 21 distinct catalase phosphorylation sites (Y84, S114, T115, Y231, S254, Y260, Y308, Y358, Y370, Y379, Y386, Y405, S417, S422, Y425, T434, T441, Y500, T511, S515, S517) [58]. However the biochemical and physiological role of these modifications remains unanswered. To our knowledge only one study has evaluated the regulation of catalase activity through phosphorylation [59]. In this work catalase was shown to be phosphorylated at Y231 and Y386 by the c-Abl and Arg protein kinases [59]. The phosphorylation of these sites was also associated with catalase activation. Unfortunately, no mechanistic explanation for the activation was provided. Indeed analyzing the crystal structure of catalase suggests that Y231 and Y386 do not appear to be accessible in the tetrameric structure. This is similar to what we have observed with S167, which is only accessible in the dimeric structure. As our data indicate that phosphorylation of S167 stimulates the assembly of the fully active catalase tetramer, perhaps the phosphorylation of Y231 and Y386 also occurs in the dimeric state and also drives tetramer assembly. However, further studies will be required to test this possibility.

Our observation that exposing wildtype catalase to PKC δ enhances tetramer assembly also raises an important question regarding the mechanism of catalase oligomerization. Interestingly, we found that both the catalase dimers and tetramers are active, while monomeric catalase was completely inactive. Catalase is a unique enzyme that has a very large oligomerization interface (10000Å² or 40% of the subunit's surface) and a very sophisticated inter-subunit threading is required for the tetramer assembly [60, 61]. However, very little is known about the catalase assembly process. Inter-subunit threading could be accomplished if there was coordination between the folding and oligomerization process. However, for this to occur it would likely require the assistance of specific chaperone proteins. But this type of post-translational folding machinery has so far not been identified for catalase. Further, recent studies demonstrate that Pex5, a peroxisome targeting cargo protein, binds to monomeric catalase and facilitates its translocation into the peroxisome [62]. Thus, a monomeric form of catalase is present in the cytosol for sufficient time to bind to Pex5 and suggests that the folding and oligomerization processes are not coordinated. Oligomerization is also important for the heme internalization process [60] and heme cavity formation [60]. Interestingly, the inter-subunit threading required for oligomerization involves the N-terminal arm of one subunit and the loop of an adjacent subunit. Thus, the N-terminal region appears to play a key role in catalase oligomerization. This is supported by our data in which a catalase mutant containing an S-D point mutation in the N-terminal S167, to mimic phosphorylation, has higher tetramer levels and higher catalase activity than wildtype catalase.

Thus, in conclusion, our data indicate that the PKC δ -dependent phosphorylation of catalase at S167 enhances catalase tetramer assembly and this in turn increases the catalytic activity of the enzyme. Further, we speculate that this type of phosphorylation event can also be used to regulate NO signaling through the modulation of cellular H₂O₂ levels. It is also interesting to speculate that the S167D-catalase mutant protein could be used as a therapy in disease states where catalase expression or activity are decreased. For example, hypocatalasemia, where cellular catalase expression [63] or its stability [64] are reduced or in HIV patients where catalase activity has been shown to be decreased in white blood cells [65]. Similarly, as H₂O₂ levels are associated with decreased catalase activity in neonatal lambs models of pulmonary hypertension S167D-catalase could have therapeutic potential [66]. However, more work in animal models of disease will be required before clinical trials in humans should be attempted.

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- PKC δ phosphorylates catalase at S167 and this enhances its catalytic activity.
- S167 is being located on the dimeric interface.
- S167 phosphorylation enhances the formation of catalase tetramers.
- A phospho-mimic (S167D) catalase exhibits increased catalytic activity.

F

Figure 1. ET-1 increases catalase activity in pulmonary arterial endothelial cells through serine phosphorylation

PAEC were exposed to ET-1 (100nM) for 0-4h. Catalase activity (H_2O_2 degraded/min/mg protein) was then determined in whole cell lysates. Catalase activity is enhanced after 4h of ET-1 exposure (A). Western blot analysis on whole cell extracts (20 μg) revealed no change in catalase protein levels (B). The levels of H_2O_2 in the medium, as determined using the Amplex red assay were significantly decreased after 4h of ET-1 exposure (C). The increase in catalase activity correlated with an increase in phospho-serine catalase levels (D). ET-1 treatment does not change the subcellular localization of catalase (Red) with the peroxisome (PMP70, green). This was confirmed by calculation of Pearson correlation coefficient for co-localization of catalase and peroxisome marker (F). Data are mean $\pm$ SEM; n=6. *P<0.05 vs. untreated.

