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Threshold of peroxynitrite cytotoxicity in bovine pulmonary artery endothelial and smooth muscle cells

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ABSTRACT

Peroxynitrite is widely reported as highly cytotoxic; yet recent evidence indicates that at certain concentrations, it can induce pulmonary cell hyper-proliferation and tissue remodelling. This study aimed to establish the threshold concentration of peroxynitrite to induce functional impairment of bovine pulmonary artery endothelial (PAEC) and smooth muscle cells (PASMC). PAEC or PASMC were exposed to solution of peroxynitrite or 3-morpholinosydnonimine (SIN-1). Twenty-four hour cell viability, DNA synthesis, and protein biochemistry were assessed by trypan blue dye exclusion, [^{3}H] thymidine incorporation and western blot analysis, respectively. Threshold concentration of peroxynitrite to significantly impair viability of PAEC and PASMC was 2 μM peroxynitrite. In PASMC and PAEC, low concentrations of peroxynitrite (2 nM–0.2 μM) increased cell proliferation and did not activate p38 MAP kinase. The decrease in DNA synthesis and cell viability caused by 2 μM peroxynitrite was associated with caspase-3 cleavage but not p38 activation. Also, 2–20 μM peroxynitrite significantly activated poly ADP ribose polymerase and stress activated kinase JNK in PAEC. However, the higher concentration of 20 μM peroxynitrite did cause a threefold increase in p38 activation. In conclusion, the threshold for the cytotoxic effects of peroxynitrite was 2 μM; which caused apoptotic cell death independent of p38 MAP kinase activation in pulmonary artery cells.

1. Introduction

There is extensive evidence for peroxynitrite formation in cardiovascular disease. For example, protein tyrosine nitration, a hallmark of peroxynitrite formation, has been reported in lung sections of infants with pulmonary hypertension (Wadsworth et al., 2004) and in patients with long standing severe forms of the pulmonary disease (Bowers et al., 2004). Furthermore, peroxynitrite ($ONOO^-$) is known to cause in several cell types, DNA fragmentation, activation and inactivation of enzymes and ion channels via protein oxidation and nitration, inhibition of mitochondrial respiration, apoptosis and impaired tissue function (Szabó, 2003; Szabo et al., 2007; Virág et al., 2003). However, the cytotoxic effects of $ONOO^-$ appear to be at odds with clinical evidence for the increased generation of the anion in pathological conditions of oxidative stress such pulmonary hypertension (Rabinovitch, 2008), since $ONOO^-$ induced cytotoxicity does not account for the unique vascular changes evident in the patho-biology of the disease. This variance may be explained by the observation that most laboratory studies on the biological actions of $ONOO^-$ have often involved the use of concentrations (5 μM–50 mM) irrelevant to the pathological formation of the anion. If $ONOO^-$ were important in the development and or progression of pulmonary hypertension, lower concentration of $ONOO^-$ may stimulate pulmonary cell proliferation, similar to the vascular changes seen in pulmonary hypertension. In addition, the stimulatory response of pulmonary artery cells must occur at $ONOO^-$ concentrations not more than the lower limits of cytotoxicity. Additionally, recent evidence demonstrate that particular concentrations of peroxynitrite can induce pulmonary intimal and medial cell hyper-proliferation, by mechanism involving activations of growth factors, extra-cellular regulated MAPK and protein kinase C (Agbani et al., 2011; El-Remessy et al., 2007). Overall, these studies indicate that peroxynitrite is capable of effects other than its widely reported cytotoxicity and its minimal cytotoxic concentration remains unknown.

The present study establishes the threshold concentration for peroxynitrite induced functional impairment in bovine pulmonary artery endothelial (PAEC) and smooth muscle cells (PASMC). In addition, we demonstrate that peroxynitrite induced cytotoxicity occurs via both necrotic and apoptotic pathways, and involves activation of p38 MAP kinase, JNK, caspase-3 and PARP. We report on peroxynitrite activity derived from bolus addition of peroxynitrite solution and in situ generation of peroxynitrite from 3-morpholinosydnonimine (SIN-1).

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2. Materials and methods

2.1. Method of adding peroxynitrite

3-Morpholinosdynonimine (SIN-1) was purchased from Sigma (UK) and dilutions were made in water. Peroxynitrite was supplied (Calbiochem, United Kingdom and Ireland) as a 170 mM solution in 4.7% NaOH. Based on an extinction coefficient of 1670 M^{-1} cm^{-1} at 302 nm in 1 M NaOH, concentration of peroxynitrite solution was determined in aliquots thawed just before use by means of a spectrophotometer (Ultrospec 2000; Pharmacia Biotech). Generation of peroxynitrite from SIN-1 was measured in cell culture medium by quenching NADH fluorescence. Peroxynitrite in this study was administered by the pre-dilution method. This involved pre-diluting authentic $ONOO^-$ to desired concentration in cell culture medium prior to exposing cells to the mixture. Each experiment had control wells exposed to equal volumes of NaOH solution (peroxynitrite vehicle).

2.2. Cell harvest and culture

Bovine pulmonary artery endothelial cells (PAEC) were obtained by a method earlier described (Ryan and Maxwell, 1986). Lungs of cows under 24 months of age were obtained from a local abattoir within 1 h 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. This was done using light, single strokes, covering each area only once. Cells were thereafter subcultured in medium comprising a 50:50 mix of Ham F-12 (Invitrogen) and Waymouth MB752 media (Invitrogen) to which 15% foetal bovine serum (FBS) and 5% penicillin (5000 U/ml):streptomycin (5000 μg) (PEN-STREP® BioWhittaker™) were added. Endothelial cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, and used between passages 2 and 6.

