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Original Article

Oxidative stress induced pulmonary endothelial cell proliferation is not mediated by superoxide

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ABSTRACT: Cellular hyper-proliferation, endothelial dysfunction and oxidative stress are hallmarks of the pathobiology of pulmonary hypertension. Indeed, pulmonary endothelial cells proliferation is susceptible to redox state modulation. Some studies suggest that superoxide stimulates endothelial cell proliferation while others have linked the proliferative response to an up-regulation of peroxynitrite in lungs under oxidative stress. Given the divergence of opinion on the subject, it is important to establish the agents mediating cellular hyper-proliferation under oxidative stress. Using the combination of xanthine and xanthine oxidase, the current study demonstrates that neither superoxide nor hydrogen peroxide stimulated pulmonary endothelial cell proliferation. Alone, low level superoxide (100 RLU/s) did not alter DNA synthesis in endothelial cell and high concentration (500 RLU/s) superoxide decreased DNA synthesis to 31.8±3%, 30.4±2%, and 53.8±5% control at 0.1, 0.5 and 2.5% basal growth stimulation, respectively. Nonetheless, the formation of peroxynitrite under this condition stimulated proliferation to 49.2±9%, 51.1±8% and 71.2±2%, respectively. Taken together, pulmonary endothelial cell proliferation occurred only under conditions producing nitric oxide and superoxide in concert.

KEYWORDS: Oxidative stress, superoxide, endothelial cells, peroxynitrite, proliferation

INTRODUCTION

Endothelial dysfunction and oxidative stress are hallmarks of the vascular pathologies associated with pulmonary hypertension. Oxidative stress is characterised by a positive shift in cellular redox status as a result of exposure of cells to conditions generating reactive oxygen species (ROS) such as superoxide and hydrogen peroxide or reactive nitrogen species (RNS) such as peroxynitrite (ONOO-) (Salganik, 2001). Peroxynitrite, formed by reaction between superoxide (O_2^{-}) and nitric oxide ($NO^{\bullet}$) is a powerful oxidant that reacts with different biomolecules including amino acids such as cysteine, methionine, tryptophan, and tyrosine, thiol groups in proteins leading to changes in protein structure and function as well as cellular responses (Alvarez and Radi, 2003, Beckman and Crow, 1993, Darley-usmar *et al*., 1992, Liaudet *et al*., 2000). Using authentic and in-situ generated ONOO- from 3-morpholinosydnonimine (SIN-1), the stimulatory effects were reported, of 2-200 nM ONOO- in pulmonary artery endothelial (PAEC) and smooth muscle cells under oxidative stress (Agbani *et al*., 2011a, Agbani *et al*., 2011b). However, other studies have linked superoxide generation with endothelial cell proliferation. For example, treatment of coronary microvascular endothelial cell with pyrogallol or bovine aortic endothelial cells with glucose/glucose oxidase, resulted in increased cell number and increased DNA synthesis (Ruiz-Gines *et al*., 2000, Bayraktutan, 2004). Also, pulmonary artery smooth muscle cells showed increased proliferation following treatment with hydrogen peroxide and Cu/Zn superoxide dismutase (SOD) inhibitor (Wedgwood *et al*., 2001). A recent work equally reported on superoxide induced neural stem cell proliferation (Topchiy *et al*., 2013)

Using the combination of xanthine and xanthine oxidase, the current study demonstrates that neither superoxide nor hydrogen peroxide stimulated pulmonary endothelial cell

proliferation under oxidative stress. Instead this work confirms that pulmonary cell proliferation occurred only under condition favouring the generation of peroxynitrite.

MATERIALS AND METHODS

Cell harvest and culture: PAEC

Primary bovine pulmonary artery endothelial cells were obtained and characterised by methods previously described (Agbani *et al*., 2011a). Briefly, lungs of cows less than 24 months of age were obtained from a local abattoir within 1h of slaughter. Lungs were dissected to free main and large lobular (>5-2 cm diameter) pulmonary arteries. Extraneous fatty and connective tissues were then gently removed. The arteries were sliced opened in a sterile petri dish, so that they laid flat with initimal surface upwards. Endothelial cells were thereafter harvested by gently scraping and scooping (into culture flasks) the luminal surface of the longitudinally opened pulmonary arteries with a sterile scapel (no. 10 blade, blunt end). This was done using light, single strokes, covering each area only once. Cells were thereafter sub-cultured in medium comprising a 50:50 mix of Ham F-12 (cat.#: 21765-037, Invitrogen) and Waymouth MB752 media (cat.#: 31220-072, Invitrogen) to which 15% foetal bovine serum (FBS) and 5% penicillin (5000 U/ml):streptomycin (5000μg) (PEN-STREP® BioWhittaker™) were added. Endothelial cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, characterised by eNOS expression and used between passages 2 and 6.