Figure 2. Attenuating PKCδ attenuates the ET-1-mediated increase in catalase activity in pulmonary arterial endothelial cells

PAEC were treated with ET-1 (100nM, 4h) in the presence or absence of the PKCδ inhibitory peptide, δV1.1 (1μM). Whole cell extracts (20μg) were then subjected to Western blot analysis to determine changes in the levels of phospho-tyr311 PKCδ and total PKCδ. ET-1 increases the levels of phospho-tyr311 PKCδ in PAEC and this is attenuated by δV1.1 (A). δV1.1 also blocked the ET-1 mediated increase in catalase activity (B) and prevents the decrease in H₂O₂ (C). Data are mean ± SEM; n=4-9. *P<0.05 vs. untreated, †P<0.05 vs. ET-1 treated.

Figure 3. PKC δ phosphorylates catalase at serine 167

Purified human recombinant catalase was incubated with PKC δ . A tryptic digest of the resulting products were analyzed for serine phosphorylation events by MS. This identified a 1609 m/z fragment that was subjected to MS/MS. The fragmentation pattern produced identified the phosphorylation modification at S167 within the peptide DPILFPSFIHS*(PO₄)QK (A). MS analysis of PAEC exposed to with ET1 (100nM, 4h) identified the same 1609m/z peptide fragment in trypsin digest of immunoprecipitated catalase (B). Using an antibody raised against pS167 catalase, Western blot analysis demonstrated that p167-catalase levels were increased by ET-1 and this increase was attenuated by δ V1.1 (C). The ET-1 receptor antagonist, tezosentan also attenuated phospho-Tyr311 PKC δ levels (D) and pS167-catalase levels in lambs with acute increases in PBF (E). Data are mean $\pm$ SEM; n= 3–6. *P<0.05 vs. untreated or acute shunt post, †P<0.05 vs. ET-1 treated.

Figure 4. A phospho-mimic catalase mutant exhibits enhanced catalytic activity

The phospho-mimic catalase mutant, S167D was expressed and purified from *E.coli* (A). The S167D mutant recombinant catalase has a decreased Michaelis-Menten constant, K_m compared to wildtype (K_m S167D=52.4mM vs. K_m =82.3mM for wildtype catalase, B). A H₂O₂ decomposition assay demonstrated that the activity of S167D-catalase is approximately 2-fold higher than wildtype catalase (C). COS-7 cells were also transfected with wildtype- and S167D-catalase. After 48h, the cells were harvested and whole cell extracts (20μg) subjected to Western blot analysis. There was a similar increase in catalase protein levels in the cells transfected with both wildtype- and S167D-catalase (D). However, the cellular catalase activity was significantly higher in the cells transfected with S167D-catalase (E). Data are mean± SEM; n=3–6. *P<0.05 vs. control, †P<0.05 vs. wild type catalase.

A

D

Figure 5. Phosphorylation of catalase at serine 167 required for the tetramerization of this enzyme

Using semi-native gel electrophoresis we found that recombinant wildtype catalase contained monomeric, dimeric, and tetrameric form (A, right panel). While zymography demonstrated that catalytic activity as present only in the dimeric and tetrameric fractions (A, left panel). Red, green, blue and yellow colored on the figure monomers represent the tetrameric organization of catalase, with the higher magnification demonstrating that S167 is buried within the tetrameric structure (B). However S167 is present on the surface of the dimeric form of catalase (C). Gel filtration analysis shows that recombinant wildtype catalase is predominantly in a dimeric form (D, upper panel). Incubation of wildtype catalase with PKC δ enhances the assembly of the catalase tetramer (D, middle panel). The S167D-catalase protein is predominantly tetrameric even in the absence of PKC δ (D, lower panel). Data are representative of n=3.

Figure 6. Complex role of ET-1 signaling in the pulmonary endothelium

Due to activation of ETB receptor, EC would experience an increase in intracellular calcium levels leading to the rapid activation of eNOS, NADPH oxidase (NOX) and PKC δ . Reactive oxygen species produced by NOX further stimulate eNOS through activation of pp60^{Src} and may additionally stimulate PKC δ by oxidizing Zn-S bonds. The stimulation of PKC δ would lead to the phosphorylation and subsequent activation of catalase reducing H_2O_2 levels and stimulating a negative feedback loop to reduce eNOS activity.

Table 1

Summary of gel filtration analysis. Data are mean $\pm$ SEM, N=3. P<0.05 vs. wildtype (WT) catalase.

	Tetramer(%)	Dimer (%)	Monomer (%)
WTCatalase	4.78 $\pm$ 2.82	52.97 $\pm$ 4.05	38.12 $\pm$ 5.17
WTCatalase+PKC δ	51.83 $\pm$ 4.12*	6.23 $\pm$ 4.15	12.56 $\pm$ 12.02*
S167D mutant	44.4 $\pm$ 1.49*	9.2 $\pm$ 5.75	0 $\pm$ 0*