Bovine PASMC were cultured from explants. The intimal cell layer and residual adventitial tissue were stripped off lobular pulmonary arteries (diameter: 5–10 mm) using scapel and forceps respectively. The vessels were cut into small rings (3–5 mm), and transferred into a 25 cm^2 cell-culture flasks containing culture medium as described above. After 21 days of incubation, artery rings were discarded and the seeded PASMC were transferred into new cell-culture flasks. Cultured PASMC were used between passages 2 and 6.

2.3. Cell characterisation by immunocytochemistry and immunofluorescense

PAEC and PASMC were characterised by immunocytochemistry and immunofluorescense as previously reported (Agbani et al., 2011). Briefly, PAEC confirmation was based on the expression and localisation of endothelial nitric oxide synthase 3 (eNOS), the characteristic cobble stone morphology, low intensity of vimentin protein expression as well as the distribution of the intermediate filament protein. The characteristic hill and valley formation as well as the ubiquitous presence of the specific α-smooth muscle actin served to positively identify the PASMC.

2.4. Trypan blue dye exclusion assay for cell viability

The percentage PAEC and PASMC becoming non-viable after treatment for 24 h, with a range of peroxynitrite and SIN-1 concentrations was estimated. Cells were detached by trypsin, centrifuged and re-suspended in phosphate buffer solution (PBS) containing 0.4% Trypan blue dye (Sigma). The number of cells stained blue ("non-viable" cells) was counted and noted against the number of cells unstained ("viable"). Cells were counted using a haemocytometer viewed by phase contrast microscopy (Nikon, Japan) at ×10 magnification.

2.5. Lactate dehydrogenase (LDH) assay for cell lysis

Following treatment of PAEC and PASMC with peroxynitrite solution or SIN-1, the release of lactate dehydrogenase (LDH) into pulmonary artery cell culture medium was detected by colorimetric assay (Roche: Cat #:11 644 793 001) according to the manufacturer's instructions.

2.6. DNA synthesis measured by [^{3}H]-thymidine incorporation assay

The proliferative response of PAEC and PASMC under growth conditions was determined following exposure to peroxynitrite solution or SIN-1. Cells were grown to approximately 70% confluency in 24-well plates at 37 °C and were quiesced with 0.1% foetal bovine serum (FBS) for 24 h. The quiescent cells were maintained at 0.1%, 2.5% and 15% FBS for 24 h with or without exposure to peroxynitrite solution or SIN-1. Cells were pulsed with [methyl-^{3}H] thymidine (GE Healthcare) at 0.1 μCi/well from 18 h until 24 h after peroxynitrite addition. Pulmonary cells were washed with ice cold phosphate buffered saline followed by 10% trichloroacetic acid and then by lauryl sulphate (10%) plus sodium hydroxide (0.2 M) and radioactivity was quantified by liquid scintillation. DNA synthesis was expressed as percentage proliferation relative to control groups. Experiments were conducted in quadruplicate.

2.7. Western blot analysis

Since cytotoxicity was similar for peroxynitrite and SIN-1, peroxynitrite was selected for western blot analysis of p38 MAP kinase and stress activated JNK. PAEC and PASMC were grown in 0.1% FBS and peroxynitrite was applied for various time period before cell lysis. Following treatments, cells were scraped in lysis buffer (63 mM Tris–HCl pH 6.8, 2 mM $Na_4P_2O_7$, 5 mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM DTT, 0.007% (w/v) bromophenol blue). Proteins (25 μg) were separated by SDS–PAGE, transferred to nitrocellulose membrane, and blocked for 1 h at room temperature with 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20. The membrane was then incubated overnight at 4 °C with an appropriate dilution of one of the following: anti-phospho JNK1/2 (New England Biolabs, UK), Total JNK (Santa Cruz Biotechnology), anti-phospho-p38 (Biosource, UK), anti-total p38 (Santa Cruz Biotechnology, INC) cleaved caspase-3, total caspase-3 and PARP antibody (Cell Signaling, Boston, USA). This was followed by incubation for 1 h with an appropriate dilution of horseradish peroxidase-conjugated secondary antibody (Jackson Laboratories PA, USA). The immunoblots were visualised using enhanced chemiluminescence. Blots were exposed to Kodak X-OMAT LS film and developed by a KODAK M35-M X-OMAT processor. Densitometric analysis of ECL autoradiographs were performed using a calibrated densitometer (GS 800, Bio-Rad™). Fold change in phosphorylated JNK or p38 MAP for each treatment was calculated relative to the respective total JNK or p38 MAP.