Cell proliferation assay: DNA synthesis measured by [^{3}H]-thymidine incorporation assay

DNA synthesis was used as a measure of PAEC proliferation. Cells were grown to approximately 70% confluency in 24-well plates at 37 ^{0}C and were quiesced with 0.1% foetal bovine serum (FBS) for 24 h. The quiescent cells were maintained at 0.1, 0.5, 1, 1.5, 2.5 and 15% FBS baseline stimulation for 24h. Cells were stimulated with either 2, 20 or 200 μM of SIN-1 at each of the baseline FBS stimulation used namely 0.1, 0.5, 1, 1.5, 2.5 and 15% for 24h. PAEC were pulsed with [methyl-^{3}H] thymidine aqueous solution (GE Healthcare® Cat #: TRK 120) at 0.1 μCi/well for 5-6h before the end of the 24 h of stimulation to allow estimation of DNA synthesis by the PAEC. At 24 h the medium was removed and the cells were washed twice with 1 ml PBS. This was followed by 4-6 1 ml washes with 10% trichloroacetic acid (TCA) at intervals of 15 min. The remaining cell contents were dissolved in 250 μL 0.1% NaOH/Sodium lauryl sulphate solution. The contents of each well were then transferred to scintillation vials, to which was added 2ml of Emulsifier-safe™ scintillation fluid (PerkinElmer, Boston, USA). Vials were vortexed thoroughly before radioactive counts were measured by scintillation counter (Packard 1500 TRI-CARB®). Counts were converted to DPMs (disintegrations per minute); each experiment had 4 replicates.

Chemiluminescence

Phosphate buffered solution (PBS) was used to make test solutions up to 1ml in clear plastic cuvettes. Lucigenin solution added to make a final cuvette concentration of 5 μM was used for the detection of superoxide using a Berthold™ chemiluminometer. Measurements of superoxide generation from various xanthine concentrations combined with a fixed amount of xanthine oxide were recorded after chemiluminescence signal was observed for 70 seconds. Time dependent superoxide generation for a fixed combination of xanthine/xanthine oxidase was monitored every 20 seconds for 15 min. Sodium salt of xanthine dissolved in warm distilled water was used and the xanthine oxidase was dissolved in phosphate buffer solution. Measurements were charted as chemiluminescence signal in relative light units per second (RLU/s). Other experiments involved luminol (10^{-3} M)-enhanced chemiluminescence for the detection of hydrogen peroxide.

Drugs

Bovine superoxide dismutase (cat.#: S2515-75KU), xanthine (cat.#: X0626-5G) xanthine oxidase (cat.#: X4376-5UN), bovine liver catalase (cat.#: C1345). 3-morpholinosdynonimine (SIN-1) was purchased from Sigma (UK; Cat # M184-25mg). Stock solutions of SIN-1 in distilled water were made and added directly to cells with gentle swirling.

RESULTS

Effect of FBS on PAEC proliferation

Preliminary work demonstrated endothelial cells proliferation can be stimulated with foetal bovine serum (FBS) in a concentration-dependent manner (results not shown). The results showed that 15% FBS produced maximal stimulation; however, this did not differ significantly from growth rates at 2.5% FBS. Accordingly, cell proliferation was also investigated at serum levels (0.1 and 0.5%) allowing for further increase in DNA synthesis.

Superoxide and hydrogen peroxide generation

Initial experiments confirmed the generation of superoxide, using lucigenin chemiluminescence, from the reaction of xanthine and xanthine oxidase (Figure 1A). The concentration relationship showed graded superoxide generation with 0.3 μM – 10 μM xanthine. The chemiluminescence signal was confirmed to be due to superoxide, since it was completely abolished by superoxide dismutase (Figure 1B). Over 75% of initial level of superoxide remained after 15min of real time decay (Figure 1C). This indicated that there was significant amount of superoxide in the cell culture medium during experimentation.