2.8. Statistics

Data are expressed as mean ± S.E.M. Statistical analysis was by 1-way ANOVA followed by Dunnett's post hoc test to compare treatment groups with the relevant control group (Prism 5;

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GraphPad Software, San Diego, CA). Differences at $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of peroxynitrite on pulmonary artery cell viability

Cells with ruptured or impaired membrane were visibly stained blue while cells with intact membrane excluded the dye and were judged as 'viable'. Control cells showed approximately 5% loss in viability (Fig. 1A and C), probably caused by the trypsinization and centrifugation steps. Twenty-four hour exposure to peroxynitrite at 0.02 μM (PAEC) or 0.2 μM (PAEC or PASMC) or to peroxynitrite vehicle (NaOH) did not significantly affect cell viability in comparison with untreated cells over the same period (Fig. 1A and C). The lowest concentration of peroxynitrite that significantly reduced PAEC and PASMC viability after 24 h was 2 μM (viability was reduced to 84.9 ± 1.8% and 74.7 ± 5.1%, respectively; Fig. 1A and C; $p < 0.05$). Cell viability was impaired without any loss of cell number in PAEC (Fig. 1B); however, in PASMC peroxynitrite 2 μM reduced both cell number and cell integrity (Fig. 1C and D). Peroxynitrite 20 μM caused further cell membrane damage (Fig. 1C). Interestingly, treatment of PAEC with 0.2 μM peroxynitrite, which is below the concentration that reduced cell viability, stimulated cell proliferation and increased cell number in both PAEC and PASMC (Fig. 1B and D; $p < 0.01$).

3.2. Effect of SIN-1 on pulmonary artery cell viability

The peroxynitrite generator SIN-1 caused significant loss of cell viability after 24 h exposure period (Fig. 2A and C; $p < 0.01$). The threshold concentration for SIN-1 induced cytotoxicity was 2 μM in PAEC and 20 μM in PASMC (Fig. 2A and C). Although the viability of PAEC was reduced by 2 μM SIN-1 (Fig. 2A), the cell number increased (Fig. 2B); PASMC cell number was also increased by 2 μM SIN-1 (Fig. 2D). The decrease in PASMC number following treatment with 200 μM SIN-1 (Fig. 2D) was due to cell detachment.

3.3. Effect of peroxynitrite on DNA synthesis

In PAEC, peroxynitrite 2 μM and SIN-1 20 μM significantly inhibited DNA synthesis whether measured under low growth conditions (0.1% FBS) or maximal growth conditions (15% FBS) (Fig. 3). These concentrations of peroxynitrite and SIN-1 impaired cell viability but did not reduce PAEC number – see Figs. 1B and 2B.

3.4. Peroxynitrite induced cytotoxicity: the role of p38 MAPK and JNK

There was no activation of p38 MAPK by peroxynitrite 2 μM in either PASMC (Fig. 4A) or PAEC (Fig. 4C) even though it had impaired membrane integrity (Fig. 1A and C). However activation of p38 MAP kinase did occur at the higher concentration of peroxynitrite 20 μM (Fig. 4A and C), which was similar to 10 μM anisomycin a well known p38 MAPK stimulant (Fig. 4A). In agreement with these results the p38 MAP kinase inhibitor SB23508 did not avert pulmonary cell impairment by 2 μM peroxynitrite (Fig. 4B). Similarly, it was also found that peroxynitrite 2 μM did not activate JNK in PAEC (Fig. 4D) nor in PASMC (fold change in phosphorylated JNK ranged from 0.85–1.2 relative to untreated cells). However, the phosphorylation of stress activated JNK was significantly increased following 15 min exposure to 20 μM $ONOO^-$ (Fig. 4D).

3.5. Activation of caspase-3 and LDH release

Peroxynitrite 2 μM caused significant release of cytosolic LDH from PASMC into the culture medium; this increased with 20 μM

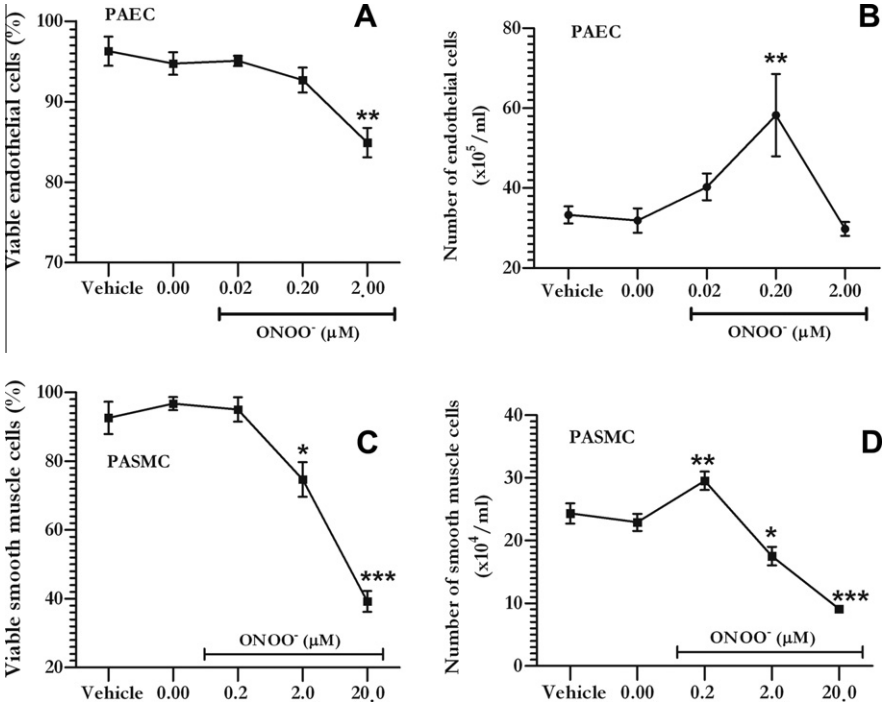

Fig. 1. Cytotoxic effect of authentic peroxynitrite on bovine pulmonary artery cells. Trypan blue assay was conducted 24 h after peroxynitrite ($ONOO^-$) treatment of cells under 15% serum condition. The figure shows the percentage viable cells (A, C) and the number of cells determined by direct cell counting (B, D). Pulmonary artery endothelial cell viability (A) and number (B) following treatment with of 0.02, 0.2 and 2 μM peroxynitrite ($ONOO^-$). Pulmonary artery smooth muscle cell viability (C) and number (D) following treatment with 0.2, 2 and 20 μM peroxynitrite ($ONOO^-$). Significantly different from untreated cells $^{***} = p < 0.001$; $^{**} = p < 0.01$; or $^{*} = p < 0.05$; $n = 8–10$.

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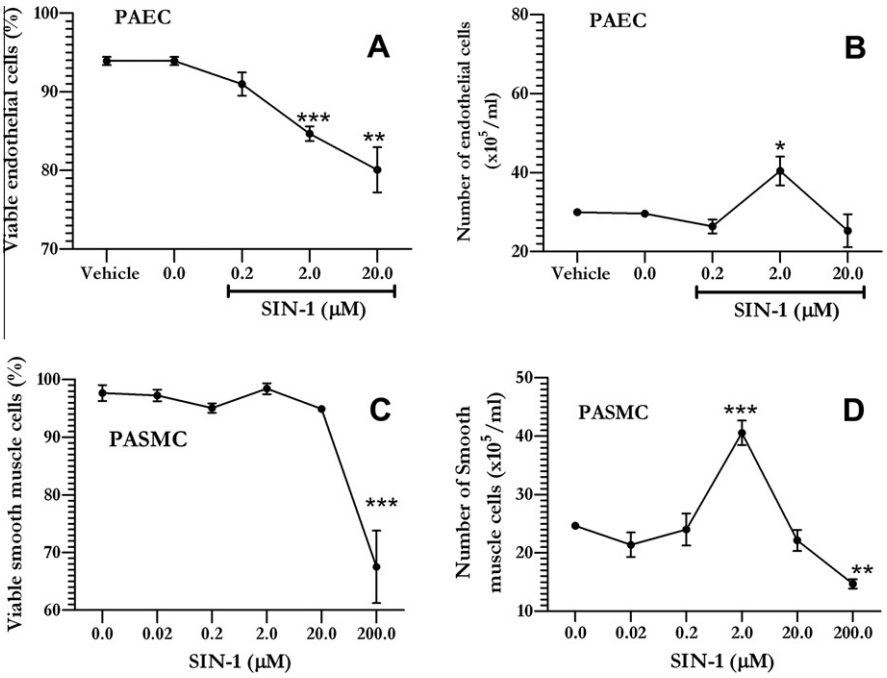

Fig. 2. Cytotoxic effect of SIN-1 generated peroxynitrite on bovine pulmonary artery cells. Trypan blue assay was conducted 24 h after SIN-1 treatment of cells under 15% serum condition. Pulmonary artery endothelial cell viability (A) and number (B) following treatment with of 0.2 and 2 and 20 μM 3-morpholinosydnonimine (SIN-1: peroxynitrite generator). Pulmonary artery smooth muscle cell viability (C) and cell number (D) following treatment with 0.2, 2, 20 and 200 μM SIN-1. Significantly different from untreated cells *** = $p < 0.001$; ** = $p < 0.01$; or * = $p < 0.05$; $n = 8–10$.

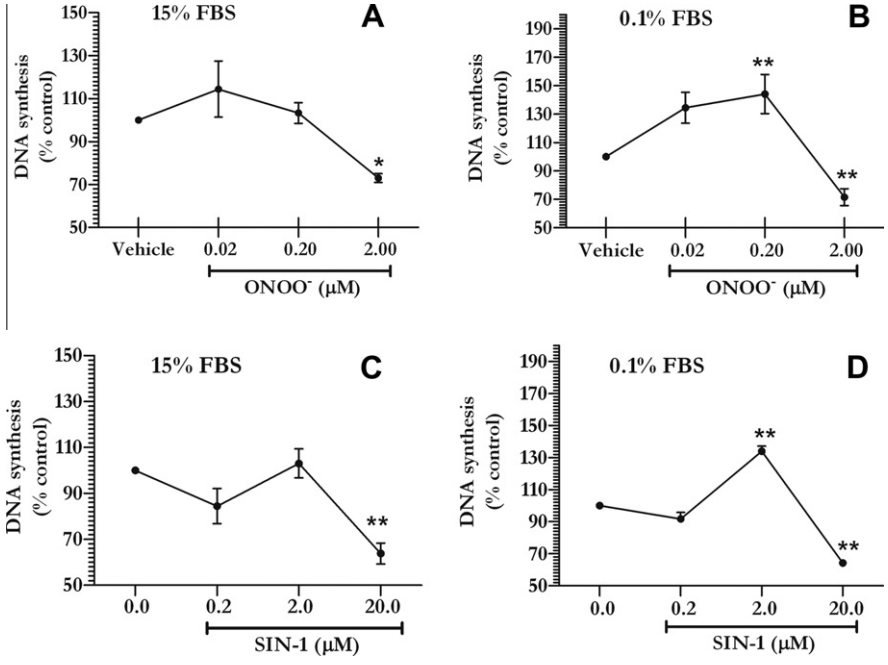

Fig. 3. Effect of peroxynitrite (A, B) or 3-morpholinosydnonimine (SIN-1) (C, D) on pulmonary artery endothelial cell DNA synthesis. DNA synthesis under maximum serum (A, C) and basal serum (B, D) conditions was evaluated by ^{3}H-thymidine incorporation assay, Counts were recorded in DPM (disintegrations per minute) and normalised to cells in other wells on the same plate that received no treatment or peroxynitrite vehicle over the same time course as the treated wells (±S.E.M.). Significantly different from untreated or vehicle treated cells *** = $p < 0.001$, ** = $p < 0.01$ or * = $p < 0.05$; $n = 5$.