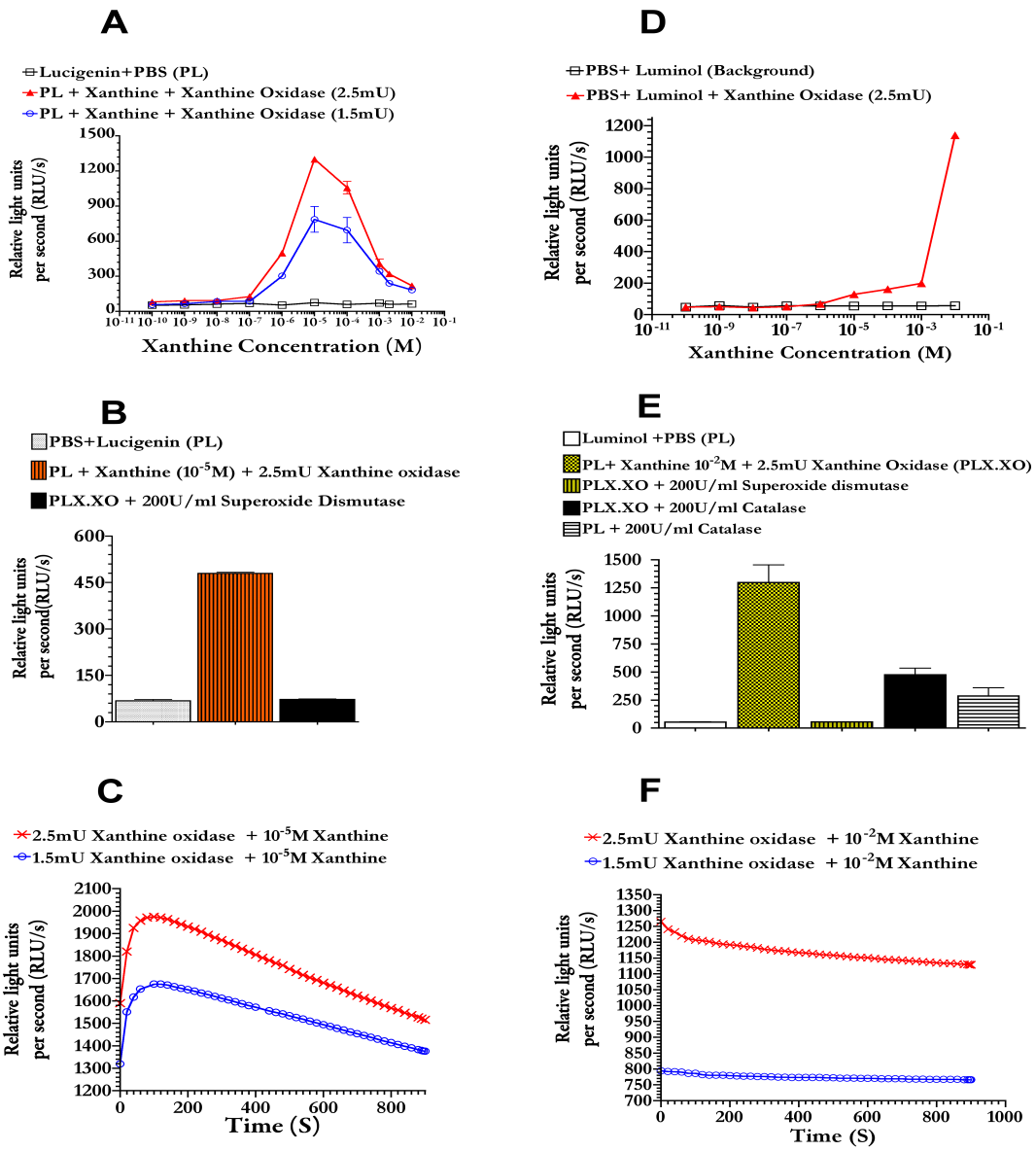

Figure 1: Superoxide and hydrogen peroxide generation. (A): Generation of superoxide by xanthine oxidase with graded xanthine concentration combined with 1.5 or 2.5mu xanthine oxidase. 5μM Lucigenin enhanced chemiluminescence was used to detect superoxide production. Measurements were taken 60 seconds following a 10 seconds delay after reactants have been added into vials and charted as chemiluminescence signal in relative light units per second (RLU/s). (B): Show total obliteration of superoxide signal by 200U/ml Superoxide dismutase (C): Example record show 15 min real time measurement of superoxide production. Measurements were taken every 20 seconds and charted as chemiluminescence signal in relative light units per second (RLU/s). (D): Example record show generation of hydrogen peroxide by graded xanthine concentration in combination with 2.5mu xanthine oxidase. Chemiluminescence was enhanced by Luminol (10^{-3}M) and charted as chemiluminescence signal in relative light units per second (RLU/s). (E) Show total and partial obliteration of hydrogen peroxide signal by 200U/ml superoxide dismutase and catalase respectively. (F): Example record show 15 min real time observation of luminol (10^{-3}M) enhanced chemiluminescence.