peroxynitrite treatment to levels approaching those found with the cytotoxic agent staurosporine (Fig. 5A). Lower concentrations (0.2 μM or below) of peroxynitrite did not cause LDH release (Fig. 5A). LDH release in PAEC followed a similar trend (Fig. 5B). In addition, fold change in cleaved pro-apoptotic caspase-3 significantly increased following 15 min treatment of PAEC with 2 or 20 μM $ONOO^-$ (Fig. 5C). Experiments investigating nuclear activation of poly ADP ribose polymerase (PARP) showed that PARP was significantly activated following 15 min 2 or 20 μM $ONOO^-$ treatment of PAEC (Fig. 5D).

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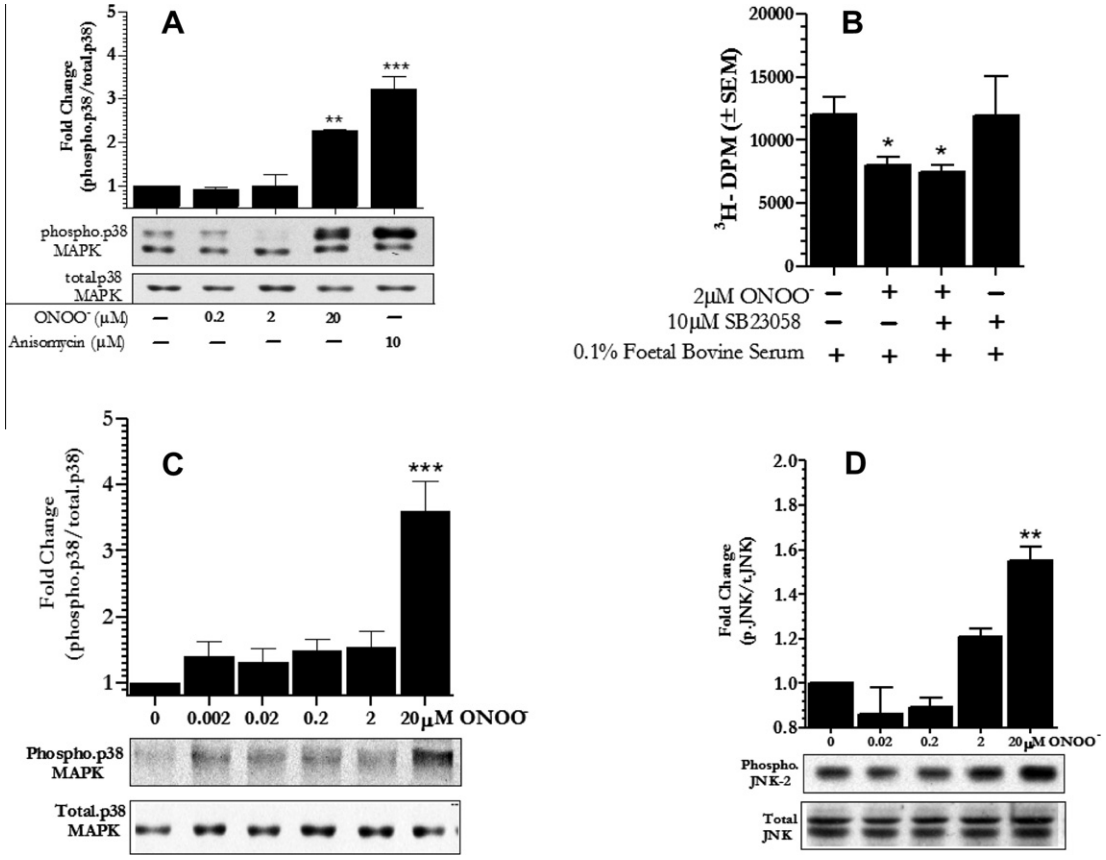

Fig. 4. Effect of peroxynitrite on phosphorylation of p38 MAPK and JNK in PASMC (A, B) and PAEC (C, D). Expression of total and phosphorylated p38 mitogen activated protein kinase or JNK was determined by western blotting; quantification was by densitometry. Cells were maintained under 0.1% serum. In B, DNA synthesis was evaluated by ^{3}H-thymidine incorporation assay. Counts were recorded in DPM (disintegrations per minute) and normalised to untreated group (±S.E.M.). Significantly different from untreated cells *** = $p < 0.001$, ** = $p < 0.01$ or * = $p < 0.05$; $n = 3$.

4. Discussion

It is well established that 5–50 μM peroxynitrite is cytotoxic by multiple mechanisms (reviewed in (Szabó, 2003; Szabo et al., 2007). This study has utilised a range of $ONOO^-$ concentration and has confirmed these cytotoxic effects in pulmonary artery cells. In addition, for the first time, this study reports a threshold concentration of 2 μM. Cytotoxicity at this concentration was mild affecting less than 20% of cells (Fig. 1). Furthermore a lower concentration caused pulmonary cells hyper-proliferation. It is expected that SIN-1 generated $ONOO^-$ over a period of time (unpublished data), this may account for the profile of SIN-1 cytotoxicity. The pattern however appeared similar to authentic $ONOO^-$. In this study, decrease in cell viability (Fig. 2) and impairment of DNA synthesis by SIN-1 (Fig. 3) were significant under basal and high serum conditions.

Increased peroxynitrite formation has been linked to the pathogenesis of several diseases (Pacher et al., 2007; Szabo et al., 2007). Nitrotyrosine, deposited in tissue as a result of peroxynitrite exposure, has been found in patients with long standing pulmonary hypertension (Bowers et al., 2004) and in lung sections of infants with pulmonary hypertension (Wadsworth et al., 2004). Some evidence also suggest that cellular destruction could be involved in the development of pulmonary hypertension; thus the ratio of pulmonary arterioles to pulmonary alveoli is reduced in certain experimental models of pulmonary hypertension (Hislop and Reid, 1976; Partovian et al., 2000; Rabinovitch et al., 1979) and by contrast the death of cells in pulmonary arteries may stimulate a phase of cell proliferation and medial hypertrophy (Golpon et al., 2004; Sakao et al., 2006). Whilst $ONOO^-$ is capable of both oxidative and nitration actions, it is not clear from the current study whether one or both actions are directly linked with the growth inhibitory effects observed.

Mitogen activated protein kinase (MAPK) activation occurs coincidentally with cell death in many situations but this does not make them causally related and the consensus is that activation of extra-cellular regulated MAPK is usually associated with cell proliferation (Brown and Sacks, 2008; Geer et al., 1994). Furthermore, the duration and or intensity of stimuli may underlie the specific response of a cell (Marshall, 1995). Using concentration higher than our threshold concentration, several studies have implicated p38 MAPK and stress activated protein kinase (JNK) in peroxynitrite impairment of cell function or mechanisms of cell death (Ali et al., 2008; Nabeyrat et al., 2003; Oh-hashi et al., 1999; Pesse et al., 2005). However, at threshold concentration (2 μM) PASMC membrane damage (Fig. 1C and D) associated with 24 h exposure to $ONOO^-$ were not mediated by early onset p38 MAPK activation nor attenuated by 24 h p38 MAPK inhibition (Fig. 4A and B). Similarly, the current study showed that the significant damage in PAEC membrane (Fig. 1A and B) following 24 h exposure to 2 μM $ONOO^-$ may not involve the activation of p38 MAPK (Fig. 4C). It seems though that this concentration of $ONOO^-$ may increase the phosphorylation of stress activated JNK at least in PAEC (Fig. 4D).

This study demonstrates caspase-3 activation in PAEC (Fig. 5C and D) in addition to the significant release of cytosolic LDH following treatment of PASMC and PAEC with 20 μM $ONOO^-$ (Fig. 5A and B). The executional phase of apoptosis has been shown