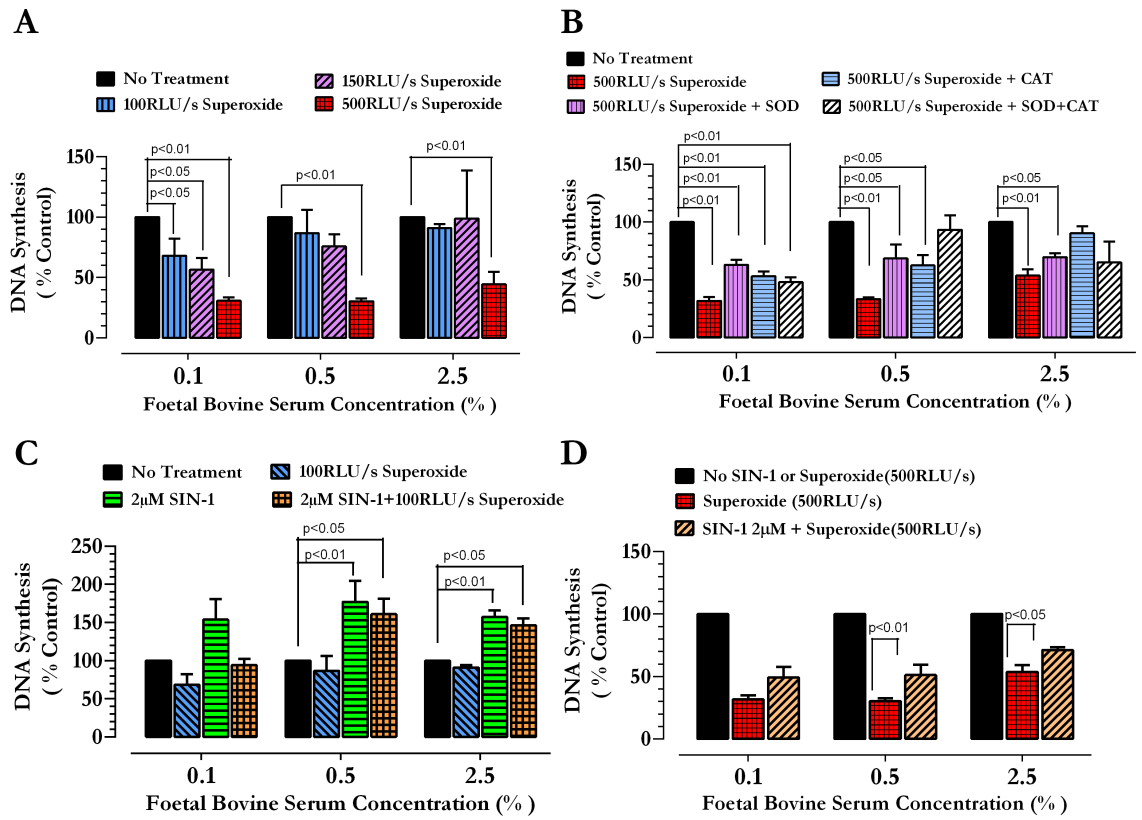

Figure 2: SIN-1 but not superoxide nor hydrogen peroxide stimulated endothelial cell proliferation. (A): Show effect of 500, 150 and 100 RLU/s superoxide alone on endothelial cell proliferation. Superoxide was generated by 10^{-6}, 10^{-7}, or 10^{-8}M xanthine (X) and 2.5mU xanthine oxidase (XO). (B): Cell were pretreated with catalase and superoxide dismutase prior to exposure to 10^{-6}M xanthine and 2.5mU/ml xanthine oxidase which together generated 500RLU/s product. Figures C and D show the effect of 2μM SIN-1 on endothelial cell proliferation in the presence of low (100 RLU/s – Fig 2C) and high (500RLU/s –Fig 2D) concentration superoxide. In A-D, DNA synthesis was measured in DPMs (disintegrations per minute) and expressed as percentage proliferation (± SEM) relative to proliferation in control cells. Statistical evaluations were by 1-way analysis of variance at each FBS concentration. Post hoc investigations were by Dunnett's test that compares treatments with control group. $p< 0.01$ or $p< 0.05$ was considered significant; experiments were conducted in quadruplicate; n=4-6.

Maximum amount of superoxide was generated with 10^{-5} M xanthine and either 2.5 or 1.5 mU xanthine oxidase. Further increases in xanthine concentration beyond 10^{-5} M appeared to have resulted in corresponding decreases in superoxide generated possibly because xanthine quenches the radicals (Figure 1A). It is also plausible that high concentration of xanthine generates hydrogen peroxide preferentially (Figure 1D).

It was confirmed that the xanthine-xanthine oxidase system generated not only superoxide but also hydrogen peroxide (H_2O_2). Luminol was used as it gives chemiluminescence in response to H_2O_2 as well as superoxide. The signals from these set of experiments which involved higher xanthine concentration were primarily due to H_2O_2, as shown by the near complete quenching with catalase (Figure 1E). The residual chemiluminescence in the presence of catalase was presumably due to superoxide. The luminol signal was abolished by superoxide dismutase as expected, since superoxide is the primary product of the xanthine-xanthine oxidase reaction and is subsequently converted to H_2O_2. Levels of H_2O_2 were slowly declining when compared to superoxide level over the same period of time; declining only by 4-11% after 15min in real time (compare Figure 1C and Figure 1F). Although these experiments were not designed to calculate the number of units of superoxide and H_2O_2 generated by 1 unit of the enzyme at any concentration of xanthine, the results however suggests that the ratio of H_2O_2 to superoxide generated by this system may range from 1:1.6 to 1:2.

Effects of superoxide on endothelial cell proliferation

Alone, neither 10^{-6}M xanthine nor 2.5mU/ml xanthine oxidase had effect on endothelial cell proliferation in preliminary experiments. Together, they generated in these experiments, the highest concentration of superoxide (500RLU/s) and inhibited PAEC proliferation at all serum concentrations investigated (Figure 2A). The effects of superoxide 150

RLU/s and 100 RLU/s generated from 10^{-7}M and 10^{-8}M xanthine, respectively on cell proliferation appeared to be insignificant, except under conditions of low growth factors (0.1% FBS; Figure 2A). Superoxide 500RLU/s significantly inhibited endothelial cell DNA synthesis at all serum concentrations investigated. Pre-treating endothelial cells with catalase and SOD combined completely abolished these inhibitory effects at all FBS concentration investigated (Figure 2B). The study thus confirmed that a combination of superoxide and H_2O_2 induced the inhibition of cell proliferation associated with oxidative stress in this study. In addition, the results showed that alone, either superoxide or H_2O_2 may produce growth inhibitory effect under conditions of low growth factors.

SIN-1 but not superoxide or hydrogen peroxide stimulated endothelial cell proliferation

As previously reported 2 μM peroxynitrite generator SIN-1 stimulated PAEC proliferation; this effect has been demonstrated by a quantification of DNA synthesis and whole cell count in previous experiments (Agbani *et al*., 2011a, Agbani *et al*., 2011c). In this study, we investigated the stimulatory effects of 2 μM SIN-1 in the presence of 100 and 500 RLU/s superoxide. The results (Figure 2C, 2D) showed that 2 μM SIN-1 remained able to stimulate DNA synthesis in viable PAEC; this effect was found to be significant at 0.5 and 2.5% FBS baseline stimulation. Alone, superoxide 100RLU/s did not affect endothelial cell DNA synthesis and the significant increases in cell proliferation associated with SIN-1+100RLU/s superoxide were likely the effects of SIN-1 alone (Figure 2C). Using 500RLU/s superoxide decreased DNA synthesis to 31.8±3%, 30.4±2%, and 53.8±5% control at 0.1, 0.5 and 2.5% FBS, respectively. However, SIN-1 remained able to stimulate proliferation to 49.2±9%, 51.1±8% and 71.2±2% control under the same condition, respectively (Figure 2D). SIN-1 did not abolish the inhibitory effects of 500RLU/s superoxide at any of the basal FBS concentrations studied (Figure 2D).