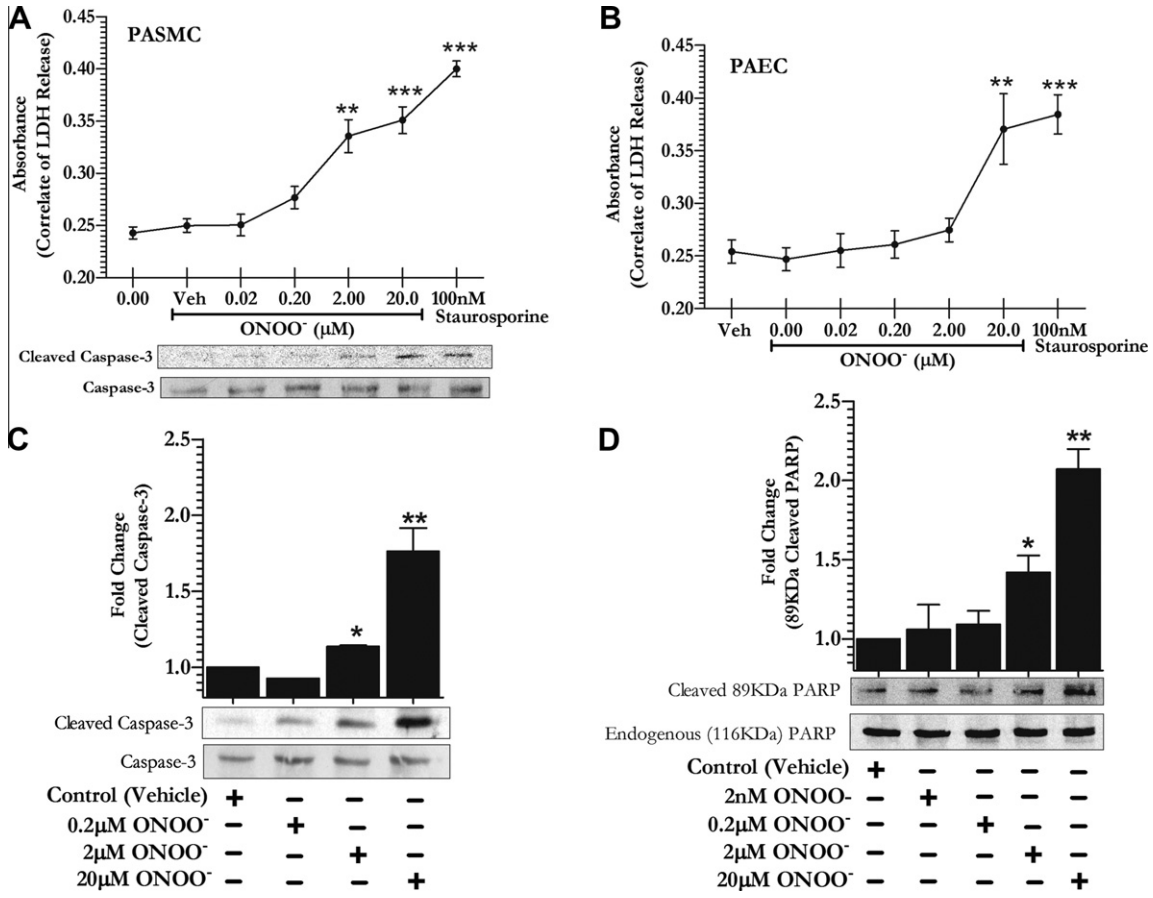

Fig. 5. Peroxynitrite concentration dependent cytotoxic effects on necrotic and apoptotic indicators. Release of cytosolic lactate dehydrogenase in pulmonary artery smooth muscle (A) and endothelial cells (B). Peroxynitrite concentration dependent induction of apoptosis in pulmonary endothelial cells (C and D). Release of cytosolic lactate dehydrogenase was evaluated by Roche kit. Statistical analysis was by 1-way ANOVA, post hoc test by Dunnett's test that compares treatment groups with relevant control. *** = $p < 0.001$; ** = $p < 0.01$; or * = $p < 0.05$ were considered significant relative to untreated cells; $n = 4$. For the assessment of caspase-3 activation ($n = 3$), PASMC were exposed to $ONOO^-$ for 15 min and prepared for western blot analysis; typical blot showed. (C) shows cleavage of capase-3 and (D) shows activation of poly ADP ribose polymerase (PARP) in pulmonary artery endothelial cells. Expression of pro-apoptotic proteins was determined by western blotting. Quantification was densitometry; cells were maintained under 0.1% serum; *** = $p < 0.001$ or ** = $p < 0.01$ were considered significant relative to untreated cells; $n = 3$.

to be via caspases likely activated by mitochondria-derived apoptogenic factors in peroxynitrite treated cells (Green and Kroemer, 1998). Although the exact mechanism of peroxynitrite-induced apoptosis is unclear from the current data, independent studies have also reported caspase-3 cleavage during the course of peroxynitrite induced apoptosis (Lin et al., 1998; Virag et al., 1998; Zhuang and Simon, 2000). The Leakage of LDH from pulmonary cells into the surrounding milieu (Fig. 5A and B) is evidence of cell death via necrosis. At 20 μM $ONOO^-$, this was associated with the activation of p38 MAPK in PASMC and PAEC (Fig. 4A and C). In addition, this study provides initial evidence for the activation of PARP in PAEC following exposure to 2 and 20 μM $ONOO^-$ (Fig. 5C and D). This likely is the result of DNA single strand breakage as seen in other cell types (Virag and Szabo, 2002). Virág et al. (2003) postulated a model in which a DNA nick sensor enzyme, poly(ADP-ribose) polymerase-1 (PARP-1) becomes activated upon sensing DNA breakage. Activated PARP-1 cleaves NAD^+ into nicotinamide and ADP-ribose and polymerises the latter on nuclear acceptor proteins. Peroxynitrite-induced overactivation of PARP consumes NAD^+ and consequently ATP culminating in cell dysfunction, apoptosis or necrosis-depending on whether the cascade of events was initiated by low or high concentration $ONOO^-$, respectively. This cellular suicide mechanism has been implicated among others as one mechanism of cardiovascular dysfunction (Virag et al., 1998, 2003). This will at best in this study, simulate lung injury due to necrosis since apoptotic cells are rapidly cleared from the tissues by macrophages. Taken together, the results provide evidence for apoptotic and necrotic pathways of cell death in pulmonary cells treated with ⩾2 μM $ONOO^-$. While 20 μM $ONOO^-$ did not activate JNK in PASMC (data not shown), it appears that this concentration of $ONOO^-$ may in PAEC, increase the levels of stress activated protein kinase JNK (Fig. 4D). Using 50–500 μM $ONOO^-$, Pesse and coworkers demonstrated concentration dependent JNK activation in primary ventricular cardiomyocytes (Pesse et al., 2005). Thus it is conceivable, at least in PAEC but not PASMC, that cytotoxicity of 20 μM $ONOO^-$ is JNK linked.

5. Conclusion

The study reports potent cytotoxic effects of authentic $ONOO^-$ and in situ generated from SIN-1. We determined for the first time, the threshold for the cytotoxic effects of authentic $ONOO^-$ to be 2 μM in pulmonary artery cells. Apoptotic cell death at 2 μM $ONOO^-$ may be caspase-3 dependent but independent of p38 activation. Cell death at >2 μM $ONOO^-$ may involve activation of caspase-3 in both apoptotic and necrotic pathways.

6. Conflict of interest

None declared.

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References

Agbani, E.O., Coats, P., Mills, A., Wadsworth, R.M., 2011. Peroxynitrite stimulates pulmonary artery endothelial and smooth muscle cell proliferation: involvement of ERK and PKC. Pulmonary Pharmacology and Therapeutics 24, 100–109.

Ali, T.K., Matragoon, S., Pillai, B.A., Liou, G.I., El-Remessy, A.B., 2008. Peroxynitrite mediates retinal neurodegeneration by inhibiting nerve growth factor survival signaling in experimental and human diabetes. Diabetes 57, 889–898.

Bowers, R., Cool, C., Murphy, R.C., Tuder, R.M., Hopken, M.W., Flores, S.C., Voelkel, N.F., 2004. Oxidative stress in severe pulmonary hypertension. American Journal of Respiratory and Critical Care Medicine 169, 764–769.