DISCUSSION

Cellular hyper-proliferation underlie the thickening of the pulmonary vascular medial layer and the formation of lesions obliterating vessel lumen; hence controlling cell proliferation represents an important strategy for the resolution of vascular remodeling associated with pulmonary hypertension (Rubin *et al*., 2007, Ghofrani *et al*., 2005, Schermuly *et al*., 2005, Balasubramaniam *et al*., 2003). While some studies suggest that oxidative stress stimulates cell proliferation (Ruiz-Gines *et al*., 2000, Bayraktutan, 2004), others have demonstrated a strong link to the up-regulation of ONOO in lungs under oxidative stress (Bowers *et al*., 2004, Wadsworth *et al*., 2004). Given the divergence of opinion on the subject, it is important to establish the agents mediating cellular hyper-proliferation under oxidative stress.

Alone, low (100RLU/s) and high (500RLU/s) levels superoxide or H_2O_2 generated by the combination of xanthine with xanthine oxidase did not stimulate bovine pulmonary endothelial cell proliferation (Figure 2A). Indeed, high level of superoxide or H_2O_2 inhibited endothelial cell growth (Fig 2B, 2D). This is in good agreement with earlier observation that excess superoxide anions inhibited proliferation of human aortic and umbilical vein endothelial cells (Zanetti *et al*., 2001). In addition, increases in superoxide formation have been recently shown to significantly decrease the viability of human umbilical vein endothelial cells (Karbach *et al*., 2012). Together, these findings indicate that superoxide alone is non-mitogenic in vascular cells under oxidative stress. Nonetheless, reports have shown that superoxides are capable of oxidizing cell constituents such as DNA, proteins, and lipids, leading to impairment of cell functions and the development of morbid conditions (Ames *et al*., 1993, SzÖcs, 2004).

On the other hand, the present study demonstrated increased endothelial cell proliferation (Figure 2C, 2D) but only under oxidative stress conditions producing nitric oxide and superoxide in concert. This condition was simulated by the use of 3-morpholinosydnonimine (SIN-1), which in culture medium generates peroxynitrite by decomposing into superoxide and nitric oxide (Feelisch *et al*., 1989, Holm *et al*., 1998, Martin-Romero *et al*., 2004). Recent findings showed that low concentration peroxynitrite (ONOO-) activated extracellular signal-regulated MAP kinases and stimulated cell proliferation (Agbani *et al*., 2011a, Ichikawa *et al*., 2008, El-Remessy *et al*., 2007). The rate constant for the reaction of superoxide and nitric oxide (~ 10^{10} $M^{-1}s^{-1}$) makes it the fastest reaction known in biology and virtually assures that ONOO- will be formed in any cell, tissue or medium where both radicals exist simultaneously and in proximity (Crow, 2002). Interestingly, superoxide was reported to stimulate microvascular endothelial cell growth in association with increase in eNOS and NAD(P)H oxidase enzyme activity and expression (Bayraktutan, 2004); together however, both enzymes will modulate the formation of ONOO-. Once formed ONOO- can induce change in redox state via nitration and or oxidation (Beckman and Crow, 1993). In addition, ONOO- anion is small sized, diffuses easily, can be generated rapidly in response to extra or intra cellular stimuli, and is short-lived under physiological conditions. Together, these suggest that the molecule can act as an intracellular messenger. We have previously observed that the mitogenic action of ONOO- in pulmonary endothelial cell is dependent on Ras, Raf, MEK and PKC, suggesting that it may be activating an upstream target (Agbani *et al*., 2011a). Furthermore, a preliminary study suggests that 0.2 μM ONOO- caused increased phosphotyrosine formation at PDGF receptors (data not shown).

Conclusion

The proliferation of pulmonary endothelial cells is susceptible to modulation by redox state; however, endothelial dysfunction and hyper-proliferation associated with oxidative stress in pulmonary hypertension are likely mediated by peroxynitrite and not superoxide.

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