Brown, M.D., Sacks, D.B., 2008. Compartmentalised MAPK pathways. In: Klussmann, E., Scott, J., Aandahl, E.M. (Eds.), Protein–Protein Interactions as New Drug Targets, Vol. 186. Springer, Berlin, pp. 205–238.

El-Remessy, A.B., Al-Shabrawey, M., Platt, D.H., Bartoli, M., Behzadian, M.A., Ghaly, N., Tsai, N., Motamed, K., Caldwell, R.B., 2007. Peroxynitrite mediates VEGF's angiogenic signal and function via a nitration-independent mechanism in endothelial cells. FASEB J. 21, 2528–2539.

Geer, P.V., Hunter, T., Lindberg, R.A., 1994. Receptor protein-tyrosine kinases and their signal transduction pathways. Annual Review of Cell Biology 10, 251–337.

Golpon, H.A., Fadok, V.A., Taraseviciene-Stewart, L., Scerbavicius, R., Sauer, C., Welte, T., Henson, P.M., Voelkel, N.F., 2004. Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. FASEB J., 04-1853fje.

Green, D., Kroemer, G., 1998. The central executioners of apoptosis: caspases or mitochondria? Trends in Cell Biology 8, 267–271.

Hislop, A., Reid, L., 1976. New findings in pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. British Journal of Experimental Pathology 57, 542–554.

Lin, K.T., Xue, J.Y., Lin, M.C., Spokas, E.G., Sun, F.F., Wong, P.Y.K., 1998. Peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease. American Journal of Physiology Cell Physiology 274, C855–C860.

Marshall, C.J., 1995. Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179–185.

Nabeyrat, E., Jones, G.E., Fenwick, P.S., Barnes, P.J., Donnelly, L.E., 2003. Mitogen-activated protein kinases mediate peroxynitrite-induced cell death in human bronchial epithelial cells. American Journal of Physiology Lung Cellular and Molecular Physiology 284, L1112–L1120.

Oh-hashi, K., Maruyama, W., Yi, H., Takahashi, T., Naoi, M., Isobe, K.i., 1999. Mitogen-activated protein kinase pathway mediates peroxynitrite-induced apoptosis in human dopaminergic neuroblastoma SH-SY5Y cells. Biochemical and Biophysical Research Communications 263, 504–509.

Pacher, P., Beckman, J.S., Liaudet, L., 2007. Nitric oxide and peroxynitrite in health and disease. Physiological Reviews 87, 315–424.

Partovian, C., Adnot, S., Raffestin, B., Louzier, V., Levame, M., Mavier, I.M., Lemarchand, P., Eddahibi, S., 2000. Adenovirus-mediated lung vascular endothelial growth factor overexpression protects against hypoxic pulmonary hypertension in rats. American Journal of Respiratory Cell and Molecular Biology 23, 762–771.

Pesse, B., Levrand, S., Feihl, F., Waeber, B., Gavillet, B., Pacher, P., Liaudet, L., 2005. Peroxynitrite activates ERK via Raf-1 and MEK, independently from EGF receptor and p21Ras in H9C2 cardiomyocytes. Journal of Molecular and Cellular Cardiology 38, 765–775.

Rabinovitch, M., 2008. Molecular pathogenesis of pulmonary arterial hypertension. The Journal of Clinical Investigation 118, 2372–2379.

Rabinovitch, M., Gamble, W., Nadas, A.S., Miettinen, O.S., Reid, L., 1979. Rat pulmonary circulation after chronic hypoxia: hemodynamic and structural features. American Journal of Physiology 236, H818–H827.

Ryan, U.S., Maxwell, G., 1986. Isolation, culture, and subculture of bovine pulmonary artery endothelial cells: mechanical methods. Methods in Cell Science 10, 3.

Sakao, S., Taraseviciene-Stewart, L., Wood, K., Cool, C.D., Voelkel, N.F., 2006. Apoptosis of pulmonary microvascular endothelial cells stimulates vascular smooth muscle cell growth. American Journal of Physiology Lung Cellular and Molecular Physiology 291, L362–L368.

Szabó, C., 2003. Multiple pathways of peroxynitrite cytotoxicity. Toxicology Letters 140–141, 105.

Szabo, C., Ischiropoulos, H., Radi, R., 2007. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nature Reviews Drug Discovery 6, 662.

Virag, L., Scott, G.S., Cuzzocrea, S., Marmer, D., Salzman, A.L., Szabo, C., 1998. Peroxynitrite-induced thymocyte apoptosis: the role of caspases and poly (ADP-ribose) synthetase (PARS) activation. Immunology 93, 345–355.

Virag, L., Szabo, C., 2002. The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. Pharmacological Reviews 54, 375–429.

Virág, L., Szabó, É., Gergely, P., Szabó, C., 2003. Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention. Toxicology Letters 140–141, 113–124.

Wadsworth, R.M., Hoehn, T., McPhaden, A., Stiller, B., Preston, A., 2004. Upregulation of nitrotyrosine, a marker for peroxynitrite, in pulmonary arteries of pulmonary hypertensive patients. In 8th International Symposium on Resistance Arteries (June 20–23) A. Daniel Henrion, France, ed., p. 85. KARGER, Angers, France.

Zhuang, S., Simon, G., 2000. Peroxynitrite-induced apoptosis involves activation of multiple caspases in HL-60 cells. American Journal of Physiology. Cell Physiology 279, C341–C351.

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